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Influence of Various Dust Sampling and Extraction Methods on the Measurement of Airborne Endotoxin

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The influence of various filter types and extraction conditions on the quantitation of airborne endotoxin with the *Limulus* amebocyte lysate test was studied by using airborne dusts sampled in a potato processing plant. Samples were collected with an apparatus designed to provide parallel samples. Data from the parallel-sampling experiment were statistically evaluated by using analysis of variance. In addition, the influence of storage conditions on the detectable endotoxin concentration was investigated by using commercially available lipopolysaccharides (LPS) and endotoxin-containing house dust extracts. The endotoxin extraction efficiency of 0.05% Tween 20 in pyrogen-free water was seven times higher than that of pyrogen-free water only. Two-times-greater amounts of endotoxin were extracted from glass fiber, Teflon, and polycarbonate filters than from cellulose ester filters. The temperature and shaking intensity during extraction were not related to the extraction efficiency. Repeated freeze (−20°C)-and-thaw cycles with commercial LPS reconstituted in pyrogen-free water had a dramatic effect on the detectable endotoxin level. A 25% loss in endotoxin activity per freeze-thaw cycle was observed. Storage of LPS samples for a period of 1 year at 7°C had no effect on the endotoxin level. House dust extracts showed a decrease of about 20% in the endotoxin level after they had been frozen and thawed for a second time. The use of different container materials (borosilicate glass, "soft" glass, and polypropylene) did not result in different endotoxin levels. This study indicates that the assessment of endotoxin exposure may differ considerably between groups when different sampling, extraction, and storage procedures are employed.

Because of their ubiquitous nature, bacterial endotoxins are commonly found in various environments. Several epidemiological and experimental studies have focused on the health effects of airborne-endotoxin exposure in both occupational and nonoccupational environments. Endotoxins are believed to play an important role in the development of organic-dust-related diseases in exposed workers. Exposure to airborne endotoxins can cause acute fever and lung function alterations accompanied by respiratory complaints such as chest tightness, cough, shortness of breath, and wheezing (1, 2, 17, 20). Chronic endotoxin exposure may lead to chronically decreased pulmonary function, byssinosis, and chronic bronchitis in workers exposed to cotton dust and grain dust (10, 11, 22).

Discrepancies in the dose-response relationships between endotoxin exposure and health effects observed by different groups may partly be explained by the use of different techniques to measure environmental endotoxin and of different sampling media, extraction methods, and storage conditions. In most studies the *Limulus* amebocyte lysate (LAL) assay is commonly used to quantify environmental endotoxin. Few comparative studies using different analytical methods and different LAL assays for endotoxin analysis have been performed. Variable results were found in comparisons of a gas chromatography-mass spectrometry method with a LAL assay (23, 25). A recent study comparing two LAL methods showed variable results (19). While in general there appeared to be agreement between the two LAL assays, some discrepancies were found in some specific environments.

In regard to sampling media, extraction media and methods, and storage conditions for endotoxin, there are no generally accepted procedures, and thus different practices continue to exist. Few studies on the optimization of filter choice, filter extraction methods, extraction buffers, and choice of glassware have been published (5, 14, 15, 16). Generalization from these studies is difficult, since the interaction of endotoxin with the above-mentioned variables often depends on the aerosol type. Thus, analytical procedures as well as sampling, extraction, and storage methods need further validation in order to establish a standard protocol for measuring airborne endotoxin, which is necessary in order to obtain results that are comparable between studies.

This study was conducted to determine the influence of various sampling media and different extraction and storage conditions on the endotoxin analysis of airborne dusts and commercially available lipopolysaccharide (LPS). For this purpose a large number of airborne dust samples from a potato processing plant were collected and analyzed for their endotoxin contents. These data were statistically evaluated to compare a number of different extraction and sampling protocols. The influence of storage conditions on the detectable endotoxin concentration was studied by using commercially available LPS and endotoxin-containing house dust extracts.

MATERIALS AND METHODS

Parallel samples. (i) Air sampling. Sampling of inhalable dust was carried out in a fiber dehydration department of a potato processing plant. A previous study showed that the personal endotoxin exposure in this department was relatively high (24.5 to 489 ng/m^3) (8).

Series of 16 parallel samples were collected by using four 25-mm filter types

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commonly used in endotoxin exposure studies (7, 13, 19, 22): glass fiber (Whatman GF/A), Teflon (Millipore; pore size, 1 μm), polycarbonate (Millipore; pore size, 1.2 μm), and cellulose mixed ester (Millipore; pore size, 1.2 μm). Sample collection was performed with PAS-6 filter holders at a flow rate of approximately 2 liters/min (24). The filter holders were mounted in an apparatus (Fig. 1), described by Eduard et al. (4), that was designed to provide parallel samples. The parallel-sampling apparatus was constructed by the technical service of the Agricultural University Wageningen. A vacuum pump was used to provide a sufficient airflow through the filters. The airflow through each filter was controlled by critical orifices and was measured before and after the sampling period. The sampling time was 8 h.

