Endotoxin exposure assessment - measurement and characterization

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S. Spaan, 2008
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Thesis Utrecht University
ISBN: 978-90-393-4923-6

Cover by Marije Brouwer and Lisanne van der Voort, Multimedia Centre Veterinary Medicine, Utrecht University
Lay-out by Harry Otter, Multimedia Centre Veterinary Medicine, Utrecht University
Printed by Ridderprint Offsetdrukkerij BV, Ridderkerk, the Netherlands

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Beoordeling van blootstelling aan endotoxinen - monstername en karakterisering

(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht
op gezag van de rector magnificus, prof.dr. J.C. Stoof,
ingevolge het besluit van het college voor promoties
in het openbaar te verdedigen op dinsdag 11 november 2008
des middags te 2:30 uur

door

Suzanne Spaan

geboren op 23 februari 1979 te Hoorn

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Contents

Abstract

Endotoxin, one of the specific agents in organic dust that cause health effects, is part of the cell wall of Gram-negative bacteria. Gram-negative bacteria are present on for instance the surfaces of plants and in animal feces, and their occurrence, growth and amplification is influenced by many factors. As a consequence, endotoxin levels may be highly variable and occurs exposure to endotoxin in various industries. Inhalation exposure is thought to be the major route of exposure.

Endotoxin exposure was investigated among workers of various agricultural industries and sewage treatment plants. Exposure was found to be generally high in agricultural industries, and moderate to low in sewage treatment workers. These studies were combined with eight other exposure studies in a database, which showed that endotoxin exposure was highly variable. In general more variability between workers than with workers (from day to day) was found. Several determinants of exposure could be determined. For example, dustiness of the product, contact with animals or plant material and bulk production were associated with higher endotoxin exposure, which 'wet' processes were associated with lower exposure levels. Information about exposure levels and variance components were input for the formulation of a measurement strategy for the assessment of endotoxin exposure. This strategy has a tiered approach, and every phase can result in a conclusion that the exposure situation is acceptable, advice on control measures or a more precise estimation of the exposure situation. The emphasis is on control measures when the circumstances indicate (elevated) endotoxin exposure.

For exposure assessment and possible comparison with an occupational exposure limit (OEL) the procedure for measurement and analysis of airborne endotoxin is very important, and preferably standardized. Existing gaps in European guidelines were explored and investigated by means of full experimental designs and the use of parallel collected inhalable dust samples, in which the effects on and interactions with airborne endotoxin concentration of filter type, transport conditions, sample storage, extraction solution, extract storage and assay solution were investigated in several working environments. This led to the recommendation to use glass-fiber filters, transport with desiccation, frozen sample storage, extraction in PFW with 0.05% Tween-20 with rocking/shaking, frozen storage of extracts, and dilution and LAL analysis in PFW. With this information a protocol for standardization of endotoxin exposure assessment can be fully specified.

Although it is yet unknown whether endotoxin will be part of the public or private part of the current (new) Dutch occupational exposure limit system, both the government and industry can use the intended health-based recommended OEL of the Health Council, which is a tool for controlling exposure and reduce the risk of health effects for workers. Standardization of methods is important to be able to compare results and for compliance testing, and this thesis provides information on some of the issues around introducing an OEL for endotoxin. Generally, one should be aware that endotoxin exposure poses a potential threat to the working population, which should be rendered into monitoring and controlling this exposure.

Chapter 1

General introduction

Organic dust

Several terms, like organic dusts and bioaerosols, are used in literature to refer to plant, animal and microbial matter. Organic dust or bioaerosols are ubiquitous in the environment and form a complex mixture of pathogenic or non-pathogenic live or dead bacteria, fungi and viruses, high molecular weight allergens, bacterial endotoxins, mycotoxins, peptidoglycans, β(1→3)-glucans, pollen, plant fibers, etc. Microorganisms often form an important component of organic dust, even when little or no plant or animal material is present. Under normal conditions, organic dust does not pose a threat to health, but when they are aerosolized and inhaled in concentrations that overload the defense systems of the human body, exposure can result in disease.[13,15,26,47] Dust has the potential to cause (lung) disease if the particles are small enough to penetrate the respiratory organ, the agents are water or lipid soluble, and inflammatory reactions occur at realistic concentrations of the agent.[45] Many people worldwide are exposed to (high levels of) organic dusts during work and in their home environments.[47]

To cause an effect organic dust must be aerosolized, which can be achieved by e.g. movement of humans or animals, machinery or ventilation air. The particle size range is known to be wide, but smaller particles are always an important fraction. Exposure to organic dust containing microorganisms may lead to infectious diseases (like tuberculosis or measles in health care workers, influenza in workers clustered in the workplace, Legionnaires disease, and cattle-associated leptospirosis in farmers), respiratory diseases (non-allergic asthma or rhinitis, chronic bronchitis, organic dust toxic syndrome (ODTS), a systemic flu-like reaction, or allergic asthma, rhinitis, hypersensitivity pneumonitis), and cancer.[13,47] Non-infectious diseases have received most attention and these are usually caused by biologically active, specific agents in the dust, which may either be a part of the dust particle itself or a contamination on the dust particle. Several causative agents have been identified, like bacterial endotoxin, the cell wall component (1→3)-β-D-glucan of fungi, urinary proteins from animals, aflatoxin (in case of cancer) and bacterial enzymes.[12,13,26,46,47,66]

Endotoxin

Endotoxins are components of the cell walls of Gram-negative bacteria and some blue-green algae, which are both common in nature. Their cell walls can be divided into three layers or membranes.[43] Endotoxins are an integral part of the outer layer and are composed of proteins, lipids and lipopolysaccharides (LPS) or lipooligosaccharides (LOS).[60] The term ‘endotoxin’ refers to the toxin present, which is often liberated as a result of cell lysis. The terms ‘endotoxin’ and ‘lipopolysaccharide’ or ‘lipooligosaccharide’ are often used interchangeably. However, ‘endotoxin’ should be used to refer to the fragments of the Gram-negative bacterial cell wall that contain lipopolysaccharide as well as all other naturally occurring compounds in the cell wall. ‘Lipopolysaccharide’ should be reserved for the chemically pure substances.[36] LOS is structurally different from LPS and originates from other species.[22] In this thesis, the focus is only on endotoxin and the LPS part thereof; LOS is not considered.

LPS from Gram-negative bacteria is an amphiphilic and heat-stable macromolecule responsible for most of the biological properties of bacterial endotoxins. It consists of a hydrophilic polysaccharide part and a covalently bound hydrophobic lipid component (‘lipid A’, a phosphoglycolipid). In water, LPS molecules aggregate with the Lipid A part shielded in micelles and the polar oligo- or polysaccharide facing the aqueous environment, making

them water-soluble. The polysaccharide part can be divided into two domains, the core region (sugars) and O-specific side chain. The O-specific chain shows the greatest structural diversity of all molecular components of LPS, and is composed of a sequence of identical polysaccharides. The core region is divided into the inner core, attached to lipid A, and the outer core, attached to the O-specific polysaccharide. Structural variation of the core region within bacterial species tends to be low. The lipid A component anchors LPS in the outer layer of the cell wall and shows the least variation of all components of LPS in all bacterial families.[20,42] Toxicity is associated with the lipid A component, whereas the immunogenicity is associated with the polysaccharide component of the LPS-molecule.[9,60]

Gram-negative bacteria are commonly present in various environments, for example on the surfaces of plants and in animal feces. They also inhabit the oral cavities and intestinal tracts of humans and animals.[50] The occurrence of Gram-negative bacteria in the environment is influenced by environmental factors like substrate availability, temperature and water activity. As a consequence, endotoxin levels may be highly variable in soil, air and water. Based on the concentrations of bacteria and endotoxins, levels of contamination in environments can be divided into four main categories, namely high contamination (like livestock barns, harvesting crop, agricultural transfer and processing, processes supporting active microbial growth), moderate contamination (like food processing, cotton textile industry, industries with machines using metal working fluids and domestic environments with significant sources), relatively low contamination (like medical and dental clinics, office environments, homes and schools) and deliberate very low contamination (like industrial clean rooms, pharmaceutical manufacturing areas, surgical suites and some research laboratories).[59]
For (occupational) health effects mainly airborne endotoxins seem relevant. Endotoxins become airborne during manufacturing or handling of organic materials and therefore endotoxin exposure is most prevalent in agricultural and related industries, although air humidifiers in buildings, recycled industrial process water or wash water and industrial oil emulsions may also be an important source of airborne endotoxin exposure. In many industries, endotoxin exposure is closely associated with exposure to organic dust.

Effects of endotoxin exposure

Inhalation exposure is thought to be the most important route of exposure. Airborne aerosols or dust particles containing, amongst others, bacterial components such as endotoxins are of a size that can deposit at each level of the respiratory tract. Endotoxins have been found in both inhalable (penetrating the respiratory system) and respirable (penetrating the alveolar region) dust fractions, but are predominantly measured in the inhalable fraction.[11,38] Whole bacteria and aggregated fragments, if deposited in the trachea and large bronchi, are eliminated by mucociliary transport or through phagocytosis and enzymatic digestion by macrophages. Smaller particles deposit in the deeper airways like small bronchi, bronchioli and alveoli, where endotoxin can generate its (inflammatory) effects. This may alter the para-cellular and trans-cellular permeability of the epithelium, allowing endotoxins or other toxins in organic dust to cross this barrier. Furthermore, several mechanisms can liberate inactive cell-bound endotoxins deposited in the lungs, which then become biologically active: 1) during lysis of bacteria by complement or antibiotics, 2) during phagocytosis of bacteria by macrophages and polymorphonuclear leucocytes (PMN), which results in both phagocyte-bound endotoxins and free endotoxins with similar or even increased toxicity, and 3) during reproduction of bacteria.[21] The responses to endotoxins are not induced directly, but are

rather mediated by immune modulator molecules such as tumor necrosis factor α (TNFα), members of the interleukin family (IL-1, IL-6, IL-8, IL-12), interferon γ, reduced oxygen species, and lipids. These mediators are released mainly by monocytes or macrophages, but also other cells participate in the response to LPS, for instance vascular cells, polymorphonuclear cells and T-cells.[20,21] The individual immunological response to endotoxin is the result of a complex interaction between dose, timing of exposure, effects of other environmental factors and genetic predisposition.[61]

First attention for endotoxin exposure in the workplace

The concept that organic dust exposure is related to health effects goes back to 1555.[31] Neal et al. were probably the first to relate exposure to microorganisms present on cotton with byssinosis, an occupational lung disorder in cotton workers.[37] The first recognition of the role of endotoxin in this so called 'Monday morning malaise' in cotton workers was probably in the sixties.[5,39] In following years, endotoxin from Gram-negative bacteria, especially from *Enterobacter agglomerans*, which are in high amounts present on cotton fibers and plant material, was considered to play an important role in the etiology of byssinosis.[3,17,44] Inhalation of cotton, flax or hemp dust is commonly observed in textile workers.

Health effects

Since then, positive but mostly negative health effects of endotoxin exposure have been described.[30,41] Both experimental and field studies have shown several negative health effects induced by endotoxin exposure,[29,50] which can be divided into:

- Inflammatory reactions in the airways, which may lead to respiratory symptoms like dry cough, shortness of breath and wheeze, clinical conditions such as non-allergic asthma, followed by accelerated lung function decline, and byssinosis in cotton workers.[64]
- Systemic reactions, resulting in a range of symptoms including fever, shivering, joint aches, myalgia, malaise and other influenza-like symptoms usually referred to as organic dust toxic syndrome (ODTS).[52,54,57]

Protective effects have more recently been described in relation with especially environmental, but also occupational endotoxin exposure and the development of allergy.[14,40,63] In a recent study, an inverse relationship between occupational endotoxin exposure and risk of atopic sensitization in adults has been found.[58] However, the protective effect with regard to allergy was paralleled by an increased risk of wheeze and bronchial hyper-responsiveness in non-atopics. This applies partly to the children studies as well. Furthermore, protective effects with regard to cancer risk have been described recently,[1,25,27,32] although the experimental evidence for a relationship between endotoxin exposure and cancer is limited and by no means consistent.

Neutrophil mediated airway inflammation has been observed in naïve, healthy subjects and swine confinement workers after exposure that is limited to periods of a few hours.[28,33,51,65] Experimental studies have shown a dose-related acute lung function decline after endotoxin exposure in cotton dust,[4,17,49] in which no-observed effect levels (NOELs) of 33 ng/m^3 (~330 endotoxin units (EU)/m³) for already occupational exposed cotton workers [49] and 9 ng/m^3 (~90 EU/m³) for FEV_1 (forced expiratory volume in one minute) in healthy naïve subjects [4] were found. Long-term exposure may have caused tolerance for acute effects, obscuring the actual dose-response relationship,[16] which may in part explain the differences in NOELs.

Several (epidemiological) field studies among cotton workers,[2,23,48,64] animal feed workers,[55,56] pig farmers,[10,18,62] potato processing workers [67] and glass fiber manufacturing workers [34,35] showed acute and chronic adverse respiratory health effects of airborne

endotoxin exposure, although the measured endotoxin levels and NOELs varied. The (chronic) lung function effects were more clearly associated with endotoxin exposure than with dust exposure.
Both the experimental and the epidemiological studies strongly support the hypothesis that inhaled endotoxin has a causative role in the etiology of occupational related (chronic) respiratory diseases in a dose-dependent way. However, because organic dusts are heterogeneous mixtures of a great variety of components, the presumed endotoxin effect could be dependent on other toxins present in the dust, which vary by origin. In addition, other factors like individual susceptibility [24,53] and effect modification may be of importance as well.

Proposed guidelines for no-effect levels for environmental endotoxin
Experimental as well as epidemiologic studies suggest that the various diseases caused by endotoxins are related to different levels of exposure. Furthermore, large inter-individual variation in sensitivity to endotoxin exposure exists. Based on the relationship between pure endotoxin and environmental endotoxin (1 µg/m³ environmental endotoxin ~ 10 µg pure endotoxin (species not specified)), values for persons with a history of atopy or asthma, and a certain analytical procedure, the following NOELs for environmental endotoxin have been proposed:[50]

- airways inflammation 10 ng/m³ (~100 EU/m³)
- systemic effects 100 ng/m³ (~1000 EU/m³)
- toxic pneumonitis (ODTS) 200 ng/m³ (~ 2000 EU/m³)

Occupational exposure limit – the Dutch situation

Although it is generally recognized that endotoxins pose a threat to a substantial part of the (working) population, no occupational exposure limit (OEL) has been introduced yet, which makes regulation of endotoxin exposure difficult.
In the Netherlands, an Expert Committee of the National Health Council (DECOS) has suggested a health-based recommended occupational exposure limit (HBROEL) for endotoxin of 50 EU/m^3 (~5 ng/m^3) in 1998.[9,19] This proposal was primarily based upon a study of Castellan et al., who showed a no-effect level of 90 EU/m^3 for selected sensitive healthy subjects in a mock 6-hr work exposure.[3,4] After applying assessment factors for differences in exposure duration and susceptibility between individuals, a HBROEL of 50 EU/m³ was proposed, relative to international reference standard endotoxin (RSE). Subsequently, the Committee of the Dutch Social and Economic Council (SER) responsible for OEL's requested branch organizations to put relevant information considering the feasibility of this proposed HBROEL at their disposal. Based on the received information the SER concluded that immediate introduction of the HBROEL in the short run would result in major financial and technical problems for the branches involved. Nevertheless, attention for the subject was thought to be of importance to tackle the problem and to prevent very high exposures. Therefore, a temporary legal limit for endotoxin of 200 EU/m^3 over an 8-hr work shift was proposed at the end of June 2000, with a transitional period of 6 months to enable the relevant industries to comply with this legal limit. It was also advised, if feasible, to further lower this legal limit of 200 EU/m³ to 50 EU/m³ within two years. The introduction of this legal limit by the Ministry of Social Affairs and Employment was postponed from July 2001 to January 2003, one year after publication of a guidance concerning the measurement method for endotoxin exposure by the European Committee for Standardization (CEN). This delay

would enable organizations to screen year-round exposure levels and if necessary take adequate control measures. Meanwhile, based on information gathered by the sectors involved, the SER committee concluded that introduction of a legal limit was not feasible, from a technical as well as a social-economical point of view. Based on this advice, the Ministry withdrew the temporary legal limit for endotoxin exposure in July 2003, with the obligation that within six months, before January 1st 2004, the sectors involved should formulate a strategy to approach endotoxin exposure in the work environment under the following conditions:

- Within six months, organizations involved had to describe the approach to minimize exposure to endotoxins. Apart from the application and possibly development of (adjusted) control measures, this plan also provided in investigating the state of the art with regard to control measures and instruction. The execution of this method of approach, in which a covenant was used as an instrument, was expected to take 2-4 years, depending on the sector. The parties were obliged to report to the committee about the method of approach taken and they had to regularly inform about the execution and progress of the plan. The committee used the received information in due course for her advice.
- The new measurement method, enclosed in the CEN guidance 14031 (Determination of airborne endotoxin) [8] in combination with CEN 13098 (Guidelines for measurement of airborne microorganisms and endotoxin),[7] should be introduced and applied in a proper way. The Ministry ought to take the right steps so that the method could be introduced as a standardized method and make quality demands on the laboratories performing the endotoxin measurements and analyses. Only then a measured endotoxin concentration can be compared with an exposure limit.
- The current measurement strategy, CEN 689,[6] which is formulated for chemical substances, should be made suitable for the problems concerning exposure to biological agents, endotoxins in particular.

Furthermore, the Health Council was asked to consider whether the (proposed) HBROEL of 50 EU/m³ should be adjusted on the basis of new information, of which the report is due the end of 2008.

Aim and outline of the thesis

This thesis aimed to provide information on some of the major issues around introducing an OEL for endotoxin exposure in the Netherlands. The main objectives of this study were to give insight in levels of and variability in endotoxin exposure and to optimize the measurement and analysis of airborne endotoxin. To address this aim, the following three subjects were distinguished:

- To investigate levels of inhalable dust and endotoxin exposure in several industries, namely agricultural industries (chapter 2.1) and sewage treatment (chapter 2.2), as well as exploring determinants of exposure (chapter 2 and 3.1) and comparing techniques for the measurement of microbial load (chapter 2.2)
- To study inhalable dust and endotoxin exposure variability (chapter 3.1), in order to adapt the existing measurement strategy for chemical agents (CEN 689) for endotoxin exposure assessment (chapter 3.2)
- To investigate the gaps present in the CEN guidance for measurement of endotoxins (CEN 13098 and CEN 14031) to give input for a standardized protocol for the measurement and analysis of endotoxin exposure (chapter 4).

Finally, in the general discussion (chapter 5), the main findings of the studies presented in this thesis are discussed in context of previous studies, implications of the outcomes and recent insights.

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Chapter 2

Exploring endotoxin exposure

Chapter 2.1

Exposure to inhalable dust and endotoxins in agricultural industries

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Published in Journal of Environmental Monitoring, 2006; 8 (1): 63-72. *Reproduced by permission of The Royal Society of Chemistry.*

Abstract

Endotoxin is a well-known bacterial toxin that causes several health effects. Animal feces and plant materials contaminated with bacteria have been identified as important determinants of organic dust related endotoxin exposure. Although high exposure to organic dust and endotoxins has been described regularly in agricultural industries, a detailed overview of levels of airborne exposure to endotoxins in the agricultural industry, as well as a systematic comparison between several specific branches using the same exposure assessment protocols are lacking. In this study, personal endotoxin exposure in a broad spectrum of agricultural industries was investigated and possible determinants of exposure were explored.

601 personal inhalable dust samples were taken in 46 companies of three agricultural industrial sectors: grains, seeds & legumes sector (GSL), horticulture sector (HC) and animal production sector (AP), with 350 participating employees. Dust and endotoxin levels were determined gravimetrically and by using the *Limulus* Amoebocyte Lysate (LAL) assay, respectively. Basic descriptive analysis and elaborate analysis of variance were performed.

Mean exposure levels were high, with large differences between sectors and between companies within the sectors. Highest dust and endotoxin exposures were found in companies of the GSL sector. In all three sectors exposure was higher in the primary production part compared to the (industrial) products processing part of the sector. The Dutch proposed health based occupational exposure limit (50 EU/m^3) and temporary legal limit (200 EU/m^3) for endotoxin were often exceeded. Differences in exposure between workers were larger than the day-to-day variability. Identified determinants increasing exposure levels were company, dustiness of the product and contact with animals/feces. 'Wet' processes resulted in less dusty working environments and thus lowered endotoxin exposure.

Overall, exposure to endotoxins over the whole range of agricultural industries is high. A 10-1000 fold reduction in exposure is needed to reduce endotoxin related health risks.

