Applied and Environmental Microbiology, Oct. 2007, p. 6134–6143 Vol. 73, No. 19
0099-2240/07/$08.00+0 doi:10.1128/AEM.00851-07

Optimization of Airborne Endotoxin Exposure Assessment: Effects of Filter Type, Transport Conditions, Extraction Solutions, and Storage of Samples and Extracts▽

Suzanne Spaan,[1,2] Dick J. J. Heederik,[1] Peter S. Thorne,[3] and Inge M. Wouters[1]*

Division Environmental Epidemiology (EEPI), Institute for Risk Assessment Sciences (IRAS), Utrecht University, Utrecht, The Netherlands[1]; Food and Chemical Risk Analysis, TNO Quality of Life, Zeist, The Netherlands[2]; and Environmental Health Sciences Research Center, University of Iowa, Iowa City, Iowa[3]

Received 16 April 2007/Accepted 28 July 2007

Endotoxin exposure occurs in homes and occupational environments and is known to cause adverse health effects. In order to compare results from different studies and establish standards, airborne endotoxin exposures should be assessed using standardized methods. Although the European Committee for Standardization (CEN) developed guidelines for endotoxin exposure assessment, these leave room for individual interpretation. The influence of methods of sampling, extraction, and analysis has never been investigated in a full experimental design. Thus, we sought to fully elucidate the importance of all facets of endotoxin assessment. Inhalable dust samples collected simultaneously were used to investigate the effects on and interactions with airborne endotoxin concentration in two working environments of filter type (glass fiber or Teflon), transport conditions (with/without desiccant), sample storage (−20 or 4°C), extraction solution (pyrogen-free water [PFW] or PFW plus 0.05% Tween 20), extract storage (−20 or 4°C), and assay solution (PFW or PFW plus 0.05% Tween 20). Four hundred samples were collected and randomly distributed over the 20 combinations of treatments. There were no differences found for transport conditions and storage temperature of extracts. Also, no interactions between study variables existed. Sampling on glass-fiber filters, storage of samples in the freezer, and extraction in PFW plus 0.05% Tween 20 resulted in 1.3-, 1.1-, and 2.1-fold-higher estimated endotoxin concentrations, respectively. Use of PFW plus 0.05% Tween 20 in the assay solution had an additive effect. Thus, this study investigated gaps in the CEN protocol and provides data with which to fully specify a protocol for standardization of endotoxin exposure assessment.

Endotoxins are constituents of the outer membrane of gram-negative bacteria and occur as contaminants in organic dusts or aerosols. Endotoxin is a well-known toxin with a high proinflammatory potency. Airborne exposure has been associated with several symptoms in the respiratory tract and reductions in pulmonary function in various agricultural and industrial environments (7, 16, 30). On the other hand, it is also suggested that environmental and occupational endotoxin exposure has a possible protective effect on the risk of atopic sensitization in childhood and possibly also in an adult working population with high endotoxin exposures (18, 26, 37).

The European Committee for Standardization (CEN) developed guidelines for the assessment of workplace exposure to airborne bacterial endotoxins, using the knowledge available at that time (9, 10). These guidelines provide methods for sampling, transportation and storage of samples, and determination of endotoxins. However, the NEN-EN 14031 protocol "Workplace atmosphere—determination of airborne endotoxin" fails to clearly delineate aspects that might affect the outcome, for example, what extraction solution or storage conditions to use. There are few empirical data to support some of the assumptions in the protocol. This leaves room for individual interpretation and nonuniform methodology.

Differences exist in laboratory methods for collection of samples (filter type), transport conditions and storage of samples, processing and analysis of samples (extraction medium, rocking, sonication, temperature, type of assay, and control standards), and reporting of results (units) (29). Previous investigations of interlaboratory differences in endotoxin analyses showed that results could differ by a factor of 10 to 1,000 between the minimum and maximum concentrations of cotton dust samples, a factor which was reduced to a 5- to 12-fold difference when the extraction protocol and assay were standardized (3). Another study showed that when further restrictions were applied (e.g., same assay supplier, same dilutions, and inclusion of results with valid spike results only), interlaboratory differences could become even smaller (two- to threefold), suggesting that interlaboratory differences might be explained to a large extent by the effects of varying procedures (17).

Several studies investigated how changes in procedures affect the endotoxin concentration in occupational settings (5, 6, 12, 15, 19–21, 23, 31, 33, 35, 38) and in house dust (11, 13, 14, 22, 24). Most of these studies investigated only one or two of the factors possibly influencing the measured endotoxin concentration and in a limited number of samples, although the high variability in the endotoxin content of dust calls for experiments with a large number of samples. Therefore, the combined influence of different factors and

* Corresponding author. Mailing address: Institute for Risk Assessment Sciences, Division Environmental Epidemiology, Utrecht University, P.O. Box 80178, 3508 TD Utrecht, The Netherlands. Phone: 31 30 253 9449. Fax: 31 30 253 9499. E-mail: i.m.wouters@iras.uu.nl.
▽ Published ahead of print on 3 August 2007.

TABLE 1. Overview of combinations of factors of interest; the number of samples per combination; and their geometric means, geometric standard deviations, and ranges in endotoxin concentration

Combination no.	Filter type	Transport condition (desiccant)[b]	Sample storage temp (°C) before extraction	Extraction solution[c] (Tween)	Extract storage temp (°C) before analysis	No. of samples	Endotoxin concn (EU/m^3)		
							GM[d]	GSD[e]	Range (minimum to maximum)
1	Glass fiber	+	4	+	4	16	2,569	1.57	930–7,104
2	Glass fiber	+	4	−	4	19	1,466	1.88	507–5,284
3	Glass fiber	+	4	+	−20	19	2,840	1.74	939–9,705
4	Glass fiber	+	4	−	−20	18	1,427	1.85	572–4,795
5	Glass fiber	+	−20	+	4	19	3,236	1.72	1,346–7,235
6	Glass fiber	+	−20	−	4	19	1,558	1.86	690–5,868
7	Glass fiber	+	−20	+	−20	18	3,266	1.74	1,137–6,649
8 (CEN[a])	Glass fiber	+	−20	−	−20	20	1,334	1.77	518–4,745
9	Glass fiber	−	4	+	4	19	2,802	1.60	1,192–8,938
10	Glass fiber	−	4	−	4	20	1,500	1.88	408–5,232
11	Glass fiber	−	4	+	−20	20	3,060	1.60	1,361–7,995
12	Glass fiber	−	4	−	−20	20	1,241	1.65	495–3,345
13	Glass fiber	−	−20	+	4	19	2,865	1.67	1,206–8,050
14	Glass fiber	−	−20	−	4	20	1,552	1.98	232–5,277
15	Glass fiber	−	−20	+	−20	20	3,191	1.56	1,257–6,821
16	Glass fiber	−	−20	−	−20	20	1,571	2.02	515–7,277
17	Teflon	−	4	+	−20	20	2,285	1.68	1,015–6,875
18	Teflon	−	4	−	−20	20	1,093	1.94	332–3,877
19	Teflon	−	−20	+	−20	20	2,440	1.86	726–7,301
20	Teflon	−	−20	−	−20	20	1,046	1.76	404–3,309

[a] CEN, combination of variables which is comparable to the CEN protocol (reference category).
[b] +, with desiccant; −, without desiccant.
[c] Extraction solution is PFW, with (+) or without (−) 0.05% Tween 20.
[d] GM, geometric mean.
[e] GSD, geometric standard deviation.

their interaction is still unknown. In most of the studies only one type of dust was investigated. Recent studies showed that variability between labs also depended on the source of dust that was analyzed (28, 29), Thus, the environment sampled needs to be taken into account when effects of different procedures are investigated.

