4 DISCUSSION

Endotoxin is suspected in some environmental investigations as a possible cause for the sick building syndrome. The hypothesis is that gram negative bacteria were sucked onto the filters of HVAC systems. When they die fragments of their cell walls (LPS) are released and contaminate the indoor air. For indicating the time for changing the filter a method is needed to determine the endotoxin activity on used filters. Therefore the three most common filter types of HVAC systems in Germany were investigated. Cause endotoxin seems to attend to the filter material [21] the recovery rate when extracting the filter is unclear. A reliable and precise method is needed to introduce endotoxin limits.
For example Douwes [21] uses the quantitative kinetic chromogenetic Limulus amebocyte lysate test with shaking the samples for 1 hour in 5 ml distilled water as well as in 5 ml 0.05% Tween 20 solution. Additionally he centrifuges the samples for 10 minutes. Unlike him, Reynolds [95] uses the chromogenic endpoint test with Limulus amebocyte lysate substrate and shaking as well as sonication (ultrasound waves in a water bath) the samples in both Triethylamine phosphate buffer and distilled water. Additional to this these investigators use different reference endotoxin each with different biological activity that make it hard to compare the results [3, 13].

4.1 MATERIAL UNDER INVESTIGATION

Original filter material from HVAC systems in use is contaminated with dust. To investigate rates of recovery used filters free of endotoxin were needed. Obtaining this obligation detoxification of filter material with heat and γ-radiation were investigated.

4.1.1 DEPYROGENIZATION WITH DRY HEAT

Heating material at 180°C for 4 hours is a sufficient method to depyrogenize material [85-90]. This was confirmed for the oven used in this investigation with endotoxin challenge vials (2.2.4.1). Filter material turned out to be thermolabile. When heating the material to 180°C for 4 hours it was visible at least under the electron microscope that the structure of the filter was changed afterwards. If the property of the filter is changed its affinity to endotoxin may also differ. It was decided not to use dry heat for depyrogenizing the filter material.

4.1.2 DEPYROGENIZATION WITH RADIATION

The use of γ-radiation to render material free of endotoxin is not clearly described in the literature. A Medline research (Table 4-1) about endotoxin and γ-radiation only showed studies of two research groups [96-98]. These groups found different results for dried LPS. Csako et al.[97] found no decrease but Tsuji et al. [96] found a 90% decrease in endotoxin activity. Even a 90% activity loss would not be enough to fulfill the recommendations of the FDA.

TABLE 4-1 MEDLINE RESEARCH FOR DETOXIFICATION OF ENDOTOXIN BY γ-RADIATION

Dose of γ-Radiation	Condition of Endotoxin	Decrease of Endotoxin Activity	Author
40 KGray	• lyophilized	no	Csako [97]
	• dissolved in H_2O	85%	
42 KGray	• dissolved in 0.9% NaCl	99.99%	Csako [98]
47 KGray	• dried	90%	Tsuji [96]

It was decided to investigate the effect of γ-radiation with a dose of 25 KGray usually applied for sterilization. Section 3.2.1.1.2 shows the inability of γ-radiation to render ECV and contaminated filter material free of endotoxin. Probably it would be possible to destroy endotoxin by higher doses but higher expenses would arise from this [99].

4.1.3 DETERMINATION OF BASIC CONTAMINATION

The difficulty in obtaining used filters that are endotoxin free made it necessary to take brand new filter and to accept basic contamination. To prevent larger contamination the filter material came directly from the manufacturer. Basic contamination of endotoxin was determined prior to start the main investigation (3.2.2). Only low precontamination was found.

4.2 SUSPENSION FOR INOCULATION OF THE FILTERS

To attain most realistic conditions the filters were contaminated with several solutions containing different kinds of endotoxin. 0.5 ml of inoculation fluid placed on half a filter piece was taken to ensure that the desiccating procedure was completed after 20 hours in the laminar flow bench. A pipette was used to distribute the suspension in order to moisten the whole filter. The cellulose fiber filter showed a different behavior than the others. Because of material impregnation the inoculation fluid did not infiltrate the filter immediately (3.2.6). This could lead to inconsistent endotoxin uptake of this kind of filter. Activities of approximately 200 EU per filter were chosen because they were found on filters of HVAC systems in prior investigations. Approximately 2,000 EU per filter was chosen in order to guaranty a retrievable activity throughout all dilution procedures.

4.2.1 DUST CONTAINING LIQUID

Dust out of a HVAC system was used to approximate the conditions found in such a system. The activity of endotoxin for inoculation (≈ 200 EU) was chosen like found before (2.1.3.2.4). The dust was sieved, so larger contamination with filter components was avoided and better distribution in suspension was achieved.

4.2.2 LIQUID WITH BACTERIAL CONTENT FOR INOCULATION

A bacteria suspension was taken because environmental LPS is present together with whole bacterial cells or their fragments, in form of particles which also contain protein and other cell constituents. These circumstances have an effect on the detection of endotoxin in the environment [13]. *Escherichia coli*, the only viable germ found on a filter of an HVAC system by the institute of hygiene, Berlin was used for contaminating the filters with approximately 200

EU. The solution was obtained as described in section 2.1.3.2.2 by suspending non-viable bacteria in water. Bacterias were inactivated to avoid an increase in endotoxin activity by growing colonies. Furthermore the cell walls were fragmented to simulate the conditions on used filters. It is not possible to obtain a suspension with a stable endotoxin activity [28]. The activity of the inoculation fluid was adjusted at an approximate level and the 100% recovery rate was determined for every set of 5 samples by the PTT. *Pseudomonas aeruginosa* prefers wet conditions that can exist on filters and it was found in the water of HVAC systems [49]. An ATCC strain was applied for better standardization. The activity level was the only difference in generating Pseudomonas *aeruginosa* containing fluid (2.1.3.2.2).

4.2.3 LIQUID CONTAINING LPS

LPS was used for highest standardized circumstances. It contains pure LPS and guarantees stable distribution and contamination. There are no differences from day to day in activity. On the other hand it might tend to form micelles more frequent than LPS attached to cell wall constituents. Two different activities (200 EU and 2,000 EU) were used for contaminating the filters (2.1.3.2.1).