The experiment was set up by using an incomplete factorial design (12) in which 32 possible combinations of sampling and extraction variables were investigated. Table 1 shows the sample and extraction variables studied. Four filters per type were randomly allocated to the 16 available places in the parallel-sampling device. The samples were then equally distributed per filter type over a preselected combination of extraction procedures in such a way that each different combination occurred only once. Only 16 combinations could be covered per measurement series, while 32 combinations were investigated. Thus, 12 series were run in order to get six repeated measurements per sampling-extraction combination, resulting in a total of 192 samples. Each series was sampled under similar conditions on 12 sampling days. After the sampling, the filters were stored at −20°C until analysis.

(ii) Extraction. Samples were extracted in 5 ml of either pyrogen-free water (catalog no. H1201; NPBI, Emmer-Compascuum, The Netherlands) or 0.05% (vol/vol) Tween 20 (polyoxyethylenesorbitan monolaurate; Merck Schuchardt) in pyrogen-free water. Endotoxin levels in dust extracts were compared with standards reconstituted and diluted in the same solution as the sample to establish the extraction efficiency. Samples were rocked either vigorously (level 8) or quietly (level 4) with a Gerhardt type LS-20 rocking apparatus for 1 h. The rocking procedure was performed either at room temperature or at 68°C. After extraction, the suspension was centrifuged at 1,000 × *g* for 10 min. The supernatant was stored in several small portions (for duplicate analyses) in "soft" glass culture tubes (Rofa-mavi; catalog no. 773119) at −20°C and analyzed within 1 month.

Field samples. Dust was sampled from floors of living rooms and bedrooms, from rugs (if present), and from mattresses in 147 homes as part of an epidemiological study on childhood respiratory disease. Sampling was performed according to an internationally standardized protocol (18) with vacuum cleaners (Philips Topomatic T518; 1,000 W) equipped with a special nozzle (ALK, Horsholm, Denmark) to collect dust on paper filters (589 black ribbon, 70 mm; Schleicher & Schuell). An area of 1 m^2 per floor was sampled for 2 min. The entire upper mattress surface, with an area of approximately 1.6 m^2, was vacuum cleaned for 2 min after the bedding had been removed.

The dust samples (n = 487; 288 floor, 57 rug, and 142 mattress samples) were extracted in 2.5 to 20 ml (depending on the dust weight) of 0.05% Tween 20 in pyrogen-free water (<0.2 g, 2.5 ml; 0.2 to 0.5 g, 5.0 ml; 0.5 to 1.0 g, 10.0 ml; and >1.0 g, 20.0 ml). They were rocked for 2 h (level 8) at room temperature. After the extraction procedure, the suspension was centrifuged at 1,000 × *g* for 10 min, and the supernatant was stored in soft glass culture tubes at −20°C. The endotoxin analyses were performed within 1 month after the extraction. Duplicate analyses were performed within 14 days on the same dust extracts frozen and thawed twice (compared with only once in the first analysis).

Endotoxin assay. Glassware was rendered sterile and pyrogen free by being heated to 190°C for 4 h. New glassware, pipette tips (Gilson; type Tipac), and microtiter plates (96 well, flat bottomed, sterile polystyrene; Costar 3596) were used.

Endotoxin was assayed with a quantitative kinetic chromogenic LAL method (Kinetic-QCL no. 50-650U; BioWhittaker; LAL lot no. OL1920) at 37°C with an automated microtiter plate reader. *Escherichia coli* O55:B5 endotoxin (BioWhittaker; lot no. OL1460) was used as standard endotoxin. The endotoxin potency of this standard was 17 endotoxin units (EU)/ng. The data processing part of the assay was a modification of the supplier's version described earlier by Hollander et al. (9). All samples were vortexed for 30 s prior to dilution or analysis. Data from the samples were compared with the standard curve, which ranged from 0.01 to 100 EU/ml. Parallel samples were analyzed in duplicate on different days, with one portion of each sample per analysis, and were frozen and thawed only once. The concentration was calculated as the mean of the two values and was expressed in EU/per milliliter. The house dust samples were analyzed only once per freeze-thaw cycle. Parallel and house dust samples did not show inhibition or enhancement of the LAL assay tested as described by Hollander et al. (9).