Therefore, in this study a full experimental design was implemented to investigate the combined influence of all gaps in the CEN protocol, namely, transport conditions, storage of samples, extraction solution, storage of extracts, filter type, and assay solution, as well as their individual and interactive influence. The effect of changes in sampling, extraction, and analysis procedures on the endotoxin concentration was investigated in two representative work environments to give input for the further development of a standardized method for the measurement and analysis of endotoxin so that exposure levels can be compared between studies and with established exposure limits.

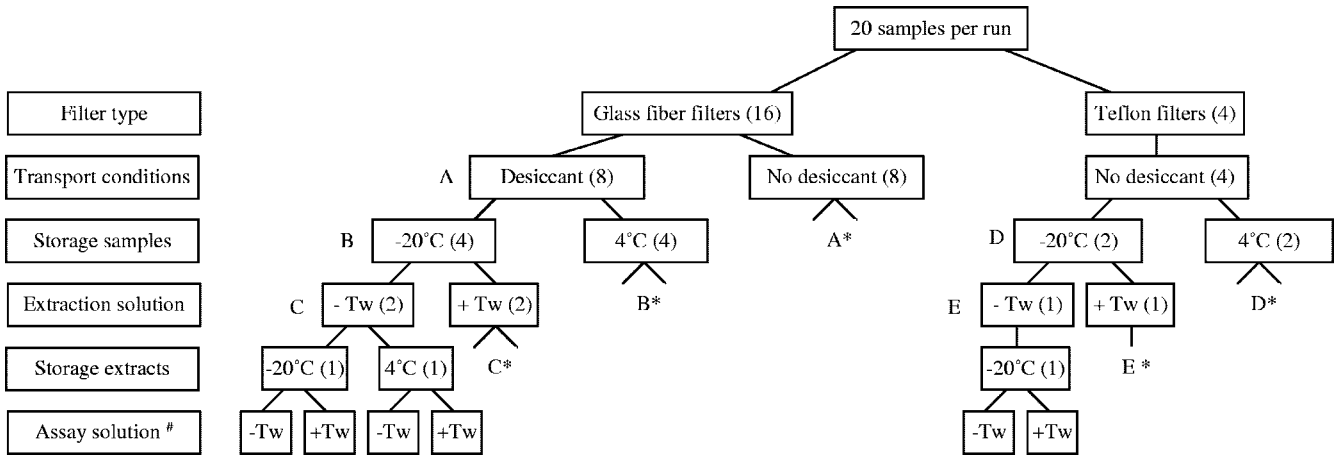

FIG. 1. Schematic overview of the design of the experiment. Asterisks mark places from which the scheme follows the same route as is written out from the stage with the corresponding letter besides the design step. The number sign indicates that the influence of assay solution was investigated in part of the data (136 out of 386 samples). Numbers in parentheses indicate the number of samples that undergo that particular step of the scheme. Tw, Tween 20.

MATERIALS AND METHODS

Design of the study. This study focused on determining the influence of and the interaction between four primary parameters on the measured endotoxin concentration: transport conditions of filters (with or without a silica dehumidifier), storage conditions of samples before extraction (at 4°C or −20°C), extraction solution (pyrogen-free water [PFW] with or without 0.05% Tween 20), and storage conditions of extracts before analysis (at 4°C or −20°C).

These four primary factors of interest and their interactions resulted in 16 combinations that were studied using glass-fiber filters, the preferred filter type in the CEN protocol. Since samples were collected with parallel samplers that had the capacity for 20 parallel samples to be collected simultaneously, an additional four combinations of factors could be investigated. We choose to study selected factors (storage of samples and extraction solution) with another filter type (Teflon), since Teflon filters are used regularly when allergens and endotoxins are measured simultaneously. Teflon filters were transported without desiccant, and extracts were stored at −20°C. Furthermore, since there has been debate on the use of Tween 20 in measuring endotoxin, we decided to analyze part of the samples both with and without use of Tween 20 in the assay solution to investigate its influence on the outcome in combination with the other parameters. Thus, two secondary parameters were also included in the experiment: filter type (glass-fiber or Teflon filters) and assay solution (PFW with or without 0.05% Tween 20). An overview of the distribution of samples over the combinations is given in Table 1 and Fig. 1. The 20 combinations of the above factors were assigned randomly to the 20 parallel sampling positions available per run.

Two representative work environments were chosen for this study, namely, pig farming and grass seed processing, representing different sources of endotoxin exposure (animal excretions and growth of bacteria on plant material). Due to the large amount of samples needed for this full experimental design, this study was restricted to these work environments. All combinations of factors were measured 10 times per worksite. Sampling time varied (measurement durations of 1, 2, 3, 5, or 6 h) to ensure that a sufficient range of concentrations was obtained. Each time interval was represented twice per worksite. Air samples were collected during 10 days, 5 days on each location, with two sampling events on each day, in two consecutive weeks in November 2005. In total, 400 samples were collected, of which 320 were on glass-fiber filters and 80 were on Teflon filters. In addition, on every sampling day a field filter blank was collected, which underwent the same steps as the other samples did except for the actual sampling. A priori conditions were that samples must be extracted within 2 weeks after sampling and endotoxin analysis must be performed within 24 h after extraction.

Collection of inhalable dust samples. Two parallel samplers, which each enabled the simultaneous collection of 10 close-to identical samples of inhalable dust, were used to collect air samples. The samplers were developed within the European MOCALEX project according to a design published by Eduard et al., modified for the simultaneous collection of 10 airborne samples using PAS-6 sampling heads (1, 8). Ten conical PAS-6 sampling heads for inhalable dust (32) were positioned in an annular chamber (outer cone diameter, 20 cm; inner cone diameter, 12 cm), which provided nearly symmetrical flow at the PAS-6 sampling head inlets. The overall flow rate was 40 liters/min. Critical orifices provided a flow of 2 liters/min at the inlet of the sampling heads (Fig. 2). The flow was checked at the PAS-6 sampling heads before and after sampling with a rotameter and showed virtually no decline over time. The filters were put in individual petri dishes after sampling, sealed with tape, and placed in a Ziploc bag. In case of desiccant use, a small bag with 15 mg silica gel drying pearls (Fluka, Germany) was added.

During a run, the two parallel samplers were positioned next to each other to collect 20 uniform air samples per run. The sampling heads were equipped with 25-mm glass-fiber filters (Whatman GF/A; United Kingdom) or 25-mm Teflon (polytetrafluoroethylene) filters (Millipore FALP2500; United Kingdom). The filters were pre- and postweighed on an analytical balance in a conditioned room meeting U.S. Environmental Protection Agency criteria, to determine the amount of dust on the filters gravimetrically. Inhalable dust concentrations below the limit of detection (LOD) were assigned a value of two-thirds of the LOD of the balance.

