

Endotoxin measurements in the PM10 fraction of ambient air

I. Laufer, U. Jäckel, H. Kühne, N. Hüttig, N. Heubach

ABSTRACT As part of the outer cell wall of gram-negative bacteria endotoxins can be found ubiquitous in nature. So far most studies on airborne endotoxins focussed on occupational environments. In Germany IFA-Arbeitsmappe 9450 gives recommendations for sampling and analysis of endotoxins at workplaces. However, apart from background measurements for workplace evaluations, there are currently no guidelines in Germany for endotoxin measurements at environmental settings. Therefore, the aim of this study was to test whether PM10 sampling on filters with a high volume sampler and determination of endotoxin concentration via LAL assay are suitable methods for a new guideline for measurement of endotoxin concentration in ambient air. Over the course of one year endotoxin concentration was investigated at three different rural sampling spots. Annual median endotoxin concentration over all samples was 0.143 EU/m³. Between different sampling spots no significant differences in endotoxin concentrations were observed, yet endotoxin concentration in ambient air showed an obvious seasonal course. The applied methods have proven suitable for the analysis of endotoxins in the outdoor air, although further standardization is needed before implementation in a guideline.

Endotoxinmessungen in der PM10-Fraktion der Außenluft

ZUSAMMENFASSUNG Endotoxine kommen als Teil der äußeren Zellwand von gram-negativen Bakterien ubiquitär in der Natur vor. Viele Studien zu luftgetragenen Endotoxinen haben sich bisher mit Arbeitsplätzen beschäftigt. In Deutschland gibt die IFA-Arbeitsmappe 9450 Empfehlungen zur Probenahme und Analyse von Endotoxinen am Arbeitsplatz. Hingegen existieren für Endotoxinmessungen in der Umwelt, abgesehen von Hintergrundmessungen für Arbeitsplatzbewertungen, in Deutschland bisher keine Richtlinien. Daher war das Ziel dieser Studie zu untersuchen, ob die Methodik der PM10-Probenahme auf Filtern mit einem High-Volume-Sampler und die anschließende Bestimmung der Endotoxinkonzentration mittels LAL-Assay für eine neue Richtlinie zur Untersuchung der Endotoxinkonzentration in der Außenluft geeignet ist. Über ein Jahr wurde dafür die Endotoxinkonzentration an drei Standorten im ländlichen Raum untersucht. Die jährliche mediane Endotoxinkonzentration lag bei 0,143 EU/m³. Zwischen verschiedenen Standorten konnte kein signifikanter Unterschied in der Endotoxinkonzentration festgestellt werden. Jedoch wurde für die Endotoxinkonzentration in der Außenluft ein saisonaler Verlauf beobachtet. Insgesamt haben sich die angewandten Methoden für die Untersuchung von Endotoxinen in der Außenluft als geeignet erwiesen, auch wenn weitere Standardisierungen vor der Implementierung in einer Richtlinie nötig sind.

1 Introduction

Endotoxins are lipopolysaccharides (LPS) from the outer membrane of gram-negative bacteria. LPS are composed of the O-antigen region, a core oligosaccharide and the highly conserved Lipid A part, which is responsible for their endotoxic activity [1]. Endotoxins are continuously released into the cells' environment during cell lysis, growth and division [1; 2]. Because of the ubiquity of gram-negative bacteria endotoxins are commonly detected as part of aerosol particles. At high concentrations endotoxins can lead to adverse lung function effects, induction of inflammation and dysfunction of airways after inhalation and deposition in the lungs [3]. In contrast, Mutius et al. proposed that exposure to low levels of endotoxin in childhood can protect against the development of atopic diseases. However, in this study dust samples of mattresses from farmers' and nonfarmers' children were collected and the geometric mean of endotoxin concentrations were determined instead of aerosol exposure. It could be shown that levels of endotoxin were significantly higher for farmers' children [49,479 EU/m²] as compared to the control group from nonfarming families [9,383 EU/m²] [4]. Those findings correlated with previous findings of lower abun-

dances of atopic sensitization of children from farming families [5]. However, not only farming is associated with endotoxin exposure. High occupational exposure to airborne endotoxins has been demonstrated, for example, at workplaces in waste recycling and cotton processing as well as agriculture [6]. Here concentrations can be a thousand times higher than the concentrations measured in the corresponding outdoor air, where the concentration seems to fluctuate over the seasons [7]. In contrast to the enormous number of exposure studies at workplaces, only few studies exist for endotoxin measurements in the PM10 fraction of ambient air. In particular, investigations in rural areas are rare.

