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IMPORTANCE OF SAMPLING, EXTRACTION AND PRESERVATION FOR THE QUANTITATION OF BIOLOGICALLY ACTIVE ENDOTOXIN

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Abstract: The influence of filter media, extraction solution and preservation method on detection of biologically active endotoxin in the LAL assay was studied with air samples collected from wastewater treatment plants. The four most common types of filters were used as collection media. The extraction solutions compared were nonpyrogenic water, KH_2PO_4-triethylamine and Trizma buffers. The effect of preservation on endotoxin air samples was ascertained by storing both the filters without extraction, and samples extracted in the collection day for a few weeks at various temperatures. Samples collected on glass fibre filters showed the highest amounts of detectable endotoxin, while the concentrations of endotoxin were significantly lower when cellulose-mixed esters, polycarbonate or polyvinyl chloride membrane filters were used for air sampling. After collection, the best efficiency for glass fibre filters was attained by extraction with nonpyrogenic water within 8 hours after sampling and storage of the extracts at 4ºC until they were analysed. If the filters were stored without extraction, the reduction in endotoxin levels of the sample was about 30% after 1 week preservation and about 70% after 2 weeks. The study shows that the effect of the filter material and preservation practice was significant. These factors play critical roles in assessing exposure to bacterial endotoxins within wastewater aerosols.

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INTRODUCTION

High levels of inhalable endotoxins, the lipopolysaccharides of the outer cell membrane of Gram-negative bacteria, have been found in various occupational environments [10, 11]. Experimental studies on humans and animals have indicated that exposure to endotoxins injures the airways and may cause many other types of health hazards due to activation of inflammatory cells [1]. However, in epidemiological studies, it has been difficult to show any relationship between health effects and exposure to endotoxin-containing aerosols [4]. One reason for this is imprecise assessment of exposure to endotoxins due to the lack of standardized methods for monitoring of environmental endotoxins, although the European Committee for Standardization (CEN/TC 137) is formulating guidelines for measurement of airborne microorganisms and endotoxin in the work environment (prEN 13098). In addition, in 1994 Walters *et al.* [14] proposed a standard method for sampling and analysis of airborne endotoxin.

Several modifications of the *Limulus* amebocyte lysate (LAL) assay are commonly used for quantification of endotoxin levels, but part of the variability is related to methods of collection and efficiency of extraction associated with use of the LAL assay [8, 13]. Filter media are one potential source of variation in results. Milton *et al.* [8] found that the inactivation of endotoxin in solution during incubation, as well as the extent of inactivation, depended on the type of filter media used. Gordon *et al.* [3] reported that the extraction efficiency of endotoxin removed from filters depended both on the composition of the filter and on the type of aerosol being sampled.

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Both of the above-mentioned studies were carried out in an experimental laboratory with a purified standard endotoxin, although Gordon *et al.* also used generated cotton dust and machining oil aerosols contaminated by endoxin. Hollander *et al.* [5] found that the environmental samples show more variability because they can include components that may inhibit or enhance endotoxin in the LAL assay. Only two studies have been conducted in which the influence of various filter media on the quantitation of endotoxin in occupational environments have been investigated, one in a potato-processing plant [2] and the other in animal-confinement facilities [13]. Therefore, we still know little about the ability of different filter media to detect endotoxin from the various environments where the endotoxin is associated with bacterial cell walls and other organic material. In addition, the importance of the filter sample preservation in detecting endotoxins has not been well-known.

The purpose of this study was to compare how various filter media and their different extraction and preservation methods after sampling influence the biologically active amount of endotoxin in the LAL assay. All filter samples were collected during normal occupational conditions at wastewater treatment plants.

MATERIAL AND METHODS

Materials. Replicate samples were collected as close to each other as possible with sterile 37 mm diameter filters in plastic filter holders (Millipore Corp., USA) using calibrated suction pumps (SKC, Model 222-3) at a flow rate of 2 litres/min. Sampling times varied from 0.5–2 hours, but for replicate samples they were the same. Before collection, the plastic filter holders were cleaned by sonication for 30 min in 1.0% triethylamine (Fluka Chemie AG, Buchs, Switzerland) and dried at 70ºC in an oven. All glassware and pipette tips were autoclaved at 121ºC for 20 min or heat-sterilised at 180ºC for 4 hours before use. A blank sample was used as a control to check for endotoxin (pyrogen) contamination during analysis.