Extraction and analysis. Samples were stored 12 to 14 days prior to extraction. Extraction of endotoxin was done as described previously, under pyrogen-free conditions (5). Briefly, filters were immersed in 5 ml extraction solution (being either PFW or PFW plus 0.05% Tween 20) and rocked vigorously for 1 h at room temperature on a horizontal shaker (160 reciprocations/min; deflection, 15 cm). After 15 min of centrifugation at 1,000 × *g*, 1 ml supernatant per sample was collected and vortexed, and four aliquots of 0.1 ml and the remaining 0.6 ml were stored until analysis. Storage temperature was either 4°C or −20°C, depending on the assigned treatment.

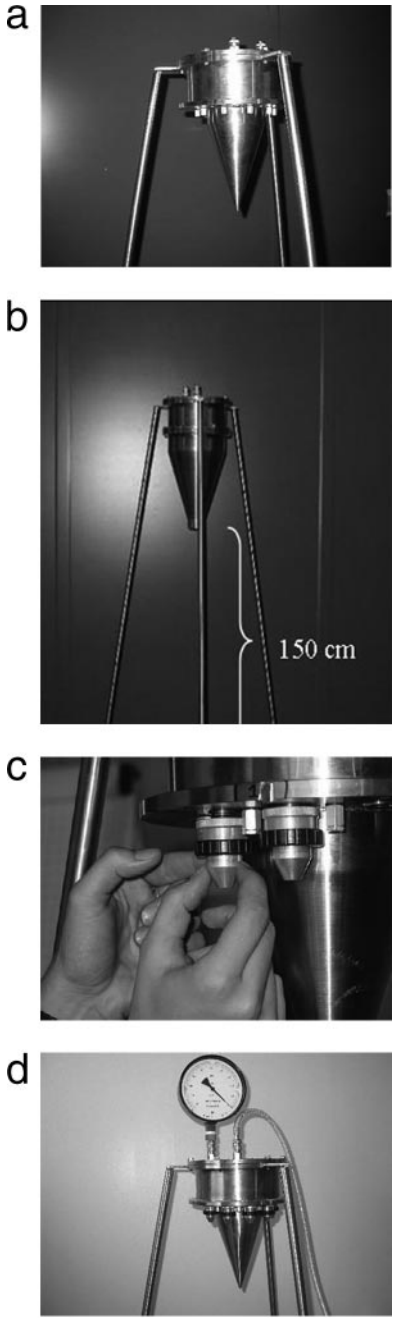

FIG. 2. Pictures of the parallel sampler, which contains 10 sampling heads positioned in an annular chamber between the inner and outer cone. (a) Parallel sampler without outer cone; (b) parallel sampler with outer cone; (c) placement of sampling heads in parallel sampler; (d) parallel sampler with vacuum monometer and tube for connection with pump, but without outer cone attached.

The endotoxin concentration in extracts was assayed using a kinetic chromogenic *Limulus* amoebocyte lysate method (Cambrex, Verviers, Belgium; lysate lot no. 3L433E, standard lot no. 3L2950 [reference standard endotoxin/control standard endotoxin ratio, 10 ng/0.90 ml = 100 endotoxin units (EU)/ml]) (5). One of every eight samples was randomly selected for analysis in duplicate to assess the coefficient of variation (CV%). The lower LOD ranged from 0.043 to

TABLE 2. Means and ranges of dust levels (mg and mg/m^3) and mean CV%s within a run of dust concentrations (based on mg/m^3), overall and stratified by filter type and work environment[a]

Stratification	No. of samples	Dust level						CV%/run	
		mg			mg/m^3			Mean	Min–max
		AM	SD	Min–max	AM	SD	Min–max		
Overall	382	0.54	0.43	ND–1.74	1.41	0.95	ND–3.78	32.7	10.9–76.2
Filter type									
Glass fiber	303	0.53	0.45	ND–1.74	1.37	0.98	ND–3.78	30.7	8.3–77.3
Teflon	79	0.56	0.33	0.08–1.38	1.55	0.80	0.40–3.69	13.2	5.7–28.4
Work environment									
Pig farm	196	0.82	0.40	ND–1.74	2.15	0.67	ND–3.78	17.0	10.9–37.0
Grass seed processing plant	186	0.24	0.22	ND–0.96	0.62	0.45	ND–1.89	48.4	19.9–76.2

[a] Abbreviations: AM, arithmetic mean; Min–max, minimum to maximum value; ND, below limit of detection.

0.064 EU/ml depending on the particular assay run. Duplicate analyses took place in the same week.

All sample extracts were analyzed with 0.05% Tween 20 in the assay solution (PFW). In addition, a randomly chosen subset of the samples was also analyzed in PFW without Tween 20 at the same dilutions.

Statistical analysis. The variation in dust levels within a sampling run and between sampling runs was investigated by means of descriptive statistics (SAS version 8e; SAS Institute, Cary, NC). Concentrations of endotoxin (EU/m^3 and EU/mg dust) fitted a log-normal distribution; therefore, data were log transformed prior to analyses. Descriptive statistics (geometric mean, geometric standard deviation, and range) of endotoxin concentrations were calculated for every combination of factors of interest. The influence of and interaction between the different variables were determined by applying mixed-effects models with run as a random factor in order to correct for possible correlation between measurements in the same run. Assuming that two repeated measurements of the same run have equal correlation (a compound symmetric covariance structure), between- and within-run components of variance were estimated by using a restricted maximum likelihood method. Determinants influencing endotoxin concentration were explored by introducing them as fixed effects (25, 27). Separate models were constructed for endotoxin and endotoxin per mg dust exposure.

Finally, the influence of the measured dust concentration on the filters and thus on the homogeneity of the samples was evaluated by adding the log-normally transformed dust concentration to the various mixed-effects models as a fixed effect.

RESULTS

Overview of samples. Of the 400 samples collected in this experiment, 18 dust and 14 endotoxin samples were compromised during weighing, extraction, or analysis, leaving 382 dust and 386 endotoxin samples for statistical analysis. Of these samples, 37 were below the LOD (0.05 mg) for dust weight. All samples were detectable for endotoxin. The lost samples originated from different runs and different combinations of variables (Table 1). The mean endotoxin level on the field filter blanks ($n = 24$) was 0.78 EU/ml (range, 0.06 to 3.83). Since the minimum of the samples was 9 EU/ml, contamination during assembling of the sampling heads and parallel samplers was unlikely. A random subset of the endotoxin samples ($n = 56$) was analyzed in duplicate, which resulted in an average CV% of 21.5 (range, 0.2 to 71.3). Endotoxin levels per combination of variables are summarized in Table 1. The geometric mean concentration varied from 1,000 to 3,200 EU/m^3 and showed relatively little variation in endotoxin levels per combination (geometric standard deviation range, 1.6 to 2.0).

Uniformity of parallel samples. Dust levels were generally higher at the pig farm than at the grass seed plant. Teflon filters yielded slightly higher dust levels than glass-fiber filters did (Table 2). The overall difference in measured dust levels (maximum/minimum ratio) within a sampling run was on average a factor of 5 and a factor of 3 and 6 for sampling runs at the pig farm and the grass seed plant, respectively (data not shown).