In Germany the method from IFA-Arbeitsmappe 9450 is recommended for the measurement of airborne endotoxins at all types of work environment and gives recommendations for sampling and analysis of endotoxin [8]. According to this method, endotoxins are quantified by the Limulus Amoebocyte Lysate (LAL) assay, preferably by the kinetic chromogenic method, for endotoxin determination in the particulate matter of the inhalable aerosol particle fraction, collected on filters and extracted in water. The LAL assay has been the most used assay for endotoxin analyses in the past. It is a biological test which is based on the activation of factor C of the enzymatic coagulation

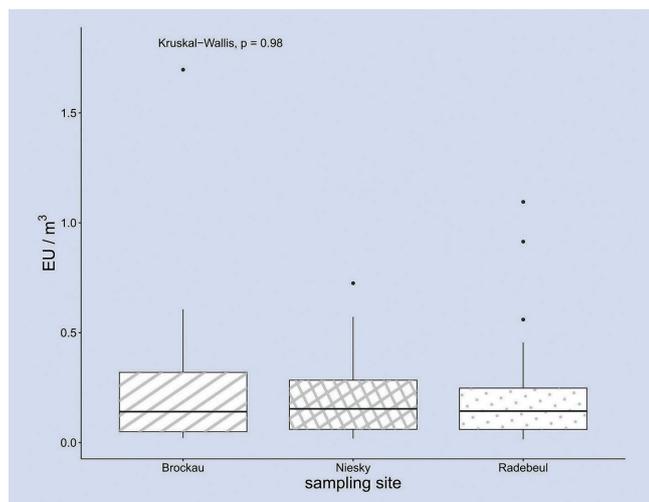


Figure 1 Endotoxin concentrations in outdoor air sampled at different sampling spots in the course of one year (n(Brockau, Niesky) = 24, n(Radebeul) = 23). Graphic: Authors

cascade in the lysate of amoebocytes of the horseshoe crab *Limulus polyphemus* in presence of endotoxin [9]. Results are expressed as Endotoxin Units (EU) by comparing the reaction of an unknown sample with a defined amount of Control Standard Endotoxin (CSE) from *E. coli*. IFA-Arbeitsmappe 9450 recommends for the assessment of endotoxin load in the inhalable dust fraction at workplaces a background endotoxin measurement in the environment with the same methods as used at the workplace [8]. In contrast, in Germany there are currently no guidelines for sampling and analyses of endotoxin concentration in ambient air at environmental settings e.g. in PM10. However, VDI 2463 Part 11 and DIN EN 12341 describe a gravimetric measurement for the determination of particulate matter in ambient air after sampling on filters with high volume samplers which probably could be the basis for endotoxin analyses [10; 11]. VDI 2463 Part 11 has been withdrawn recently and the sampling method is now described in VDI 2463 Part 2 [12].

In this study the PM10 particle fraction was sampled with high volume samplers on filters over a period of one year at three rural sampling spots in a monitoring network and endotoxins are determined in the dust extracted from filters. The aim of this study is to test whether the applied methods are suitable for a new guideline for measurement of endotoxin concentrations in ambient air.

2 Methods

2.1 Sampling

Three different sampling spots in Saxony, Germany, were chosen: Radebeul (51°7'10" N, 13°40'30" E, 246 m AMSL), Brockau (50°36'29" N, 12°12'40" E, 430 m AMSL) and Niesky (51°17'7" N, 14°44'59" E, 148 m AMSL). These sampling spots belong to the air monitoring network of the Saxon State Office for Environment, Agriculture and Geology [13]. Brockau and Niesky are defined as rural, Radebeul as rural and peri-urban. In a 500 m radius the land-use around the sampling spots is mainly dominated by agriculture, residential buildings and recreational areas. The volume of traffic in a 100 m radius is <2,000 vehicles/day, <100 vehicles/day and no traffic for Brockau, Niesky and Radebeul, respectively. According Zensus 2011 Atlas the popula-