Filter media. The following filters were evaluated: a glass fibre (GF) filter (about 0.3 µm pore; 85/220, Macherey-Nagel, Düren, Germany) and three membrane filters on cellulose backing pads (Millipore). These included mixed cellulose esters (CE: 0.45 µm pore; Millipore), polyvinyl chloride (PVC: 5 µm pore; VM-1, Gelman Sciences Inc., USA) and polycarbonate membrane (PC: 0.2 µm pore; Nuclepore Corp., USA). These four filters were selected for the study because they are generally used in endotoxin sampling in occupational environments.

Preservation experiment. Replicate samples with GF filters were taken simultaneously from the air of six plants in various phases of the wastewater treatment process. One of the replicate samples was stored in the polystyrene filter holder without extraction and other was placed in 25 ml nonpyrogenic polystyrene or glass tubes with 10 ml of nonpyrogenic water within 8 hours after sampling. Both samples were kept at 4ºC for 1-14 days. All samples were controlled for bacterial growth at the same time as endotoxin concentrations of the extracts were analysed. Bacterial growth was tested by plating 0.1 ml of the extraction solution onto eosin methylene blue agar (EMB medium) and the plates were incubated in the dark at 37ºC for 48 hours (EMB; Becton Dickinson Microbiology Systems, USA).

Another experiment was established with six air samples collected from one wastewater treatment plant. After collection, these samples were extracted and divided into two groups. One group of extracted samples was stored at -20ºC to compare them with the other group stored at 4ºC.

Extraction solutions and endotoxin analysis. The GF filters were extracted with 10 ml of nonpyrogenic water by shaking them horizontally (90 shakes/min) at room temperature for 60 min. Part of the filters (n = 35 of the total 176 samples) were extracted in 0.05M potassium dihydrogen phosphate - 0.01% triethylamine nonpyrogenic water solution (pH 7.5; KH_2PO_4, Merck, Darmstadt, Germany) or in 0.01M Trizma nonpyrogenic water solution (pH 9.0; Tris(hydroxymethyl)aminomethane, Sigma, USA). All extracts were centrifuged at 112 × *g* for 10 min after shaking. The supernatant was then diluted to achieve a linear working concentration for the LAL assay (0.0125-0.075 ng/ml). 50 µl sample of the supernatant fraction was analysed in duplicate at multiple dilutions for the presence of endotoxin with the end-point chromogenic LAL assay (Coatest® Endotoxin, Chromogenix, Mölndal, Sweden). All standard curves were made by reconstituting the endotoxin standard *Escherichia coli* O111:B4 with nonpyrogenic water. Endotoxin values of standards, samples and blanks in the LAL assay are accepted according to directions of Coatest®. Twelve endotoxin units (EU) of this endotoxin standard are assumed to equal 1 ng and the EU has been standardized against Reference Standard Endotoxins EC-5 and EC-6 of the U.S. Food and Drug Administration. The results are expressed as nanograms of endotoxin per cubic meter of air.

A coefficient of variation (CV) for the endotoxin measurements at wastewater treatment plants was estimated by collecting five replicate air samples at the same time in the same place. The CV of the preservation at 4ºC for the samples, which were extracted with nonpyrogenic water within eight hours after sampling, was estimated by analysing five replicate samples in duplicate the day after collection, then 1 week later, and a third time 2 weeks after collection.

Experimental laboratory test. The interaction between the filter media and the endotoxin standard solution was studied in a small-scale laboratory experiment. The test was started by pipetting 0.1 ml of the endotoxin standard solution (*E. coli* O111 : B4, Sigma) onto duplicate filters.

All four types of filter media (GF, CE, PC and PVC) were included in the test. One filter was placed in nonpyrogenic water immediately after pipetting, and the other was kept without extraction at room temperature for 1.5 hours. After 1.5 hours, nonpyrogenic water was added to the dry filters. Cellulose backing pads of membrane filters were analysed by pipetting 0.1 ml of the endotoxin standard solution (*E. coli* 055 : B5, Chromogenix) onto the membrane filters in plastic holders simultaneously by use of the suction pumps at a flow rate of 2 l/min for one hour, after which the backing pads were extracted with nonpyrogenic water. All the extracts were shaken for 60 min and analysed with the LAL assay using two dilutions (1 : 5 and 1 : 20) of these experimental samples.

Statistical analysis. The statistical significance of the difference in concentration of the endotoxin was analysed by Wilcoxon matched-pairs signed-rank test. Linear regression analysis was used to determine the relationship between replicate air samples.