4.3 EXTRACTION PROCEDURE

A survey of the literature showed that a variety of methods have been used by different groups (Table 4-2) [21, 23, 26, 27, 30, 34, 74, 95, 100-103]. For example, Douwes [21] used the quantitative kinetic chromogenetic LAL test with shaking the samples for 1 hour in 5 ml distilled water as well as in 5 ml 0.05% Tween 20 solution. He also centrifuged the samples for 10 minutes. In contrast, Reynolds [95] applied the chromogenic endpoint test with LAL substrate and shaking as well as sonication of the samples in both Triethylamin phosphate buffer and distilled water. Moreover, different investigators used different reference endotoxin with different biological activity. This makes it difficult to compare results [13]. Different recovery rates point out the necessity of standardizing methods to detect endotoxin on filters for better comparison of findings [21, 72, 95].

TABLE 4-2 METHODS FOR EXTRACTING FILTER MATERIAL USED FOR ENVIRONMENTAL AIR-SAMPLING IN AIRBORNE ENDOTOXIN DETERMINATION STUDIES

Investigator	Study aim	Sampling area	Endotoxin Detection		Extraction Protocol			Essentials of Filter Extraction
			Method	Test for Interference	Physical Extraction	Solvent	Others / Filter Material	
Douwes et al. [21]	Dust sampling and extraction	homes	KQCL	Serial dilutions	• quietly shaking for 1h • vigorously shaking for 1h	•5 ml H2O • 5 ml 0.05% Tween 20	• c at 1,000g for 10 min. • GF, Teflon, PC and CF filter used	Tween 20 best
Gordon [23]	Influence of sampling media	Artificial Aerosols in a Laboratory	QCL	Not shown	Incubating in 68°C water bath and intermittent gently agitation for 30 min.	30 ml H_2O	GF, CF, PC, Polysulfone, Polytetrafluoroethylene, Silver and Nylon used	GF and CF fiber show highest extractable activities
Hollander et al. [73]	Inhibition and enhancement	Animal-, agricultural- and textile industry	KQCL	Serial dilutions	shaking 1h	5 ml H_2O	• c at 1,000g for 10 min. • GF filter used	Test of validation is needed
Laitinen et al. [100]	Workers exposure of endotoxin	Savage Plant	KQCL	Not shown	shaking 1h	10 ml H_2O	• c at 112g for 10 min. • GF filter used	
Laitinen et al. [104]	Importance of sampling and extraction	Savage Plant	QCL	Not shown	shaking 1h	• 10 ml H_2O • 10 ml TAP • 10 ml Trizma buffer	• c at 112g for 10 min. • polyvinyl chloride, CF, GF, PC	• GF highest endotoxin recovery • best efficiency using GF filters and extraction with water
Mårtensson et al. [26]	Comparison of GCMS and LAL results	Animal care area	• GCMS • QCL	Not shown	shaking 5 min	5 ml H_2O	• Extracted filter analyzed by GCMS • PC and CF filter used	25% of endotoxin remained on filter after extraction
Milton et al. [72]	Apply precise and sensitive LAL test for environmental measurements	Animal care area	KQCL / KLARE	Serial dilutions	bath sonicator for time intervals of 5, 20 and 60 min.	9 ml TAP	PC, Polyflon, Teflon and CF filter used	• Activity of extraction does not increase after 5 of sonication • Filter media reduce recovered activity

Investigator	Study aim	Sampling area	Endotoxin Detection		Extraction Protocol			Essentials of Filter Extraction
			Method	Test for Interference	Physical Extraction	Solvent	Others / Filter Material	
Olenchock et al. [31]	Report endotoxin levels	Cotton textile mills	QCL	Not shown	shaking for 1h	10 ml H_2O	• c at 1,000g for 10 min. • PC filter used	
Reynolds and Milton [95]	Compare two LAL-based methods	Animal care area	• KLARE • QCL	• Serial dilutions • spike	• sonication for 1h • shaking for 2h	• 5 ml TAP buffer • 10 ml H2O	• c at 1,000g for 10 min. • Teflon filter used	Recovery from Teflon filter of 67%
Rylander and Morey [105]	Report endotoxin levels	Vegetable fiber processing units	Gel clot	yes	Shaking	10 ml H_2O	CF filter used	
Schimberg [34]	role of exposure to organic matter in farmer's lung disorder	Animal care area	QCL	spike	Shaking	10 ml H_2O	GF filter used	
Sonesson et al. [38]	Comparison of GCMS and LAL results	Animal care area	• GCMS • QCL	Not shown	Eluting for 1h	10 ml H_2O	CF filter used	• GCMS twice activity than LAL in CF filter • GCMS 10^2 to 10^5 times higher with PC filters
Teeuw et al. [101]	Role of endotoxin in SBS	Office buildings	QCL	Not shown	Shaking	10 ml H_2O	Millipore filter used	
Thorne et al. [102]	Evaluation of endotoxin assay and extraction methods	Animal care area	• KLARE • QCL	Serial dilutions	• Rocking for 30 min. at 68°C • Vortexing for 2h at 22°C • Sonication for 1h at 20°C	• 30 ml H2O • 5 ml TAP	GF and PC filter used	• GF filter less variability than PC filter • Rocking and Vortexing same results • KLARE sign. Different than QCL in some samples
Walters et al. [103]	Evaluation of endotoxin assay and extraction method	Fiber glass manufacturer	• KLARE • GCMS	Serial dilutions	Sonication for 1h	5 ml TAP	PC filter used	same results with GCMS and KLARE

KQCL = Kinetic quantitative chromogenetic LAL, QCL = endpoint chromogenic LAL, c = centrifugation, PC = polycarbonate, min. = minute

In this study dry filters were placed into extraction vials and 100ml of extraction fluid was poured on it. This amount of fluid was necessary because in contrast to the investigations in Table 4-2 filter material from HVAC systems is much thicker than for air sampling settings. Tween 20 and TAP were used as solutions for extraction. Vials were shaken or sonicated for predetermined periods of time (2.1.4).