Storage conditions for commercial LPS. The following container materials were compared in the storage experiment: Duran borosilicate glass culture tubes (Schott; catalog no. 530K12), soft glass culture tubes (Rofa-mavi; catalog no. 773119), and polypropylene tubes (Greiner; catalog no. 121261). The polypropylene tubes were sterile and new, and testing showed that they were endotoxin free. Glassware was rendered pyrogen free before use. Commercial LPS (*E. coli* O55:B5 endotoxin; BioWhittaker; lot no. OL1460) was reconstituted and diluted in pyrogen-free water to obtain 5-, 0.5-, and 0.05-EU/ml solutions. These three endotoxin dilutions were made separately in the three preselected container materials. A total of 53 test tubes per type were filled with 250 μl of one of the three possible endotoxin dilutions, and 6 test tubes per type were filled with 2 ml

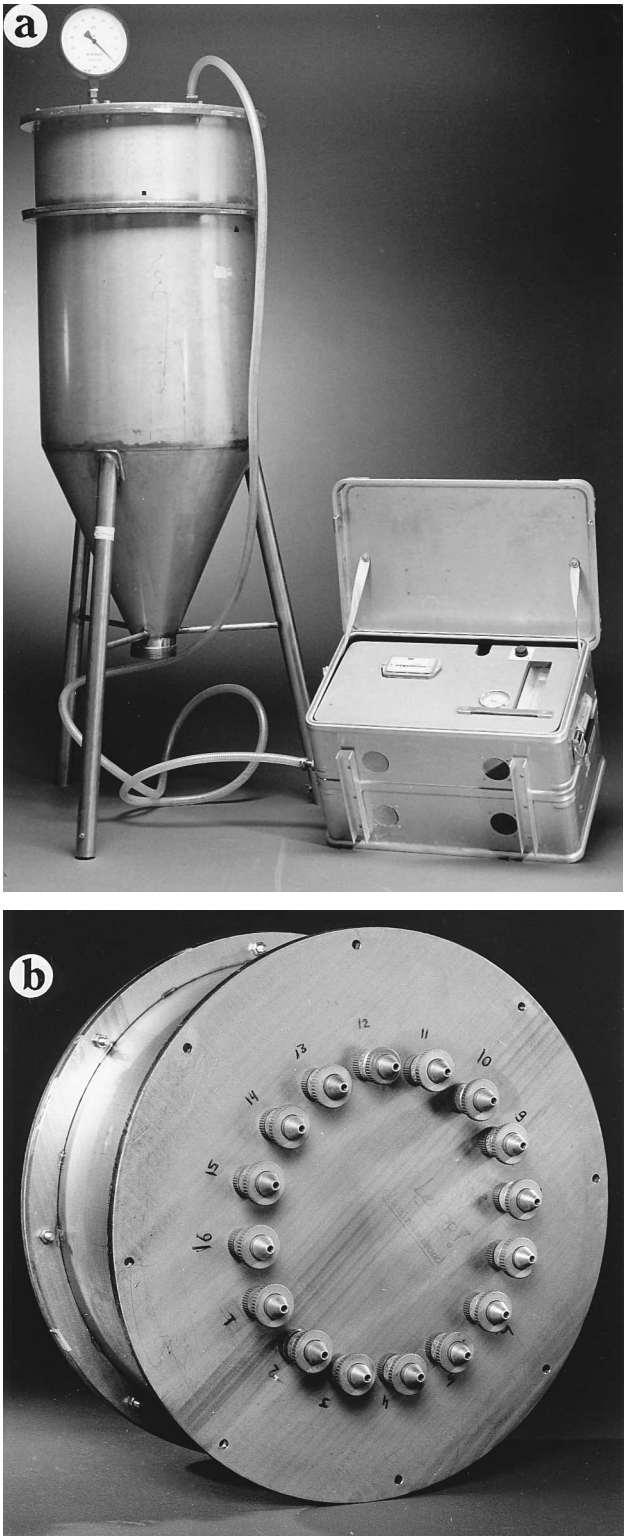

FIG. 1. (a) Apparatus and pump for collection of parallel samples (height, 170 cm; diameter, 50 cm; inlet 37 cm above floor level; inlet diameter, 10 cm). (b) Detached upper section of parallel-sampling apparatus with 16 mounted PAS-6 filter holders (height, 22 cm; diameter, 50 cm).

TABLE 1. Sample and extraction variables studied[a]

Category	Variables
Filter type	Glass fiber, Teflon, polycarbonate, cellulose mixed ester
Extraction medium	Pyrogen-free water, 0.05% Tween 20 in pyrogen-free water
Temp	Room temp, 68°C
Rocking conditions	Vigorous, quiet

[a] Combination of the sample and extraction variables studied results in 32 ($4 \cdot 2 \cdot 2 \cdot 2$) possible variations.

of one of the three endotoxin dilutions. Half of the test tubes, equally representing all other variables, were stored frozen at −20°C, while the other half were stored in the refrigerator at a temperature of 7°C. The endotoxin analyses were performed at days 0, 1, 3, 7, 14, 28, 90, 180, and 364. The test tubes filled with 2 ml were reused during the nine analyses; thus, these samples underwent eight repeated freeze-thaw cycles. The other samples (250 μl per test tube) were discarded after the endotoxin analysis. The dendrogram in Fig. 2 explains the distribution of the different test tubes over the variables used in this study.