The uniformity in the samples collected by the parallel sampling was investigated further by calculating the CV% between the replicate samples within a sampling run. The overall CV%, reflecting the sampling and analytical error, of dust levels in 20 parallel samples ranged from 11 to 76 (Table 2) and showed a decline with increasing dust levels. This variability is most likely caused by measurement error.

Influences of transport conditions, storage conditions before and after extraction, extraction solution, and filter type. In Table 3 the effect estimates of all possible combinations of variables for the samples collected on glass-fiber filters ($n = 320$) relative to the CEN protocol (desiccant, samples in freezer, extraction using PFW, extracts in freezer) are presented for both airborne endotoxin concentration (EU/m^3) and endotoxin concentration in dust (EU/mg dust). Fourteen of the 15 combinations of variables resulted in a higher exposure level than did the reference combination with ratios ranging from 1.2 to 2.5, although they were not all statistically significant. Combination 5 (desiccant, storage filter at −20°C, extraction in PFW with 0.05% Tween 20, and storage extracts at 4°C) resulted in the highest endotoxin concentration levels (for both EU/m^3 and EU/mg dust). Generally, the combinations containing extraction in PFW with the addition of Tween 20 to the solution resulted in significantly higher concentrations. The within-run variability for the endotoxin concentration (0.08) and endotoxin in dust concentration (0.23) was smaller than the between-run variability (0.24 and 0.88 for EU/m^3 and EU/mg dust, respectively). Because the estimates of endotoxin and endotoxin per mg dust were in agreement with each other, further analyses in this part of the data set focused only on airborne endotoxin concentrations. The total variability was higher in endotoxin concentrations in dust than airborne endotoxin concentrations, which is largely due to the measurement error that occurs in sampling dust.

In Fig. 3 the factors relative to the CEN reference are

TABLE 3. Relative effects and 95% confidence intervals of changes in procedures (combinations of variables) compared to the CEN protocol on endotoxin concentration

Combination	Description[a]	Endotoxin concn[b]			
		EU/m³[c]		EU/mg dust[d]	
		e^{β}	95% CI	e^{β}	95% CI
Intercept		1,334*	1,024–1,738	1,389*	847–2,276
1	D-R-WT-R	2.02*	1.67–2.44	1.92*	1.40–2.65
2	D-R-W-R	1.10	0.92–1.31	1.17	0.86–1.59
3	D-R-WT-F	2.13*	1.78–2.55	2.00*	1.47–2.73
4	D-R-W-F	1.07	0.89–1.29	1.23#	0.91–1.68
5	D-F-WT-R	2.51*	2.10–3.01	2.59*	1.91–3.52
6	D-F-W-R	1.16#	0.97–1.39	1.31**	0.97–1.78
7	D-F-WT-F	2.50*	2.09–3.01	2.45*	1.80–3.34
8	D-F-W-F	Ref		Ref	
9	nD-R-WT-R	2.10*	1.75–2.51	2.07*	1.53–2.81
10	nD-R-W-R	1.12#	0.94–1.34	1.31**	0.97–1.77
11	nD-R-WT-F	2.29*	1.92–2.74	2.55*	1.89–3.44
12	nD-R-W-F	0.93	0.78–1.11	1.03	0.76–1.39
13	nD-F-WT-R	2.23*	1.86–2.66	2.45*	1.81–3.32
14	nD-F-W-R	1.16**	0.97–1.39	1.23#	0.91–1.67
15	nD-F-WT-F	2.39*	2.00–2.86	2.30*	1.70–3.12
16	nD-F-W-F	1.18**	0.99–1.41	1.27#	0.94–1.71

[a] D, desiccant during transport; nD, no desiccant during transport; R, refrigerator; F, freezer; WT, PFW with 0.05% Tween 20; W, PFW alone.
[b] Symbols and abbreviations: *, $P < 0.05$; **, $0.05 < P < 0.10$; #, $0.10 < P < 0.20$; Ref, reference category (=1.0); e^{β}, relative effect; 95% CI, 95% confidence interval.
[c] Between-run variability, 0.2375; within-run variability, 0.0810.
[d] Between-run variability, 0.8752; within-run variability, 0.2260.

shown, for both Teflon and glass-fiber filters (whole data set). Extraction of Teflon filters with PFW resulted in lower endotoxin levels than when Tween 20 was included, similar to what was observed for glass-fiber filters.

Next, the individual effects of the four investigated parameters were studied by applying them as fixed effects in a mixed-effects model for the glass-fiber filters, both overall and stratified for the kind of dust (Table 4). Addition of Tween 20 in the extraction solution was the only parameter resulting in significantly higher airborne endotoxin concentrations. Transport conditions and storage of extracts did not have any major impact on endotoxin concentration, although storage of the

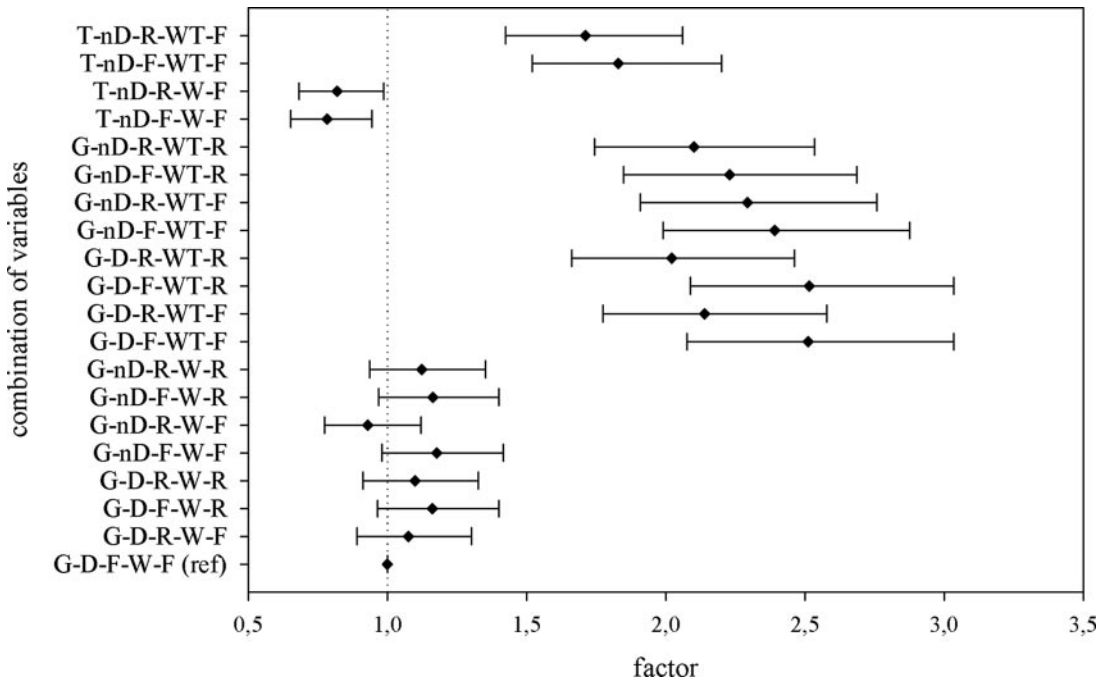

FIG. 3. The factor of influence and 95% confidence interval for the effect on endotoxin exposure levels in EU/m³ per combination of variables changed compared to the CEN protocol as a reference. T, Teflon filter; G, glass-fiber filter; nD, no desiccant during transport; D, desiccant during transport; R, refrigerator; F, freezer; WT, PFW with 0.05% Tween 20; W, PFW alone.