4.3.1 TWEEN 20 AS EXTRACTION FLUID FOR AMPHIPHILIC LPS

Amphiphilic molecules combine to form micelles. There exists a critical micellar concentration (CMC) for every amphiphilic substance. CMC means that 50% of molecules forming micellar structures and the other halve is present as single molecules.
CMC of *Escherichia coli* 0111:B4 was found to be 22 micrograms LPS/ml [91] which corresponds to 220,000 EU/ml (1ng = 10EU). This means that in the normal range of the standard curve the LPS is mostly in a non aggregation state. This conflicts with the experience of LAL test manufacturers and the inferences of other investigators like Douwes and Olenchock [21, 30, 68]. They recommend to vortex samples vigorously and / or use of detergents like Tween to avoid micellar formation.

Olenchock investigated Tween 20 and Tween 80 in 1% concentration [30] and found an interference with the dose-response slope of the test. Later Douwes et al. [21] made their tests with Tween 20 in 0.05% concentration and found a slight decrease in the slope but no effect on the test. The Health Council of the Netherlands: Dutch Expert Committee on Occupational Standards (DECOS) [106] recommends a concentration of 0.05% Tween for analyzing the samples. In this study the recovery of endotoxin in Tween 20 solutions was tested like described in section 2.2.3. Because the critical miscellaneous concentration (CMC) of Tween 20 is attained at 0.0059% [84] and the LAL activity is less affected at a concentration of 0.005% Tween 20 (3.2.3.1) this concentration was used in the present investigation.

4.3.2 TAP AS EXTRACTION FLUID

Beneficial effects of triethylamine phosphate buffer on endotoxin recovery were shown [72, 81]. TAP decrease the interference, increases sensitivity of the assay and may bring LPS in a uniform aggregation size and confirmation state [72].

4.3.3 SHAKING THE SAMPLES

To extract the endotoxin from the filter material one standard procedure is to agitate the extraction vials for certain times [21, 23, 26, 31, 34, 73, 95, 100-102, 105]. In preliminary investigations it was shown that the recovery is best at the times used in this study.

4.3.4 SONICATION OF SAMPLES

Ultrasound has been used for cleaning equipment since a long time [107, 108]. Researchers used it for filter extraction in environmental air sampling [72, 95, 102, 103]. It was investigated if it is advantageous upon shaking.

4.4 ASSAY WORK

The decision was made to use the chromogenic kinetic LAL test because of its wide range of activities to detect endotoxin. It is the standard detection method and is widely used. It is also easy to perform in all laboratories. GCMS require more extensive technical support and it

detects all LPS even if not biological active [38]. On the other hand some authors have found similar amounts of endotoxin with GCMS and LAL-test [43, 102, 103].

4.4.1 STANDARD CURVE

Pedersen et al. [68] found a lower reaction rate for LPS when measuring the samples with a water standard curve instead of using the solvent in a tissue culture media study. Olenchock et al. [30] made a standard curve with 1% Tween 20 and found an interference with the LAL test. The Tween standard curve changed the curve's slope thus they revealed different findings when calculated with a Tween or water standard curve. Douwes et al. [21] made a standard curve with 0.05% Tween 20 and showed no significant differences in the slope of the curves. With respect to the above and the findings in section 3.2.5 it was decided to use a standard curve made with the solution media.

4.4.2 BLINDING

The LAL assay requires meticulous care to produce high quality results. Even minor irregularities would influence the endotoxin determination. To avoid bias of unconscious irregularities the experimental work was split. One person did the inoculation and extraction procedure and a second person did the assay work at the microtiter plate.

4.4.3 LOT OF LAL TESTS

LAL is formed out of the blood of *Limulus Polyphemus*. Unique animals have different activity of their blood cells (amebocytes) and thus LAL substrate is different from one sample to the next. It is of course the multi-enzyme nature of the LAL reaction that results in complex kinetics, and the natural variability between raw LAL extracts (season, location, fitness of the horseshoe crab, etc.) that results in different characteristics for different LAL lots [109]. To form one lot the manufacturers have to pool the blood from many animals. The next lot is made from different animals with different activity of LAL [110]. This shows the importance to use one lot for comparative studies [3, 28, 109, 111-113].

4.4.4 TEST FOR INTERFERING SUBSTANCES

The LAL test tends to be affected by a lot of substances [1, 5, 23, 28, 33, 68, 70, 72-74, 76, 81, 95, 103, 112, 114]. Every sample has to be checked for its validity. Spiking the samples as described in section 2.1.1.2.2 is the only officially permitted form (4.4.4.1) and was used in this study. When measuring small activities serial dilutions could lead to not detectable activities of endotoxin.

In this investigation, a high dilution-dependent inhibition (DDI) was found. It was necessary to dilute the samples up to 10^{-3}. Inhibition was detected by spiking the probes. Obviously, much higher activities of endotoxin are obtained, if the wrong dilution step is chosen. In general, investigations of filter material requires the interference test to be reliable [1, 5, 70, 72-74, 112]. Name of the manufacturer should also be mentioned and whether the same lot was chosen for all investigations so that major differences can be taken into account [3, 28, 95].

4.4.4.1 SPIKE METHOD

As test of validity a spike was used to check all samples for enhancement and inhibition. The Food and Drug Administration (FDA) of the USA has permitted this procedure for testing interference of the LAL test. Control of drug manufacture is responsibility of the FDA. Phar-

maceutical manufacturers have to guarantee endotoxin limits as described in FDA guidelines for every substance. It is necessary to recover an endotoxin spike within ±50% [94]. This is a wide range for a very precise method with a lower detectable limit of 0.005 EU/ml but the manufacturers only have to show that their product contains less than 0.05 EU/ml. When they spike their product and it shows 49% inhibition for a measured sample of 0.049 EU/ml this would mean that the sample is approximately 0.024 EU/ml higher than the result has shown. The real sample value of this sample without inhibition would be 0.073 EU/ml in maximum. This deviation seems to be tolerable to show the absence of endotoxin. In indoor air measurements it is necessary to determine higher activities and backwards calculation to i.e. 1 m^3 of air or m^2 of filter would lead to major differences. Tolerated spike recoveries of ±50% do not seem to be acceptable if one wants to determine exact activities of endotoxin. For this purpose a more precise method for testing validity has to be used. The dilution method could be a possible alternative.