Statistical analysis. To evaluate the efficiency of the sampling and extraction conditions for the endotoxin concentration obtained, data from the parallel-sampling experiment were statistically analyzed by an analysis of variance. Statistical analyses were carried out with SAS statistical software (21). Like most air pollution data, endotoxin measurements were characterized by a right-skewed distribution. For this reason the data were ln transformed. The analysis of variance was then performed with the normally distributed ln-transformed data by using SAS PROC GLM. The analysis was started with a full model as expressed in the following equation, which contains filter and extraction variables and all interactions between these variables: $\text{LEC} = \text{Int} + \beta_1 \cdot \text{Day} + \beta_2 \cdot \text{Filter holder} + \beta_3 \cdot F + \beta_4 \cdot E + \beta_5 \cdot T + \beta_6 \cdot R + \beta_\tau \cdot F/E/T/R$, where LEC = ln-transformed endotoxin concentration; Int = Intercept; F, E, T, and R = main effects (filter type, extraction medium, temperature, and rocking conditions, respectively); $\beta_{1,2,3,4,5,6}$ = regression coefficients for main effects, including day (of sampling) and (location of) filter holder; $F/E/T/R$ = all possible interactions between the main effects F, E, T, and R; and β_τ = regression coefficients for the interaction effects (β_7 to β_{17}). The sampling day ($\beta_1 \cdot$ Day) was brought into the model to adjust for differences in endotoxin concentration caused by daily changes in airborne endotoxin levels in the sampling area. The model also adjusted for differences in endotoxin concentrations due to variation in dust collection by the 16 filter holders mounted on fixed places inside the parallel-sampling apparatus ($\beta_2 \cdot$ Filter holder). Variables and interactions that were not statistically significant ($P > 0.1$) in the full model were omitted and were no longer considered in the subsequent statistical analysis.

Linear regression was performed with SAS PROC REG to evaluate freeze-thaw effects on both commercial LPS solutions and house dust extracts that were frozen and thawed twice compared with those subjected to only one freeze-thaw cycle.

RESULTS

Parallel samples. No significant difference between the slopes of standard curves for endotoxins from the same lot that were reconstituted and diluted in either pyrogen-free water or 0.05% Tween 20 was observed. A slight decrease (<0.2 maximum velocity units) in the intercept of the standard curve was observed with 0.05% Tween 20. Tween 20 (0.05%) did not show endotoxin contamination.

Because of analytical errors, 2 of the 192 endotoxin measurements had to be deleted from the data set. A full model, including all main and interaction effects, could explain 94% ($P < 0.01$) of the variation in ln-transformed endotoxin concentrations. More than 50% ($P < 0.01$) of the variance in endotoxin concentration was explained by the extraction medium. The filter type explained approximately 6% ($P < 0.01$), and only 2.8% of the variance was explained by statistically significant interaction effects ($P < 0.01$). The variables of temperature and rocking conditions did not significantly contribute to the explained variance in endotoxin concentration. The day of sampling explained 30.9% ($P < 0.01$), while only 0.7% (not significant) was explained by the location of the filter holder inside the apparatus. Thus, good parallel samples were obtained independent of the location of the filter holder inside the parallel-sampling device. A reduced model in which only the significant main variables of extraction medium and filter type were considered, adjusted for sampling day, was then applied. In Table 2 the antilogs of the regression coefficients (e^β) are given for each main effect; these represent differences in geometric mean levels, since ln-transformed concentrations were used in the model. Significant interaction effects ($P < 0.01$) were no longer considered in the reduced model because of their small contribution to the extraction efficiency compared with that of the main effects. It is concluded that the extraction medium and filter type are the most important variables in determining the endotoxin extraction efficiency. The addition of 0.05% Tween 20 resulted in an extraction efficiency approximately seven times better than that with pyrogen-free water only ($P < 0.01$). The use of glass fiber, Teflon, and polycarbonate filters resulted in a significant ($P < 0.01$) approximately twofold increase in detectable endotoxin compared with the use of cellulose filters. There were no significant