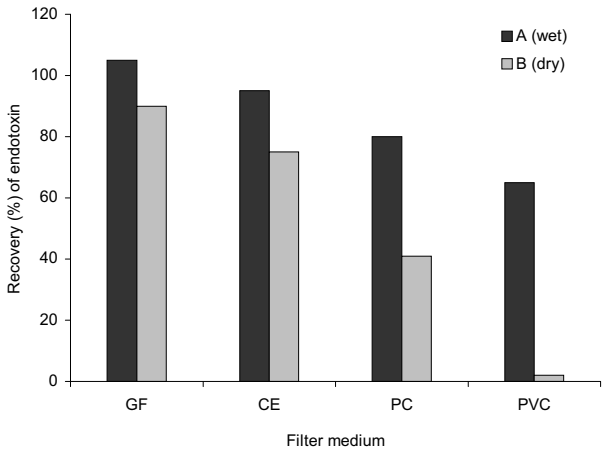

Figure 1. Recovery (%) of standard endotoxin from different filter media. The filters (A) were placed in 10 ml of nonpyrogenic water immediately after pipetting of endotoxin onto the filters; the others (B) were kept without extraction at room temperature for 1.5 hours after pipetting of endotoxin onto the filters.

RESULTS

Comparison of the four various filter media showed that the concentrations of endotoxin at wastewater treatment plants were the highest when the GF filters were used for air sampling (Tab. 1). The endotoxin concentrations determined from air samples collected on the CE filters were, on average, 53% and on the PC filters 26% of those collected on the GF filters, while the lowest concentrations of endotoxin were detected on the PVC filters.

In the experimental laboratory test no significant change was observed in the amount of biological available endotoxin in the LAL assay when the standard endotoxin solution was pipetted onto the GF filters (Fig. 1). Similar results were obtained with the hydrophilic CE filters, although the recovery of the endotoxin from the dried CE filters after pipetting the standard solution was lower than that from the GF filters. With the CE filters, however, the changes were smaller than with the PC and PVC filters, by which reduction in the recovery of the pipetted amount of endotoxin was over 50% when those filters were kept without extraction at room temperature for 1.5 hours. Backing pads of the CE, PC and PVC filters were also analysed and the concentrations of endotoxin were under 10% of the pipetted amount of endotoxin on those; the highest amounts were found from below the PC filters and the lowest amounts from below the PVC filters. Endotoxin concentrations on backing pads of the field samples were, in most cases, under the limit of detection.

Wastewater aerosols including GF filters were used in a test of an extraction solution. The largest amounts of endotoxin were detected when nonpyrogenic water was used as the extraction solution (Tab. 2). The 0.01M Trizma buffer, which is used as a buffer solution for chromogenic substrate in the LAL assay, was almost as good as nonpyrogenic water for extracting endotoxins from the filters. However, if the endotoxin standard was reconstituted and diluted with the Trizma buffer instead of nonpyrogenic water, on average, 17% decrease was observed in the concentrations of endotoxin. The smallest

Table 1. Concentration of endotoxin on the glass fibre (GF) filters compared with the cellulose mixed esters (A), polycarbonate (B) and polyvinyl chloride (C) membrane filters. Compared pair samples were collected at the same time from the air of wastewater treatment plants, but A, B and C groups were collected separately.

Comparison	Filter type	Concentration of endotoxin (ng/m^3)		
		AM[a]	MD[b]	Range
A (n = 6)	Glass fibre	38	19	7.8-92
	Cellulose mixed esters	7.1*	10	0.5-22
B (n = 10)	Glass fibre	8.7	6.6	0.7-25
	Polycarbonate	1.9*	1.7	0.03-5.6
C (n = 17)	Glass fibre	53	28	0.4-320
	Polyvinyl chloride	0.07*	< 0.01	< 0.01-0.4

[a]Arithmetic mean; [b]Median; * $p < 0.05$ compared with glass fibre filters

Table 2. Concentration of endotoxin on replicate glass fibre filters, which were extracted with different solutions: nonpyrogenic water, 0.05M KH_2PO_4 and 0.01M Trizma buffers.

Comparison	Extraction solution	Concentration of endotoxin (ng/m^3)		
		AM[a]	MD[b]	Range
1. (n = 14)	Nonpyrogenic water	44	23	0.7–140
	0.05M KH_2PO_4	7.4*	7.7	0.4–21
2. (n = 21)	Nonpyrogenic water	59	19	0.5–350
	0.01M Trizma	50	25	0.5–300

[a]Arithmetic mean; [b]Median; *$p < 0.01$ compared with nonpyrogenic water.