Shaking of the spike prior to measure the plate seems to prevent inhomogeneous distribution of the spike (see 3.2.4). It does not take significantly more time for the analysis and is not able to disturb the test. Because of this purpose it should be used for routine investigations.

4.4.4.2 SERIAL DILUTION METHOD

This procedure uses a series of sample dilutions and a mathematical procedure applying the internal evidence for the test for validity.

A better method for detecting endotoxin is by using the Kinetic Limulus Amebocyte Lysate Assay with Resistant Parallel Line Estimation (KLARE) method (Milton et al. 1992) [74]. It is described that TAP and sonication with serial dilutions reveals good recovery and precise measurement. Five 1:6 dilution steps were used. When measuring small activities it is possible that endotoxin activity is below the detection limit when disturbing factors are diluted out of the sample. Higher costs will arise by this procedure because every sample has to be measured 6 times. These costs can be reduced if only halve of sample volume and LAL-Reagent volume is used for running the test, like recommended by Don Milton [115]. It is suggested that one measurement per set of samples is enough to show the absence of interference [73]. This study shows frequent interference even if one sample out of five presents no disturbance the other four may do so.

4.4.5 INTERVAL OF MEASURING

Major differences were observed when measuring an activity of 4.5 EU/ml of standard endotoxin with an onset time at 0.2 OD and a reading interval of 1 minute. The first calculation showed an activity of 5.37 EU/ml and the second of 3.69 EU/ml (Table 4-3). The difference of 1.68 EU/ml is explained by different times for exceeding the threshold of 0.2 OD. The first sample reached the threshold one minute prior to the second sample. Probably sample 1 reached the threshold immediately before the microplate was read and sample 2 reached it seconds later. This difference of seconds can lead to a difference of 1.68 EU/ml in the determined activity of endotoxin when reading the plate every minute. Walters et al. [103]chose a 15 second interval for reading the microtiter plate.

TABLE 4-3 DEPENDENCY OF ENDOTOXIN RECOVERY OF IDENTICAL SAMPLES (4.5 EU/ML) FROM THE TIME NEEDED TO EXCEED THE THRESHOLD OF 0.2 OD

Number of Readings to Exceed Threshold of 0.2 OD[a]	Recovered Activity (EU/ml)	Time Difference Between Readings
11th	5.37	} Δ t = 1 minute
12th	3.69	

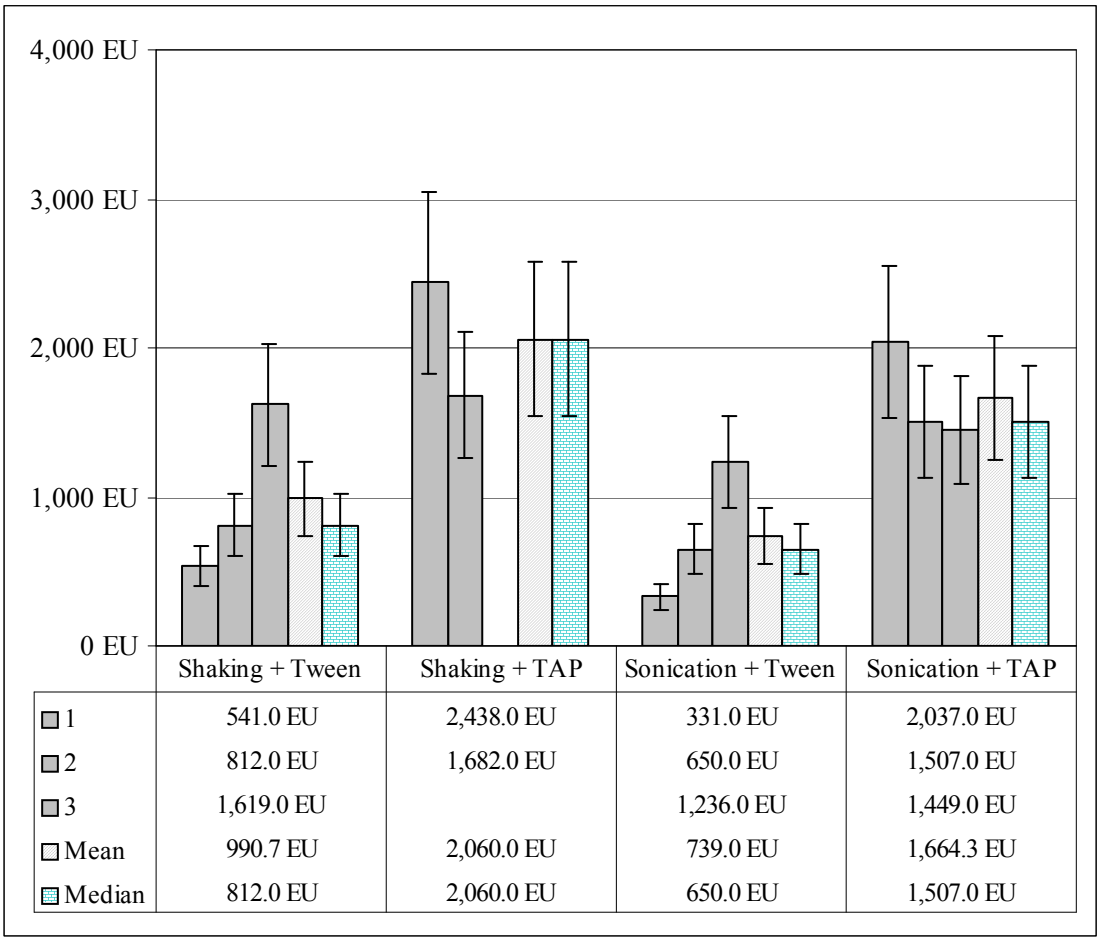
[a] Time interval for reading the microplate = 60 seconds