Background

Many workers in the agricultural industry are exposed to organic dusts, which are known to be harmful to the respiratory tract. Endotoxins are ubiquitous contaminants of organic dusts and are probably a major causative agent in health problems associated with organic dust exposure.[24,25,38,39] Endotoxins are chemically complex constituents of the outer membrane of Gram-negative bacteria and airborne endotoxins are directly related to the occurrence of these bacteria. During cell growth and after cell death lysis occurs, resulting in the release of endotoxins into the environment. Lipopolysaccharides (LPS) are responsible for most of the biologic properties characteristic of bacterial endotoxins.[28,36] Animal faeces and plant materials contaminated with bacteria are known important determinants of organic-dust-related endotoxin exposure.[19] Microbiological growth can occur during culturing, processing, storage, and transport of agricultural products, under specific conditions in which bacteria thrive well. High occupational endotoxin exposure is therefore prevalent in agricultural and related industries.[11,14,19,24,25]

Inhalation is thought to be the major route of endotoxin exposure in the working environment. Inhaled endotoxin causes respiratory and systemic inflammatory responses. Acute symptoms after inhalation of high levels of endotoxin are dry cough and shortness of breath, accompanied by a decrease in lung function, fever reactions, shivering and malaise. Dyspnoea, headache and joint aches may also occur a few hours after exposure. Furthermore, epidemiological studies suggest that chronic exposure, to on average much lower levels, may lead to accelerated lung function decline and Chronic Obstructive Pulmonary Disease (COPD).[18,37,40,48] On the other hand, recent literature has suggested a possible protective effect of environmental and occupational endotoxin exposure on the risk of atopic sensitization.[26,30,50]

In The Netherlands, the Dutch Expert Committee on Occupational Standards has recommended a health based exposure limit of 50 EU/m³ for exposure to airborne endotoxin in the working environment, averaged over an 8-hour working day. Several studies, experimental as well as epidemiological, have shown that endotoxins can cause respiratory effects at concentrations around this standard (50-100 EU/m^3).[4,8,52]

During the 80's and begin 90's, exposure to endotoxin has been investigated in several agricultural industries. However, comparison of exposure levels in the agricultural industry at large is difficult, as only certain branches have been investigated. Additionally, most studies were performed by different laboratories using different measurement and analytical techniques. More importantly, most studies comprised small measurement series and important information about sampling and analytic methods was either lacking or differed between studies.[3,5,7,9,17,20,27,29,41,47] For example, measured dust fractions differed between studies or were unknown, and not always personal exposure measurements were performed. Some large scaled studies are available for specific agricultural industries like pig farming, dairy barns, animal feed industry and potato industry, but studies were limited by investigating only one industry at the time.[1,23,32,45,46,51] Only twice comparison of endotoxin exposure in a limited number of different industries have been reported.[33,44]
Therefore, this study investigated exposure to endotoxins in a broad spectrum of agricultural industries, using personal exposure measurements and similar sampling and analytical methods. Results are compared with proposed exposure limits, and possible determinants of exposure are explored.

Materials and Methods

Study population and design

This study was conducted in a total of 46 companies in the Netherlands, with collaboration of three national employers' organizations: 'Grains, Seeds and Legumes sector' (GSL, 14 companies), 'Horticulture sector' (HC, 21 companies) and 'Animal Production sector' (AP, 11 companies) (Table 1). The GSL sector exists of the culturing, harvesting, (industrial) processing and trade of grains, seeds, legumes, derivatives and related products. The HC sector contains indoor nurseries and outdoor culturing of flowers, vegetables and plants, preparation and trade of mushroom compost, and industrial processing and trade of horticulture products. The AP sector consists of production of dairy products, meat and eggs on farms and the (industrial) processing of these products, with emphasis on abattoirs.

Representative companies within the relevant sectors were contacted to participate in the study. During the selection procedure companies with technology that reflected future trends were preferred, which led to a bias in favor of more modern companies. Furthermore, measurements were partly performed during selected activities when exposure was expected to be high, for example during cyclic activities like harvesting, based on information from previous studies and literature.

The study was conducted over a 10-month period (December 2001 - September 2002).

In principle all workers of a company were included in the study. In large companies (> 10 employees), 10 subjects were selected to be included in the study. Selection was based on relevant work areas and jobs in the companies to obtain a representative overview of exposure to organic dust and endotoxin during a typical work shift for each industry. Sampling was performed on two days, in most companies on two consecutive days with, as many repeated measurements as possible, depending on the availability and willingness of workers. In total 350 workers participated and 601 measurements were collected, of which 251 twice repeated measurements on one subject. Mean sampling time was 7.3 hours (range 1.8 – 10.1 hours).

Exposure measurements

Full-shift personal inhalable dust samples were collected using Gilian Gilair5 portable constant-flow pumps at a flow rate of 3.5 L/min, in combination with conductive plastic conical inhalable samplers (CIS), manufactured after example of the German GSP (JS Holdings, UK). Samplers were equipped with 37 mm glass fiber filters (Whatman GF/A, UK). These sampling heads are less sensitive to changes in wind speed, an important factor when measuring in open air. Moreover, they maintain adequate performance, and sample in agreement with the inhalable dust convention.[21] The sampling head was placed on the shoulder of the worker, near the breathing zone, with the inlet facing forward. Each sampling day a control filter was included. Dust samples were stored at -20ºC after collection until further processing. Duration of storage ranged from a week until a few months because extractions and analyses were performed after collection of all samples. A previous study showed for house dust that storage at -20ºC before extraction does not affect endotoxin concentration.[15] Even so, storage of extracts for several months at -20ºC before analysis does not affect endotoxin concentrations.[12,15] although repeated freeze and thaw cycles of extracts lower endotoxin concentrations.[12,35] In this study extracts were stored in several aliquots and each aliquot is used only once to avoid repeatedly freezing of extracts.

The amount of dust on filters was determined gravimetrically by pre- and post-weighing of filters on an analytical balance in an EPA (US) criteria conditioned room. Extraction was done as described previously, under pyrogen-free conditions.[12] Briefly; filters were immersed in 5 ml 0.05% Tween20 in pyrogen-free water and rocked vigorously for one hour at room temperature. After 15 minutes of centrifugation at 1000G (=2094 rpm), supernatant was harvested and stored in 0.1 ml aliquots at -20ºC until analysis.
Endotoxin concentration in extracts was assayed using a quantitative kinetic chromogenic Limulus Amoebocyte Lysate (LAL) method (BioWhittaker; lotnr. lysate 1L6765, lotnr. standard 2L0090 (RSE/CSE ratio 11.5 EU/ng)).[12] Samples were assayed at an initial dilution of 1:20, and when the measured concentration was too close to the upper detection limit of the assay, retested at higher dilutions up to a maximum of 1:1000. Potential enhancement or inhibition was evaluated by testing samples in serial dilutions, but no significant deviation from parallelity to the calibration line was observed.

Worker and company information
A self-administered checklist was used to obtain information from the workers included in the study on job, job title, workplace, work activities, work environment and use of protective equipment. In each company information about process characteristics and other possible determinants of organic dust and/or endotoxins exposure was gathered by interviewing someone from the executive staff with use of a for this purpose developed checklist.

Statistical analysis
Data were analyzed with SAS statistical software (version 8e; SAS Institute, Cary, NC, USA). Inhalable dust and endotoxin concentrations below the limits of detection (LOD) were assigned a value of two third of the detection limit, which was 0.01 mg in the case of dust. For endotoxin the detection limit varied from 1.3 to 3.0 EU per filter, depending on day of analysis and the plate the analyses were performed on.
Levels of exposure were natural log transformed before statistical analysis. Distributions of dust and endotoxin exposure were examined to ascertain lognormal distributions. Crude descriptive exposure levels were calculated as geometric mean (GM) with geometric standard deviation (GSD) for each sector and company. GM and GSD of sectors and subsectors were used to calculate the chance of exceeding the Dutch occupational exposure limit of nuisance dust (10 mg/m^3), the proposed health based occupational exposure limit for endotoxin (50 EU/m^3), and the as of January 1st 2003 implemented temporary legal limit for endotoxin (200 EU/m^3),[11] as described in Boleij et al.[2] Spearman correlations were calculated between inhalable dust and endotoxin concentrations.

Determinants of exposure were explored by mixed effect analysis of variance in order to correct for possible correlation between repeated measurements.[34] Sector, company and process characteristics or activities were introduced as fixed effects, while worker identity was introduced as a random effect. The mixed-effect models are specified by the following expression: $Y_{ij} = \mu_y + \beta_1 + \ldots + \beta_p + \chi_i + \varepsilon_{ij}$
for i = 1, …, k (workers) and j = 1, …, n_i (repetitions of the ith worker), where Y_{ij} is the log-transformed exposure level. In this model, μ_y represents an overall intercept for the group that corresponds to mean background exposure (log-transformed); β_1, …, β_p are fixed effects; χ_i is the random effects of the ithworker; and ε_{ij} is the random effect of the jth measurement effect of the Ith worker. It is assumed that $\chi_{i(k)}$ and $\varepsilon_{j(ik)}$ are each normally distributed and mutually independent, with zero means and between-worker ($_{bw}\sigma^2$) and within-worker ($_{ww}\sigma^2$) variances. Separate models were constructed for inhalable dust and

endotoxin exposure. Variances are estimated as between-worker and within-worker variance components.

Results

Exposure to inhalable dust and endotoxin

Of the 601 collected samples, 10 dust and 14 endotoxin samples were lost during extraction and analysis. Thus, dust and endotoxin data were available for 591 and 587 samples, respectively. Of these samples, 7 were below the LOD of dust and 49 below the LOD of endotoxin. All control filters resulted in endotoxin concentrations below LOD, thus contamination during mounting of the samplers did not occur. Inhalable dust and endotoxin exposure is summarized in Figures 1&2 and described in more detail in Table 1. The overall geometric mean concentration was 0.8 mg/m^3 for inhalable dust and 230 EU/m^3 for endotoxins, with distinctly more spreading in endotoxin exposure (GSD 4.5 for dust vs. 8.6 for endotoxin). These large variances were also observed within the sector, indicating considerable variation in exposure between workers or between days for a worker in all sectors, especially for endotoxin. Overall, highest mean exposure levels were found in the GSL sector. Dust and endotoxin exposure levels were slightly higher in the HC sector than in the AP industry. However, large differences in exposure were found between companies within each sector.

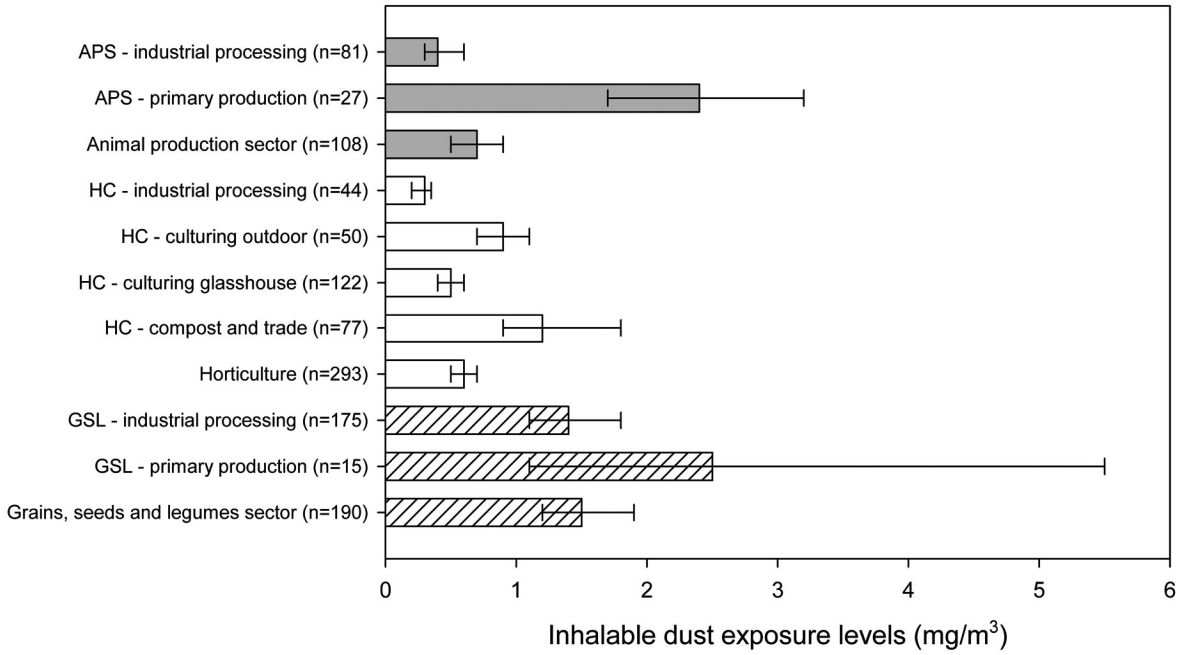

Figure 1: Inhalable dust exposure (GM and 95% CI) levels in three sectors and subsectors of the agricultural industry

In the GSL sector (Table 1A, Figure 1 & 2) a difference between exposure during primary production (culture and harvest of the products) and further industrial processing and trade could be observed. In primary GSL production, endotoxin exposure was high in almost every company and function (GM=2700 EU/m^3). This was due to both a fairly high dust exposure (GM=2.5 mg/m^3), and relatively high amounts of endotoxin per mg dust (Figure 3).

Table 1: Personal geometric mean (GM) and geometric standard deviation (GSD) of endotoxin, inhalable dust and relative amount of endotoxin per mg dust in agricultural industries. Results are expressed overall and per sector

	Endotoxin (EU/m³)			Inhalable dust (mg/m³)			Endotoxin/mg dust	
Sector / *subsector* / company	**N**	**GM (GSD)**	**Range**	**N**	**GM (GSD)**	**Range**	**N**	**GM (GSD)**
OVERALL	587	230 (8.6)	1.6-191430	591	0.8 (4.5)	< 0.1[a]-99	587	270 (4.4)
A. GRAINS, SEEDS AND LEGUMES SECTOR	188	580 (8.5)	2.3-149060	190	1.5 (5.3)	< 0.1-99	188	375 (4.9)
Primary production	*15*	*2700 (4.5)*	*96-41200*	*15*	*2.5 (4.3)*	*0.3-56*	*15*	*1090 (2.9)*
Potato cultivation	2	310 (5.4)	96-1030	2	2.0 (3.9)	0.8-5.2	2	160 (1.4)
Flax culture and processing	10	4470 (3.7)	685-41200	10	4.1 (4.0)	0.6-57	10	1090 (1.7)
Arable farming, grain harvest	3	2100 (2.5)	1032-5790	3	0.5 (2.1)	0.3-1.2	3	3980 (1.6)
(Industrial) processing	*173*	*500 (8.4)*	*2.3-149060*	*175*	*1.4 (5.4)*	*< 0.1-99*	*173*	*340 (4.9)*
Meal/flour tillage and processing	16	280 (7.7)	19-28240	17	1.5 (3.0)	0.2-7.3	16	200 (3.4)
Animal feed industry	20	470 (4.4)	24-4930	20	1.1 (3.7)	< 0.1-7.5	20	520 (2.3)
Grinding industry	17	2810 (4.1)	257-35940	18	2.4 (5.5)	< 0.1-17	17	800 (5.9)
Rice hulling plant	16	1110 (7.6)	95-149060	16	3.1 (6.0)	0.3-80	16	360 (1.9)
Industrial bakery	12	49 (7.4)	2-3030	12	1.2 (3.0)	0.3-11	12	40 (5.5)
Corn processing	14	710 (7.3)	36-30720	14	7.4 (3.6)	0.7-42	14	90 (4.8)
Grain transshipment and derivatives	19	2150 (9.0)	113-131480	19	6.7 (5.1)	0.8-99	19	320 (3.4)
Malting plant	8	3720 (4.3)	291-20030	8	0.7 (1.5)	0.4-1.3	8	5125 (3.0)
Grass drying plant	5	2900 (6.2)	179-20180	5	3.7 (4.0)	0.5-18	5	780 (1.6)
Coffee-roasting plant and tea trading	19	140 (3.4)	12-2030	19	0.7 (2.5)	0.2-2.7	19	200 (3.1)
Sugar production (sugar beets)	27	130 (4.0)	9-2520	27	0.2 (2.7)	< 0.1-1.3	27	575 (3.9)
B. HORTICULTURE	291	170 (6.9)	1.6-191430	293	0.6 (3.7)	< 0.1-35	291	265 (3.8)
Culturing vegetables, flowers and plants (glasshouse)	*120*	*110 (4.3)*	*1.6-4130*	*122*	*0.5 (3.2)*	*< 0.1-11*	*120*	*205 (3.1)*
Mushroom nursery/growing	17	81 (4.0)	3-1350	17	0.2 (4.2)	< 0.1-0.9	17	375 (5.6)
Chicory nursery/growing	19	140 (2.6)	35-770	19	0.8 (1.6)	0.4-2.0	19	165 (2.0)
Cut flowers nursery/growing (tulips)	13	66 (1.9)	30-330	13	0.3 (1.4)	0.2-0.6	13	195 (1.8)
Cut flowers nursery/growing (roses)	18	27 (2.8)	5-180	18	0.3 (1.5)	0.1-0.7	18	90 (2.0)
Pot-plants nursery (ficus)	8	48 (6.7)	2-1490	8	0.3 (2.5)	0.1-2.4	8	155 (3.6)
Tomatoes nursery	10	69 (2.5)	14-340	10	0.8 (1.7)	0.4-1.9	10	83 (1.8)
Cucumber and paprika nursery	14	160 (2.2)	36-650	16	0.3 (6.3)	< 0.1-2.4	14	275 (2.0)
Flower bulbs nursery	15	430 (3.5)	10-1930	15	1.0 (2.1)	0.3-4.1	15	410 (3.0)
Flower bulbs nursery	6	1120 (4.5)	108-4130	6	2.6 (2.3)	1.1-11.4	6	435 (3.3)
Culturing vegetables, flowers and plants (outdoor)	*50*	*110 (2.5)*	*8.6-450*	*50*	*0.9 (2.4)*	*0.1-9.2*	*50*	*120 (2.5)*
Hardy nursery stock and trading	19	130 (1.9)	25-310	19	1.4 (1.8)	0.3-3.2	19	90 (1.5)
Hardy nursery stock	10	110 (2.4)	19-350	10	1.2 (2.8)	0.5-9.2	10	95 (3.2)
Gardening company	5	150 (2.3)	55-450	5	0.9 (1.9)	0.4-1.7	5	175 (1.3)
Gardening company	16	75 (3.2)	9-450	16	0.4 (2.0)	0.1-1.2	16	170 (3.5)

Sector / *subsector* / company	Endotoxin (EU/m³)			Inhalable dust (mg/m³)			Endotoxin/mg dust	
	N	GM (GSD)	Range	N	GM (GSD)	Range	N	GM (GSD)
Compost preparation/trade and trade	*77*	*860 (9.8)*	*14-191430*	*77*	*1.2 (5.1)*	*0.1-35*	*77*	*680 (2.6)*
Mushroom compost preparation	20	240 (3.1)	18-2430	20	0.6 (2.7)	0.1-2.6	20	380 (2.4)
Flower bulb trade	16	390 (1.8)	107-1220	16	1.7 (1.9)	0.2-2.7	16	655 (1.7)
Onion trade	20	25930 (2.7)	4025 -191430	20	14.4 (1.5)	6.7-35	20	1795 (2.0)
Mushroom compost preparation	21	210 (3.3)	14-1780	21	0.4 (1.7)	0.1-1.2	21	535 (2.3)
Industrial processing	*44*	*61 (4.9)*	*4.9-1200*	*44*	*0.3 (1.9)*	*< 0.1-1.5*	*44*	*240 (5.9)*
Vegetable slicing plant	9	39 (3.9)	9-590	9	0.1 (4.2)	< 0.1-0.5	9	270 (6.8)
Dried subtropical fruit	15	19 (2.3)	5-150	15	0.4 (1.8)	0.2-1.5	15	50 (2.0)
Vegetable and fruit canning industry	19	140 (3.4)	12-2030	12	0.3 (1.2)	0.2-0.3	8	255 (3.9)
Vegetable and fruit freezing industry	8	49 (3.1)	11-280	8	0.2 (1.5)	0.1-0.3	12	1575 (2.2)
C. ANIMAL PRODUCTION SECTOR	108	110 (9.3)	2.0-8120	108	0.7 (4.0)	< 0.1-21	108	170 (4.9)
Primary production	*27*	*1190 (3.1)*	*62-8120*	*27*	*2.4 (2.2)*	*0.4-14*	*27*	*505 (2.4)*
Dairy farming	8	560 (3.9)	62-2230	8	1.3 (1.8)	0.4-2.3	8	440 (2.9)
Dairy farming and cattle breeding	4	1570 (2.5)	444-3860	4	1.5 (6.1)	0.7-2.7	4	1030 (1.7)
Poultry farm (eggs)	2	2090 (1.3)	1716-2550	2	9.5 (1.7)	6.6-14	2	220 (2.2)
Poultry farm (chickens for meat)	2	880 (2.1)	520-1500	2	4.2 (1.1)	4.0-4.4	2	210 (2.0)
Poultry farm (free-range hens)	5	2140 (3.6)	360-8120	5	3.6 (2.1)	1.6-11	5	600 (2.4)
Pig farm (with own pulp feed installation)	6	1510 (2.1)	992-6970	6	2.6 (1.6)	1.6-5.4	6	575 (1.7)
(Industrial) processing	*81*	*51 (6.8)*	*2.0-6230*	*81*	*0.4 (3.7)*	*< 0.1-21*	*81*	*115 (5.0)*
Poultry abattoir	14	310 (7.0)	27-6230	14	1.5 (5.3)	0.2-21	14	210 (1.6)
Calf abattoir	12	120 (11.8)	3-3480	12	0.2 (4.9)	< 0.1-2.1	12	510 (5.0)
Cow/Cattle abattoir	19	31 (5.2)	2-820	19	0.3 (1.9)	0.1-1.9	19	110 (5.4)
Pig/Swine abattoir	16	28 (3.4)	2-220	16	0.3 (1.6)	0.1-0.6	16	90 (3.1)
Meat processing	20	23 (3.6)	3-1420	20	0.6 (3.3)	0.1-11	20	400 (4.9)

[a] Non-detectable dust concentration, <0.1 mg/m³

It should be noted that the measurements in the primary production part of this sector were worst-case scenarios, namely the measurements during the harvesting of grain (n=3) and flax (n=10) and the cultivation of potatoes (n=2). Mean endotoxin and dust exposure levels during processing in the GSL sector were lower, being 500 EU/m^3 and 1.4 mg/m^3,

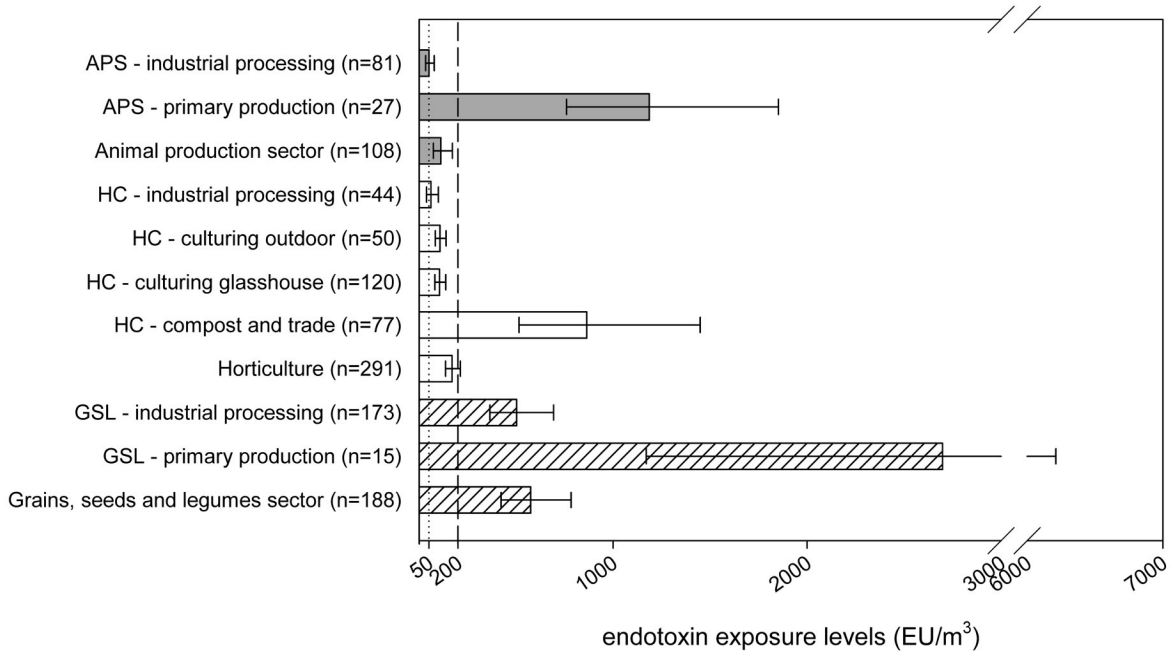

Figure 2: Endotoxin exposure (GM and 95% CI) levels in three sectors and subsectors of the agricultural industry

Inhalable dust exposure was low in most HC companies (GM=0.6 mg/m^3), except for the onion trade (GM=14.4 mg/m^3). In contrast, exposure to endotoxins varied greatly, with highest exposure in the onion trade company (GM>25000 EU/m^3) and fairly low exposures in other companies, including the industrial processing of vegetables (GM=61 EU/m^3) (Table 1B). The ratio of endotoxin per mg dust varied as well, with most endotoxin per mg dust in onion trading (Figure 3). In general, endotoxin exposure levels were similar for different jobs within a company. However, some companies had a few highly exposed jobs, e.g. in the mushroom nursery, during mushroom compost preparation, in the flower bulb nursery and the cucumber & paprika nursery (data not shown). Roughly, endotoxin exposure seemed to depend on type of process, the handled products and thus most likely the occurrence of microbiological growth in the products, and level of dust exposure.