TABLE 4. Relative effects and 95% confidence intervals of changes in transport dehumidifier, sample storage before extraction, extraction solution, extract storage before analysis, and work environment on endotoxin concentration, overall and stratified for work environment[a]

Model and description	Endotoxin concn (EU/m^3)[b]					
	Overall (n = 306)[c]		Pig farm (n = 158)[d]		Grass seed plant (n = 148)[e]	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
Intercept	1,487*	1,054–2,097	1,503*	1,120–2,017	1,492*	959–2,320
Transport conditions						
No desiccant	1.00	0.94–1.07	0.97	0.89–1.07	1.03	0.95–1.12
Desiccant	Ref					
Storage before extraction						
Refrigerator (4°C)	0.91*	0.86–0.98	0.85*	0.77–0.94	0.99	0.91–1.08
Freezer (−20°C)	Ref					
Extraction solution						
Water-Tween	2.09*	1.96–2.23	2.35*	2.14–2.58	1.84*	1.70–2.00
Water	Ref					
Storage before analysis						
Refrigerator (4°C)	1.02	0.96–1.09	1.01	0.92–1.12	1.03	0.95–1.12
Freezer (−20°C)	Ref					
Work environment						
Pig farm	1.01	0.65–1.58				
Grass seed plant	Ref					

[a] Only results for glass-fiber filters are given. Abbreviations: e^{β}, relative effect; 95% CI, 95% confidence interval; Ref, reference category (=1.0).
[b] *, $P < 0.05$.
[c] Between-run variability, 0.2515; within-run variability, 0.0819.
[d] Between-run variability, 0.1407; within-run variability, 0.0904.
[e] Between-run variability, 0.3595; within-run variability, 0.0630.

filters at 4°C seemed to slightly lower the endotoxin concentration. However, when the data were stratified for work environment, this effect was seen only in pig farm samples. No significant interactions between the parameters were found (data not shown).

In a subset of the data (n = 160) with comparable combinations for glass-fiber and Teflon filters, the influence and interactions of filter type, storage of samples, and extraction solution were explored. The combination of variables most similar to the CEN protocol, apart from desiccant use during transport, was used as the reference category (combination 16). Of the seven possible combinations of parameters, three resulted in significantly lower airborne endotoxin concentrations and two in significantly lower endotoxin concentrations in dust relative to the reference category, of which most combinations were samples collected on Teflon filters (Table 5). For both glass fiber and Teflon filters, use of Tween 20 in the extraction solution resulted in the highest airborne endotoxin concentrations and endotoxin concentrations in dust. For Teflon filters, these levels approximated the reference values.

TABLE 5. Relative effects and 95% confidence intervals of changes in procedures (combinations of variables) on endotoxin concentration compared to a reference close to the CEN protocol in a subset of the data set (glass-fiber and Teflon filters with corresponding combinations of variables [n = 160])

Combination	Description[a]	Endotoxin concn[b]			
		EU/m^3[c]		EU/mg dust[d]	
		e^{β}	95% CI	e^{β}	95% CI
Intercept		1,572*	1,205–2,050	1,757*	1,133–2,723
11	G-nD-R-WT-F	1.95*	1.60–2.37	2.01*	1.53–2.65
12	G-nD-R-W-F	0.79*	0.65–0.96	0.81#	0.62–1.07
15	G-nD-F-WT-F	2.03*	1.67–2.47	1.84*	1.39–2.44
16	G-nD-F-W-F	Ref		Ref	
17	T-nD-R-WT-F	1.45*	1.20–1.77	1.05	0.80–1.38
18	T-nD-R-W-F	0.70*	0.57–0.85	0.46*	0.35–0.61
19	T-nD-F-WT-F	1.55*	1.28–1.89	1.02	0.77–1.35
20	T-nD-F-W-F	0.67*	0.55–0.81	0.43*	0.33–0.57

[a] Abbreviations: G, glass-fiber filter; T, Teflon filter; nD, no desiccant during transport; R, refrigerator; F, freezer; WT, PFW with 0.05% Tween 20; W, PFW alone.
[b] Symbols and abbreviations: *, $P < 0.05$; #, $0.10 < P < 0.20$; Ref, reference category (=1.0); e^{β}, relative effect; 95% CI, 95% confidence interval.
[c] Between-run variability, 0.2253; within-run variability, 0.0974.
[d] Between-run variability, 0.6813; within-run variability, 0.1957.

TABLE 6. Relative effects and 95% confidence intervals of change in filter type, sample storage before extraction, extraction solution, and work environment on endotoxin concentration, overall and stratified per kind of dust, for glass-fiber and Teflon filters with corresponding combinations of variables

Model and description	Endotoxin concn (EU/m^3)[a]					
	Overall (n =160)[b]		Pig farm (n = 80)[c]		Grass seed plant (n = 80)[d]	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
Intercept	1,553*	1,103–2,185	1,403*	1,011–1,949	1,508*	994–2289
Filter type						
Teflon	0.76*	0.69–0.84	0.66*	0.57–0.76	0.88*	0.79–0.98
Glass fiber	Ref		Ref		Ref	
Storage before extraction						
Refrigerator (4°C)	0.93#	0.84–1.02	0.83*	0.72–0.96	1.03	0.93–1.15
Freezer (−20°C)	Ref		Ref		Ref	
Extraction solution						
Water-Tween	2.22*	2.02–2.45	2.71*	2.35–3.12	1.82*	1.64–2.03
Water	Ref		Ref		Ref	
Work environment						
Pig farm	1.14	0.57–1.36				
Grass seed plant	Ref					

[a] Symbols and abbreviations: e^{β}, relative effect; 95% CI, 95% confidence interval; *, $P < 0.05$; #, $0.10 < P < 0.20$; Ref, reference category (=1.0).
[b] Between-run variability, 0.2338; within-run variability, 0.0975.
[c] Between-run variability, 0.1608; within-run variability, 0.1000.
[d] Between-run variability, 0.3117; within-run variability, 0.0560.

The further statistical analysis of this subset focused on the airborne endotoxin concentrations, since the results for endotoxin concentrations and endotoxin concentrations in dust pointed in the same direction. Subsequently, the individual effect of the three parameters was investigated (Table 6). In the whole subset, sampling with Teflon filters and storage of samples in the refrigerator (4°C) resulted in significantly lower endotoxin concentrations, whereas extraction in PFW with addition of Tween 20 resulted in significantly higher endotoxin concentrations. After stratification for work environment, the direction of the effects of the parameters was mostly unchanged, although the positive effect of extraction in PFW with Tween 20 and the negative effect of sampling on Teflon filters on measured endotoxin concentrations were larger in pig farm samples than in grass seed plant samples. Storage conditions of samples before extraction did not affect endotoxin concentrations in the samples from the grass seed plant, although storage of samples at 4°C significantly lowered endotoxin concentrations in pig farm samples.