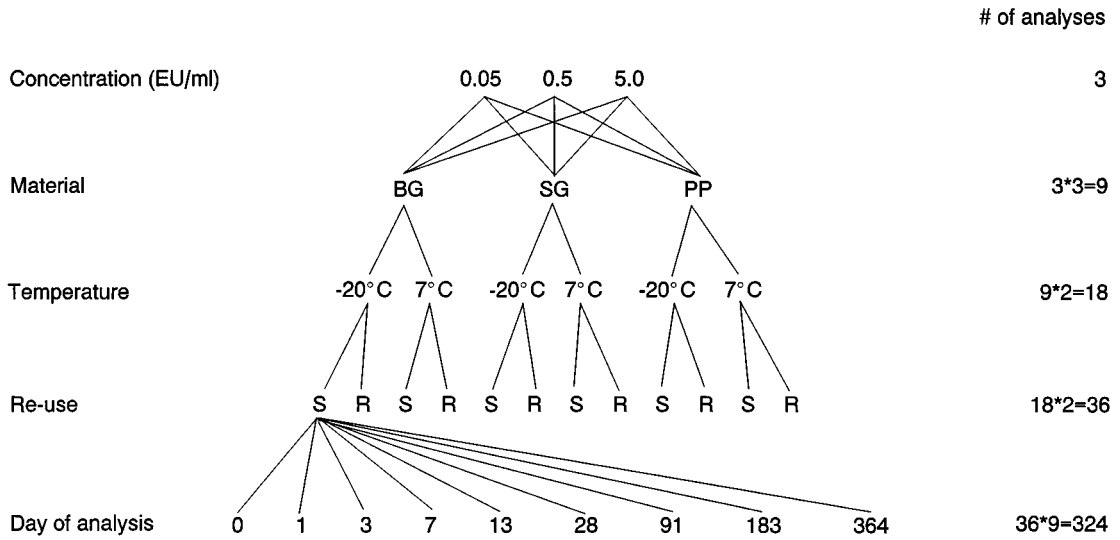

FIG. 2. Dendrogram of storage condition variables studied. BG, borosilicate glass; SG, soft glass; PP, polypropylene; S, single use; R, reused.

TABLE 2. Ratios from a reduced model with only significant main effects, corrected for sampling day[a]

Main effect	Partial r^2 (%)[b]	Condition	Ratio (e^{β})	95% confidence interval
Extraction medium	52.6[c]	0.05% Tween 20	7.24[c]	6.34–8.28
		Pyrogen-free water	1.00[d]	
Filter type	5.6[c]	Glass fiber	2.34[c]	1.94–2.82
		Teflon	2.05[c]	1.70–2.48
		Polycarbonate	1.84[c]	1.52–2.22
		Cellulose ester	1.00[d]	
Sampling day	30.9[c]			

[a] Analysis of variance: degrees of freedom for model = 15; degrees of freedom for error = 174; sum of squares for model = 315.10; sum of squares for error = 38.25; mean square for model = 21.01; mean square for error = 0.21; $F = 95.56$; $P = 0.0001$; $r^2 = 89\%$; and $n = 190$.
[b] r^2, Explained variance.
[c] $P < 0.01$.
[d] Arbitrarily chosen as a reference level.

differences in the extraction efficiencies of glass fiber, Teflon, and polycarbonate filters.

The endotoxin concentration in the fiber dehydration department where sampling took place ranged from 71.5 to 1,770 EU/m^3 (4.2 to 104.1 ng/m^3) during the 12 sampling days. This was calculated by using geometric mean concentrations ($n = 8$) determined for each sampling day and only for samples that were extracted in pyrogen-free water. A range of 681.3 to 13,279 EU/m^3 (40.1 to 781.1 ng/m^3) was calculated when endotoxin concentrations in the Tween 20 (0.05%) extracts were used.

Storage conditions. Reused samples that were repeatedly frozen (−20°C) and thawed showed a continuous decline in endotoxin concentration, up to 90% and more after eight freeze-thaw cycles. The semilogarithmic relationship between the observed endotoxin concentration and the number of freeze-thaw cycles (Fig. 3; shown only for 5 EU/ml) indicates that each freeze-thaw cycle results in a loss of endotoxin activity of approximately 25%. The regression coefficients thus estimated for the starting concentration of 5 EU/ml stored in three different container materials (borosilicate glass, soft glass, and polypropylene) differed significantly from 0 and were, respectively, −0.20 (standard error = 0.019), −0.25 (standard error = 0.018), and −0.29 (standard error 0.025). A half-life for endotoxin activity was calculated to be approximately three freeze-thaw cycles. The steady decrease in endotoxin activity after repeated freeze-thaw cycles was observed for all endotoxin concentrations and container materials used. The estimated regression coefficients for the starting concentration of 0.5 EU/ml were all statistically significant ($P < 0.05$) at −0.22, −0.21, and −0.33. The regression coefficients for the starting concentration of 0.05 EU/ml showed a similar trend but did not reach statistical significance because of detection limitations in this concentration area (<0.05 EU/ml). Single-use samples stored at −20°C did not show a significant decrease in endotoxin concentration during the year of the experiment compared with the concentration measured on day 1 (Fig. 3). An approximately 25% decrease, however, was observed for single-use samples analyzed on days 1 to 364 compared with day 0 (not ever frozen), which was in accordance with the effect observed in the reused samples after one freeze-thaw cycle. The endotoxin concentration measured in the soft glass, single-use samples of 5 EU/ml appeared to be slightly elevated (Fig. 3). This might be due to the container material, but this explanation seems improbable since this phenomenon