In the AP sector, exposure to dust was overall moderate (GM=0.7 mg m^{-3}), except for farming (GM=3.6-9.5 mg/m^3) (Table 1C, Figure 1). There was a wide range in endotoxin exposure (GM=110, range 2.0 - 8120 EU/m^3), with highest exposure levels found in poultry and dairy farming (Table 1c, Figure 2). During primary production, almost all farm workers were highly exposed to dust (GM=2.4 mg/m^3) as well as endotoxin (GM=1190 EU/m^3), whereas during further (industrial) processing high exposures were only found in small specific parts of the companies where workers had direct contact with animals (front end of the process) or animal waste (data not shown). The amount of endotoxin per mg dust was also higher for primary production compared to exposure during industrial processing of products of the AP sector (Figure 3).

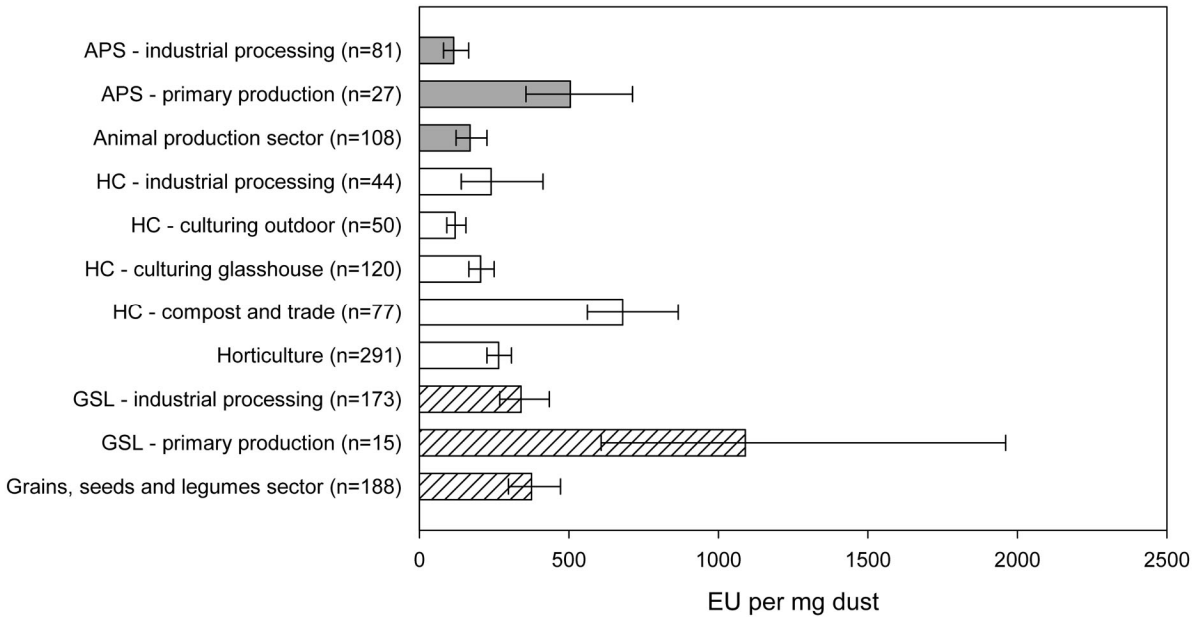

Figure 3: Relative amount of endotoxin (EU per mg dust) (GM and 95% CI) levels in three sectors and subsectors of the agricultural industry.

Correlation

The correlation coefficient (r) between inhalable dust and endotoxin was 0.69 for all measurements, and 0.67, 0.59 and 0.66 for the 'Grains, Seeds and Legumes', 'Horticulture' and the 'Animal Production', respectively. This coefficient squared gives the explained variance (R^2), which for dust levels explained at maximum 48 % of the variance in endotoxin exposure levels. Indeed, there were large differences in exposure levels between companies of a sector and in the ratio endotoxin per mg dust.

Comparison with exposure limits

In Table 2 the chances of exceeding occupational exposure limits for endotoxin and dust are presented. While only 5% of all inhalable dust measurements were above the occupational exposure limit for nuisance dust of 10 mg/m^3, 53% of the endotoxin measurements exceeded the temporary legal limit of 200 EU/m^3 and 76% was above the proposed health based exposure limit of 50 EU/m^3. Both limits for endotoxin were exceeded in all sectors, with the highest chance in primary production companies.

Table 2: Calculated percentage of exceeding exposure limits for endotoxin and inhalable dust, overall and per sector

	Endotoxins		**Inhalable dust**
	> 50 EU/m^3	**> 200 EU/m^3**	**> 10 mg/m^3**
Overall	**76 %**	**53 %**	**5 %**
Grains, seeds and legumes sector	**87 %**	**69 %**	**13 %**
Primary production	100 %	96 %	17 %
Industrial processing	86 %	67 %	13 %
Horticulture	**74 %**	**47 %**	**2 %**
Industrial processing	55 %	23 %	0 %
Compost & trade	89 %	74 %	10 %
Culturing glasshouse	69 %	33 %	0 %
Culturing outdoor	80 %	25 %	0 %
Animal production sector	**64 %**	**40 %**	**3 %**
Primary production	100%	95 %	3 %
Industrial processing	50 %	24 %	1 %

Determinants of exposure
Day-to-day and between-worker variance of exposure were 0.9 and 3.7 for endotoxin, and 0.6 and 1.8 for inhalable dust exposure, respectively. Thus, differences in exposure between workers were considerably larger than variation in exposure from day to day. Dustiness of the product processed and 'short versus long work cycles' explained some but only little of the day-to-day variability in a worker (data not shown). Presence of waste water, dustiness of the product, and contact with animals explained differences between workers in dust and endotoxin exposures. Effect estimates on exposure levels of above described and other possible determinants are presented in Table 3. Presence of waste water, process water, exhaust ventilation, a cyclic process, an industrial scale process and continuous exposure patterns were associated with lower exposure levels of both dust and endotoxin. Presence of faeces was associated with lower dust and higher endotoxin exposure. Type of company, presence of animals and a prolonged cycle (with seasonal variation) were associated with higher exposure levels of both dust and endotoxin.

Stratified analysis for the three sectors and subsectors generally showed a similar pattern for the effect of determinants, although some determinants disappeared due to a lack of diversity within the sector. The strongest determinants explained some of the observed dissimilarities between sectors. For example, exposure in the GSL sector was increased by the presence of remnant products (products that remain during the process and in some cases can be used in other industries like animal feed) and bulk product and decreased by presence of recirculating process water. In the HC sector remnant products, dustiness of the product and process water were important explanatory variables, and in the AP sector contact with living animals explained most of the differences.

Discussion

In this study, exposure to inhalable dust and endotoxin in a broad spectrum of agricultural industries has been investigated. Mean inhalable dust and endotoxin exposure levels were highest in the grains, seeds and legumes (GSL) sector. Exposure in the horticulture (HC) sector is slightly higher than in the animal production (AP) sector. Within the different sectors large differences between companies and between jobs were noted. Additional subdivision within the sectors revealed that highest exposures occur in the primary production phases of grains, seeds, legumes and animal products, mushroom compost preparation and trade of horticulture products: both dust as well as endotoxin exposure levels were high. The lowest concentrations were found in the industrial processing of animal as well as horticulture products, with exception of the front end of the abattoirs, when workers have contact with living animals or animal waste. The industrial processing of grains and related products results in fairly high exposure levels, where in most cases the endotoxin exposure is high when dust exposure is high.

The sample of companies included in the study was not random, as the width of the study and time available limited the number of companies included in the study, which might have resulted in selection bias. This was not likely to have happened as a qualitative walk through in comparable companies of a number of industries showed no large differences between those companies included for sampling in comparison with the others.

Table 3: Relative effect (compared to the reference) of exposure determinants on endotoxin and inhalable dust levels with all variables in the mixed regression model

	Endotoxin (EU/m³)				Inhalable dust (mg/m³)			
	Overall	**GSL [a]**	**H [a]**	**AP [a]**	**Overall**	**GSL [a]**	**H [a]**	**AP [a]**
Working mainly inside (I), outside (O) or both (IO) on worker level	I: 0.9 O: 0.7 IO = ref.	I: 0.5 ** O: 1.7 IO = ref.	I: 1.2 O: 0.3 * IO = ref.	I: 3.3 * O: 0.9 IO = ref.	I: 0.9 O: 0.8 IO = ref.	I: 0.6 O: 0.9 IO = ref.	I: 1.2 O: 0.6 IO = ref.	I: 1.8 O: 3.0 IO = ref.
Working mainly inside (I), outside (O) or both (IO) on company level	I: 0.9 O: 1.3 IO = ref.	I: 0.4 O: - IO = ref.	I: 0.8 O: 2.0 IO = ref.	I: 1.3 O: - IO = ref.	I: 0.9 O: 2.5 * IO = ref.	I: 5.0 O: - IO = ref.	I: 0.5 ** O: 2.5 ** IO = ref.	I: 2.3 O: - IO = ref.
Contact with living animals [b]	6.8 *	-	-	15.8 *	3.9 *	-	-	4.5 *
Remnant products [b]	2.5 *	116 *	6.0 *	1.1	0.9	2.0	1.5	1.1
Waste water [b]	0.3 *	0.9	0.1 *	-	0.3 *	0.5 **	0.1 *	-
Process water [b]	0.7	0.4	0.6	-	1.7	1.1	1.1	-
Recirculating process water [b]	0.9	0.03 *	-	-	0.4 *	1.4	-	-
Ventilation [b]	0.3 *	0.4	0.5	-	0.5 *	0.1 *	0.4 *	-
Faeces [b]	1.9 *	-	1.2	1.1	0.9	-	0.5 **	0.6
Cyclic process (company) [b]	0.7	5.3 **	1.5	1.2	0.5 *	0.5	0.6	0.5 *
Bulk product [b]	3.3 *	8.1 *	3.5 *	-	0.7 *	0.8	0.4 *	-
Dustiness product [b]	5.2 *	-	10.4 *	1.6	2.9 *	-	7.6 *	1.6
Industrial process [b]	0.3 *	-	1.7	0.3	0.6 *	-	1.3	0.2
Exposure variable vs. continued	0.2 *	-	0.3 *	-	0.4 *	-	0.8	-
Cycle short vs. prolonged	4.3 *	-	4.8 *	-	1.8 *	-	1.6	-

[a] GSL: grains, seeds and legumes sector, H: horticulture, AP: animal production sector
[b] (0/1) dummy variable: present versus absent (absent is reference)
ref.: reference variable, *: $p < 0.05$, **: $0.05 < p < 0.1$

Also, not all workers of a company were included in the study, but selected workers taking into account as much relevant functions and activities as possible. Therefore, no distortion of the results from this perspective is expected. On the other hand, most data in primary production of the GSL sector came from worst-case measurements. In these industries exposure occurs during specific activities, for example during harvesting, which is conducted during a limited period, and the exposure pattern might be quite different during the rest of the year because of other activities and/or crops. Determined exposure levels thus only represent specific periods. In contrast, the processing of these products continues throughout the year, as well as the work on animal farms.

Both inhalable dust and endotoxin showed a reasonably large variability, but the variation in endotoxin was much larger (GSD 8.6 versus 4.5 for dust levels). This difference may be partly due to a larger analytical error, since the assessment of endotoxin requires a much more complicated procedure involving extraction of filters, storage of extracts, and dilution and testing in the LAL assay, compared to 'only' weighing of filters for dust analyses. A larger analytical error however does not explain all of the difference in variance of exposure between endotoxins and dust. The larger variance in endotoxin exposure is most likely due to large variation in microbiological activity in the products and processes of the different branches. This is confirmed by the varying endotoxin content of the dust, which showed considerable differences between and within the various sectors.

It is known that assessment of endotoxin exposure may differ considerably between groups when different sampling, extraction, analysis, and storage procedures are employed. Differences between laboratories are usually within an order of magnitude, and vary according to the type of dust,[6,12,35] which compromises comparisons between results obtained by different groups. Nonetheless, in the Netherlands comparable techniques have been used in the past, which simplifies comparison with previous Dutch studies in animal production and GSL sectors, although for other industries data is lacking. The exposure levels found in the animal production companies are comparable with measurements conducted in pig farmers in the Netherlands with mean exposure levels of about 1820 EU/m^3 (56-8250 EU/m^3; n=182) in Summer and 1680 EU/m^3 (11-15030 EU/m^3; n=168) in Winter.[32,49] In other studies in pig farmers outside the Netherlands comparably high endotoxin exposures were found.[33,44] Previous studies in poultry farmers also showed high exposure levels ranging from 0.24-39167 EU/m^3 for total endotoxin and 0.35-694 EU/m^3 for respirable endotoxin.[10,47] Total endotoxin exposure levels found in poultry slaughter houses for workers handling living poultry ranged from 200 to 15000 EU/m^3, which is in the same range as we found,[17] although even higher exposure levels have been found.[44] The results from the animal feed industry are also within the range of earlier investigations in and outside the Netherlands, although the range in exposure in the Dutch studies was larger (2 to 18700 EU/m^3).[44-46] The endotoxin concentrations in the sugar beet processing company were even higher than those in an earlier study in the same industry (range 9-2521 versus 25-350 EU/m^3)[16] Comparable results are also found in studies that investigated a few different agricultural industries at the same time.[33,44]
In Horticulture only a few comparisons can be made. The concentrations found in mushroom growing are comparable with an earlier study.[44] However, endotoxin exposure in glasshouses was higher than has been found in Spanish measurements (GM 110 EU/m^3 vs. 0.36 ng/m^3), but here difference in technique used to assess endotoxin levels may also account for much of the apparent differences.[33]

It can be concluded that in general exposure levels derived from this current study are in agreement with earlier investigations in similar agricultural settings. Albeit a more thorough and detailed comparison of exposure levels in the future would require standardization of measurement and analytical methods for endotoxin exposure.

Several determinants were associated with exposure, e.g., the presence of waste water, process water, ventilation, cyclic process and an industrial scale process are associated with lower dust and endotoxin exposure. Contact with faeces was associated with higher endotoxin but lower dust levels. As information about most determinants of exposure was only available at the company level, interpretation of differences between workers was not possible.
Presence of water in the process of industries was expected to increase endotoxin exposure, as previously reported for potato processing [13,52] and the paper industry.[31,42] Surprisingly, the presence of water in the industries in the current study seemed more important to reduce dust and endotoxin exposure levels. Since production of consumption goods and the use of water in the process are bound to strict hygienic rules, water recycling was not common. This time water itself appeared not to be a source of microbes, but aided reducing exposure levels.
The type of company, presence of animals, dustiness of the product, bulk production and prolonged exposure are associated with a higher exposure level, as might have been expected at forehand. Endotoxins that originate from faeces, microbial growth in contaminated plant material on the land or during storage have been associated previously with high organic dust exposure.[19] Type of company explained most of the variability between workers, suggesting that together with the mentioned specific determinants, other unidentified determinants of exposure play a role. This was further supported by the fact that inclusion of company decreased the effect estimates of all other variables (data not shown). However, there is still little knowledge about the origin of endotoxin exposure in the studied sectors. There are large differences in amount of microbiological growth and different sources of exposure might play a role.[19] Improvement of the explanatory models may be obtained by including more personal information and detailed descriptive information on microbial growth and determinants of microbial growth, which were unfortunately not available.
There was only little day-to-day variability in exposure within workers, and we were not able to find factors that explained variation in exposure from day-to-day. This might be due to the fact that repeated measures were derived from two successive days with almost no change of working conditions. In future studies, more repeated measurements over a larger time period have to be performed to be able to distinguish possible determinants of within-worker variance.

From several studies, experimental as well as epidemiological, endotoxins appear to be related to (respiratory) health effects at relatively low concentrations (50-100 EU/m^3).[4,25,52] Since many measurements were above the temporary Dutch legal limit of 200 EU/m^3 as well as the proposed health based exposure limit of and 50 EU/m^3, a potential health risk exists. In every company workers were exposed to concentrations above 200 EU/m^3, and in the companies of the GSL sector almost all jobs had an exposure above 200 EU/m^3. Even considering overestimation of exposure in primary production of the GSL sector, considerable exceedance of limits occurs. It is clear that efforts should be made to lower the exposure to endotoxins drastically.

During the study, a qualitative assessment of currently applied exposure control measures was executed. Control measures like forced ventilation and local exhaust ventilation were mostly not available and if present, frequently not sufficient. Personal protective devices were often present. However, based on theoretical protection factors and determined exposure levels in these companies, they are not able to protect workers enough. In addition, many workers do not use them properly, which would even lower theoretical protection factors. Thus, to create a healthy working environment for workers in these industries considering endotoxin exposure, exposure levels need to be reduced with a factor 10-100 and sometimes with a factor 1000 or more. The current control measures do and cannot result in such reduction factors. Changes of processes, procedures and control measures will be necessary and this will require large (technical) interventions and investments from the companies.

Investigation of health effects was not the scope of the current investigation. Previous studies in pig farmers, the animal feed industry and potato industry showed adverse respiratory health effects of endotoxin exposure.[35,45,52] More recently, a possible protective effect of endotoxins in the development of atopy and asthma in children has been found.[26,50] A recent study showed that this might apply in an adult population as well.[30] Thus, to fully understand the impact of occupational exposure to endotoxins in these sectors, future investigations should focus on both protective and adverse effects and should also take into account individual sensitivity of people after endotoxin exposure.[22,43]

Conclusion

This study gives insight into endotoxin exposure in a broad spectrum of agricultural industries. Overall, it can be concluded that exposure to endotoxin in the many different parts of the agricultural industry is high. Inhalable dust and endotoxin exposure is the highest in the primary production cycle of a sector, and lower in the following cycle: (industrial) processing and trade. However, the exposure to dust and endotoxin varies greatly and seem to be dependent of the process and the products processed and produced in the company. More detailed information about possible exposure determinants is needed to fully understand differences in exposure between industries and between workers within an industry. Moreover, exposure levels exceed health-based exposure limits, indicating a possible health risk for workers in these industries. In the current situation ‘good housekeeping’ and the present control measures are not enough to realize a desirable reduction of the exposure; this can only be realized through structural exposure control measures.

Acknowledgements

The authors are indebted to the participating companies and employees. Furthermore, the authors thank Griet Terpstra and Olaf Welting for their technical and analytical assistance during the study.

The studies were financially supported by the three employer organizations of the agricultural sectors: ‘the grains, seeds and legumes sector’, ‘the horticulture sector’ and ‘the animal production sector’.

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Chapter 2.2

Endotoxin exposure in sewage treatment workers: investigation of exposure variability and comparison of analytical techniques

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Accepted for publication in Annals of Agricultural and Environmental Medicine after revision.