Our conclusion was to choose a shorter reading interval of 10 seconds.4.4.5

4.5 LOOKING AT RAW DATA

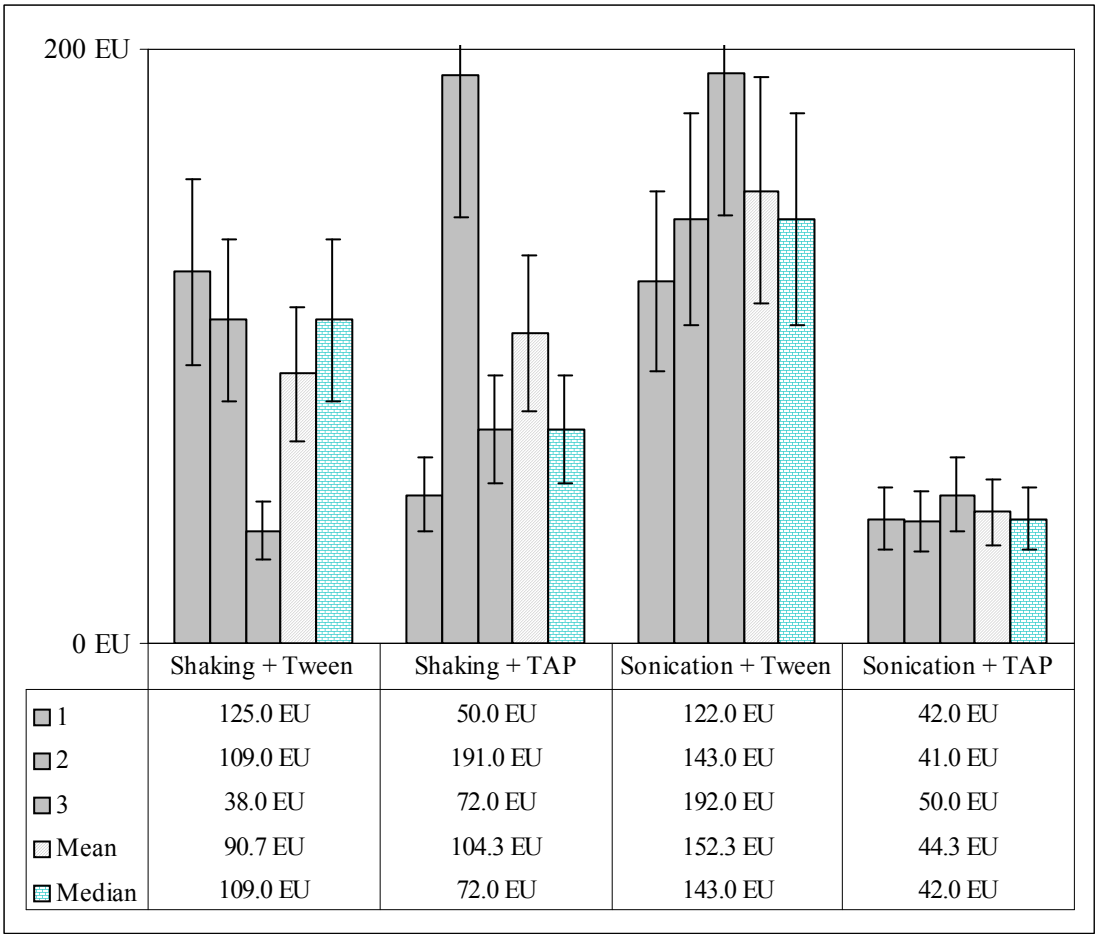
When looking at the bar diagrams of 3.1.1 "Measured Activity on Filter Pieces" it is visible that the intra-sample (between set of 5 filter samples) variation is mostly small. Inter-sample variation (from one extraction method to the next) is huge.

Figure 4-1 to Figure 4-2 show all available PTT activities for LPS 2,000 and LPS 200 for different extraction methods. Mean and median of these activities are shown and error bars indicate the allowable variation of 25% between two measurements.



	Shaking + Tween	Shaking + TAP	Sonication + Tween	Sonication + TAP
1	541.0 EU	2,438.0 EU	331.0 EU	2,037.0 EU
2	812.0 EU	1,682.0 EU	650.0 EU	1,507.0 EU
3	1,619.0 EU		1,236.0 EU	1,449.0 EU
Mean	990.7 EU	2,060.0 EU	739.0 EU	1,664.3 EU
Median	812.0 EU	2,060.0 EU	650.0 EU	1,507.0 EU

FIGURE 4-1 ALL PTT'S OF CONTAMINATION WITH LPS 2,000 EU FOR DIFFERENT EXTRACTION METHODS. MEAN AND MEDIAN ARE SHOWN . ERROR BARS INDICATE ALLOWED VARIATION OF 25%



	Shaking + Tween	Shaking + TAP	Sonication + Tween	Sonication + TAP
1	125.0 EU	50.0 EU	122.0 EU	42.0 EU
2	109.0 EU	191.0 EU	143.0 EU	41.0 EU
3	38.0 EU	72.0 EU	192.0 EU	50.0 EU
Mean	90.7 EU	104.3 EU	152.3 EU	44.3 EU
Median	109.0 EU	72.0 EU	143.0 EU	42.0 EU

FIGURE 4-2 ALL PTT'S OF CONTAMINATION WITH LPS 200 EU FOR DIFFERENT EXTRACTION METHODS. MEAN AND MEDIAN ARE SHOWN . ERROR BARS INDICATE ALLOWED VARIATION OF 25%

When looking at raw data and focusing on the most standardized conditions (LPS 200 + LPS 2,000 without a filter sample (PTT)) (see Figure 4-1 and Figure 4-2) it is possible to identify: For the extraction with TAP and Tween 20 it can be it can be shown a difference between LPS 200 and LPS 2,000. LPS 2,000 show small difference between PTT's and the original activity (2,000 EU ± 25%) when extracted with TAP and a reduction of > 25% for 4 of 6 PTT's if treated with Tween 20. Focusing on LPS 200 more percentage reduction is obvious. With TAP 5 of 6 PTT's present a reduction of > 50% other than Tween 20 with a decrease of less than 50%. The measured reduction in recovered endotoxin activity in PTT-samples can only be attributed to the extraction itself. To exclude this fact from our recovery rate the difference of PTT and sample values from filter extraction (measured values (MV)) were calculated for analyzing the data.