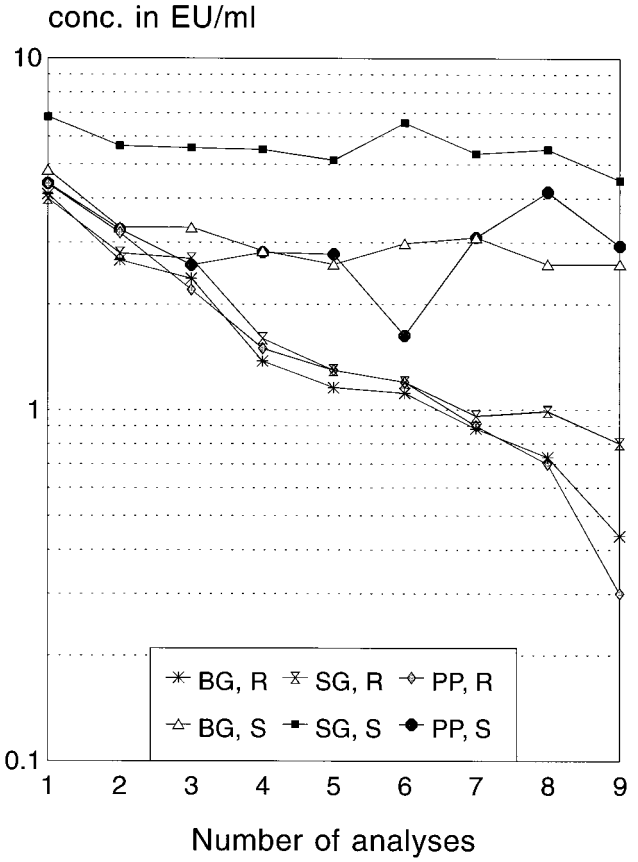

FIG. 3. Effect of repeated freeze-thaw cycles (−20°C) and duration of frozen storage (−20°C) on the recovery of commercial LPS in pyrogen-free water with a stock solution of 5 EU/ml. BG, borosilicate glass; SG, soft glass; PP, polypropylene; R, reused; S, single use.

was not observed for samples in soft glass containing 0.5 and 0.05 EU/ml. No significant deviations in endotoxin concentration were detected in both the single-use and reused samples that were stored for 1 year at 7°C (data not shown). This was the case for all container materials and endotoxin concentrations used.

There was no difference in the detectable endotoxin level between samples stored in different container materials, independent of the rest of the variables discussed above. This suggests that the container materials used did not irreversibly adsorb LPS during the year of the study.

Freeze-thaw effects were also demonstrated with endotoxin activity measured in house dust extracts. The endotoxin concentration in the house dust extracts ranged from 0 to 10,000 EU/ml. Linear regression showed a significant ($P < 0.01$) decrease in endotoxin concentration of approximately 20% when duplicate analyses of 487 samples were compared after the samples had been frozen and thawed a second time ($y = 0.77x + 107$, where x = once frozen and thawed and y = twice frozen and thawed) (Fig. 4). The correlation (r^2) between the two analyses was 84%. The omission of seven outliers of this data set (Cook's distance, >0.1) resulted in a slightly higher r^2 (0.88) and a regression coefficient and intercept of 0.83 and 48, respectively. The intercept deviates from zero, but it is relatively small compared with the range of measured endotoxin concentrations and is therefore not important in the interpretation.