Abstract

Objectives were to give an overview of endotoxin exposure and its determinants in sewage treatment workers, and to study exposure to culturable and non-culturable microorganisms and the applicability of the LAL assay in this work environment.
In 43 Dutch sewage treatment plants 470 full-shift, 123 task-based personal and 54 stationary inhalable dust samples were collected. Endotoxin concentration was determined with the LAL-assay. Mixed effects models were used to investigate possible determinants of exposure. Simultaneous parallel filters samples, impinger samples and viable total bacteria and Gram-negative bacterial samples were taken to compare analysis techniques. Filter and impinger samples were analyzed with the LAL-assay, gas chromatography-mass spectrometry (GC-MS) and fluorescence microscopy.
Endotoxin exposure levels were moderate to low (geometric mean personal exposure 27 EU/m³, stationary 33 EU/m³, task-based 64 EU/m³), yet differences between jobs and sources and some determinants of exposure were identified. Exposure varied more from day to day than between workers. Concentrations in filter samples were higher and more consistent than in impinger samples. Fungi and Gram-positive bacteria were found in higher levels than Gram-negative bacteria. The LAL assay and GC-MS showed comparable endotoxin levels.
Exposure to inhalable dust and endotoxin in Dutch sewage treatment workers was relatively low. Comparison of sampling and analytical techniques suggests that the LAL-assay did not result in much exposure misclassification. It thus seems justified to perform filter measurements in combination with the LAL-assay to measure endotoxin exposure in sewage treatment plants.

Introduction

Sewage treatment is one of the components of waste control to decrease the environmental burden and to control disease in the human population. Sewage, originating from domestic habitats and/or industrial facilities is collected in sewerage and routed to a sewage treatment plant in the vicinity. The sewage treatment process, as schematically shown in Figure 1, removes human pathogens and other physical, chemical and biological contaminants from wastewater by physical, chemical and biological processes, ultimately resulting in a waste stream, sludge and effluent.[17] Much of the biological matter is converted by microorganisms, which thus are present in the sewage treatment plant environment. Sewage and sludge produce a number of gases such as hydrogen sulfide, ammonia, and carbon monoxide. Furthermore, chemicals are used for the treatment of liquid waste and in the cleaning and maintenance of the plants. Consequently, workers in sewage treatment plants are exposed to a large variety of chemicals and microorganisms and their products, among which are endotoxins.[17]

Endotoxins are lipopolysaccharides (LPS) present in the outer membrane of Gram-negative bacteria, and inhaled endotoxin is a well-known toxin with high pro-inflammatory potency. Exposure to endotoxin has been associated with several health effects in various agricultural and industrial environments.[14,26] An increased prevalence of (work-related) airway, flu-like, gastrointestinal and neurological symptoms and joint pain has been observed in sewage workers,[2,5,8,13,15,16,24,30,35] and in several studies endotoxin exposure has been suggested as the most probable cause of these symptoms.[2,24,30,35] Inhalation is thought to be the most important route of exposure, e.g. after aerosol formation, although contact with raw sewage or sludge (dermal and ingestion) might also play a role.[20] An experimental study showed that cleaning activities with effluent and relatively high pressure resulted in the highest endotoxin exposures.[36] Aeration of sewage may also result in the formation of bioaerosols when air bubbles burst, and thus in exposure to endotoxin.

In view of the planned introduction of a health based occupational exposure limit for endotoxin, the aim of this study was to give an overview of exposure to endotoxin in sewage treatment plants. Since determinants of exposure had not been studied in this industry before, this was also incorporated in the study. Although exposure was exceptionally low, the occurrence of health effects was directly related to the endotoxin exposure measured.[30] This might be an indication that either the LAL assay underestimates exposure to endotoxin, at least in this occupational environment, or that other microorganisms with comparable health effects occur in conjunction with the determined endotoxin exposure. Therefore, an additional experiment was performed to explore exposure to (viable) microorganisms and endotoxin at sewage treatment plants, and to investigate the relative amount of Gram-negative bacteria to the total microbial load. Furthermore, the performance of the LAL assay relative to the chemical analysis of endotoxin in this work environment was investigated in filter and impinger samples, in order to compare sampling and analytical techniques. In addition to the LAL-assay, samples were analyzed with gas chromatography–mass spectrometry (GC-MS) and fluorescence microscopy (FM). Analysis of chemical markers with GC-MS is proposed as alternative for the LAL assay. Although the technique does not quantify the endotoxin activity as the LAL assay, it measures LPS, and is known to be less sensitive for interferences. Furthermore, apart from the measurement of 3-hydroxy fatty acids (3-OH FAs) as chemical marker for endotoxin, also muramic acid (MuAc) for peptidoglycan can be measured in the same sample, which gives information about the presence of Gram-positive

bacteria.[27,29] By means of FM it is possible to measure the total microbial load, thus both viable and non-viable bacteria and fungi instead of only the culturable fraction. Both bacteria and fungal spores can be counted, the fluorescence staining causes recognition of microorganisms between other particles and microorganisms present in complex aggregates. Bacteria are classified based on their morphology as spherical or rod shaped, but viable and non-viable bacteria cannot be distinguished.[4]

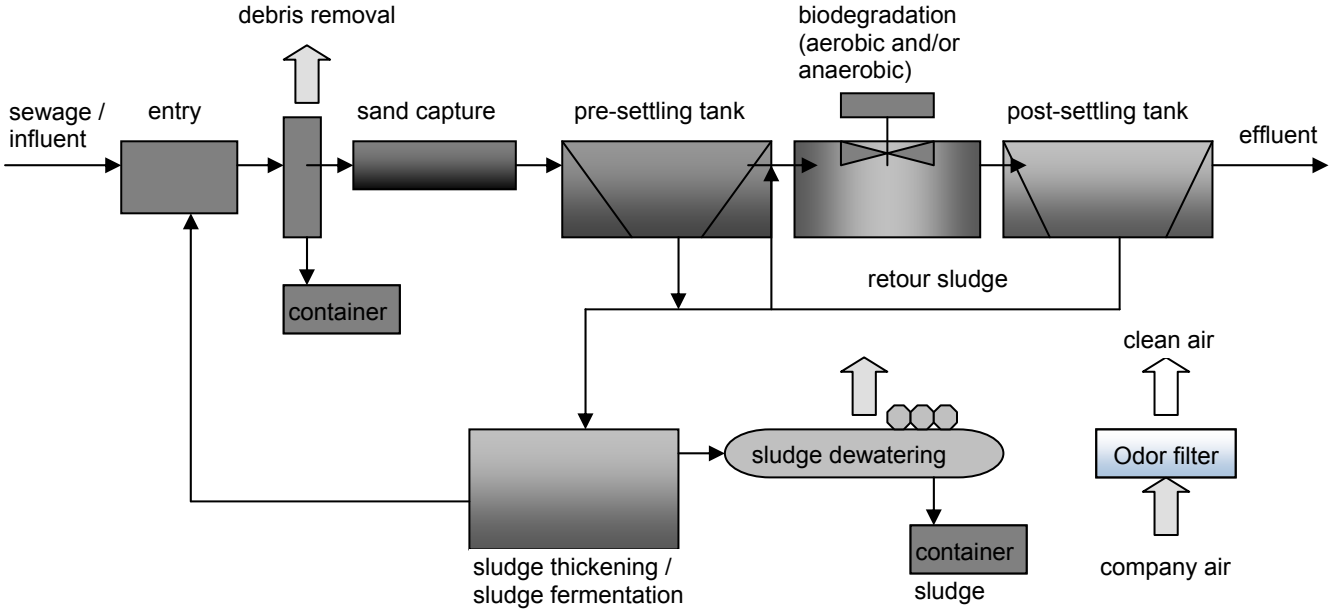

Figure 1: Schematic overview of sewage treatment process in the Netherlands

Material and methods

Exposure study

From the 27 Dutch Water Boards, which are responsible for the treatment of sewage in the Netherlands, 21 agreed to participate in the study. Personal inhalable dust and endotoxin exposure was measured in 225 workers from 43 sewage treatment plants. The measurements were performed in three periods (June-July, August-October and November 2003), and 8 individual measurements were taken in July 2004. One to six measurements per worker were obtained, depending on availability of the workers in the three sampling periods, leading to a total of 470 measurements. Furthermore, 54 stationary and 123 task-based measurements were performed to identify possible sources of endotoxin exposure or activities leading to a high endotoxin exposure.

Full-shift and task-based personal inhalable dust samples were collected using GSP sampling heads (JS Holdings) with 37 mm glass fiber filters (Whatman GF/A) in combination with Gilian GilAir5 portable pumps at a flow of 3.5 liter/min. The sampling head was placed on the shoulder of the worker, near the breathing zone, with the inlet facing forward. For the stationary measurements the same equipment was used, with the sampling head placed on a tripod (height 1.5 m) during a full shift. On each sampling day a control filter (field blank) was included, which was handled as the other samples except for the actual sampling. The loaded filters were stored at -20 ˚C prior to extraction.

The filters were pre- and post-weighed on an analytical balance in a conditioned room with stable temperature and humidity meeting U.S. EPA criteria, to determine the amount of dust on the filters gravimetrically. Inhalable dust concentrations below the limit of detection (LOD) were assigned a value of 2/3 of the LOD of the balance, which was 0.05 mg. For extraction, each filter was immersed in 5 ml pyrogen-free water with 0.05% Tween-20 in a glass tube and rocked vigorously for 1 hr at room temperature on a horizontal shaker. After 15 minutes of centrifugation at 1000xG, 1 ml supernatant per sample was collected, vortexed, and four aliquots of 0.1 ml and the remaining 0.6 ml were stored in pyrogen-free glass tubes at -20˚C until analysis. The endotoxin concentration in extracts was assayed with the kinetic chromogenic Limulus *Amoebocyte* Lysate (LAL) method (Cambrex, Verviers, Belgium; lot no. lysate 1L676S, lot no. standard 2L20090 (RSE/CSE ratio 11.5 EU/ng)), in which pyrogen-free water+0.05% Tween-20 was used as assay solution. Samples were assayed at an initial dilution of 1:5, and retested at higher dilutions (up to 1:100) when the measured concentration was too close to the upper detection limit of the assay. 95% of all samples were analyzed in duplicate. Samples with endotoxin levels below the limit of detection (LOD) were assigned a value of 2/3 of the mean LOD of the sampling runs in that period, which was 0.05 EU/ml for all three assay periods.

Information about job title, workplace, work activities during the measurements, work environment etc. was obtained from the workers included in the study. Furthermore, additional information about job, work activities in general and use of personal protective equipment was available from a questionnaire, which is described elsewhere.[30] In each plant information about process characteristics and other possible determinants was gathered with a company checklist. Information about weather characteristics for each sampling day was obtained from the website of the Royal Netherlands Meteorological Institute (www.knmi.nl).

Data were analyzed with SAS statistical software (version 9e; SAS Institute, Cary, NC, USA). Endotoxin concentrations were log-normally distributed. Therefore, all calculations were performed with natural log-transformed concentrations. Crude descriptive endotoxin and inhalable dust exposure levels were calculated as arithmetic mean (AM), geometric mean (GM) and geometric standard deviation (GSD) for the full-shift and task-based personal and stationary measurements. Spearman correlations were calculated between inhalable dust and endotoxin concentrations.

Between-worker and day-to-day (within-worker) variance in exposure were determined by applying mixed effects models, with worker identity as a random factor in order to correct for possible correlation between repeated measurements in the same worker. Any two repeated measurements of the same worker were assumed to have equal correlation (a compound symmetric covariance structure). Between- and within-variance components were estimated by using a restricted maximum likelihood method (REML). Process characteristics and information about job, work activities, workplace, weather conditions, etc. were introduced as fixed effects to investigate possible determinants of exposure.[19,22] For this analysis part of the dataset (417 out of 470 measurements) was used; the measurements with missing data for one or more of the determinants of interest were removed from the dataset for stability of the analysis.

Comparison of methods

An additional experiment was carried out to compare several sampling and analytical methods. Five measurement series of 4.5-6 hours (for the filter and impinger measurements)

were performed on three days in August 2004, two at the sludge dewatering department and three at the debris removal department of a sewage treatment plant. Each measurement series was placed close to each other and consisted of:

- Two simultaneous measurements with the N6-modification of the Andersen sampler and Becker VT3 pumps at a flow of 28.3 L/min. One sampler was equipped with a Tryptone Soya Agar (TSA, for total bacteria, measurement duration 8 minutes) and one with a Tryptone Soya Agar + 0.001% Kristal violet (TSA+KV, for Gram-negative bacteria, measurement duration 10 minutes). The measurements started at the same time, were positioned on a tripod at 1.5 m., and were performed shortly after the start of the other measurements at the same location. The plates were incubated at 37 ˚C for 48 hours, and the colonies were counted at 24 and 48 hours. The number of colonies was corrected according to the positive hole conversion method, and then converted to colony forming units per m³ (CFU/m³) on the basis of the sampling volume.
- Three measurements with liquid impingers, two filled with 15 ml pyrogen-free water (PFW) and one filled with 15 ml 9% saline solution, in combination with Gilian GilAir5 portable pumps set at a flow of 2 liter/min, positioned at 1.5 m on tripods. After the measurement on the same day, the amount of liquid was checked, filled up to 20 ml, vortexed, and divided in the following way:
  - 2 x 1 ml was stored in tubes to which 0.05% Tween was added, vortexed, and stored at -20 ˚C until analysis with the LAL-assay
  - 5 ml was stored in two 5-ml tubes, and stored at -20 ˚C until transport on dry-ice to Lund, Sweden, for analysis with gas chromatography-mass spectrometry (GC-MS)
  - 5 ml was filtered through a 25 mm polycarbonate membrane filter (Whatman Nuclepore®, 0.2µm pore size), the filter dried and stored in a cassette at room temperature until transport to Oslo, Norway, for analysis with fluorescence microscopy (FM)
  - 4 x 1 ml was plated on 2 TSA agars and 2 TSA+KV agars, and after that the agars were treated as described above.

  On 2 of the 3 measurement days also an impinger field blank was collected, of which the liquid was divided over the analytical methods in the same way as the other samples.
  From the liquid impingers with the saline solution, two times 2 ml was stored in tubes to which 0.05% Tween was added, vortexed, and stored at -20 ˚C until analysis with the LAL-assay.
- A sampling run with a previously described parallel sampler,[4,31] which enabled the simultaneous collection of 10 close to identical airborne samples using PAS6 sampling heads, of which eight were equipped with 25 mm glass fiber filters (Whatman, GF/A) and two with 25 mm polycarbonate membrane filters (Whatman Nuclepore®). The filters were then divided over the various treatments:
  - 2 glass fiber filters were stored in petri dishes at -20 ˚C until further extraction and analysis with the LAL assay
  - 2 glass fiber filters were stored in cassettes at -20 ˚C until transport on dry-ice to Lund, Sweden, for analysis with GC-MS
  - 2 polycarbonate filters were stored in cassettes at room temperature until transport to Oslo, Norway, for analysis with FM

  The other 4 glass fiber filters were used in another experiment which will not be discussed in this paper. Furthermore, per analytical method two field blanks were collected over the measurement days.

The filters assigned to the LAL-assay were extracted and analyzed together with the impinger samples in our laboratory in the same way as described earlier in this paper.
With GC-MS, the samples were analyzed for the chemical markers 3-hydroxy fatty acids of various length (3-OHFAs C_{10}-C_{18}, marker for endotoxin) and muramic acid (MuAc, marker for peptidoglycan) as described previously. The LPS concentration was computed as the sum of nanomoles of individual 3-OHFA with chain lengths 10-16 divided by 4 to account for the 4 molecules of 3-OHFAs assumed per molecule of LPS.[27,29,33]
With FM, the fluorescence staining causes recognition of microorganisms between other particles and microorganisms present in complex aggregates.[4] Both viable and non-viable bacteria and fungi were counted. The particles on the filter were resuspended and analyzed using the modified CAMNEA method by staining with acridine orange and counting with an epifluorescence microscope.[6]

Results

In total 647 measurements in 43 sewage treatment plants were performed, of which 470 full-shift personal, 123 task-based personal and 54 stationary measurements. More characteristics of the measurements are given in Table 1. For part of the samples no dust weight could be estimated due to errors during the weighing procedure, and for 2 samples the endotoxin concentration could not be estimated. The endotoxin concentration of 115 out of 124 field blanks was below the LOD (range detectable field blanks 2.0-5.3 EU per sample). Thus, contamination during handling of the filters and assembling of the sampling heads was unlikely. Of the loaded samples, 21 were below the LOD for endotoxin and 142 below the LOD for inhalable dust, most of them being task-based measurements. The average coefficient of variation (CV%) for duplicate analyses was 18%.

Table 1: Characteristics of personal, stationary and task-based measurements in sewage treatment plants

	Personal	Stationary	Task-based
# measurements	470	54	123
# sewage treatment plants	43	22	28
# workers	225	-	82
# measurements per worker	1-6	-	1-5
Sampling time (hr)	6.4 (range 1.3-8.2)	5.9 (range 0.5-8.7)	0.9 (range 0.1-5.0)
Missing endotoxin concentration	2	-	-
<LOD for endotoxin	2	3	16
CV% of duplicate endotoxin analyses	19.0 (range 0-90)	19.2 (range 0-90)	18.9 (range 0-150)
Missing dust concentration	75	4	6
<LOD for dust	26	31	85
Correlation dust and endotoxin exposure	0.37 (p<0.0001)	0.27 (p=0.056)	0.14 (p=0.127)

#: number, LOD: limit of detection, CV%: coefficient of variation

Table 2 shows the endotoxin and inhalable dust exposure levels for the different types of measurements, overall and divided over respectively functions, locations and tasks. The geometric mean personal, stationary and task-based endotoxin concentrations were moderate, with levels of 27 EU/m³, 33 EU/m³ and 64 EU/m³, respectively. The highest personal exposure levels were found in operators and sludge workers, which were statistically higher than the reference group management (Table 3). The highest dust concentrations were found in mechanics and sludge workers. The results of the stationary

measurements indicate that the highest endotoxin levels are found in the front end of the process, whereas the highest dust concentrations were found during sludge dewatering. Sludge dewatering and manufacturing of polymers showed the highest task-based endotoxin and dust levels, respectively. Overall, the correlation between measured dust and endotoxin exposure levels was low (Table 1).

Table 2: Endotoxin (EU/m³) and inhalable dust (mg/m³) exposure levels for personal, stationary and task-based measurements in sewage treatment plants

	Endotoxin (EU/m³)					Inhalable dust (mg/m³)		
	N	**AM**	**GM**	**GSD**	**range**	**N**	**AM**	**range**
Personal measurements								
Overall	*468*	*71.0*	*26.9*	*3.7*	*0.2-2093*	*394*	*0.4*	*0.0-23.5*
Office workers	21	14.9	8.5	2.7	1.6-97.9	18	0.1	0.0-0.2
Management / office	18	15.1	8.0	2.9	1.6-97.9	15	0.1	0.0-0.2
Administration / house keeping	1	20.6				1	0.2	
Analist	2	10.3	9.9	1.5	7.4-13.3	2	0.1	0.1-0.1
Technician	82	46.6	24.9	2.8	2.9-702	69	0.6	0.0-15.7
Electrical engineer	15	27.6	19.5	2.4	6.2-86.1	12	0.2	0.0-0.9
Mechanic	67	50.8	26.3	2.8	2.9-702	57	0.7	0.0-15.7
Operator	258	77.5	28.0	4.0	0.2-2093	211	0.3	0.0-3.8
Sludge worker	107	84.8	32.4	3.7	1.4-1506	96	0.5	0.0-23.5
Stationary measurements								
Overall	*54*	*110.3*	*32.9*	*6.4*	*0.2-1397*	*50*	*0.3*	*0.0-12.6*
Supply sewage and debris removal	12	180.5	51.3	5.3	1.8-1397	10	0.1	0.0-0.2
Sewage treatment process	12	36.3	12.6	4.8	2.1-204	11	0.0	0.0-0.1
Sludge dewatering	30	111.9	40.4	7.1	0.2-458	29	0.5	0.0-12.6
Task-based measurements								
Overall	*123*	*178.6*	*64.4*	*4.3*	*2.2-2135*	*117*	*0.5*	*0.1-4.3*
Supply sewage & debris removal	29	151.7	86.6	2.6	15.6-1100	27	0.4	0.1-1.7
Sewage treatment process	41	140.0	40.9	4.6	2.2-1317	40	0.5	0.1-2.8
Manufacture polymers	3	29.3	19.3	3.4	5.4-60.8	3	1.8	0.2-4.3
Sludge dewatering	40	272.6	112.9	4.1	2.2-2135	37	0.4	0.1-0.8
Sludge drying	2	271.0	199.5	3.2	87.6-454	2	0.5	0.1-0.8
Sludge transfer/reloading	8	37.1	15.9	4.1	3.0-123	8	0.6	0.2-1.4

N: number of measurements, AM: arithmetic mean, GM: geometric mean, GSD: geometric standard deviation

Day-to-day variability in endotoxin exposure was larger than differences between workers in average exposures, with the between- and within-worker variance components being 0.39 and 1.37, respectively (Table 3). No clear determinants of day-to-day variability in exposure could be identified; some climate characteristics (precipitation, wind direction, and relative humidity), month in which the measurements took place and measured dust concentration explained only 1 to 7% of the variability over time (Table 3). The combination of all climate variables available from the national survey points of the KNMI (mean temperature, duration sunshine, relative duration sunshine, minimal sight, air pressure, precipitation, length precipitation, wind speed, wind direction, relative humidity) explained 11% of the variability in endotoxin exposure over time. Between-worker variability was mainly reduced by introducing function category, plant and cleaning during the measurement day as fixed effects (Table 3).