tion density in a 500 m radius around the sampling spots for Brockau is <250 residents, in Niesky <150 residents and in Radebeul <600 residents [14]. In terms of routine air monitoring these sampling spots are regarded as background monitoring spots. In the course of one year (between September 2018 and September 2019) approximately every two weeks PM10 samples were taken with a high volume sampler (HVS, DIGITEL DHA-80, Fa. Riemer Messtechnik, Hausen/Röhn) at a flow rate of 500 L/min for 24 h according to Annex B.2.2.1 of DIN EN 12341 and VDI 2463 Part 11, in parallel to routine monitoring of PM10, NO_x, NO, NO₂ and ozone as well as monitoring of meteorological parameters (temperature, humidity, radiation, air pressure, wind speed) [10; 11; 15 to 22]. Daily data for rain fall and snow height were obtained from the German National Meteorological Service from monitoring sites <9 km distance from the bioaerosol monitoring sites (Station ID: 7329, 1693, 7423) [23; 24]. Glass fibre filters (MN85/90 BF, Fa. MACHEREY-NAGEL, Düren) used for aerosol particle sampling were heated beforehand at 180 °C for 3 h and transported sterile to the sampler. After sampling the filters remained in the sampler for 8 to 10 days before being transported back to the laboratory where they were stored in a desiccator (room temperature; relative humidity ≤ 25 %) for 3 to 20 days until analysis.

2.2 Determination of endotoxins

For ease of use, the filters were cut into small pieces before analysis. After cutting the filters the filter pieces were suspended in 50 ml pyrogen-free water (Fa. ACC Europe, Mörfelden-Walldorf). The analysis of endotoxins has been performed according to IFA-Arbeitsmappe 9450 [8] using the kinetic chromogenic *Limulus* Amoebocyte Lysate (LAL) assay (Pyrochrome®, Fa. ACC Europe, Mörfelden-Walldorf) according to the manufacturer's instructions in a microplate reader in absorption mode (Biotek Synergy H1 with Gen5 software, Fa. BioTek Instruments, Bad Friedrichshall) (in duplicates). According to manufacturer's specifications, we can quantify in the range of 0.005–50 EU/ml in aqueous solution. The detection limit in air is as follows:

$$LOD \text{ in air } \left[\frac{EU}{m^3} \right] = \frac{0.005 \frac{EU}{ml} * 50 ml}{720 m^3} = 0.00035 \frac{EU}{m^3}$$

Statistical Analyses/Visualisation

All statistical analyses were conducted in R version 4.0.3 [25]. For plotting group comparisons ggpubr version and ggforce were used [26; 27]. The Kruskal-Wallis test was used for multiple group comparisons and Wilcox test with Benjamin & Hochberg adjustment for post hoc pairwise comparisons. Correlation analysis was carried out with Hmisc and corrplot [28; 29].

3 Results

Over the course of one year PM10 samples were taken for 24 h approximately every two weeks at three different sampling spots (Brockau, Niesky, Radebeul) and investigated for endotoxin concentration. All 71 samples were above the quantification limit of 0.00035 EU/m³. Annual median endotoxin concentration over all samples was 0.143 EU/m³. Between different sampling spots no significant differences in endotoxin concentrations were observed (Figure 1). In contrast, endotoxin concentrations of

different seasons varied statistically significant (**Figure 2**) with an obvious seasonal course. Seasonal median concentrations of 0.131, 0.186, 0.297 and 0.050 EU/m³ were observed in spring (March to May), summer (June to August), autumn (September to November) and winter (December to February), respectively. Endotoxin concentration correlates weakly with relative humidity ($r_s = -0.35$, $p < 0.01$), temperature ($r_s = 0.34$, $p < 0.01$), PM10 ($r_s = 0.27$, $p < 0.05$) and global radiation ($r_s = 0.28$, $p < 0.05$) (data not shown).

4 Discussion

In the present study endotoxin concentrations varied between 0.015 to 1.7 EU/m³ in the PM10 fraction with annual median concentrations of 0.14 EU/m³ at three different sampling spots in rural areas in Saxony, Germany. These results are comparable to the few previously published data. Mean and median concentrations of 0.051 to 1.424 EU/m³ e.g. have been reported after sampling of the PM10 fraction of ambient air in rural surroundings [30 to 35]. The above mentioned sampling campaigns varied in respect to sampling device, flow rates (2 to 1,270 l/min), sampling durations (0.33 h to one week), sampling heights from the ground (1 m to five story building), sampling intervals (months, seasons, years) and country in which they were conducted. For livestock dense areas mean endotoxin concentrations of 0.46 to 0.66 EU/m³ have been observed in the PM10 fraction of outdoor air [31]. *Mahapatra et al.* (2018) measured endotoxin concentrations of 0.29 to 0.53 EU/m³ in the PM10 fraction of outdoor air in rural or semi-rural regions of the Hindu Kush Himalayan region of Nepal [32]. *Kolk et al.* investigated background endotoxin concentrations related to workplace investigations from 1999 to 2007, that are recorded in the exposure database MEGA [36]. They detected monthly median endotoxin concentrations between 0.75 and 1.99 EU/m³ and monthly arithmetic mean endotoxin concentrations between 1.99 and 17.22 EU/m³ in the inhalable aerosol fraction in background air. Moreover, annual median endotoxin concentration of 2.9 EU/m³ were measured in the outdoor air at an agricultural field in the inhalable aerosol fraction [7]. So, the higher concentrations found in the inhalable aerosol fraction by *Kolk* and co-workers [36] as well as *Madsen* [7] may be explained by sampled and investigated particle fraction. The physical sampling efficiency of both systems is quite different.