4.6 ANALYSIS OF DATA

Modi were established, descriptive statistics and generalized linear model calculation was displayed.

4.6.1 SPECIFICATION OF MODI

For analyzing the data some factors had to be established.

4.6.1.1 DETERMINATION OF RECOVERY RATE

The recovery rate can be measured by the difference of every single measurement and the maximum retrievable activity (MRA). The advantage is the large number of 273 samples. On the other hand it can be attained by the mean out of 5 filter samples and the MRA. This is more stable against outliners but the number of results is only 60. However, it was decided to use the mean out of 5 filter samples for the statistical analysis.

4.6.1.2 DEFINITION OF 100% RECOVERY

For every set of 5 samples there was one positive test tube (PTT) without a piece of filter to determine the 100% recovery. Because every combination of extraction method was tested on three types of filters there were 3 identical PTT's for every kind of contamination. Two of these PTT's were made on one day. The third was normally made the next day. To define the 100% recovery of a sample the term "true amount" (TA) was established. It had to be decided if a combination out of the three PTT's should construct the TA. In 10% of cases there were only two PTT's determined per extraction method (Figure 4-3 (pointed)).

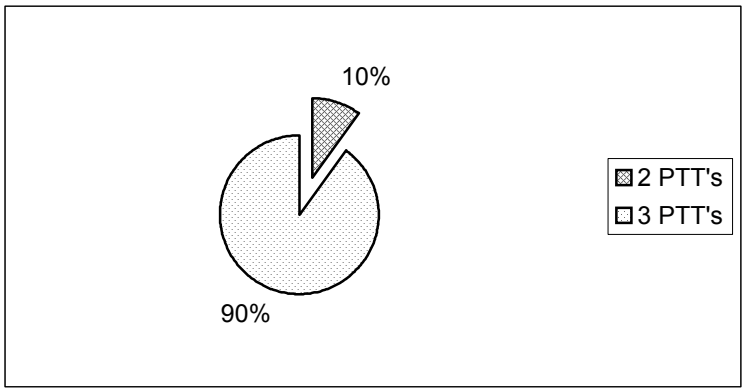


FIGURE 4-3 QUANTITY OF PTTS PER EXTRACTION MANNER

Feasible TAs are:
First: mean or median of all 3 PTT's per extraction manner (TA 1). As an example see Table 4-4 (for complete data see Table 6-2). Tables are classified by kind of contamination and show a statistical analysis of PTT recovery. Second: Only the corresponding PTT which was done for every 5 filter samples (TA 2). Third: The corresponding PTT which was done for every 5 filter samples and if there is a second PTT done exactly the same time in the same manner then the mean out of these two (TA 3).

TABLE 4-4 ENDOTOXIN ACTIVITY IN PTT'S FOR DIFFERENT EXTRACTION METHODS FORMING TA 1

	Extraction procedure			
	T/U/*E.coli*	T/S/*E.coli*	P/U/*E.coli*	P/S/*E.coli*
Mean	107.5 EU	120.0 EU	84.3 EU	33.8 EU
Standard error mean	12.5 EU	81.5 EU	21.02 EU	5.41 EU
Median	107.5 EU	67.0 EU	100.8 EU	32.1 EU
Standard deviation	17.7 EU	141.2 EU	36.4 EU	9.4 EU
Number of vials	2	3	3	3
Confidence interval (95%)	±24.5 EU	±159.7 EU	±41.2 EU	±10.6 EU

T = Tween 20
P = TAP buffer
U = sonication
S = shaking

The coefficient of correlation served as a decision criterion. Correlations between the mean measured activities of 5 filter samples and the corresponding TAs were calculated. Table 4-5 shows the best coefficient of correlation (r) for Pearson's correlation with (0.755) for TA 1, 0.596 for TA 2 and 0.605 for TA 3. Kendall-Tau-b correlation reveals similar coefficients of correlation between 0.539 for TA 1 and 0.586 for TA 2. Confidence intervals indicate no significant difference in the investigated correlations for the different TAs. TA 2 guaranties exactly the same treatment of sample and PTT, thus it is the best predictor for the 100% recovery rate.

TABLE 4-5 CORRELATION OF PEARSON AND KENDALL-TAU-B IN-BETWEEN TA AND MEANS RECOVERED ON FILTER PIECES

Used Correlation	Tested "True amount"	r	Confidence Interval 95%	
			Lower Bound	Upper Bound
Pearson	TA1	0.755	0.608	0.902
	TA2	0.596	0.231	0.961
	TA3	0.605	0.240	0.970
Kendall-Tau-b	TA1	0.539	0.394	0.684
	TA2	0.586	0.443	0.729
	TA3	0.575	0.432	0.718

r = coefficient of correlation

4.6.1.3 DETERMINING THE MATHEMATICAL EXPRESSION THAT CORRESPONDS CLOSEST TO THE STUDY AIM

There are six different expressions to analyze the data for recovered endotoxin (see Table 4-6).