The additional effect of the use of Tween 20 in the assay was studied in a random subset of glass-fiber filters for which the extract was processed both with and without addition of Tween 20 in the assay (136 samples). The samples in this subset were sufficiently distributed over all sampling runs to be representative. Mixed-effects models with the individual effects of transport conditions, storage before extraction, extraction solution, and storage before analysis, and with the addition of the parameter assay solution, were formulated (Table 7). In the model without the parameter assay solution, only extraction in PFW with Tween 20 resulted in a significantly higher endotoxin concentration. The other parameters did not have a significant effect. Analyzing extracts when Tween 20 was added to the assay resulted in a 1.5-fold-higher endotoxin concentration.

TABLE 7. Relative effects and 95% confidence intervals on endotoxin concentration of change in transport conditions, sample storage before extraction, extraction solution, and extract storage before analysis in a model with and without assay solution included, for a random subset of glass-fiber filters (136 samples analyzed both with and without Tween 20 in the assay solution)

Model and description	Endotoxin concn (EU/m^3) for model[a]:			
	Without assay solution[b]		With assay solution[c]	
	e^{β}	95% CI	e^{β}	95% CI
Intercept	1,102*	863–1,408	907*	712–1,155
Transport conditions				
No desiccant	0.99	0.90–1.09	0.99	0.91–1.07
Desiccant	Ref		Ref	
Storage before extraction				
Refrigerator (4°C)	0.95	0.86–1.04	0.95#	0.87–1.03
Freezer (−20°C)	Ref		Ref	
Extraction solution				
Water-Tween	2.55*	2.31–2.80	2.55*	2.35–2.76
Water	Ref		Ref	
Storage before analysis				
Refrigerator (4°C)	1.03	0.93–1.13	1.03	0.95–1.11
Freezer (−20°C)	Ref		Ref	
Assay solution				
Water-Tween			1.48*	1.37–1.60
Water			Ref	

[a] Symbols and abbreviations: e^{β}, relative effect; 95% CI, 95% confidence interval; *, $P < 0.05$; #, $0.10 < P < 0.20$; Ref, reference category (=1.0).
[b] Between-run variability, 0.2114; within-run variability, 0.1522.
[c] Between-run variability, 0.2142; within-run variability, 0.1107.

Addition of the parameter assay solution did not change the effects of the above-mentioned parameters. Adding Tween 20 to the assay solution likely affects only the measured endotoxin concentration and does not interfere with upstream parameters.

Determinants of within- and between-run variability. Inclusion of either a fixed-effects variable representing the different treatments or a combination of fixed-effects variables for filter type, transport conditions, storage conditions of samples and extracts, extraction conditions, and assay solution explained much of the within-run variability and almost no between-run variability, as was expected. Adding the dust concentration to the different models had no or very little effect on both the within- and between-run variability, suggesting good uniformity of samples. Furthermore, estimates for the different parameters did not change after adding dust concentration to the models (data not shown).

DISCUSSION

In the field of endotoxin exposure assessment almost every institute has its own sampling and analysis protocol. Various filter types, extraction and assay solutions, transport conditions, and storage temperatures are used. In Europe, CEN has formulated guidelines for the assessment of airborne endotoxin to standardize exposure assessment. However, these guidelines leave room for individual interpretation and thus varying methodology. This study investigated the influence of various factors using an experimental design to cover all combinations of factors and their possible interactions. Of the five initial variables in our experiment (four primary parameters and filter type), extraction solution influenced the airborne endotoxin concentration the most. Addition of Tween 20 to PFW yielded significantly more endotoxin and thus resulted in an improvement of the extraction efficiency, which has been found before (5). This may be caused by disruption of hydrophobic interactions between endotoxin and filter material or by disaggregating of endotoxin micelles or dissociation of cell-wall-bound endotoxin (4, 5). Transport conditions, in this case use of a desiccant or not, did not influence the measured endotoxin concentration, suggesting that further microbial growth did not occur during transport. Storage of extracts by different temperatures (−20 or 4°C) did not influence the estimated endotoxin concentration. Storage of filters in a freezer yielded about 10% higher estimated airborne endotoxin than did storage in a refrigerator. Freeze-thaw cycling of bacteria is known to lyse bacteria, and therefore more lipopolysaccharide (LPS) may be available in the assay after extraction (2, 36). There was no significant effect of storage temperature for the extracts, perhaps because these had been centrifuged and there were no bacterial cells to lyse. Sampling on glass-fiber filters results in a higher endotoxin concentration than does sampling on Teflon filters, as previously reported (5, 12, 15, 31, 35). Interaction between filter type and assay methodology has been reported (35), as well as inactivation of LPS in solution by a variety of filter media (20). It is suggested that in the latter study the LPS was adsorbed to the surfaces of the filter material and thus not available to the *Limulus* amoebocyte lysate reagent and that the extraction procedure was not sufficient (30).

In addition to the initial five factors under investigation, the influence of assay solution was also investigated in a subset of the data. Use of Tween 20 in the assay yielded a higher airborne endotoxin concentration than did the use of only PFW. This seemed to be an additive effect and did not depend on the extraction solution used (no interactions).

The type of dust sampled had a clear but small effect on the effect estimates of the different variables that were studied. The influence on extraction efficiency of adding Tween 20 to the extraction solution was higher in samples from the pig farm than in grass seed plant samples. Also, the "freezing" effect (higher endotoxin concentration when the sampled filters were stored in the freezer than when they were stored in a refrigerator) occurred only in the pig farm samples. Furthermore, the factor for sampling on glass-fiber filters compared to Teflon filters was higher for pig farm samples than for grass seed plant samples. Gordon et al. found that the endotoxin extraction efficiency of different filter types was dependent on the aerosol type (12). Confirmation of these observations in other environments might be needed. However, the work environments included in this experiment are representative for different types of endotoxin exposure, namely, those originating from animal and from plant material. Since the estimates of the variables studied do not vary much for the different types of dust and the directions of the models remain approximately the same, these results are thought to represent the general underlying effects of the studied variables on the measured endotoxin concentration.

Several studies showed differences between laboratories when endotoxin samples were analyzed (3, 17, 28). One of these showed that the generally high variations between laboratories were reduced by using a common extraction protocol and endotoxin assay kit, although differences remained (3). When further limitations were dictated, interlaboratory differences became even smaller (17), suggesting that differences in endotoxin exposure estimation are caused mostly by procedural differences. Further standardization (training, use of identical equipment, tubes, etc.) may thus lead to comparable interlaboratory analysis of samples.

This experiment is a vigorous attempt to come to an optimized protocol for airborne endotoxin measurements in occupational settings, which may be expanded to other settings. The experiment was designed to look at key variables and their interactions established a priori as opposed to consideration of one or a few variables at a time, as was done in most previous studies (6, 11–15, 19–24, 31, 33, 35, 38). Furthermore, some of these studies used commercial LPS (5, 15, 19, 20, 23) or house dust (5, 11, 13, 14, 24, 34) instead of rather homogeneous parallel occupational dust samples for (part of) their research, which reduces the applicability of their results for work environments. Nevertheless, to a large extent this full design appeared to confirm and extend earlier findings.