In addition, it is difficult to compare measurement results from sampling with different sampling durations. Moreover, storage in the sampler might have an influence on the endotoxin concentration. It can be speculated that humidity and temperature influences during the sampling or storage period have an effect on the chemical state of the endotoxins. For example, the formation of endotoxin monomers instead of endotoxin aggregates leads to lower activity in LAL assay [37]. In previous studies several other factors influencing endotoxin measurements have been revealed. The sampling device and fraction are important determinants in endotoxin measurements [30; 38]. Therefore, it is necessary to define convention-related parameters within the scope of standardization.

In this measuring campaign significantly lower endotoxin levels were observed in winter compared to spring, summer and autumn. Furthermore, endotoxin concentration was significantly lower in spring compared to autumn. Similarly, in previous stu-

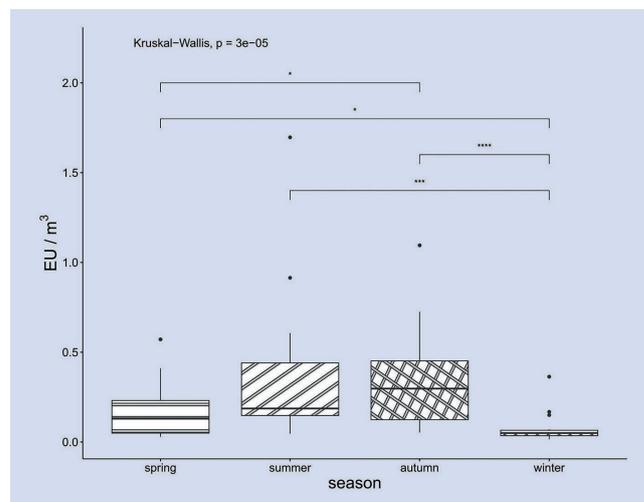


Figure 2 Endotoxin concentrations in outdoor air at different seasons (n(spring) = 17, n(summer, autumn, winter) = 18). Results of each season contain data from three sampling spots. Mean comparisons showed significant differences between endotoxin concentrations in different seasons (* $p \leq 0.05$; *** $p \leq 0.001$; **** $p \leq 0.0001$). Graphic: Authors

dies lowest endotoxin concentrations in outdoor air have been detected in winter [7; 30; 34; 39 to 42]. Soil and plants can serve as substrate for gram-negative bacteria, e.g. *Enterobacter* and *Pseudomonas*, and are therefore a potential source for outdoor atmospheric endotoxins [43; 44]. Moreover, pollen may act as vector for airborne endotoxins [45]. Although most pollen's sizes lay above the PM10 cut-off it cannot be excluded that a certain amount of pollen or parts of pollen is still deposited on the filter. Given the higher vegetational activity in spring, summer and autumn the observed seasonal pattern might not be so surprising. In addition (1,3)- β -D-glucans can lead to false positives in the LAL assay [46]. (1,3)- β -D-glucans are cell wall components of the fungal cell wall. Concentrations of fungi in outdoor air are also highest during summer and fall and lowest in winter and spring [47]. So the seasonal concentrations of airborne fungi might influence the LAL activity. Furthermore, higher endotoxin levels in summer might be connected to higher agricultural activities. Often in rural areas a high density of farming facilities, agricultural fields and organic waste or wastewater treatment plants can be found. All of these facilities can act as source for airborne endotoxins and sampling in their vicinity might lead to higher endotoxin levels [39; 41; 48; 49].

The number of factors possibly having an influence on endotoxin background measurements in outdoor air clearly shows the necessity for standardization. The combination of a standard environmental dust analysis according to VDI 2463 Blatt 2 with the method 9450 for workplace measurements of endotoxins presented here has been a first successful attempt. ■

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