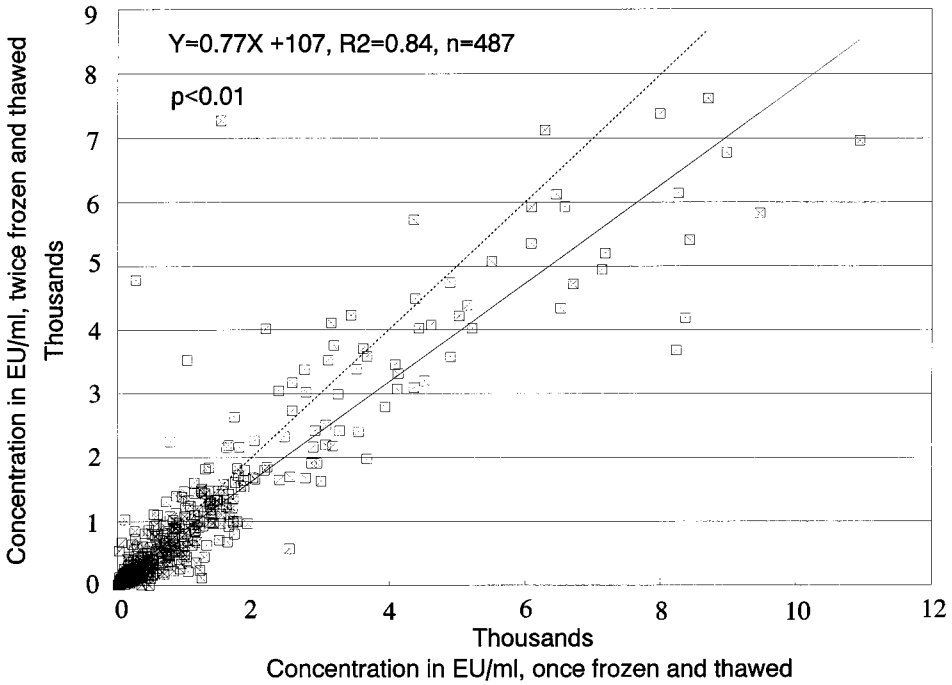

FIG. 4. Effect of freezing and thawing on the recovery of endotoxin in house dust extracts. The solid line represents the regression line $y = 0.77x + 107$. The dashed line represents the identity line $y = x$.

DISCUSSION

Several types of filter materials are commonly used for endotoxin sampling: cellulose, polyvinylchloride, glass fiber, Teflon, and polycarbonate. Using purified (no airborne) endotoxin in buffered solutions, Milton and coworkers (14) demonstrated that different filter types could inactivate LPS to different degrees, with recoveries ranging from 6 to 25%. Gordon et al. (5) examined the influence of filter type on endotoxin extraction for a variety of laboratory-generated aerosols contaminated with endotoxin. They concluded that the endotoxin extraction efficiency for different filters was dependent on the aerosol type. For extraction, no standard method exists. Most laboratories use pyrogen-free water or buffers such as Tris (26) and phosphate triethylamine (pH 7.5) (14) with or without endotoxin-dispersing agents such as Tween 20, Tween 80, Triton X-100, or saponin (16). The use of buffers and dispersing agents may be beneficial in the LAL assay in the case of deviation from the optimal pH (6.5) or increased ionic strength of the extract (3, 6). The most common method of extraction is rocking or sonication of filters in extraction medium, or a combination of both. The duration of this extraction and the temperature during the extraction may differ considerably between research groups. Olenchock and coworkers (16) demonstrated that there is a peak extractable endotoxin concentration after rocking of airborne grain dust in water for 2 h, while measured levels declined rapidly after that time. At present, however, there is still limited insight into the influence on efficiency of most of these variations in extraction procedures.

Little attention has been given to the influence of storage conditions on the yielded endotoxin level in dust extracts. Most laboratories store extracts frozen at a temperature of −20°C in either glass or plastic container materials. In experiments with grain dust extracts, the detectable endotoxin level was not significantly affected by repeated freeze (−85°C)-and-thaw procedures (16). Another study showed that the use of polypropylene may introduce recovery problems because of its capacity to irreversibly adsorb LPS (15).

In this study the addition of 0.05% Tween 20 to the regularly used extraction media resulted in a considerable improvement in the extraction efficiency. Olenchock and coworkers (16) studied the use of 1% Tween 20 in pyrogen-free water as extraction solution for endotoxins in grain dust. They demonstrated a substantial increase in the slopes of the standard curves with the use of 1% Tween 20 compared with pyrogen-free water to reconstitute and dilute the endotoxin standard. Obviously a 1% Tween 20 solution affected the kinetics of the chromogenic LAL assay used, which came from the same source and was probably essentially identical to the test used in our study. Olenchock et al. (16) were not able to show improved extraction with a 1% Tween 20 solution when sample extracts were referenced to standards reconstituted and diluted in the same Tween 20 solution. In our study the use of a 0.05% Tween 20 solution did not significantly change the slope of the standard curve. Only a slight parallel decrease in the level of the standard curve was observed, which had no consequences for the outcomes of the assays. We thus conclude that endotoxin extraction with 0.05% Tween 20 yields better results than extraction with pyrogen-free water. Disruption of hydrophobic interactions between LPS and filter material, because of the surface-active properties of Tween, is one of the possible explanations of the increased extraction efficiency. Since formation of micelles by endotoxin and cell wall-bound endotoxin lead to an underestimation of the actual endotoxin concentration (3), disaggregation of endotoxin micelles or dissociation of cell wall-bound endotoxin may be another explanation for an increased extraction efficiency.

The endotoxin extraction was significantly better for glass fiber, Teflon, and polycarbonate filters than for cellulose mixed ester filters. Apparently cellulose mixed ester irreversibly binds

more endotoxin than the other filter materials used. In this study, the glass fiber filter yielded the highest extractable endotoxin concentrations. Gordon and coworkers (5) reached the same conclusion in their study. They demonstrated, however, that the effects of different filter types and extraction media on the endotoxin analysis were strongly dependent on the matrix in which endotoxin was sampled. They concluded that differences in extraction recoveries were caused by specific interactions between endotoxin and filter type, which in turn are influenced by the sample matrix. Therefore, the results found in our study may not be able to be directly generalized to other organic dusts.