Several gaps in the CEN protocol (10) have been evaluated. With full knowledge of assay parameters that have an effect on the exposure estimate, one can clearly specify these in an agreed-upon international protocol. A fully standardized international protocol would support the establishment of an occupational exposure limit for endotoxin. Based on the outcomes of this experiment and earlier research, the following procedural steps are preferred: inhalable dust sampling on glass-

fiber filters, transport with desiccant, storage of samples at −20°C, extraction in PFW with 0.05% Tween 20 and rocking/shaking during the procedure for maximal extraction efficiency, storage of extracts at −20°C, and analysis using PFW without Tween 20 in the assay solution. No evidence was found that transport with or without desiccant and storage of extracts in a refrigerator or a freezer results in different endotoxin concentrations. However, it is preferred that possible growth of biological material be prevented by use of desiccant and storage in a freezer. Although the exact effect of repeated freezing and thawing has not been established (5, 23), storage of extracts in multiple aliquots is strongly encouraged. The 25% decline in endotoxin activity in house dust extracts after one freeze-thaw cycle that has been found elsewhere did not occur in this experiment (5). In this study all extracts were analyzed within 24 h after extraction, and thus no statements can be made about the influence of long-term storage. However, other studies have shown that long-term storage of extracts did not affect the endotoxin concentration (5, 11, 22). One study found higher endotoxin concentrations in extracts stored at 4°C than in extracts stored at −20°C for up to 20 to 30 days and in samples stored at 4°C with immediate extraction after sampling than in samples stored without extraction (15). This was possibly due to the growth of gram-negative bacteria during storage.

Douwes et al. previously showed that the endotoxin extraction efficiency of PFW with 0.05% Tween 20 was seven times higher than that of only PFW and that 0.05% Tween 20 in the assay solution did not influence the slope of the standard curve (5). We also saw an increased extraction efficiency from the addition of Tween 20, although the effect was lower. Wouters et al. found that addition of 0.05% Tween 20 to the assay mixture suppressed the assay reactivity but did not alter the slope of the standard curve (I. M. Wouters, S. Spaan, D. Heederik, and G. Doekes, data presented at the International Conference of the American Thoracic Society, 2007). The assay reactivity was affected to a larger extent for the standard curve than for the samples for at least some of the dust types (Wouters et al., American Thoracic Society), which might explain the smaller effect of the addition of Tween 20 during extraction in the current study. Therefore, it is concluded that Tween 20 enhances the extraction efficiency but should not be used during analysis because of possible interference with the assay.

This study investigated the effect of procedural changes on the endotoxin concentration in a full design including the interactions. The distributions of treatments over the samples did not introduce bias nor influence the outcome, since the 20 different combinations of treatments were randomly assigned to the 20 places in the parallel samplers and, thus, to the 20 filters available per run, using a randomizing feature in SAS software. Furthermore, the dust measurements were performed with parallel samplers to obtain a reasonably homogeneous set of samples per sampling run. Two parallel samplers were used within one sampling run in order to obtain enough samples for the design of our experiment. The results did not change when the influence of the sampling devices was investigated, suggesting that the samples were uniform.

Contrary to expectations, not all sampling runs yielded high dust concentrations. At low dust concentrations the precision of the method for dust measurement is lower. This is shown by a decline in the coefficient of variation, representing the sampling and analytical error, with increasing dust levels. However, the within-run variability was almost completely explained by the variables that we investigated, and the measured dust concentration had little effect on the within-run variability. We concluded that parallel sampling is a suitable method for collecting homogeneous samples in a manner that is comparable with personal dust sampling.

Conclusion. This study with a rigorous experimental design has investigated a large part of the gaps present in the CEN protocol for endotoxin exposure assessment and thus moved us forward toward establishing a standardized protocol for the measurement of endotoxin exposure in the work environment. Based on this study we advise that a new protocol should prescribe use of glass-fiber filters, transport with desiccation, frozen sample storage, extraction in PFW with 0.05% Tween 20 with rocking/shaking, frozen storage of extracts, and analysis in PWF.

ACKNOWLEDGMENTS

We acknowledge the following individuals for their contribution to this work: the company owners who cooperated and made this study possible, Isabella Oosting-van Schothorst and Karel van der Heijden for conducting the fieldwork, and Hans Jongsma for his analytical assistance.

This research was supported by the Ministry of Social Affairs and Employment, The Netherlands, by means of the long-term research program on biological agents in the work environment of TNO Quality of Life.

REFERENCES

1. **Bogdanovic, J., I. M. Wouters, I. Sander, E. Zahradnik, H. R. Joanne, M. J. Rodrigo, S. Gomez-Olles, D. J. Heederik, and G. Doekes.** 2006. Airborne exposure to wheat allergens: optimised elution for airborne dust samples. J. Environ. Monit. **8:**1043–1048.
2. **Chen, R. R.** 2007. Permeability issues in whole-cell bioprocesses and cellular membrane engineering. Appl. Microbiol. Biotechnol. **74:**730–738.
3. **Chun, D. T., K. Bartlett, T. Gordon, R. R. Jacobs, B. M. Larsson, L. Larsson, D. M. Lewis, J. Liesivuori, O. Michel, D. K. Milton, R. Rylander, P. S. Thorne, E. M. White, M. E. Brown, V. S. Gunn, and H. Wurtz.** 2006. History and results of the two inter-laboratory round robin endotoxin assay studies on cotton dust. Am. J. Ind. Med. **49:**301–306.
4. **Cooper, J. F.** 1990. Resolving LAL test interferences. J. Parenter. Sci. Technol. **44:**13–15.
5. **Douwes, J., P. Versloot, A. Hollander, D. Heederik, and G. Doekes.** 1995. Influence of various dust sampling and extraction methods on the measurement of airborne endotoxin. Appl. Environ. Microbiol. **61:**1763–1769.
6. **Duchaine, C., P. S. Thorne, A. Meriaux, Y. Grimard, P. Whitten, and Y. Cormier.** 2001. Comparison of endotoxin exposure assessment by bioaerosol impinger and filter-sampling methods. Appl. Environ. Microbiol. **67:**2775–2780.
7. **Dutch Expert Committee on Occupational Standards.** 1998. Endotoxins: health-based recommended occupational exposure limit. Health Council of The Netherlands, The Hague, The Netherlands.
8. **Eduard, W., G. Blomquist, B. H. Nielsen, and K. K. Heldal.** 2001. Recognition errors in the quantification of micro-organisms by fluorescence microscopy. Ann. Occup. Hyg. **45:**493–498.
9. **European Committee for Standardization.** 1998. 13098: workplace atmosphere—guidelines for measurement of airborne micro-organisms and endotoxin. European Committee for Standardization, Brussels, Belgium.
10. **European Committee for Standardization.** 2000. 14031: workplace atmosphere—determination of airborne endotoxin. European Committee for Standardization, Brussels, Belgium.
11. **Fahlbusch, B., A. Koch, J. Douwes, W. Bischof, U. Gehring, K. Richter, H. E. Wichmann, and J. Heinrich.** 2003. The effect of storage on allergen and microbial agent levels in frozen house dust. Allergy **58:**150–153.
12. **Gordon, T., K. Galdanes, and L. Brosseau.** 1992. Comparison of sampling media of endotoxin-contaminated aerosols. Appl. Occup. Environ. Hyg. **7:**472–477.
13. **Heinrich, J., B. Holscher, J. Douwes, K. Richter, A. Koch, W. Bischof, B. Fahlbusch, R. W. Kinne, and H. E. Wichmann.** 2003. Reproducibility of