Table 3: Between- and within-worker variability and percentage of explained variance, and relative effect of variables on endotoxin levels (EU/m³), in 417 out of 470 personal measurements without missing data

Variable	BW (%) [a]	WW (%) [a]	e^{β}	95% CI
Worker only	0.39	1.37		
Function	0.33 (15%)	1.37 (0%)		
- Administration			2.67	0.19-37.88
- Analist			1.28	0.19-8.88
- Electrical engineer			2.59 **	0.96-6.93
- Mechanic			3.46 *	1.61-7.45
- Operator			3.80 *	1.89-7.67
- Sludge worker			4.55 *	2.17-9.55
- Management			ref	
Function category	0.32 (18%)	1.37 (0%)		
- Operator			3.50 *	1.84-6.64
- Sludge worker			4.18 *	2.11-8.30
- Technician			2.99 *	1.50-5.98
- Office workers			ref	
Plant (n=38)	0.24 (38%)	1.38 (-1%)		
Debris removal (uncovered vs. covered)	0.34 (13%)	1.37 (0%)	1.64 *	1.22-2.20
Type of aeration tank	0.35 (10%)	1.37 (0%)		
- carrousel			ref	
- oxidation tank			4.85 *	1.58-14.83
- aeration tank			1.33	0.93-1.92
- other			1.09	0.69-1.73
Type of aeration system	0.32 (18%)	1.38 (-1%)		
- fine bubbles aeration			1.32	0.83-2.11
- point aeration (covered)			1.34	0.81-2.22
- point aeration (uncovered)			3.81 *	1.90-7.63
- other			ref	
Load of installation	0.38 (3%)	1.36 (1%)		
- high			1.87 *	1.12-3.13
- low			1.24	0.91-1.70
- ultra low			ref	
Maintenance	0.36 (8%)	1.37 (0%)		
- external			4.39 *	1.03-18.73
- external and internal			1.35 **	0.99-1.85
- internal (all workers)			ref	
Industrial clothing	0.35 (10%)	1.37 (0%)		
- changing clothes at home			2.22 *	1.36-3.61
- changing clothes at work			1.20	0.86-1.67
- both at home and at work			ref	
% supply domestic waste water [#]	0.40 (-3%)	1.38 (-1%)	0.99 *	0.978-0.998
Use personal protective devices (PPD) [$]	0.33 (15%)	1.38 (-1%)		
- yes, for specific work activities			1.23	0.56-2.70
- yes, during majority of the work day			2.45 *	1.05-5.72
- yes, both			1.18	0.49-2.83
- no use of PPD			ref	
Cleaning as part of work activities (yes vs. no) [$]	0.35 (10%)	1.37 (0%)	2.16 *	1.31-3.55
Cleaning during work day (yes vs. no) [&]	0.31 (21%)	1.38 (-1%)	1.63 *	1.26-2.10
Number of cleaning activities during day [&]	0.29 (26%)	1.40 (-2%)		
Working at sludge dewatering during day (yes vs. no) [&]	0.38 (3%)	1.37 (0%)	1.29 **	0.98-1.69
Precipitation (mm) [# %]	0.39 (0%)	1.34 (2%)	1.04 *	1.01-1.07
Length precipitation (hours) [# %]	0.39 (0%)	1.34 (2%)	1.09 *	1.03-1.16
Wind direction (16 categories measured) [%]	0.41 (-5%)	1.29 (6%)		
Relative humidity [# %]	0.39 (0%)	1.36 (1%)	1.02 *	1.002-1.04
Month in which is measured	0.39 (0%)	1.31 (4%)		
- June			5.82 *	1.40-24.15
- July			7.20 *	1.70-30.57
- August			3.93 *	1.91-16.98
- September			7.66 *	1.85-31.77
- October			4.77 *	1.10-20.65

Variable	BW (%) [a]	WW (%) [a]	e^{β}	95% CI
- November			9.41 *	2.18-40.59
- December			ref	
Dust concentration (lognormally transformed) [^]	0.40 (7%)	1.30 (7%)	1.36 *	1.21-1.54

BW: between-worker variability, WW: within-worker variability, [a]: percentage of explained variance, e^{β}: relative effect, 95% CI: 95% confidence interval, *: p<0.05, **: 0.05<p<0.10, ref: reference category, [#]: continuous variable, [$]: variables from questionnaire, [&]: variable from time observation during measurement, [%]: climate variables, [^]: in smaller part of data, without missing data for dust (n=342, worker only BW=0.43, WW=1.40)

Also process characteristics like kind of debris removal, type of aeration tank and system explained some of the between-worker variance. However, many of the available variables had no effect on the variance components and are therefore not mentioned in Table 3. The combination of function category and plant explained 56% of the between-worker variability. Furthermore, the combination of climate variables, hygiene facilities of a plant (industrial clothing, washing clothes at the plant, changing clothes before entering the canteen and the procedure for use of personal protective devices), information on tasks and task duration registered during the measurement, variables concerning process characteristics and a combination of variables from the questionnaire (cleaning activities, cleaning with effluent, eating/drinking during work, showering at end of the day, frequency washing work clothes, place washing work clothes, place changing clothes) explained 13%, 18%, 10%, 13% and 28% of the between-worker variability, respectively (data not shown).

Figure 2 shows the results of the various viable and non-viable sampling and analytical methods. Because of the limited number of observations, the data are only be presented graphically. As is shown in Figure 2A, analyses with the LAL assay resulted in higher measured endotoxin concentration per m³ for filter measurements compared to liquid impingers, with the highest levels measured in the sludge dewatering department. Almost all concentrations were above the levels measured in the field blanks. Some sampling runs resulted in fairly comparable results, others differed considerably. The saline solution of impingers was analyzed solely with the LAL assay; they resulted all in non-detectable concentrations and were not considered any further (data not shown).
The mean amount of microorganisms found per m³ with fluorescence microscopy was $4.1x10^4$ for impinger measurements and $1.4x10^5$ for filter measurements, which is slightly elevated but not very high. A small part of these microorganisms was identified as fungi in the filter samples, no fungi were found in the impinger samples (Figure 2B). For the filter measurements, there were more microorganisms per m³ found in sludge dewatering than during debris removal, and for the impinger measurements it was the other way around. Most of the concentrations microorganisms of the impinger measurements were below the concentrations measured in the field blanks (Figure 2B).
Figure 2C and 2D show the results of the GC-MS analyses for 3-OH FAs (endotoxin) and MuAc (peptidoglycan), respectively. Overall, the amounts of 3-OH FAs and MuAc were low. A reasonably high part of the impinger measurements analyzed for 3-OH FAs were below the highest value measured in the field blanks, as were most of the filter samples from the debris removal. In the filter samples the highest 3-OH FA concentrations were always samples in the sludge dewatering department, for the impinger samples this was very variable. Both for filters and impingers concentrations of longer chain 3-OH FAs were higher.
The variation in MuAc concentrations in filter samples was larger than in impinger samples, although many of the samples taken during debris removal were below the highest value measured in the filter field blanks. The impinger measurements during debris removal and sludge dewatering were more or less similar.

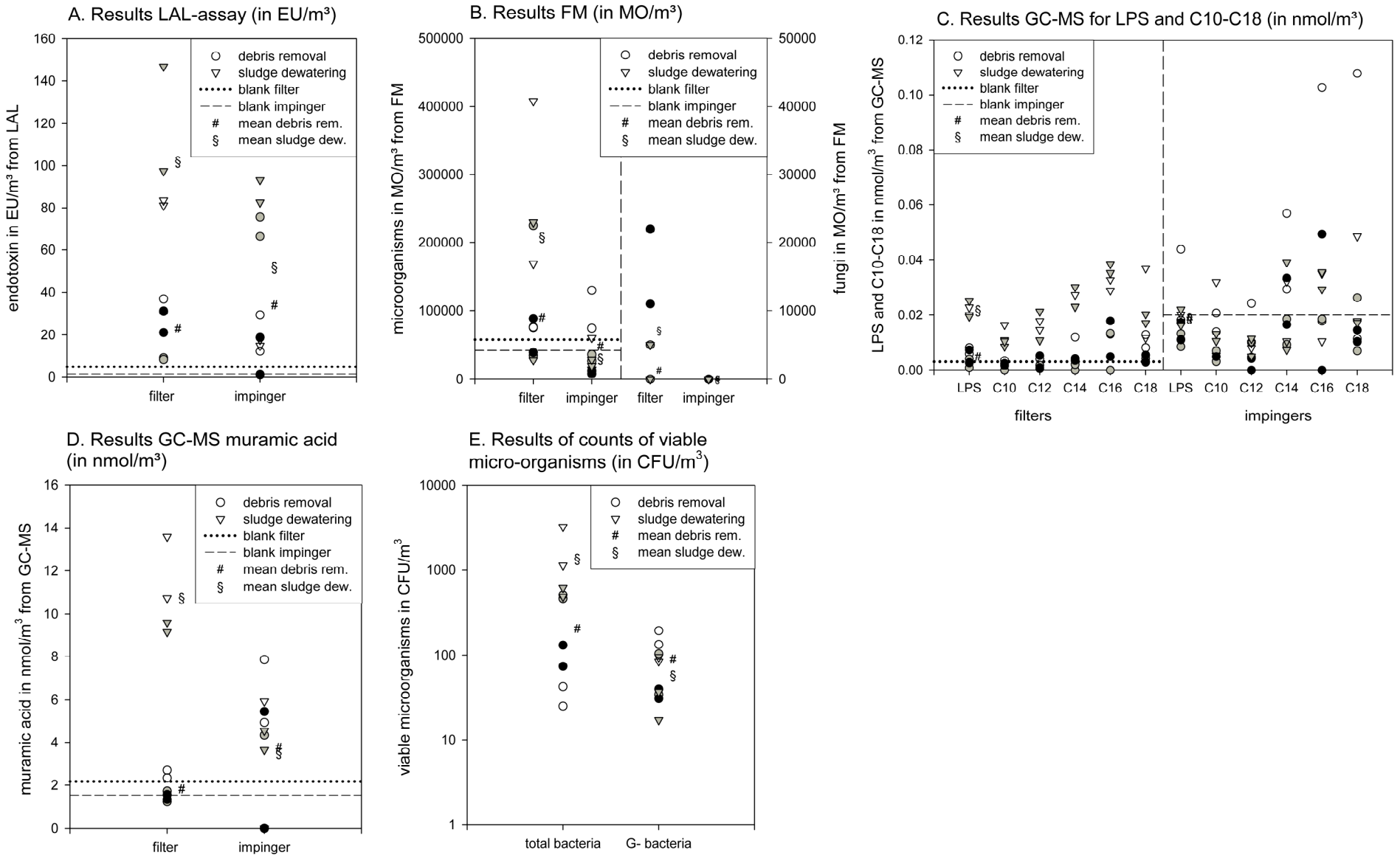

Figure 2: Results comparison two sampling methods (filters and impingers) per analytical method; the results are grouped by sampling location (● for debris removal and▼ for sludge dewatering) and per sampling run (fill color).

The results of the viable measurements are shown in Figure 2E. During the counting of the formed colonies on the plates it appeared that also many fungi colonies had formed on the plates. The number of fungal colonies in samples from debris removal often exceeded the upper detection limit (>399 colonies). In samples from sludge dewatering almost no fungi were found (data not shown). In most sampling runs the amount of total bacteria was higher than the amount of Gram-negative bacteria, although levels itself were low to moderate. The highest amounts of total bacteria were found in the sludge dewatering department, the highest amounts of Gram-negative bacteria were found in the debris removal department. Plating part of the impinger liquid on agars resulted in virtually no growth of colonies (data not shown). The total amount of both viable and nonviable microorganisms was in the order of 10^4-10^5, and thus the proportion of viable microorganisms (10^3-10^4) was 1-10%.

The ratio of the endotoxin bioactivity (EU) and the LPS concentration is referred to as the potency (EU/nmol LPS).[23] This potency ranged from 3600 to 5500 EU/nmol LPS for filters per sampling run, and from 60 to 7900 EU/nmol LPS for impinger samples. The mean potency was higher in filter samples than in impinger samples (AM 4600 vs. 2800 EU/nmol LPS) However, the mean potencies per sampling location (debris removal and sludge dewatering) were approximately the same for both collection methods (data not shown).

Discussion and Conclusions

Dust and endotoxin exposure levels in Dutch sewage treatment plants were relatively low, although differences between functions and tasks were observed. Apart from debris removal and sludge dewatering, sewage treatment was mostly situated outdoors, with some covered sections, which explains the relatively low exposure levels found. The generally higher endotoxin levels of stationary and task-based measurements compared to personal full-shift measurements suggest that working in certain parts of the installation and/or performing certain tasks is associated with higher endotoxin exposure. Yet workers normally spend only a relatively short part of their working day in these parts of the installation and/or performing these tasks. This results in relatively low exposures over the whole working day, as possible peak exposures are diluted over the rest of the day, when they work mainly in control rooms with low exposure.

The low endotoxin levels found are in accordance with those found in other studies that investigated endotoxin exposure in sewage treatment plant workers.[2,18,21,34] These studies also showed higher exposure with ambient measurements in sludge dewatering areas,[2] at specific worksites with highest values found for worksites located indoors, during agitation of wastewater,[34] and during tasks with expected high peak exposure.[18] Scandinavian studies [10,16,24], an American [12] and a Polish study [9] in sewage treatment workers, and in the wastewater treatment part of wood processing plants [28] have shown much higher endotoxin concentrations. The fact that in for instance Scandinavia many phases of the wastewater treatment process are typically located indoors due to the low ambient temperature may be an explanation for the higher endotoxin levels found there.

Day-to-day variability is the major source of exposure variability, most probably caused by a combination of variation in work activities, changing weather conditions and differences in the supply and composition of influent. The latter is for instance influenced by the origin of sewage (domestic or industrial), holiday periods (less supply) and the possible drainage of

rainwater. A combination of weather conditions also explained part of the day-to-day variability in endotoxin exposure. The plant a worker worked on in combination with function explained most of the variability between workers. However, hygiene associated variables also explained 28% of the difference between workers.
Although clear determinants of exposure were not observed, some characteristics were associated with a higher endotoxin concentration, for instance workers who changed their working clothes at home instead of on the plant. This is probably indirectly caused by the general hygiene in the sewage plant, which is related to the age and/or size of the plant. Furthermore, workers involved in cleaning activities had higher endotoxin exposures. An experimental study showed that cleaning with tap water or surface water instead of effluent and lowering the water pressure during cleaning, as well as mechanical ventilation, significantly lowered endotoxin exposure.[36] Good hygiene practices and adequate cleaning protocols could thus reduce endotoxin exposure. Furthermore, some process characteristics were associated with a higher endotoxin exposure, like the presence of an oxidation tank and use of open point aeration. The extent to which process water or sludge is being moved and/or agitated seems to have an effect on endotoxin exposure, which has been found before.[10,34] Covering sources of exposure, like the debris removal area or aeration systems, may be a way to further reduce endotoxin exposure, although working indoors resulted in higher exposure levels [10,18] and maintenance thus should be performed wit caution. Beforehand, variation in weather conditions over seasons was thought to be a determinant of exposure, but no clear differences between measurement series or seasons were found. A Swiss study also found no differences in endotoxin exposure between seasons (summer and winter).[18]

It is suggested that the wet environment and frequent generation of aerosols could cause clogging of filters.[1] Use of liquid impingers could be an alternative in these situations. Comparison of filter and impinger samplers resulted in variable outcomes with respect to the used analytical technique, i.e. LAL-assay, GC-MS and FM. Except for 3-OH FAs in impinger samples, the highest concentrations were found in filter samples, which suggests no major role of clogging. Filter samples could better distinguish the difference in composition of microorganisms in the air of the departments compared to impinger samples. Although it has been suggested that the activity of liquid inside impingers might result in lysis of microorganisms and thus more endotoxin available in the LAL assay, this did not seem to be the case here. However, part of the liquid had ‘evaporated’ from the impingers at the end of the measurements, due to the fierce bubbling of the liquid during the measurements. This loss was replenished until 20 ml with the same liquid as used during sampling to be able to divide the sample for the different applications in the experiment, which might have diluted the ultimately measured concentrations. This does not pose a problem when only liquid evaporates during sampling, but does alter the outcomes when droplets leave the impinger. Furthermore, bacteria prefer staying on the border of liquid and air and therefore could differentially disappear in larger quantities when droplets are formed. Our data, however, showed both lower bacterial and fungal levels in impinger samples, which suggests that differential loss of bacteria had not occured. The kind of liquid may also have influenced the measured concentration. The endotoxin concentrations in all 9% saline impinger samples were below LOD. Perhaps also solely pyrogen-free water is not the optimal sampling solution for impinger measurements due to osmosis or related mechanisms, thus affecting the cells in the solution. Other studies that compared filter and impinger sampling methods concluded that the performance of both methods depends on the airborne endotoxin levels. However, in

these studies impinger measurements generally resulted in higher and less variable endotoxin levels.[3,32]

In a simultaneously performed questionnaire study a dose-response relation between endotoxin exposure and systemic and flu-like symptoms, and lower respiratory and skin symptoms was found. However, these results should be treated with caution since only a small part of the workers was exposed to levels >50 EU/m^3,[30] although the symptoms found are in accordance with symptoms reported by other work populations occupationally exposed to biological agents including endotoxin.[14] The relatively low exposure levels suggest that, apart from some exceptions, endotoxin itself may play only a minor part in causing possible health effects in sewage treatment workers. Endotoxin in air may more be seen as a relatively easily measured marker of general microbial burden. The results of the additional experiment also point in that direction, with more viable Gram-positive bacteria and fungi than Gram-negative bacteria, and the presence of muramic acid in the filter and impinger samples. Prazmo et al. investigated the microbial composition of aerosols in the air on several locations of a sewage treatment plant. Here also low endotoxin concentrations were found, although several pathogenic bacteria and fungi could be measured with viable techniques, and Gram-positive bacteria were dominant.[21] Another study found (total) viable bacteria concentrations of 10^2-10^5 CFU/m^3, and viable Gram-negative bacteria concentrations of 10-8700 CFU/m^3 at wastewater treatment plants. The total numbers of bacteria in extracts of endotoxin samples, determined by epifluorescence microscopy, ranged from 10^7-10^9 bacterial cells per m^3. However, the true concentration may have been overestimated through disturbance of counting by glass fibers from the filters and other nonbacterial particles that show fluorescence.[11] In industrial wastewater treatment plants the endotoxin and bacteria concentrations varied widely between phases in the treatment process, and were lowest outdoors and where wastewater or sludge was not agitated.[10] Oppliger et al (2005) showed more cultivable bacteria indoors than outdoors. Climatic parameters seemed to have a significant effect on the mean airborne concentration of fungi (in summer higher than in winter), but not on total bacteria, Gram-negative bacteria and endotoxin.[18] Since the experimental measurements were performed in summer on relatively hot days, this could explain the rather high amounts of fungi found.

The results of the LAL-assay did not differ substantially from the GC-MS analysis. Both the analysis with FM and GC-MS showed a relatively low exposure to bacteria. Only in sludge dewatering 3-OH FAs and MuAc were found. The viable measurements accordingly showed that bacteria occurred mainly in sludge dewatering, the majority being Gram-positive bacteria, which is confirmed by the concentrations MuAc found in this department. In the debris removal department exposure to viable microorganisms was also reasonably high, but these were mainly fungi.
It is known that the LAL assay particularly measures free (unbound) endotoxin,[7] and thus the LAL assay rather reflects the bioactivity of the sampled and dissolved endotoxin. In cell-bound endotoxin most of the lipid A is a covalent part of the membrane and thus does not activate Limulus enzymes. However, experimental data suggest that cell bound LPS may still be highly biologically active when inhaled.[25] Results from the GC-MS analysis indicated a very low endotoxin exposure, whereas the LAL assay suggested also low but slightly elevated exposure levels. In combination with differences between filter and impinger samples and the fact that the viable as well as the non-viable measurement techniques showed the presence of microorganisms at sewage treatment plants, these results do not rule out that some fungi and peptidoglycans may have interfered with the LAL-assay. This

interference is of little consequence in case of high exposure levels, but may cause some exposure misclassification in case of moderate exposure levels in combination with the presence of relatively high amounts of viable Gram-positive and Gram-negative bacteria and fungi, as found in this study. It should be noted that the additional experiment consisted of only a limited set of measurements, and thus no very firm conclusions can be drawn here. Nevertheless, comparison of sampling and analytical techniques suggests that it seems justified to perform filter measurements in combination with the LAL-assay to measure endotoxin exposure in sewage treatment plants.

Acknowledgements

The authors thank the management and workers of the sewage treatment plants for their participation. Furthermore, we thank Jack Spithoven (IRAS) for his assistance during fieldwork and in the laboratory, and Karel van der Heijden and Isabella Oosting-van Schothorst from IRAS, Per-Ole Huser from STAMI and Christina Pehrson from Lund University for their analytical assistance. This research was granted by the Stichting Toegepast Onderzoek Waterbeheer (STOWA, Dutch acronym for the Foundation for Applied Water Research).

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Chapter 3

From exposure variability to a measurement strategy

Chapter 3.1

Variability in endotoxin exposure levels and consequences for exposure assessment

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Published in Annals of Occupational Hygiene, 2008; 52 (5): 303-316.

Abstract

Workers in many industries are exposed to endotoxins, which may cause adverse health effects. In exposure assessment, information about exposure variability is essential. However, variability in exposure has rarely been investigated for biological agents and more specifically for endotoxin. Therefore, variance components and determinants of exposure were studied in a large database with >2000 endotoxin measurements.

Data from 10 individual studies were combined to create a database with 2010 personal inhalable dust and endotoxin measurements, of which 1650 were repeated measurements. Exposure groups were defined based on job codes. Between- and within-worker variance components were estimated for different grouping strategies, and determinants of exposure were studied using mixed effects models.

Inhalable dust and endotoxin exposure levels are summarized for 46 industries and 4 broadly defined sectors. The between-worker variability exceeded the within-worker variability overall and within sectors and subsectors, and variance components were larger for endotoxin than for dust. Between-worker variability also exceeded within-worker variability in nearly half of the exposure groups based upon industries or job code within industries for endotoxin exposure and in 10% of the groups for dust exposure. Among other things, dustiness of the process, contact with animals, bulk production, presence of plant material or a cyclic process appeared as determinants of exposure, which largely explained the between-worker variability.