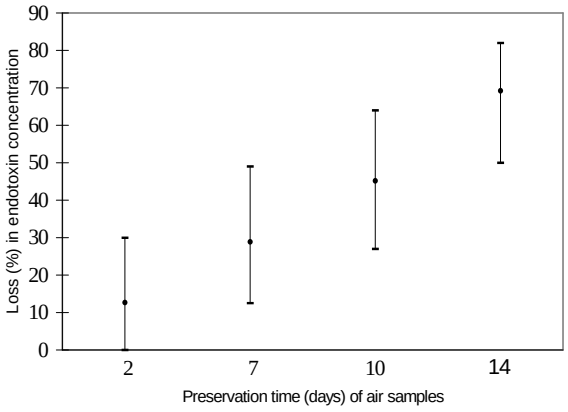

Figure 2. Loss in concentrations of biologically active endotoxins (average % ± range) on non-extracted filter samples during two weeks' preservation time compared to the replicate samples extracted on the day of air sampling.

amounts of endotoxin were detected from the filters which were extracted with the KH_2PO_4 buffer. In contrast to the Trizma buffer, if the KH_2PO_4 buffer was used to reconstitute and to dilute the endotoxin standard instead of nonpyrogenic water, the concentrations of endotoxin increased 25% on average, although the difference with nonpyrogenic water was still significant.

The effect of preservation method on detectable concentrations of endotoxin was studied using replicate filter samples collected from the air of wastewater treatment plants. According to the results of this study, the air samples extracted on the day of collection showed larger amounts of endotoxin than those stored at 4ºC without extraction (Fig. 2). With prolonged preservation time, the difference increased rapidly. Similar changes in the amount of extractable endotoxin were also found in the laboratory experiment during 1.5 hours (Fig. 1).

Six pairs of replicate air samples, which were extracted with nonpyrogenic water after collection, were also stored for 20-30 days: six samples at -20ºC and six samples at 4ºC. The endotoxin concentrations of the samples kept at -20ºC were 0.3-8.5 ng/m^3 while those kept at +4ºC were 1.0-140 ng/m^3. Thus, the endotoxin concentrations of deep-frozen samples were much lower than the concentrations of those stored at 4ºC.

The coefficient of variation (CV) for the endotoxin measurements at wastewater treatment plants was 21-38% (AM 30%) when GF filters, nonpyrogenic water extraction on the day of collection, preservation of the extracted air samples at 4ºC for two weeks, and the end-point LAL assay were used. The median CV for preservation of the extracted air samples at 4ºC for two weeks was 6%.

DISCUSSION

Comparison of filter media. Of the four filter media compared, the GF filters collected the largest amounts of extractable endotoxin from the air of wastewater treatment plants. In the replicate air samples the endotoxin concentrations on the other types of filters (CE, PC and PVC) were significantly lower. The CE, PC and PVC are membrane filters and therefore endotoxin including components, which are smaller than a pore size, might penetrate the filter. However, in the analysis of the backing pads of those filters after air sampling no significant amounts of endotoxin were detected. One possible explanation for the different results with the various filters is some kind of interaction, such as inactivation or adsorption of endotoxin to surfaces of the filter medium [6]. Aerosols at the wastewater treatment plants do not include many organic dust fibres, as is the case in cotton dust, for example; and endotoxins within the wastewater aerosol may react with the filter medium. Milton *et al.* [8] showed the reduction of endotoxin by the CE and PVC filters in laboratory experiments. They found that PVC filters produced the greatest reduction in the amount of endotoxin after incubation in solution, and CE filters produced the least. The GF and PC filters were tested in the present experimental study, which showed that the extractable amounts of the standard endotoxin solution were higher from the GF filters than from the PC, CE and PVC filters. Even if the purified endotoxin used in the laboratory study is not equal to environmental endotoxin, similar results from field and experimental studies confirm suitability of the GF filter for air sampling of airborne endotoxin in that kind of workplaces, as wastewater treatment plants are.

The PVC filter is widely used in field studies [7], and is a standard medium of the National Institute for Occupational Safety and Health (USA) for gravimetric measurement of cotton dust. In the laboratory, Gordon *et al.* [3] found that PVC filters were suitable for collection of cotton dust samples, but not for endotoxin-contaminated machine oil aerosols. The recovery of extractable endotoxin on the PVC filters was as poor as that found in our study with air samples from wastewater treatment plants. The PVC filter is hydrophobic; therefore, the surface of a PVC filter may bind the endotoxin of bacterial aerosols avidly (Milton *et al.* 1990). Insufficient disruption of hydrophobic interactions between the endotoxin and the PVC filter material by extraction solution may lead to the low recovery of extractable endotoxin.