TABLE 4-6 OPPORTUNITIES FOR ANALYZING THE DATA

	Description	Formulation
A	Difference of MV minus TA	$MV_i - TA_i$
B	Absolute difference of MV minus TA	$\lvert MV_i - TA_i \rvert$
C	Percentage deviation of TA	$\frac{MV_i - TA_i}{TA_i} \cdot 100$
D	Absolute percentage deviation of TA	$\left\lvert \frac{MV_i - TA_i}{TA_i} \right\rvert \cdot 100$
E	Mean square error (MSE)	$\frac{1}{n}\sum_{i=1}^{n}(MV_i - TA_i)^2$
F	Mean square fractional difference of TA	$\frac{1}{n}\sum_{i=1}^{n}\left(\frac{MV_i - TA_i}{TA_i}\right)^2$

MV_i = Measured value (mean of 5 filter samples)
TA_i = true amount

Ad A) The difference shows the mean value that is closest to the TA. Positive and negative amounts can neutralize each other but it indicates a more positive or negative recovery rate. It seems to be good with a great SD.
Ad B) Small absolute difference reveals the value with the smallest deviation, in mean, to TA. The advantage is that SD is taken into account.
Ad C) Percentage deviation takes into account that different activities of contamination were used. It reveals important information about the percentage that was recovered.
Ad D) Absolute percentage deviation considers different activities of contamination.
Ad E) Mean square error considers the reliability and the validity that makes it to a strong value.
Ad F) Mean square fractional difference (MSFD) takes into account reliability, validity and different activities of contamination. Best value for replying to the study aims.

It was decided:
1. Recovery is expressed as percentage difference of means (from 5 samples) and PTT.
2. The 100% recovery rate was determined from the corresponding PTT (TA2).
3. For descriptive statistic it was decided to use MSFD as the strongest value to describe the data.

4.6.2 DESCRIPTIVE STATISTICS

When analyzing the data with all contaminations it was obvious that *Pseudomonas aeruginosa* had enormous influence on the outcome of the evaluation. It was decided to analyze the data separately for *Pseudomonas aeruginosa* and for all other types of endotoxin. Tsuji and Harrison described a considerable higher variation in the LPS preparation of *Pseudomonas aeruginosa* [96]. This inconsistency could be explained by the "gelation forming nature" of this bacterium.

When studying the data of Table 3-2 till Table 3-5 one gets evidence for that endotoxin is best recovered on glass fiber filter like Laitinen et al. [104] and Thorne et al. [102] previously described. Sonication and TAP seem to be much better than compared to shaking and Tween 20, respectively. For example Tween 20 reveal an 9 fold higher MSFD than TAP that indicates a higher precision for the endotoxin retrieval with TAP. LPS 2000 presents smallest MSFD with a difference of –12.6% for the different kinds of endotoxin. It expresses a high reliability and validity with a mean reduction in endotoxin recovery of 12.6%. *Escherichia coli* show a high degree of SD that expresses the instability of the contamination fluid. Dust shows an increase in recovered endotoxin of 44.1% relative to the TA.

By contrast, *Pseudomonas aeruginosa* gave the opposite result (Table 3-6). It was best recovered on synthetic fiber filters (worst GF filter) and a better recovery was achieved by shaking the filters and the treatment with Tween 20. This might be the clue resulting in the exclusion of *Pseudomonas aeruginosa* from the analysis. Maybe its capsule forming origin lead to a different affinity to the filter material than the other contaminations do.

Looking at the combined criteria (3.1.2.2) for dust as the most realistic- and LPS 2000 as the most standardized contamination it is displayed in Table 4-7 that:
Dust is best recovered on CF and GF filters with Tween 20 and sonication. Most unfavorable MSFD was achieved for TAP and shaking. Different constellation is proved for SF filters. Unfortunately there was no uniformity shown for LPS 2000 extraction methods and the reported findings for dust contamination with respect to optimal conditions in endotoxin recovery. LPS 2000 recovery on SF filters demonstrates no bigger differences in the endotoxin removal.

TABLE 4-7 ANALYZING THE EXTRACTION METHODS FOR DIFFERENT FILTER TYPES

Endotoxin Contamination	Type of Filter	Extraction Method	
		Best	Worst
Dust	CF	Tween 20 + sonication	TAP + shaking
	GF	Tween 20 + sonication	TAP + shaking
	SF	TAP + shaking	Tween 20 + shaking
LPS 2000	CF	TAP + sonication	Tween 20 + sonication
	GF	TAP + shaking	TAP + sonication
	SF	No preference	No preference

4.6.3 GENERALIZED LINEAR MODEL

When analyzing the data there were no significant differences found. It was only possible to describe tendencies. Similarly Douwes et al. [21] found no significant differences in temperature and rocking conditions.

4.6.3.1 UNIVARIATE ANALYSIS EXCLUDING DATA FOR *PSEUDOMONAS AERUGINOSA* CONTAMINATION

Studying single factor analysis (Table 3-25) sonication reveals better recovery than shaking the samples. TAP was the more effective solution media and GF fiber filter shows smaller differences than SF and CF filters. In the kind of contamination fluid there were only minor

differences detected between LPS 2000, LPS 200 and dust suspension. Major differences but not significant were observed for contamination with E.coli that shows the highest differences from the mean.

4.6.3.2 MULTIVARIATE ANALYSIS EXCLUDING DATA FOR *PSEUDOMONAS AERUGINOSA* CONTAMINATION

In multivariate analysis (Table 3-26) emphasis was put on the contaminations of LPS 2000 and dust.
Better detection for dust was shown with Tween 20. In contrast LPS 2000 preferred TAP as solution media. The kind of physical extraction procedure revealed better results for dust when sonication was applied and LPS show no major differences in both treatments. In combination of solution media and physical extraction Tween 20 and sonication produce the best results. When Tween 20 and shaking were applied it turned out to be the worst combination. If TAP was used smaller differences in recovery were exhibited for shaking and for sonication.

Looking at the type of filter, it was shown a slightly better outcome for GF and CF filter with sonication and TAP. In contrast, SF filter were better shaken and treated with Tween 20.

4.6.3.3 UNI- AND MULTIVARIATE ANALYSIS FOR FILTER CONTAMINATION FOR *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa was better detected with Tween 20. Shaking produce better results than sonication and the germ showed smaller differences on SF- than on CF- than on GF filters, respectively. Combination of solution media and extraction method revealed a ranking from best to worst for Tween 20 and sonication as best combination, TAP and shaking, Tween 20 and shaking, TAP and sonication, respectively.