Exposure groups were much less homogeneous for endotoxin exposure than for dust exposure. This is distinctly different than for chemical exposure. Large variability in measured exposure levels is inherent to endotoxin exposure, which is caused in part by determinants that influence growth of microorganisms. These findings have major consequences for the design of future occupational intervention and epidemiological studies. The measurement effort needs to be greater than exposure assessment for chemical agents which demonstrate lower exposure variability, especially when evaluating endotoxin exposure for compliance testing. The established determinants of exposure give direction for potential exposure control, although more information about determinants of day-to-day variability in exposure is still needed to be able to effectively control endotoxin exposure.

Introduction

Organic dust, defined as airborne particulates of vegetable, animal or microbial origin, is known to be associated with respiratory symptoms. Workers in various industries are exposed to organic dusts.[25] Endotoxins are well-known contaminants of organic dust and a major causative agent for respiratory effects.[5,24] Endotoxin is a component of the outer cell wall of Gram-negative bacteria, and commonly present in a variety of occupational environments. Airborne endotoxins are related to the occurrence of these bacteria. Lysis after cell death results in release of endotoxins into the environment. Inhalation is thought to be the major route of exposure and is associated with respiratory and systemic inflammatory responses, both acute and chronic.[8,23,25] There are also indications that environmental endotoxin exposure has a potential protective effect on the risk of atopic sensitization in childhood, and possibly also in adults working with high endotoxin exposures.[10,14,26,39]

Gram-negative bacteria are ubiquitous in the environment. Their growth is dependent upon many factors, including the presence of a substrate for nutrients, favourable water activity and temperature. Aerosolization and distribution of particles are necessary conditions for exposure. Consequently, exposure to endotoxin is highly variable. The inherent exponential amplification of living microorganisms probably contributes to the environmental variability in exposure. Furthermore, possible growth of organisms on the filter or in the extract after sampling, reaction of organisms or their products with the assay, or agglomeration in the solution may cause even more variability in the results from analysis of biological agents and thus the measured exposure. This is in contrast with exposure to chemical agents, where such factors do not play a role.

Traditionally, measured exposure for compliance testing used to be assessed by sampling workers at one point in time during a worst-case exposure scenario. Yet, information about sources of between- and within-person exposure variability are needed in order to accurately assess overexposure and exceedance[36] or effectively advise on determinants of exposure and control measures. For this purpose, randomly collected repeated exposure measurements from a representative subset of workers in an occupational group are essential.[33] The first studies to evaluate exposure variability predominantly focused at airborne exposure to chemical agents.[3,18] After that, other studies also explored exposure variance components, in more industries, for other substances and for different routes of exposure.[4,32,33]

Variability in exposure to biological agents has rarely been investigated, although the total exposed population is substantial. However, knowledge about variability in exposure is a necessary requirement and vital starting point for (future) measurement campaigns. Insight into components of exposure variability and underlying determinants may also influence the design of intervention[6] and epidemiologic studies.[35] Variance components can be used in compliance testing as well.[19]

We constructed a database containing >2000 full-shift endotoxin measurements to inform research in the area of compliance testing, intervention studies and epidemiology. As far as we know, this is the first large endotoxin exposure database that has been composed and analyzed for variance components and determinants of exposure. The specific aims of this study were to:

I. give an overview of exposure to inhalable dust and endotoxin in a range of industries using comparable measurement protocols,
II. investigate determinants of exposure, to get insight in factors that influence exposure across sectors and industries, and
III. study between- and within-worker variance components as a basis for a protocol for a measurement strategy for endotoxin exposure assessment like those that exist for chemical agents, as described elsewhere.[29]

Methods

Development of database

The study was performed using a database with personal inhalable exposure data collected in 10 studies, performed over the years by the Institute for Risk Assessment Sciences (Utrecht, the Netherlands; former department Environmental and Occupational Health at Wageningen University) and TNO (Zeist, the Netherlands). The studies were conducted over the years 1991-2006 and most results have been published in open literature.[15,27,28,30,41] The original datasets of these studies were made available for this current investigation. Details of the exposure assessment of the studies have been described previously. Briefly, the measurements were performed with 25 mm Teflon filters (PAS-6 sampler, 1 study, 17% of measurements), 25 mm glass fiber filters (PAS-6 sampler, 1 study, 5%) or 37 mm glass fiber filters (GSP-sampler, 8 studies, 78%). After sampling, filters were stored at -20˚C until extraction. The filters were extracted in pyrogen-free water with (9 studies, 83%) or without (1 study, 17%) 0.05% Tween-20 and stored at -20˚C until endotoxin analysis. All extracts were analyzed with the kinetic chromogenic Limulus Amoebocyte Lysate (LAL) assay in one laboratory. In all studies LAL reagents from the same producer were used (BioWhitakker).
The original datasets included 2147 measurements. After excluding task-based and stationary measurements, and measurements without known endotoxin concentration, 2010 personal inhalable measurements were available, gathered from >1000 workers from 317 factories in 46 industries (see Table 2). Of these measurements, 1650 were repeated measurements from 730 workers.

The industries were classified into the following 4 sectors based on similarities regarding the kind of process, materials/products being used, or the manufactured end product:

I. Waste management/treatment: all industries that handle any kind of waste product, including domestic waste.
II. Grains, seeds and legumes processing: the primary production of grains, seeds, legumes etc., as well as the (industrial) processing of these materials into half-products and consumption goods.
III. Horticulture: the indoor (greenhouse) and outdoor culturing and trade of vegetables, flowers and plants, as well as the (industrial) processing of these products.
IV. Animal production: the primary production (farms) and industrial processing (abattoirs etc.) of animals or animal products.

If applicable, a further subdivision within sectors was made based on similarities between industries within a sector; these groups of industries within sectors were called subsectors.
Determinants as listed in Table 1 were either already identified at time of the study or identified afterwards by consulting the primary investigators. Nonetheless, not all information was available or could be assigned afterwards, e.g. descriptive components like tasks

performed or products handled during the measurement, due to the diverse nature and original purposes of the underlying studies.

Table 1: Information about variables in the database

Variable	Description
Survey	Study the measurement(s) originates from
Sector	Description of sector
Industry	Description of industry
Factory	Unique indication
Worker	Unique number
Job	Description of job
Job code	Classification of job
Tasks	Tasks performed (with duration) during measurement
Products	Products handled during measurement
Date	Date of measurement
Exposure concentration	Measured concentration (dust, endotoxin or glucan)
Detection limit	Below (-1) or at or above (0) the detection limit (for endotoxin)
Units	Unit of measurement (mg/m³, EU/m³ or µg/m³)
Analysis	Kind of analysis used to measure exposure
Sampling time	Duration of measurement (in minutes)
Flow	Mean flow during sampling
Sampling equipment	Kind of sampling head used: GSP (=1), PAS6 (=2)
Filter type	Kind of filter used: 37 mm glass fiber, 25 mm glass fiber, 25 mm Teflon
Use of Tween during extraction	No (0) or yes (1)
Sample of workers	Non-random (=0), random (=1), random within jobs (=2), everybody (=3)
Sample of days	Non-random, season (=0), random (=1), fixed days (=2), every day (=3)
Environment	Working environment mainly: outside (=1), inside (=2), outside & inside (=3)
Season	Winter (=1), spring (=2), summer (=3), autumn (=4)
Mechanical ventilation	Absent (=0) or present (=1)
Natural ventilation	Absent (=0) or present (=1)
Local exhaust ventilation (LEV)	Absent (=0) or present (=1)
Use PPE	Use of personal protective equipment (PPE): no (=0) or yes (=1)
Waste water	Absent (=0) or present (=1)
Process water	Absent (=0) or present (=1)
Recycling process water	Absent (=0) or present (=1)
Source of exposure	Local (=0) or general (=1)
Mobility of source	Stationary (=0) or mobile (=1)
Mobility of worker	Stationary (=0) or mobile (=1)
Process	Intermittent (=0) or continuous (=1) (coded based on the work day)
Cyclic process	Absent (=0) or present (=1) (coded based at the process cycle as a whole)
Length of process	Short (=0) or long (=1)
Bulk production/processing	Absent (=0) or present (=1)
Dusty process	Absent (=0) or present (=1)
Exposure pattern	Continued (=0) or variable (=1)
Industrial process	Absent (=0) or present (=1)
Microbial growth in process	Absent (=0) or present (=1)
Plant material	Absent (=0) or present (=1)
Formation (watery) aerosol	Absent (=0) or present (=1)
Damp environment	Absent (=0) or present (=1)
Faeces (human or animal)	Absent (=0) or present (=1)
Contact with living animals	Absent (=0) or present (=1) (at worker level)
Animals	Absent (=0) or present (=1) (at factory level)

The structural relationship of the data was organized in several levels, namely:

I. measurements within workers
II. workers within jobs
III. jobs within factories
IV. factories within industries
V. industries within subsectors and sectors

In some industries measurements were performed in several factories with analogue processes (composting, domestic waste collection, sewage treatment and pig farms), but in most industries only one or two factories were included (Table 2). The measurements were grouped on job code level within industries for statistical purposes, leading to a total of 147 groups.

Statistical analysis

Data were analyzed with SAS statistical software (version 8e; SAS Institute, Cary, NC, USA). Levels of exposure were log-normally transformed before statistical analysis. Exposure levels were calculated as geometric mean (GM) with geometric standard deviation (GSD) for each sector, subsector and industry. Spearman correlations were calculated between inhalable dust and endotoxin concentrations.

Between-worker and day-to-day (within-worker) variance in exposure were determined by applying mixed effects models. Worker identity was introduced as a random factor in order to correct for possible correlation between repeated measurements in the same worker. Any two repeated measurements of the same worker were assumed to have equal correlation (a compound symmetric covariance structure). Between- and within-variance components were estimated by using a restricted maximum likelihood method. In addition, a multilevel approach was used to investigate the manner in which the variability in exposure was distributed over the structural relationship levels in the database by adding those levels as random effects. For statistical purposes, here the maximum likelihood method was used.

Determinants of exposure were identified, and the effect of these determinants on the between- and within-worker exposure variance was investigated by introducing them as fixed effects.[13,20] For this analysis only part of the dataset was used (1757 out of 2010 measurements). Measurements with missing data for one or more of the determinants of interest were removed from the dataset to ensure stability of the analysis. A forward stepwise procedure was followed, applying the χ^2 goodness of fit test (based on -2RLL, restricted log likelihood) for all determinants. Only determinants with a significant χ^2 test were added to the model. Separate models were constructed for endotoxin and inhalable dust exposure. Graphical analyses of residuals were performed to evaluate assumptions of homoscedasticity.

Between- and within-worker variance components were estimated for each exposure group (job code within industry). Exposure groups with less than 2 workers measured twice were excluded from the analysis. As a result, 66 exposure groups were excluded from this analysis. The ratios of the 97.5th and 2.5th percentiles of the variance components of the log-normally distributed endotoxin and dust exposures were estimated for each exposure group. These ratios provide information regarding the ranges of exposures found between workers ($_{\text{between-worker}}R_{0.95}$) and within workers from day-to-day ($_{\text{within-worker}}R_{0.95}$).[17] The cumulative distributions (%) of these ratios were plotted both for endotoxin and dust.

Table 2: Characteristics of the endotoxin database – number of measurements, mean exposure levels for endotoxin (EU/m³), inhalable dust (mg/m³) and endotoxin per mg dust (EU/mg), and correlations between endotoxin and dust for (sub)sectors and industries

Sector-*subsector*-industry	C [a]	N (repeat) [b]	K (repeat) [c]	G [d]	Endotoxin (EU/m³)		Dust (mg/m³)		EU/mg dust	r [e]
					GM (GSD)	range	GM (GSD)	range	GM (GSD)	
OVERALL (total database)	**317**	**2010 (1653)**	**1089 (732)**	**147**	**160 (8.6)**	**0.6-191400**	**0.77 (4.3)**	**nd-131**	**220 (4.4)**	**0.75 ***
Waste treatment & management	**65**	**951 (751)**	**482 (282)**	**29**	**48.0 (4.9)**	**0.6-37000**	**0.40 (3.7)**	**nd-131**	**122 (4.1)**	**0.56 ***
Domestic waste collection	4	179 (162)	79 (53)	2	40.2 (3.0)	2.4-7180	0.58 (2.5)	0.15-9.1	69.7 (2.8)	0.63 *
Mushroom compost preparation	2	41 (34)	24 (17)	4	225 (3.2)	14.1-2430	0.50 (2.3)	0.08-2.6	452 (2.4)	0.62 *
Composting	13	215 (150)	115 (50)	7	155 (5.7)	1.8-37000	0.98 (2.6)	0.13-131	155 (3.4)	0.75 *
Wood power plant	1	8 (6)	5 (3)	4	102 (3.9)	11.4-438	0.33 (4.9)	0.02-2.34	306 (1.9)	0.93 *
Coal & biomass power plant	3	48 (18)	39 (9)	7	26.5 (5.7)	2.2-2100	1.37 (3.5)	0.09-13.4	19.4 (4.0)	0.57 *
Sewage treatment plant	42	460 (381)	220 (141)	5	27.2 (3.7)	0.6-2090	0.17 (3.2)	nd-23.5	150 (4.3)	0.38 *
Grains, seeds & legumes processing	**26**	**351 (292)**	**202 (145)**	**61**	**633 (8.6)**	**2.3-149000**	**1.47 (4.8)**	**nd-102**	**431 (4.6)**	**0.71 ***
Primary production	*3*	*15 (6)*	*12 (3)*	*4*	*2700 (4.6)*	*95.5-41200*	*2.47 (4.3)*	*0.27-56.5*	*1092 (2.9)*	*0.74 **
Potato cultivation	1	2 (-)	2 (-)	1	314 (5.4)	95.5-1030	1.97 (3.9)	0.76-5.2	159 (1.4)	-
Grain harvest	1	3 (2)	2 (1)	1	2100 (2.5)	1030-5790	0.53 (2.1)	0.27-1.2	3990 (1.6)	0.50
Flax culture and processing	1	10 (4)	8 (2)	2	4470 (3.7)	685-41200	4.11 (4.0)	0.59-56.5	1090 (1.7)	0.85 *
(Industrial) processing	*19*	*262 (220)*	*151 (109)*	*46*	*831 (8.3)*	*9.1-131000*	*1.76 (4.6)*	*nd-102*	*473 (4.5)*	*0.70 **
Grain trans-shipment and derivates	1	19 (14)	12 (7)	3	2150 (9.0)	113-131000	6.71 (5.1)	0.77-98.5	321 (3.4)	0.79 *
Cereal seed	1	2 (2)	1 (1)	2	3560 (2.9)	1690-7470	4.05 (1.5)	3.00-5.5	879 (1.9)	-
Grass/corn seed	2	28 (20)	18 (10)	4	1160 (6.7)	9.1-51400	1.44 (5.0)	0.13-98.1	804 (2.8)	0.89 *
Grass drying plant	1	5 (4)	3 (2)	3	2700 (6.2)	179-20200	3.71 (4.0)	0.52-18.3	780 (1.6)	1.00 *
Grass seed	3	27 (24)	14 (11)	4	5470 (4.0)	222-79900	3.37 (3.9)	0.11-34.7	1620 (2.2)	0.72 *
Vegetable seed	3	39 (34)	22 (17)	4	770 (6.4)	25.6-42200	0.96 (3.4)	0.14-14.1	803 (3.5)	0.74 *
Grinding industry	1	17 (16)	9 (8)	5	2810 (4.1)	257-35900	3.50 (2.2)	1.07-16.9	803 (5.9)	-0.38
Corn processing	1	14 (8)	10 (4)	5	710 (7.3)	35.9-30700	7.45 (3.6)	0.75-41.7	95.3 (4.8)	0.54 *
Meal/flour tillage and processing	1	16 (12)	10 (6)	4	281 (7.7)	19.1-28200	1.40 (3.1)	0.20-7.3	202 (3.4)	0.89 *
Animal feed industry	4	87 (78)	48 (39)	10	270 (7.0)	14.4-80500	1.10 (4.5)	nd-102	245 (3.6)	0.78 *
Malting plant	1	8 (8)	4 (4)	2	3730 (4.3)	291-20000	0.73 (1.5)	0.41-1.3	5130 (3.0)	0.88 *
Processing for consumption	*4*	*74 (66)*	*41 (33)*	*11*	*181 (6.6)*	*2.3-149000*	*0.70 (4.7)*	*nd-79.6*	*257 (4.7)*	*0.50 **
Industrial bakery	1	12 (10)	7 (5)	2	49.2 (7.4)	2.3-3030	1.23 (3.0)	0.27-11.2	40.1 (5.5)	0.46
Coffee-roasting and tea trading	1	19 (16)	11 (8)	2	138 (3.4)	12.4-2030	0.69 (2.5)	0.17-2.7	198 (3.1)	0.52 *
Rice hulling plant	1	16 (16)	8 (8)	4	1110 (7.6)	95.1-149000	3.06 (6.0)	0.34-79.6	362 (1.9)	0.94 *
Sugar production (sugar beets)	1	27 (24)	15 (12)	3	134 (4.0)	9.4-2520	0.23 (2.7)	nd-1.3	576 (3.9)	0.33 [#]

Sector-*subsector*-industry	C [a]	N (repeat) [b]	K (repeat) [c]	G [d]	Endotoxin (EU/m^3)		Dust (mg/m^3)		EU/mg dust	r [e]
					GM (GSD)	range	GM (GSD)	range	GM (GSD)	
Horticulture	**19**	**250 (216)**	**142 (108)**	**38**	**162 (7.6)**	**1.6-191400**	**0.67 (3.7)**	**nd-35.1**	**242 (3.9)**	**0.60 ***
Flower bulbs nursery	2	21 (16)	13 (8)	2	565 (3.9)	9.7-4130	1.35 (2.3)	0.28-11.4	419 (3.0)	0.66 *
Mushroom nursery/growing	1	17 (16)	9 (8)	4	80.8 (4.0)	2.9-1350	0.22 (4.2)	nd-0.85	375 (5.6)	0.13
Cucumber and paprika nursery	1	14 (10)	9 (5)	2	157 (2.2)	36.1-650	0.57 (2.1)	0.25-2.4	275 (2.0)	0.77 *
Pot-plants nursery (ficus)	1	8 (8)	4 (4)	2	47.6 (6.7)	1.6-1490	0.31 (2.5)	0.14-2.4	156 (3.6)	0.74 *
Cut flowers nursery/growing	2	31 (28)	17 (14)	2	39.5 (2.7)	4.9-326	0.31 (1.5)	0.11-0.68	127 (2.1)	0.76 *
Tomatoes nursery	1	10 (10)	5 (5)	3	68.7 (2.5)	13.8-342	0.83 (1.7)	0.44-1.9	83.2 (1.8)	0.75 *
Chicory nursery/growing	1	19 (18)	10 (9)	3	136 (2.6)	35.4-769	0.82 (1.6)	0.37-2.0	165 (2.0)	0.67 *
Outdoor	*4*	*50 (44)*	*28 (22)*	*4*	*107 (2.5)*	*8.6-449*	*0.89 (2.4)*	*0.10-9.2*	*120 (2.5)*	*0.52 **
Hardy nursery stock and trading	2	29 (28)	15 (14)	3	123 (2.1)	18.6-347	1.33 (2.1)	0.33-9.2	92.6 (2.1)	0.59 *
Gardening company	2	21 (16)	13 (8)	1	88.7 (3.1)	8.6-449	0.52 (2.0)	0.10-1.7	171 (3.0)	0.46 *
(Industrial) processing	*4*	*44 (34)*	*27 (17)*	*10*	*60.9 (4.9)*	*4.9-1200*	*0.25 (1.9)*	*nd-1.5*	*241 (5.9)*	*-0.03*
Dried subtropical fruit	1	15 (10)	10 (5)	3	19.4 (2.3)	4.9-148	0.40 (1.8)	0.18-1.5	49.0 (2.0)	0.63 *
Vegetable and fruit processing	2	20 (20)	10 (10)	5	177 (4.2)	11.1-1200	0.23 (1.4)	0.13-0.34	758 (4.0)	0.27
Vegetable slicing plant	1	9 (4)	7 (2)	2	38.8 (3.9)	8.5-594	0.14 (2.4)	nd-0.48	268 (6.8)	0.00
Trade	*2*	*36 (32)*	*20 (16)*	*6*	*4000 (9.8)*	*107-191400*	*3.50 (5.4)*	*0.18-35.0*	*1145 (2.2)*	*0.94 **
Flower bulb trade	1	16 (16)	8 (8)	4	388 (1.8)	107-1220	0.59 (1.9)	0.18-2.7	653 (1.7)	0.71 *
Onion trade	1	20 (16)	12 (8)	2	25900 (2.7)	4030-191400	14.5 (1.5)	6.67-35.0	1790 (2.0)	0.77 *
Animal production	**207**	**458 (394)**	**261 (197)**	**19**	**681 (5.2)**	**2.0-19500**	**1.78 (2.8)**	**nd-26.6**	**383 (3.1)**	**0.67 ***
Primary production	*202*	*377 (332)*	*211 (166)*	*3*	*1190 (2.4)*	*62.2-19500*	*2.40 (1.9)*	*0.36-26.6*	*496 (2.2)*	*0.52 **
Dairy farming	2	12 (12)	6 (6)	1	788 (3.6)	62.2-3860	1.35 (1.8)	0.36-2.7	584 (2.7)	0.41
Poultry farm	3	9 (6)	6 (3)	1	1750 (2.8)	360-8120	4.59 (2.0)	1.58-13.6	381 (2.5)	0.70 *
Pig farm	197	356 (314)	199 (157)	1	1190 (2.4)	73.0-19500	2.40 (1.9)	0.36-26.6	496 (2.1)	0.52 *
(Industrial) processing	*5*	*81 (62)*	*50 (31)*	*16*	*51.0 (6.8)*	*2.0-6230*	*0.44 (3.7)*	*nd-21.3*	*116 (5.0)*	*0.48 **
Calf abattoir	1	12 (12)	6 (6)	2	119 (11.8)	2.6-3480	0.23 (4.9)	nd-2.1	512 (5.0)	0.70 *
Poultry abattoir	1	14 (12)	8 (6)	4	308 (7.0)	26.5-6230	1.47 (5.3)	0.22-21.3	209 (1.6)	0.94 *
Cow/cattle abattoir	1	19 (14)	12 (7)	4	30.5 (5.2)	2.0-816.8	0.27 (1.9)	0.07-1.9	113 (5.4)	0.09
Pig/swine abattoir	1	16 (12)	10 (6)	2	27.5 (3.4)	2.4-218.5	0.31 (1.6)	0.15-0.61	89.1 (3.1)	0.38
Meat processing	1	20 (12)	14 (6)	4	23.3 (3.6)	3.0-1420	0.59 (3.3)	0.14-10.9	39.6 (4.9)	0.21

[a] number of companies/factories per industry included in the database
[b] number of measurements (number of repeated measurements)
[c] number of workers (number of workers with repeated measurements)
[d] number of jobs measured
[e] correlation coefficient (r) for inhalable dust and endotoxin exposure
nd: below detection limit, *: $p<0.05$, [#]: $0.05<p<0.10$

Results

Specification of data

The mean sampling time of the measurements in the database was 7.2 hours; only 1% of the observations had a sampling time of <4 h. Furthermore, 90 and 62 measurements were below the limit of detection (LOD) for endotoxin and dust, respectively. These were given a value of two-thirds the LOD for endotoxin and dust of the particular study from which they originated. For 75 measurements, the dust level was missing. In none of the industries were there >25% of the observations below the LOD. Glucan exposures were assessed in some of the studies, but insufficienty data were available for inclusion in this analysis.