allergen, endotoxin and fungi measurements in the indoor environment. J. Exposure Anal. Environ. Epidemiol. **13:**152–160.
14. **Hyvarinen, A., M. Roponen, P. Tiittanen, S. Laitinen, A. Nevalainen, and J. Pekkanen.** 2006. Dust sampling methods for endotoxin—an essential, but underestimated issue. Indoor Air **16:**20–27.
15. **Laitinen, S. K.** 1999. Importance of sampling, extraction and preservation for the quantitation of biologically active endotoxin. Ann. Agric. Environ. Med. **6:**33–38.
16. **Liebers, V., T. Bruning, and M. Raulf-Heimsoth.** 2006. Occupational endotoxin-exposure and possible health effects on humans. Am. J. Ind. Med. **49:**474–491.
17. **Linsel, G., C. Doering, S. Duggal, J. Hartung, A. Koch, A. Kottmair, M. Lohmeyer, R. Schierl, K. Weist, and B. A. Zucker.** 2002. Ergebnisse eines Ringversuches zur Messung luftgetragener Endotoxine. VDI-Berichte **1656:** 329–339.
18. **Liu, A. H.** 2002. Endotoxin exposure in allergy and asthma: reconciling a paradox. J. Allergy Clin. Immunol. **109:**379–392.
19. **Milton, D. K., H. A. Feldman, D. S. Neuberg, R. J. Bruckner, and I. A. Greaves.** 1992. Environmental endotoxin measurement: the kinetic Limulus assay with resistant-parallel-line estimation. Environ. Res. **57:**212–230.
20. **Milton, D. K., R. J. Gere, H. A. Feldman, and I. A. Greaves.** 1990. Endotoxin measurement: aerosol sampling and application of a new Limulus method. Am. Ind. Hyg. Assoc. J. **51:**331–337.
21. **Milton, D. K., D. K. Johnson, and J. H. Park.** 1997. Environmental endotoxin measurement: interference and sources of variation in the Limulus assay of house dust. Am. Ind. Hyg. Assoc. J. **58:**861–867.
22. **Morgenstern, V., W. Bischof, A. Koch, and J. Heinrich.** 2006. Measurements of endotoxin on ambient loaded PM filters after long-term storage. Sci. Total Environ. **370:**574–579.
23. **Olenchock, S. A., D. M. Lewis, and J. C. Mull.** 1989. Effects of different extraction protocols on endotoxin analyses of airborne grain dusts. Scand. J. Work Environ. Health **15:**430–435.
24. **Park, J. H., B. Szponar, L. Larsson, D. R. Gold, and D. K. Milton.** 2004. Characterization of lipopolysaccharides present in settled house dust. Appl. Environ. Microbiol. **70:**262–267.
25. **Peretz, C., A. Goren, T. Smid, and H. Kromhout.** 2002. Application of mixed-effects models for exposure assessment. Ann. Occup. Hyg. **46:**69–77.
26. **Portengen, L., L. Preller, M. Tielen, G. Doekes, and D. Heederik.** 2005. Endotoxin exposure and atopic sensitization in adult pig farmers. J. Allergy Clin. Immunol. **115:**797–802.
27. **Rappaport, S. M., M. Weaver, D. Taylor, L. Kupper, and P. Susi.** 1999. Application of mixed models to assess exposures monitored by construction workers during hot processes. Ann. Occup. Hyg. **43:**457–469.
28. **Reynolds, S. J., D. K. Milton, D. Heederik, P. S. Thorne, K. J. Donham, E. A. Croteau, K. M. Kelly, J. Douwes, D. Lewis, M. Whitmer, I. Connaughton, S. Koch, P. Malmberg, B. M. Larsson, J. Deddens, A. Saraf, and L. Larsson.** 2005. Interlaboratory evaluation of endotoxin analyses in agricultural dusts—comparison of LAL assay and mass spectrometry. J. Environ. Monit. **7:**1371–1377.
29. **Reynolds, S. J., P. S. Thorne, K. J. Donham, E. A. Croteau, K. M. Kelly, D. Lewis, M. Whitmer, D. J. Heederik, J. Douwes, I. Connaughton, S. Koch, P. Malmberg, B. M. Larsson, and D. K. Milton.** 2002. Comparison of endotoxin assays using agricultural dusts. Am. Ind. Hyg. Assoc. J. **63:**430–438.
30. **Rylander, R., and R. R. Jacobs.** 1997. Endotoxins in the environment—a criteria document. Int. J. Occup. Environ. Health **3:**S1–S48.
31. **Stephenson, D. J., D. R. Lillquist, F. D. DeRosso, D. D. Greene, and G. White.** 2004. Side-by-side comparison of three sampling methods for aerosolized endotoxin in a wastewater treatment facility. J. Environ. Health **67:**16–19.
32. **ter Kuile, W. M.** 1984. Vergleichmessungen mit verschiedenen Geräten zur Bestimmung der Gesamtsaubkonzentration am Arbeitsplatz: Teil II. Staub Rhein Luft **44:**211–216.
33. **Thorne, P. S., K. H. Bartlett, J. Phipps, and K. Kulhankova.** 2003. Evaluation of five extraction protocols for quantification of endotoxin in metalworking fluid aerosol. Ann. Occup. Hyg. **47:**31–36.
34. **Thorne, P. S., K. Kulhankova, M. Yin, R. Cohn, S. J. Arbes, Jr., and D. C. Zeldin.** 2005. Endotoxin exposure is a risk factor for asthma: the national survey of endotoxin in United States housing. Am. J. Respir. Crit. Care Med. **172:**1371–1377.
35. **Thorne, P. S., S. J. Reynolds, D. K. Milton, P. D. Bloebaum, X. Zhang, P. Whitten, and L. F. Burmeister.** 1997. Field evaluation of endotoxin air sampling assay methods. Am. Ind. Hyg. Assoc. J. **58:**792–799.
36. **van Langevelde, P., K. M. Kwappenberg, P. H. Groeneveld, H. Mattie, and J. T. van Dissel.** 1998. Antibiotic-induced lipopolysaccharide (LPS) release from *Salmonella typhi*: delay between killing by ceftazidime and imipenem and release of LPS. Antimicrob. Agents Chemother. **42:**739–743.
37. **von Mutius, E., C. Braun-Fahrlander, R. Schierl, J. Riedler, S. Ehlermann, S. Maisch, M. Waser, and D. Nowak.** 2000. Exposure to endotoxin or other bacterial components might protect against the development of atopy. Clin. Exp. Allergy **30:**1230–1234.
38. **Walters, M., D. Milton, L. Larsson, and T. Ford.** 1994. Airborne environmental endotoxin: a cross-validation of sampling and analysis techniques. Appl. Environ. Microbiol. **60:**996–1005.