4.7 ENDOTOXIN LIMITS

In 1976 Urbaschek and colleagues wrote that the humans are the species most sensitive to endotoxin [116]. Several epidemiologic studies and experiments with human volunteers showed acute reductions in pulmonary function among subjects exposed to organic aerosols containing endotoxin [19, 20, 24, 25, 35, 42, 95, 117]. Herbert et al. [118] reported no decrease in pulmonary function but inflammation at the alveolar level.

In this study filters were investigated to perform a step to introduce recommendations for levels indicating the change of filters of HVAC systems. In the literature there are no current publications on this issue.

First, there are no effect levels (NELs) needed for indoor air activity of endotoxin. Many authors discuss this [19, 20, 24, 25, 35, 42, 117, 118]. After this establishment it is possible to work out levels of endotoxin for burdened filters. The next step would be to investigate filters of HVAC systems in use of so called sick- and healthy buildings.

But even for establishing indoor air NEL this study provides help. For measurements of airborne endotoxin many investigators draw air on filters (Table 4-2) and similar problems of extraction arise from this.

Thresholds for indoor air activities of endotoxin are demanded. In the literature no effect levels for inhalatory endotoxin exposure have been calculated ranging from approximately 90 - 1800 EU/m^3 [18, 20, 24, 25, 27, 36, 37, 42] based mainly on experimental endotoxin exposure studies. Haglind and Rylander reported changes in FEV1 associated with an endotoxin dose of 170 ng/m^3 for students exposed to cotton dust and endotoxin in an experimental card room. Smoking workers demonstrated decreased FEV1 at a level of 80 ng/m^3. A threshold for acute FEV1 decrement in swine confinement workers was reported to be about 200 ng/m^3. Pulmonary responses to endotoxin-containing aerosols in enclosed chicken and turkey facilities have also been reported. However, Kennedy et al. found little correlation between respiratory disease and endotoxin exposure [119].

Calculated NELs for chronic and acute respiratory effects based on epidemiological studies in occupationally exposed populations are comparable. For the establishment of a health-based recommended occupational exposure the limit is the NEL of 90 EU/m^3 [20] based on acute respiratory effects and obtained from a large and well designed experimental exposure study in which non-symptomatic subjects from the general population were exposed to endotoxin contaminated cotton dust. Because endotoxin may have chronic pulmonary effects at levels which may be lower than for acute respiratory effects [25, 37, 117] lower levels can be proposed. For occupational exposure it was recommended a limit of 50 EU/m^3, based on personal inhalable dust exposure, measured as an eight-hour time weighted average [106].
Unfortunately, comparison of endotoxin exposures reported by various authors is problematic, since sampling and analytical methods have not been standardized [18, 95].

4.8 COSTS

Determination of endotoxin is very cost intensive. The costs per sample are between 37 DM and 45 DM. Experienced technical support is needed for maintenance. The test kits are expensive and cost 500 € for 200 wells on the microtiter plate.

4.9 CONCLUSIONS

No single method can be pointed out for best endotoxin recovery on filters of HVAC systems. Although the work was performed under optimal conditions - to standardize the procedure, to blind the assay work, to compress the investigation time and to carefully avoid endotoxin contamination - it was not possible to show statistical significant differences between detection methods nor it was possible to show reliable recovery rates for different methods. Only tendencies can be described and advice can be given for extracting filter material.

4.9.1 BIASING FACTORS OF THE INVESTIGATION

Possible biasing factors can be named. A huge variability in the results is shown.
The small number of samples for single extraction manner and the use of a biological test that were not stable itself can explain this.

Additionally the endotoxin containing test fluids were not stable. It was shown by Milton et al. that extracts stored at 4°C showed a 65% decline. When stored at -20°C an 86% decline after 4-6 weeks [28]. Douwes et al. [21] showed that dissolved endotoxin remained stable at 7°C over a period of one year. Probably lyophilized endotoxin would be better for inoculation of filter material. Walters et al. [103] used desiccation of filters and storage at 4°C. And it was shown that dust stored up to 8-10 weeks at 4°C and -20°C showed no difference [28].

Unclear interactions of endotoxin and filter material were observed. On one hand endotoxin can be bound to filter components [21] and part of it is not detectable, on the other hand it was retrieved much greater activity than in the PTT. This could be generated by contact of cell wall components and filter material with the treatment of the extraction procedure. Mechanical movement of it could crack connections of cell wall components and LPS. This could lead to a higher recovery of endotoxin [38]. Other possible causes are an inhibition of micellar formation in presents of filter material or / and substances released from the filter material that change the sensitivity of the LAL-test to factor C, that was not detected by the test of validity.

Dilution schemes are potential sources of variation in an endotoxin assay. It is generally recognized that large (>1:100) dilutions are potential sources of error [112]. In this investigation samples were diluted up to 1:1,000 to make sure that interfering substances are absent. Some variation of the samples may result out of this fact.

4.9.2 Recommendations for Endotoxin Challenge Trials

Some advises for the improvement of endotoxin recovery can be given.

4.9.2.1 General Recommendations

- All equipment coming in contact with the samples have to be free of endotoxin. For non-disposable material dry heat should be preferred. If material is thermo-labile determination of basic contamination is needed.
- Used water has to be checked for lack of endotoxin. Kinetic chromogenic LAL test should be used because of its wide range of the standard curve.
- Perform precise working.
- Test of interference is of great importance. It should be applied to every sample. Dilution series should be preferred.
- Same number of lot should be used for comparative studies.
- The type of standard endotoxin should be displayed.
- Endotoxin solutions are not stable over time. Samples should be desiccated if storage is needed prior to analysis.
- Interval of measuring the sample by the reader should be less than one minute.

4.9.2.2 Special Recommendations for Filter Extraction

- The type of extraction does not matter that much as far as displayed in this study.
- Recovery rate for the used procedure and type of filter is important and should be shown.
- Causes of high variation more than five repetitive sample extractions are needed.
- The standard curve should be made with the solution media.