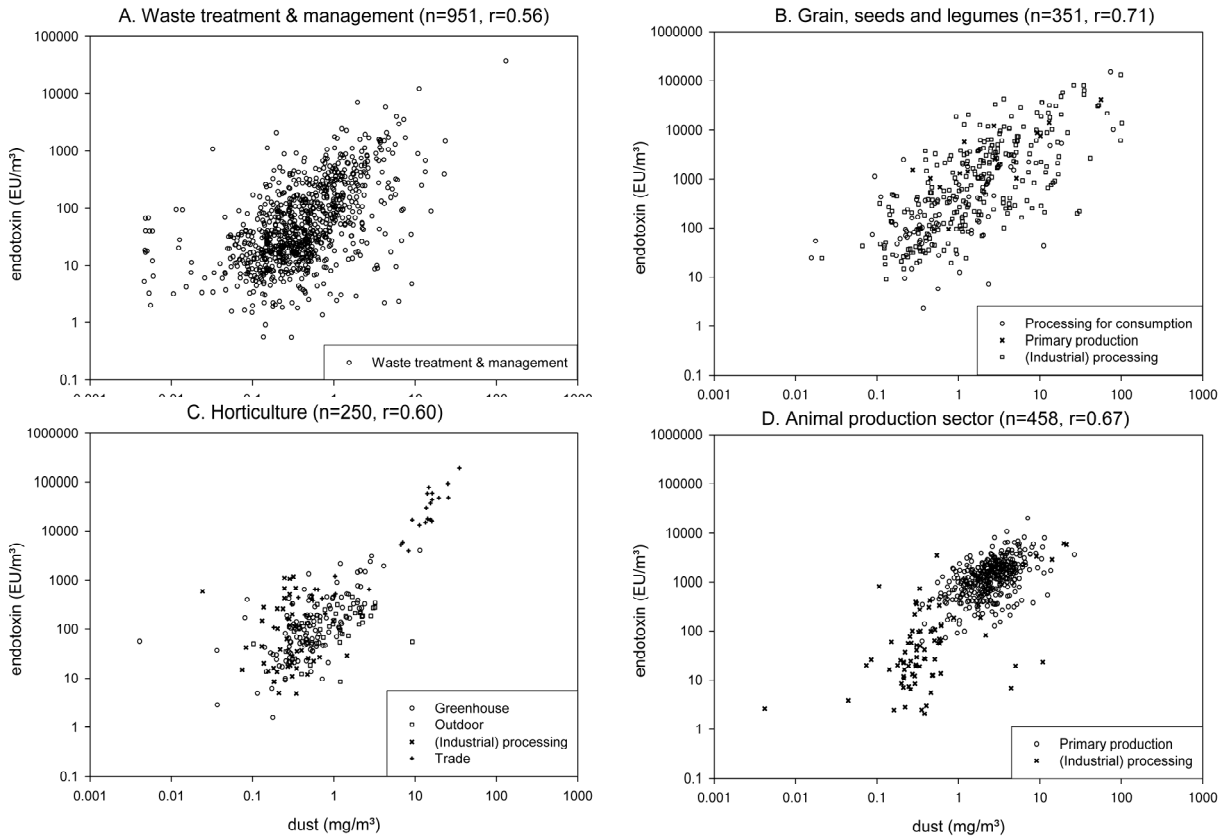

Figure 1: Dust and endotoxin levels per sector (A-D) on a log scale, with number of measurements and correlation coefficient per sector

Exposure levels

Table 2 shows endotoxin, inhalable dust and endotoxin per mg dust levels. The overall GM concentration was 160 EU/m³ for endotoxins and 0.8 mg/m³ for inhalable dust, with distinctly more spread in endotoxin exposure (GSD 8.6 for endotoxin vs. 4.3 for dust). Similarly, endotoxin exposure showed generally larger variability in sectors and industries, both between workers and between days. Highest mean exposure levels for both endotoxin and dust were found in animal production. In ‘grains, seeds and legumes processing’ and ‘animal production’, distinct differences were seen between primary production (front end) and subsequent processing. In ‘waste treatment and management’ and ‘horticulture’, exposure levels were not as clearly different between subsectors, although trade in horticulture had much higher dust and endotoxin exposure levels than the other subsectors. In general, exposure in these sectors was lower than in ‘animal production’ and ‘grains, seeds and legumes processing’.

Correlation analysis

Correlations between inhalable dust and endotoxin differed considerably between sectors and industries within sectors, ranging from virtually no correlation to very strong correlation (Table 2 and Figure 1A-D). The overall correlation coefficient was high (Spearman 0.75).

Table 3: Between- and within-worker variance components in the database, based on log-transformed data (n=2010)

	Endotoxin (EU/m^3)		Dust (mg/m^3)	
	BW	**WW**	**BW**	**WW**
Total database	3.72 *	1.00 *	1.44 *	0.71 *
Waste treatment & management	*1.42 **	*1.13 **	*0.84 **	*0.90 **
Grains, seeds & legumes processing	*3.55 **	*1.00 **	*1.67 **	*0.79 **
Primary production	1.25	1.06	1.35 #	0.67 #
(Industrial) processing	3.30 *	1.08 *	1.44 *	0.86 *
Processing for consumption	2.84 *	0.72 *	1.92 *	0.52 *
Horticulture	*3.27 **	*0.96 **	*1.27 **	*0.47 **
Greenhouse	0.67 *	1.47 *	0.40 *	0.60 *
Outdoor	0.47 *	0.37 *	0.21 #	0.54 #
(Industrial) processing	1.88 *	0.59 *	0.35 *	0.17 *
Trade	4.91 *	0.35 *	2.66 *	0.16 *
Animal production sector	*2.04 **	*0.71 **	*0.69 **	*0.39 **
Primary production	0.15 *	0.63 *	0.11 *	0.32 *
(Industrial) processing	2.56 *	1.00 *	0.91 *	0.72 *

BW: between-worker variance component, WW: within-worker variance component, *: $p<0.05$, #: $0.05<p<0.10$

Variance components

Overall, for both endotoxin and inhalable dust levels, the between-worker variability was larger than day-to-day variability. The same was seen when the data were grouped in sectors or subsectors (Table 3). This indicates that differences in exposure between workers were larger than between work shifts on different days. Exceptions were the waste treatment and management sector, outdoor horticulture and primary animal production for dust exposure, and greenhouse horticulture and primary animal production for endotoxin exposure (Table 3). The ratio of between-worker and within-worker variability was less distinct when workers were grouped in industries or in job codes within industries. Between-worker variability exceeded within-worker variability in 26 and 20 of 46 industries for endotoxin and dust, respectively. Between-worker variability exceeded day-to-day variability in 39 groups in case of endotoxin exposure and in 35 groups when looking at dust exposure in the 81 job code within-industry groups with enough repeated measurements. Both for workers grouped in industries or in job codes within industries, in almost all cases total variance was larger for endotoxin exposure than dust exposure (data not shown).

Different hierarchical levels (sector, subsector, industry, factory, job code and worker) could be distinguished in the database. To gain better insight into between- and within-worker variability, the distribution of exposure variability over these levels was investigated in the same part of the database used for the determinant-analysis (n=1757). Only the between-worker variance was affected taking into account the hierarchical structure of the data, whereas the within-worker variance component did not change noticeably. The between-worker variance component ($\sigma^2_{between\text{-}worker}$=3.6) broke down into a between-subsector, between-industry, between-factory, between-job (job code within industry) and between-worker component. After the between-job component was included ($\sigma^2_{between\text{-}job}$=3.1 and $\sigma^2_{between\text{-}worker}$=0.4), adding other levels like factory or industry did not significantly improve the model, although the individual variance components differed statistically significant from

zero. The between-worker variance component levelled off at a value of ~0.4 for endotoxin exposure and 0.2 for dust exposure, which was clearly lower than in the worker-only model (data not shown).

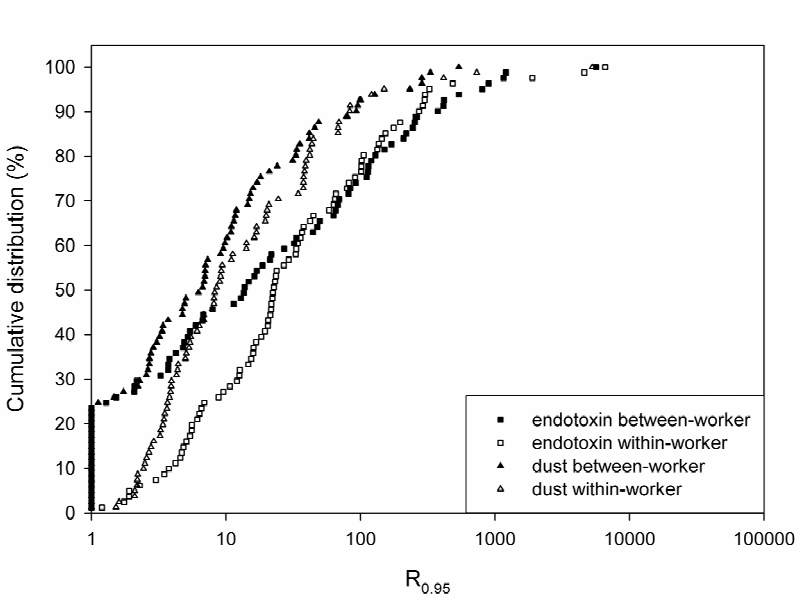

Figure 2: Cumulative distributions of $_{\text{between-worker}}R_{0.95}$ (closed) and $_{\text{within-worker}}R_{0.95}$ (open) for endotoxin (square) and dust (triangle) for 81 groups based on job code within industry

Grouping workers by job codes within industries and excluding groups with less than two subjects and two repeats left 81 groups with 975 workers and 1840 measurements. For these 81 groups, median total, between- and within-worker GSDs were 3.44, 1.95 and 2.22 for endotoxin exposure, and 2.53, 1.62 and 1.73 for dust exposure. In Figure 2, the distributions of the within- and between-worker $R_{0.95}$ for endotoxin and dust exposure are shown for these 81 groups. Generally, both the between- and within-worker values of $R_{0.95}$ were larger for endotoxin than for dust. Only 21 (26%) and 22 (27%) groups had 95% of the individual mean exposures within a factor 2 ($_{\text{between-worker}}R_{0.95} \leq 2$) for endotoxin and dust, respectively. Furthermore, 54% and 40% of the groups had values of $_{\text{between-worker}}R_{0.95} > 10$, and 35% and 12% of the groups even had values of $_{\text{between-worker}}R_{0.95} > 50$ for endotoxin and dust, respectively. Part of the groups had a $_{\text{between-worker}}R_{0.95} = 1$ for both endotoxin and dust (Figure 2). This is due to an estimation problem when calculating variance components. The between-worker variance component is estimated with a zero or negative value in case of a large within-worker geometric standard deviation or few repeated measurements.

Determinants of exposure and exposure variability

Potential determinants of exposure (see Table 1) were tested as fixed effects in a stepwise procedure to explain variability in endotoxin and dust exposure. The effect estimates for variables significantly contributing to the model are shown in Table 4. In the first model, subsector and job code were used initially to account for the influence of these two variables on exposure level.

Table 4: Relative effect of determinants of exposure on endotoxin and inhalable dust levels, in part of the dataset (n=1757)

Variables	Coding variables	1. Model with subsector, job code and determinants		2. Model with determinants only		3. Model with job code within industry and determinants	
		Endotoxin e^{β} [95% CI]	Inhalable dust e^{β} [95% CI]	Endotoxin e^{β} [95% CI]	Inhalable dust e^{β} [95% CI]	Endotoxin e^{β} [95% CI]	Inhalable dust e^{β} [95% CI]
Intercept		20.1 * [13.2-30.7]	0.3 * [0.2-0.5]	76.4 * [52.7-111]	0.7 * [0.4-0.9]	154 * [76.7-309]	0.7 [0.4-1.2]
Subsector	10	x	x	-	-	-	-
Job code	14	x	x	-	-	-	-
Job code within industry	124	-	-	-	-	x	x
Dusty process	present vs. absent	2.7 * [1.4-5.2]	2.3 * [1.1-4.9]	1.9 * [1.3-2.8]	2.2 * [1.7-2.9]	-	-
Contact animals	present vs. absent	7.5 * [3.3-17.0]	3.2 * [1.8-5.8]	3.8 * [2.5-5.9]	2.2 * [1.6-3.0]	-	-
Plant material	present vs. absent	1.6 * [1.2-2.2]	-	-	0.7 * [0.6-0.9]	-	-
Work environment	inside	2.1 * [1.4-3.0]	0.7 * [0.5-1.0]	2.1 * [1.4-3.0]	-	5.5 * [3.2-9.4]	-
	both inside and outside	1.9 * [1.3-2.9]	1.2 [0.8-1.8]	1.4 * [1.0-2.1]	-	5.7 * [2.7-12.3]	-
	outside (ref)	1.0	1.0	1.0	-	1.0	-
Cyclic process	present vs. absent	0.2 * [0.1-0.3]	0.2 * [0.2-0.3]	-	0.4 * [0.3-0.6]	-	-
Bulk production	present vs. absent	4.0 * [2.4-6.4]	-	7.0 * [4.6-10.8]	1.4 * [1.1-1.9]	-	-
Process	continuous vs. intermittent	5.2 * [3.0-9.1]	4.3 * [2.4-7.8]	2.7 * [1.8-3.9]	2.5 * [1.8-3.5]	-	-
Microbial growth	present vs. absent	0.2 * [0.1-0.4]	0.2 * [0.1-0.5]	0.2 * [0.1-0.3]	0.3 * [0.2-0.4]	-	-
Faeces	present vs. absent	2.6 * [1.6-4.1]	-	-	-	-	-
LEV	present vs. absent	0.7 * [0.5-0.9]	-	-	-	-	-
Exposure	variable vs. continuous	-	1.7 * [1.2-2.4]	-	2.0 * [1.5-2.5]	-	1.8 * [1.4-2.4]
Cycle	long vs. short	-	3.1 * [1.3-7.9]	1.9 * [1.2-3.1]	2.5 * [1.7-3.8]	-	-
Season	spring	-	1.5 * [1.2-1.8]	0.9 [0.7-1.1]	1.2 [1.0-1.4]	0.8 [0.5-1.1]	-
	summer	-	0.8 * [0.7-0.9]	0.8 * [0.6-0.9]	0.8 * [0.7-0.9]	0.7 * [0.5-0.8]	-
	autumn	-	2.0 * [1.3-3.1]	1.0 [0.7-1.6]	1.5 * [1.1-2.1]	0.4 * [0.2-0.7]	-
	winter (ref)	-	1.0	1.0	1.0	1.0	-
Formation aerosol	present vs. absent	-	-	0.6 * [0.4-0.8]	-	-	-
Waste water	present vs. absent	-	-	0.6 * [0.4-0.8]	0.7 * [0.6-0.9]	-	0.7 * [0.5-0.9]
Industrial process	present vs. absent	-	-	0.3 * [0.2-0.5]	0.7 * [0.5-0.9]	-	-
Mobility of source	mobile vs. stationary	-	-	-	0.6 * [0.5-0.8]	-	-

*: $p<0.05$, x: estimates not given , -: variable not in model

In addition to subsector and job code, a dusty process, contact with living animals and a continuous process increased both endotoxin and dust exposure, while a cyclic process and microbial growth (when the latter is an inherent part of the process) were associated with decreased exposure levels for both endotoxin and dust. Working inside was associated with increased endotoxin exposure but decreased dust exposure. Furthermore, the models for endotoxin and dust exposure consisted of different additional determinants. Presence of plant material and faeces and production in bulk were associated with higher endotoxin levels, whereas presence of local exhaust ventilation was associated with lower endotoxin levels. For dust exposure, intermittent exposure (versus continuous) and a prolonged (versus short) cycle were associated with higher dust levels, while season had a variable effect.

In a model with determinants only (Model 2), the outcomes were comparable to those of Model 1, with similar effect estimates. However, some determinants no longer had a significant effect on the measured endotoxin exposure (presence of plant material, faeces and local exhaust ventilation), while other determinants were included (a long work cycle, an industrial process, presence of waste water and formation of aerosol). For dust exposure, the work environment was no longer a part of the model, while production in bulk, presence of plant material, waste water, an industrial process, and a mobile source were added (Table 4)When job code within industry was introduced as a fixed effect (Model 3), only work environment and season remained as determinants of endotoxin exposure. As for dust exposure, exposure pattern of the worker and presence of waste water in the process were the only determinants remaining, with their effect pointing towards the same direction as in the other models (Table 4).

Since exposure variability could be distributed over more levels than worker only, we also investigated the way determinants of endotoxin and dust exposure affected a model with both worker and job categories within an industry as random effects. For both endotoxin and dust exposure, approximately similar models were found, with effect estimates analogous to that of a model with only worker as random effect (data not shown).

Because information about some possible determinants (mechanical ventilation, re-circulating process water and source of exposure) was missing for a considerable number of measurements in the database, the additional effect of these variables in the above-mentioned models was tested in a subset of the dataset with complete data for these variables. Addition of these variables did not result in significant additional effects (data not shown).

No clear determinants of day-to-day variability in exposure were identified. Models including determinants of exposure showed only minimal changes in within-worker variability for both endotoxin and dust. However, inclusion of the log-normally transformed dust concentration explained 3-18% of the within-worker variance in endotoxin exposure (Table 5). The between-worker variance was strongly reduced by introducing determinants of exposure. With regard to endotoxin exposure, inclusion of the variable subsector, industry or factory in the model explained 62%, 78% and 78% of the between-worker variability, respectively (Table 5). Model 1 explained 80% and Model 2 explained 72% of both the endotoxin and dust exposure. Introducing job categories within an industry explained 88% of the endotoxin and dust exposure, and introducing other determinants had no substantial additional effect. In the multilevel models with worker and job code within industry as random effects,

determinants mostly explained between-job variability (~60% for both endotoxin and dust exposure) and only a little between-worker variability (~5%) (data not shown).

Table 5: Percentage explained variance by certain variables or models, in part of the data set (n=1757)

Model with fixed effects	Endotoxin		Inhalable dust	
	BW (%)[a]	WW (%)[a]	BW (%)[a]	WW (%)[a]
Worker only	3.64	0.97	1.38	0.67
Model with sector	2.15 (41)	0.96 (~0)	0.91 (34)	0.67 (~0)
Model with subsector	1.38 (62)	0.96 (~0)	0.69 (50)	0.66 (~0)
Model with industry	0.79 (78)	0.98 (~0)	0.29 (79)	0.65 (3)
Model with factory	0.80 (78)	0.97 (~0)	*	
Model with job code	2.82 (23)	0.96 (~0)	0.97 (30)	0.68 (~0)
Model with job code within sector	1.32 (64)	0.96 (~0)	*	
Model with job code within industry	0.44 (88)	0.96 (~0)	0.17 (88)	0.65 (3)
Model with dust concentration	1.12 (69)	0.94 (3)	x	
Model with subsector, job code and determinants (1)	0.69 (81)	0.96 (~0)	0.28 (80)	0.66 (~0)
Model with subsector, job code, determinants and dust concentration	0.41 (89)	0.80 (18)	x	
Model with determinants only (2)	1.02 (72)	0.97 (~0)	0.38 (72)	0.67 (~0)
Model with determinants and dust concentration	0.54 (85)	0.82 (15)	x	
Model with job code within industry and determinants (3)	0.41 (89)	0.94 (3)	0.16 (88)	0.65 (3)

BW: between-worker variance, WW: within-worker variance, *: stopped because of infinite likelihood,
[a]: percentage explained variance

Discussion

A database with many measurements for inhalable dust and endotoxin exposure was set up successfully. Data from 10 individual studies comprising >2000 measurements in >1000 workers were put together in a combined database. Although endotoxin exposure levels of the individual studies have been published before, this is the first study to investigate determinants of exposure levels and more importantly of exposure variability in a broad spectrum of working environments. Measurements in the database have all been collected and analyzed within the same two closely collaborating research groups and analyzed in one laboratory, which minimized the introduction of additional variability.