Effect of extraction solution. The study of the effect of the extraction solution showed that nonpyrogenic water alone was a good choice for extracting endotoxin from GF filters. The additive agents (Tris and phosphate-triethylamine), which are used to increase the pH or ionic strength of the extraction solution, were not necessarily needed when filter samples were collected from the air of wastewater treatment plants and analysed with the end-point chromogenic LAL assay. Olenchock *et al.* [9], who have made experimental studies with grain dust, have also found that dispersing agents do not essentially improve the eluation of endotoxins from grain dust to water when

the chromogenic LAL assay is used for analysis. However, Douwes *et al.* [2] showed that the addition of a dispersing agent (0.05% Tween 20) to water considerably improved the extraction efficiency. Their airborne endotoxin samples were collected from a potato-processing plant, and their method of analysis was a kinetic chromogenic LAL assay. Similarly, in a kinetic-turbidimetric LAL assay, the 0.05M potassium phosphate - 0.01% triethylamine buffer can be used as the extraction solution [8], although the biological activity of endotoxin extracted with the same kind of buffer was very small in the chromogenic LAL assay.

Therefore, in addition to the extraction solution, both the origin of the endotoxin samples and the method of analysis influence the amount of detectable endotoxin.

Importance of preservation method. The detectable concentration of endotoxin collected on the GF filter from the air varies considerably depending on the method used to preserve the samples. After one week of preservation, the concentrations of endotoxin in the air samples which were stored without extraction were about 70% of those in the samples extracted on the day of collection. After the second week, only 30% of the endotoxin concentration recovered from the extracted samples was found in the samples stored without extraction. All replicate air samples for the preservation experiment were taken at the same time and by the same method. The concentrations of endotoxin in non-exracted and on the sampling day extracted air samples correlated well (r = 0.97) with each other, and thus the difference in results was due mainly to different preservation practices. Decreasing concentrations of detectable endotoxin in the non-extracted samples may depend on changes in the physical state of endotoxins on the dry filter and strengthened interactions between endotoxins and the filter material during the preservation.

Another explanation could be that possible growth of viable Gram-negative bacteria during storage at 4ºC might increase the amount of detectable endotoxin in extracted samples. This was tested and there were no culturable Gram-negative bacteria in the extracted air samples. Thorne *et al.* [12] reported that Gram-negative bacteria easily lose their viability when air samples are collected onto filter media. These results confirm that Gram-negative bacterial cells collected on the filter from the air are no longer culturable in the water solution from which the filter is extracted. However, difference (%) in endotoxin concentrations between non-extracted and extracted filter samples were much bigger than variation (CV 2-8%) in endotoxin concentrations during two weeks' preservation of the extracted air samples at 4ºC. Therefore, it is obvious that the possible presence of Gram-negative bacteria alone did not cause changes in endotoxin concentrations between two different preservation practices.

To ensure that bacteria could not increase in water-extracted samples, it would be possible to freeze them. However, the results of this study showed that the endotoxin concentrations of the extracted air samples decreased during storage in a deep-freeze (-20ºC), and subsequent thawing compared with the samples stored at 4ºC. Douwes *et al.* [2] found that freezing and thawing of house-dust extracts may also lead to a 20% loss in the concentration of endotoxin. Thus, freezing and thawing will also decrease the amount of biologically available endotoxin in the LAL assay.

CONCLUSIONS

The amount of detectable endotoxin in air samples depends both on the composition of the filter used for collection and on the type of aerosol being sampled. In the present study, significantly larger amounts of endotoxin could be found in the air when GF filters were used instead of CE, PC or PVC filters. Thus, the GF filter is recommended for collection of endotoxin at humid sites such as wastewater treatment plants.

The extraction solution of filters may also increase or decrease the detection of endotoxins. Nonpyrogenic water alone seemed to be a good extraction solution for the GF filter samples that included wastewater aerosols. No additive agents in water significantly improved the effectiveness of eluation of endotoxins from the GF filters.

How the air samples are stored after collection is of great importance for the accuracy of the endotoxin determination. According to the results of the present study, the best way to preserve samples is to extract the filter media on the day of sampling and to store them at 4ºC until they are analysed. The extracted samples should be stored in a refrigerator rather than in a deep-freeze because freezing and thawing decrease the concentration of detectable endotoxin in the LAL assay.

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