Temperature and rocking conditions did not significantly contribute to the explained variance determined by the analysis of variance. Differences in detectable endotoxin concentration, if any, due to these factors would therefore be very small and in this context would not be relevant compared with the relatively high coefficient-of-variation values (17.5% [9]) of the LAL assay itself.

Storage of commercially available LPS dissolved in pyrogen-free water at −20°C resulted in a reduction of approximately 25% in activity in the LAL test compared with nonfrozen samples. Each additional freeze-thaw cycle reduced the endotoxin activity by another 25%. The duration of storage at −20°C, up to 1 year, did not influence the endotoxin activity. This indicates that only the number of freeze-thaw cycles, rather than the duration of storage, explains the considerable loss in endotoxin activity observed during the year of the experiment. The observed 20% decrease in endotoxin content after freezing and thawing of the house dust extracts for a second time indicates that the endotoxin activity in environmental samples may also be negatively influenced as a result of freezing-and-thawing procedures. Olenchock and coworkers (16) showed that there was no influence on the endotoxin level in two water extracts of airborne grain dusts (spring wheat dust and oat dust) that were frozen (−85°C) and thawed 13 times during a 30-day period. The endotoxin concentrations used in their experiments were relatively high (50 mg of dust per m $\approx$ 100 EU/mg of dust; $\approx$ 55,000 EU/ml). The endotoxin concentration in almost 90% of our house dust extracts ranged from 0 to 2,000 EU/ml, and it was below 10,000 EU/ml in the remaining samples. It is therefore not clear from our data whether these findings can be extrapolated to higher concentrations as used in the study by Olenchock et al. Relative decreases in the endotoxin level after freezing and thawing may be negligible at higher concentrations. Figure 4, however, does not indicate any improvement in endotoxin recovery at higher concentrations. The differences in the results of the two studies may also be explained by the temperature difference itself (−20 versus −85°C). However, a good comparison of our results with those of Olenchock et al. is hampered by the fact that in their study only two samples from different origins were tested, with a relatively large day-to-day variation, ranging from 800 to 1,250 EU/mg ($\approx$40,000 to 62,500 EU/ml). The decreased endotoxin activity observed in our study after repeated freezing and thawing of aqueous endotoxin solutions could be caused by denaturation of the functional structure of endotoxin or by irreversible binding of endotoxin to the container material. The first explanation seems more likely, since no differences in effect were observed for the different container materials used.

Storage of LPS dissolved in pyrogen-free water at a temperature of 7°C did not interfere with the endotoxin concentration over a period of 1 year. Long-term storage of environmental samples at 4 to 7°C, however, should not be recommended, since microbial growth at these temperatures may affect the endotoxin content of the sample significantly. The addition of bactericides and fungicides to the extraction medium may be a good option for short-term storage of extracts at temperatures ranging from 4 to 7°C. Possible effects of these additives on the LAL assay should first be investigated, however.

None of the container materials used showed irreversible adsorption of aqueous LPS during storage for 1 year at 7°C, while the observed recovery problems at −20°C were similar for all container materials used. Thus, recovery problems seem to be associated with freezing and thawing of samples rather than with the material in which aqueous LPS is stored. Novitsky and coworkers showed that the use of polypropylene leads to a higher degree of nonrecoverable, adsorbed endotoxin than the use of other materials, such as borosilicate glass, flint glass, and polystyrene (15). Polypropylene exhibited an LPS recovery of less than 1% (15). In that study, however, the endotoxin solution was dried to apply endotoxin to the container surface by either lyophilizing or air drying, after which it was reconstituted in pyrogen-free water. In the same paper it was reported (as unpublished results) that polystyrene was found to adsorb negligible amounts of endotoxin from aqueous solutions. Thus, the use of different container materials to store aqueous LPS is not likely to influence the LPS recovery dramatically.

This study showed that different approaches to endotoxin sampling and the processing of these samples can lead to substantial differences in the assessment of endotoxin exposure. Differences of up to a factor of 17 (calculated from Table 2) between different protocols are possible (cellulose ester filter and pyrogen-free water versus glass fiber filter and 0.05% Tween 20, $7.24 \cdot 2.34 \approx 17$). This study indicates that the use of a 0.05% Tween 20 extraction solution may improve the endotoxin extraction compared with that with pyrogen-free water, without changing the kinetics of the LAL test. The use of a cellulose ester filter as the sampling medium, on the other hand, may lead to an underestimation of the actual endotoxin exposure compared with that with the other filters tested. Freezing, and especially repeated freezing and thawing, of sample extracts at −20°C may also lead to considerable endotoxin loss. Storage of dust extracts at 4 to 7°C for short periods prior to analysis with bactericides and fungicides may be a better approach.

Whether the results reported in this paper can be generalized for all airborne dust-associated endotoxin is not yet clear. Large international validation studies are necessary to make possible a valid comparison of the results obtained by different research groups.

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