Some of the individual studies looked, among other variables, at the influence of tasks and specific characteristics of the process on exposure levels.[15,16,28,41] Unfortunately, this detailed information could not be gathered for all measurements in the database. Nonetheless, it can be concluded that determinants of exposure identified in this database are generally in accordance with previous results and give a more complete picture of determinants of dust and endotoxin exposure. For instance, dustiness of the process and contact with animals were found to be determinants of exposure in both the study in agricultural industries [30] and this database. The study in agricultural industries comprises 'only' a fourth of the total database, which also contains data from studies in other industries with different characteristics as well as data from studies in one or a limited set of agricultural industries. Surprisingly, the previously observed exposure determinants appeared to be of importance when taking into account other industries as well. These determinants provide information for the implementation of generic control measures to lower exposure, for instance reducing the dustiness of products or dust formation by preventing leakage from the process. The

presence of determinants that are associated with aqueous media (waste water, formation of watery aerosols, and microbial growth as part of a (moist) process) seem to be associated with lower endotoxin and dust exposures.

Since job code within industry is a very specific categorization, and most of the possible determinants were classified at factory level, not many other variables added a significant effect to the model with job code within industry (Model 3). Furthermore, many of the determinants of exposure occurring in the model with subsector and job code (Model 1) also appeared in a model without subsector and job code (Model 2). This suggests that although many determinants are captured in the variable subsector, other determinants are specific enough to explain some of the differences in endotoxin and dust exposure, which enhances the reliability of the various models. The influence of variables reflecting differences in procedures between the studies (filter type, sampler type, extraction/assay medium used) was also investigated. These did not have an effect and did not change the models.

This database is a collection of individual studies that were initially not set up to be joined at a later stage, which means that they all had their own design and specific study aims. This influenced the way workers, factories and measurement days were selected and information was collected (on job code or industry level, specific or more general). The extent of detail in the information varied from almost none to elaborate information about tasks. Coding afterwards may have led to some misclassification, determinants that are left undetected and availability of information on a more general level.

Most determinants on which information was available concerned company or process characteristics and dust formation, and were coded on company and sometimes worker level. These types of determinants do not vary over time. Therefore, it is not surprising that almost no day-to-day variability could be explained by these variables. Only measured dust concentration explained some variance in endotoxin exposure, but this is expected since measured dust exposure captures day-to-day variability. Furthermore, not all exposure variability could be explained by the current determinants, compared to the models with job code or industry as fixed effects, which suggests that other determinants of exposure also play a role. For a more detailed analysis of exposure determinants, more refined information at a personal level and for instance on sources of exposure and amount of microbiological growth is needed.

When investigating determinants of exposure in the total database, homogeneity of variance components over determinants is assumed.[34,37,40] Since the variance components did not differ much between different sectors, and overall between-worker variability exceeded within-worker variability, this assumption seems reasonable. Furthermore, subsector and job code were included as fixed effects to correct for possible differences.

An important aim of this study was to analyze variance components for endotoxin and dust exposure. Overall, there was a relatively high variability in both endotoxin and inhalable dust exposure, with more variability in endotoxin levels. Furthermore, between-worker variability was generally larger than within-worker variability. This differs distinctly from the situation of exposure to chemical agents. For instance, Kromhout et al. found median values for total, within- and between-worker GSDs of 2.41, 2.00 and 1.43, respectively, in a database for inhalatory exposure to chemical agents.[3] A study on dermal exposure to chemicals showed similar results, with median values for the total, within- and between-worker GSDs of 2.55,

1.98 and 1.47, respectively.[4] The total variance for inhalable dust exposure found in our database was in the same range (median GSD_{total} 2.53), but endotoxin exposure variability was distinctly larger (median GSD_{total} 3.44). Furthermore, between-worker variance was larger as well, although there was more within-worker than between-worker exposure variability when workers were grouped by job code within industry. For both chemical exposure and endotoxin exposure, ~25% of the groups based on job code categories were homogeneous ($_{between\text{-}worker}R_{0.95}$ ≤2). However, for endotoxin 54% of the groups had a $_{between\text{-}worker}R_{0.95}$ >10 and 35% even a $_{between\text{-}worker}R_{0.95}$ >50, compared to 30% and 10% for chemical exposure, respectively [3]. In a previously published meta-analysis, day-to-day variation in exposure generally exceeded variation between workers, with exposure in the chemical industry, on average, more homogeneous than exposures in non-chemical industries. Gaseous exposures were also more homogeneous in comparison with exposures to aerosols or dermal exposures.[33] This may partly be due to a greater degree of variation from day to day in factors that influence the emission of gases/vapours (temperature and pressure) compared with those factors that govern the emission of aerosols (mechanical and physical forces) and the physical and chemical characteristics of the agent that also affect variation in exposure after emission.[11,38]

Studies have shown large inter- and intra-laboratory differences for endotoxin analysis. It has been suggested that this may explain the high variability in endotoxin exposure.[2,9,21,22] However, in the studies in this paper, methods used were fairly similar and protocols only changed marginally over time. The analytical error for endotoxin, expressed as coefficient of variation (CV%), is generally <20%. This analytical error is usually part of the within-worker variance. After correction for this analytical error, the within-worker variance component would only change marginally ($GSD_{within\text{-}worker}$ 2.4 after correction compared to 2.7 for the crude $GSD_{within\text{-}worker}$), which points out that the analytical variance is much smaller than the variability over time. This is in accordance with Nicas et al., who stated that measurement error is often small relative to exposure variability over time.[12] Thus, most of the variability in endotoxin exposure is an inherent part of the true exposure to endotoxin and is presumably caused by the fact that endotoxins originate from Gram-negative bacteria, which grow and amplify. Storage conditions, contamination of process water, differences in temperature, number of micro-organisms in the product (for instance differences between seasons caused by the conditions on the land during culturing and harvesting) and type of dust may all have an effect on the measured endotoxin level. When circumstances are optimal, microbiological activity in products and processes can increase exponentially and lead to an increased endotoxin exposure and increased exposure variability, both between and within workers, even over a very short period of time. The varying endotoxin content of dust confirms this observation. Exposure to chemical agents is not influenced by such factors and thus varies considerably less.

It is expected that workers who have similar jobs have more similar exposures (relative to the variation in exposure over time) than workers at the same location but with different jobs. However, lack of homogeneity in exposure in a considerable number of groups is reported before.[3,32] When bio-monitoring data was grouped on plant level, more variation among workers at the same plant than variation from day to day was found.[32] The same was seen in the endotoxin data, with relatively more between-worker than within-worker variability when grouping workers at sector or industry level, but vice versa when workers were grouped on a more detailed level based on job code. Hence, to arrive at relatively homogeneous exposure groups, one should use a grouping scheme based on at least job code and perhaps an even

more detailed level is needed. This information is important in designing an optimal exposure assessment approach for epidemiological studies.

The large variability in endotoxin exposure also has consequences for existing compliance strategies and the estimation of exposure for epidemiological studies. For instance, the existing CEN 689 guideline for measuring chemical components [1] needs to be modified to take into account the greater variability in endotoxin exposure. If the decision-making process is based on calculations of overexposure and exceedance,[36] for which between- and within-worker variance components from this endotoxin database are used,[29] the number of measurements available, variance around the mean exposure and selected cut-off points (e.g. ½ or ¼ of the occupational exposure limit (OEL)) determine the probability of making an accurate decision based on the number of samples taken.[7,31] Since the exposure variability for endotoxin is large, more measurements will be needed to estimate the probability of overexposure or exceedance.

Conclusions
As far as we know, this is the first endotoxin exposure database that has been created and analyzed for variance components and determinants of exposure. Data from future studies can be added in order to get a more complete picture of occupational endotoxin and dust exposure and factors that influence exposure. Variance components give insight into the variability in endotoxin and inhalable dust exposure, with more variability overall between workers than from day to day. This differs from exposures to chemical agents. Also, the total variance in endotoxin exposure was higher than for chemical exposures, with fewer and less homogeneous exposure groups. Therefore, large variability in measured exposure levels is an inherent part of endotoxin exposure, caused by many factors that influence growth of microorganisms and the process of aerosolization. This should be taken into account when assessing endotoxin exposure. It has consequences for the design of future occupational intervention and epidemiologic studies. The variables in the database may function as a guideline for information that should be gathered. The observed determinants of exposure could be a starting point for the development of control measures, although more insight into determinants that cause day-to-day variability is needed.

Acknowledgements

The authors would like to acknowledge all financers, researchers, students and laboratory workers who made the studies that are part of the database that was used in this study, in particular Lidwien Smit. Furthermore, we thank Tim Meijster and Lutzen Portengen for their assistance in the statistical analysis. This research was granted by the Ministry of Social Affairs and Employment, the Netherlands by means of the long term research program on biological agents in the work environment of TNO Quality of Life.

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Chapter 3.2

Endotoxin: from database to measurement strategy

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Published in Gafahrstoffe – Reinhaltung der Luft, 2007; 67 (9): 377-383.

Abstract

Endotoxin is a well-known toxin which has been associated with several health effects. Many factors influence airborne endotoxin exposure and can cause high variability in exposure between and within workers. Additionally, since the source of endotoxin exposure - gram-negative bacteria - grow and amplify, exposure variability is thought to be larger than for chemical exposure. We therefore explored exposure variability in a database with more than 2000 endotoxin measurements collected over the last years. Based on the outcomes, the existing measurement strategy for chemical agents, the European standard EN 689, was adapted for endotoxin exposure assessment. This measurement strategy for endotoxin exposure should be seen as a novel method taking the increased exposure variability usually encountered with endotoxin exposure into account. It can be a useful guide for occupational hygienists to assess endotoxin exposure and the probability of exceedance and overexposure in a certain working environment. The emphasis of the strategy is on control measures when the circumstances indicate (elevated) endotoxin exposure.

Background

Endotoxin is a constituent of the outer membrane of gram-negative bacteria, and occur as contaminants in organic dusts or aerosols. Endotoxin is a well-known toxin with a high pro-inflammatory potency. Airborne exposure to endotoxins has been associated with acute and chronic respiratory and systemic inflammatory responses in occupational settings.[5,10,20] Many factors might influence exposure (for instance production process, job or task, distance to the source, individual operating procedure, climatic circumstances, presence or absence of ventilation). These factors can vary from day to day and from person to person,[20] and consequently, the exposure levels between and within workers can vary considerably. For endotoxin, the exposure variability is thought to be much larger than for chemical exposure, as endotoxin derives from bacteria, a source that can grow by itself. Gram-negative bacteria have a heterogeneous source and their growth is dependent on many (environmental) factors, which enlarges exposure variability.
The current widely used European standard for the measurement of chemical components EN 689 does not take into account the larger variability in exposure levels for biological components.[1] Our aim was to develop a guideline for endotoxin exposure assessment, taking the larger variability in exposure levels into account. Changes to the EN 689 protocol were based on the information obtained from a database containing more than 2000 endotoxin measurements collected in a range of industries. The database and measurement strategy give input for compliance testing, implementation of control measures, and design of future hygiene studies in the field of endotoxin exposure.

Development of the database and calculations for measurement strategy

Available personal exposure data (airborne endotoxin and inhalable dust concentrations) from ten studies performed in the years 1991 to 2006 were combined to form a database containing 2010 measurements in 1089 workers, of which 1653 were repeated measurements. Almost all results from the ten studies have been published in open literature.[13,21-23,27] The original datasets were made available by the investigators. The mean sampling time was 7.2 hr, and all samples were analyzed in the same laboratory using the kinetic LAL-assay. Study or sample characteristics were allocated or recoded based on information obtained from the original studies and interviews with investigators. The data were classified in 46 industries, divided over ten sub-sectors in four sectors.
Descriptive exposure levels (geometric mean (GM), geometric standard deviation (GSD) and range) and Spearman correlation coefficients between endotoxin and inhalable dust were calculated. Mixed effects models were used to estimate between-worker and within-worker variance components and their contribution to the total variance.[12,17] These variance components were used to calculate the probability of overexposure and non-compliance to several proposed exposure limits.[26] The variance components combined with observed actual exposure levels were used to calculate the minimum number of subjects and measurements needed for accurate exposure assessment (sample size calculations).[16]

Content of the database

Table 1 presents an overview of the endotoxin and inhalable dust exposure levels, overall and per sector. The observations in the database originated from many different industries and companies within these industries. The observations are not evenly distributed over the sectors and industries.

Table 1: Overview exposure levels and variance components for endotoxin and inhalable dust from the database

Sector	Industries/ companies	N	Endotoxin in EU/m³				Inhalable dust in mg/m³			
			GM	GSD	σ_b^2	σ_w^2	GM	GSD	σ_b^2	σ_w^2
Overall	46 / 317	2010	160	8.6	3.65	1.00	0.77	4.3	1.44	0.71
Waste management	6 / 65	951	48	4.9	1.42	1.13	0.40	3.7	0.83	0.90
Grain, seeds and legumes	18 / 25	351	633	8.6	3.65	1.00	1.47	4.8	1.69	0.79
Horticulture	14 / 20	250	162	7.6	3.19	0.95	0.67	3.7	1.25	0.47
Animal production	8 / 207	458	681	5.2	2.03	0.71	1.78	2.8	0.68	0.39

GM: geometric mean, GSD: geometric standard deviation, σ_b^2: between-worker variance component, σ_w^2: within-worker variance component, EU: Endotoxin units

Almost 45% of the measured endotoxin concentrations were above the earlier proposed Dutch occupational exposure limit of 200 EU/m³ [6]. The variation in endotoxin exposure (GSD = 8.6) was distinctly higher than in inhalable dust exposure (GSD=4.3) (Table 1). Most (79%) of the total variability in endotoxin exposure is due to variability in exposure between workers (Table 1). This means that differences in average exposure between workers are considerably larger for endotoxin than for dust. In contrast, earlier publications on the analysis of exposure to chemical agents showed much less overall variation in measured exposure levels (median GSD = 2.41). Kromhout and colleagues showed that the median between- and within-worker variance components were 0.13 and 0.48 respectively, indicating relatively more within- worker variability as opposed to more between-worker variability for endotoxin exposure.[8]

All samples in this database have been analyzed in the same laboratory. Previous investigations have shown that large intra- and inter-laboratory differences in measured endotoxin concentration can exist,[4,11,18,19] which has been suggested to be part of the cause of the large variability in endotoxin exposure. This might be true for different studies from different groups published in the open literature, but within studies methods are very likely to have been similar. A remaining factor of importance may be the analytical or intra-laboratory variability or measurement error. For endotoxin the analytical error usually is around 20% expressed as a coefficient of variation (CV). However, the within-worker variance component would only change marginally after correction for this analytical error ($GSD_{within\text{-}worker}$ 2.4 after correction instead of 2.7 for the crude $GSD_{within\text{-}worker}$). The total variance would change even less compared to the existing variance because of the large between-worker variance component. This means that the variability in endotoxin exposure cannot be attributed to measurement error but is an inherent phenomenon, and most probably due to the fact that endotoxin originates from biological material, on which gram-negative bacteria grow and amplify. Ideal circumstances for microorganisms can cause an exponential increase in growth and amplification of bacteria, and thus, in endotoxin exposure leading to increased variability in exposure. This is obviously not the case for exposure to chemical agents.

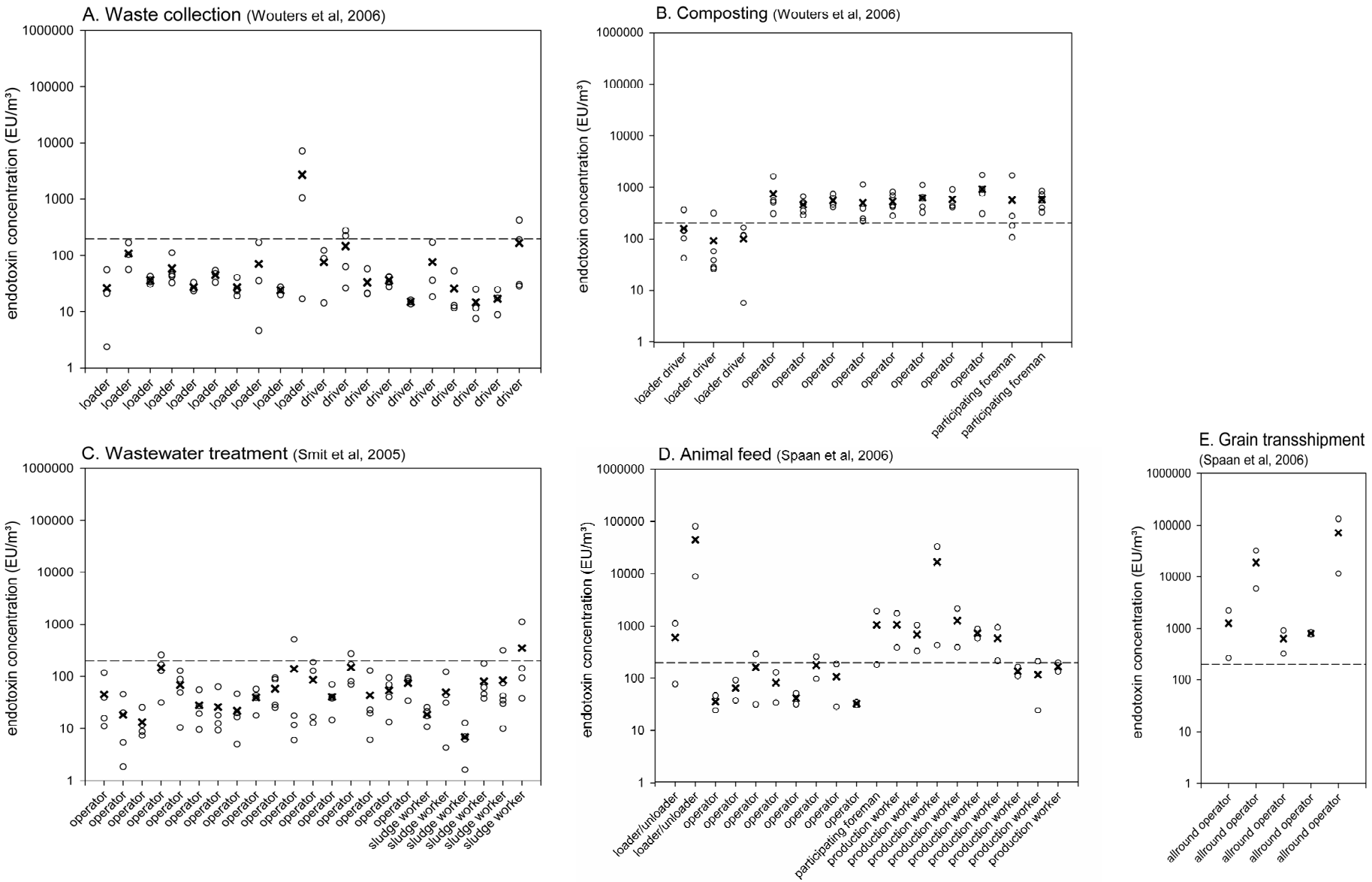

Figure 1: Observed variability in endotoxin exposure (examples of overexposure and exceedance), lognormal scale (o = data point, **x** = AM, ---- = proposed Dutch OEL of 200 EU/m³)

In compliance testing, measured exposure levels are compared with occupational exposure limits (OELs). Exceedance is the probability that a random selected worker's measurement exceeds the OEL on a randomly selected day. Overexposure is the probability that the individual long-term mean exposure of a randomly selected worker would exceed the OEL.[26] Figure 1 displays the variability in measured endotoxin concentrations in workers with a specified job in a few industries. In the industries waste collection and wastewater treatment only a few workers have measurements exceeding the proposed Dutch OEL of 200 EU/m³, and merely one worker's mean exposure is above that OEL (overexposure) (Figure 1A and C). In a series collected in the composting industry, distinct differences can be observed between loader drivers and operators, although the variability in endotoxin exposure is relatively small, with some observations of the loader drivers exceeding the OEL and overexposure for all operators (Figure 1B). In both the animal feed industry and grain transhipment there is a high variability in exposure, with large differences between jobs (Figure 1D and E).
The allround operators in grain transhipment are exposed to very high and highly variable levels of airborne endotoxin, which are all above the proposed OEL. The operators in the animal feed industry in general have exposure levels below the OEL, whereas loaders/unloaders and production workers have a large probability of overexposure.

When making a decision based on samples taken in an occupational hygiene strategy, there is always a certain probability of making a wrong decision (conclude that there is overexposure when there is not or the other way around). This probability depends on the number of measurements available, the variance around the mean exposure (standard deviation) and cut-off points (for instance ½ or ¼ of the OEL) that are usually chosen in this type of decision schemes.[9,24,25] When the variability in exposure is large and/or the mean exposure is near the proposed limit value, more measurements are needed to be able to estimate probability of overexposure or exceedance in a valid way. Furthermore, lowering the cut-off points will lead to a lower likelihood of a false positive decision at the expense that more often one will propose to take more measurements. In this case we chose to perform calculations with the proposed Dutch OEL of 200 EU/m³, but another limit value could also been used.

Measurement strategy

A risk inventory and evaluation (RIE) or health complaints from workers can be the motivation to investigate the degree of endotoxin exposure. Figure 2 shows the structure of the measurement strategy for assessment of the exposure situation. This strategy has been developed on the basis of the database and the already existing EN 689 protocol. It consists of 3 phases. Every phase can result in a conclusion that the exposure situation is acceptable, advice on control measures or a more precise estimation of the exposure situation. After implementation of control measures, the preceding phase in the strategy has to be evaluated again. Ultimately, this process will continue until an acceptable exposure situation is reached. After reaching an acceptable exposure level, one should periodically check whether the acceptable exposure situation still exists.

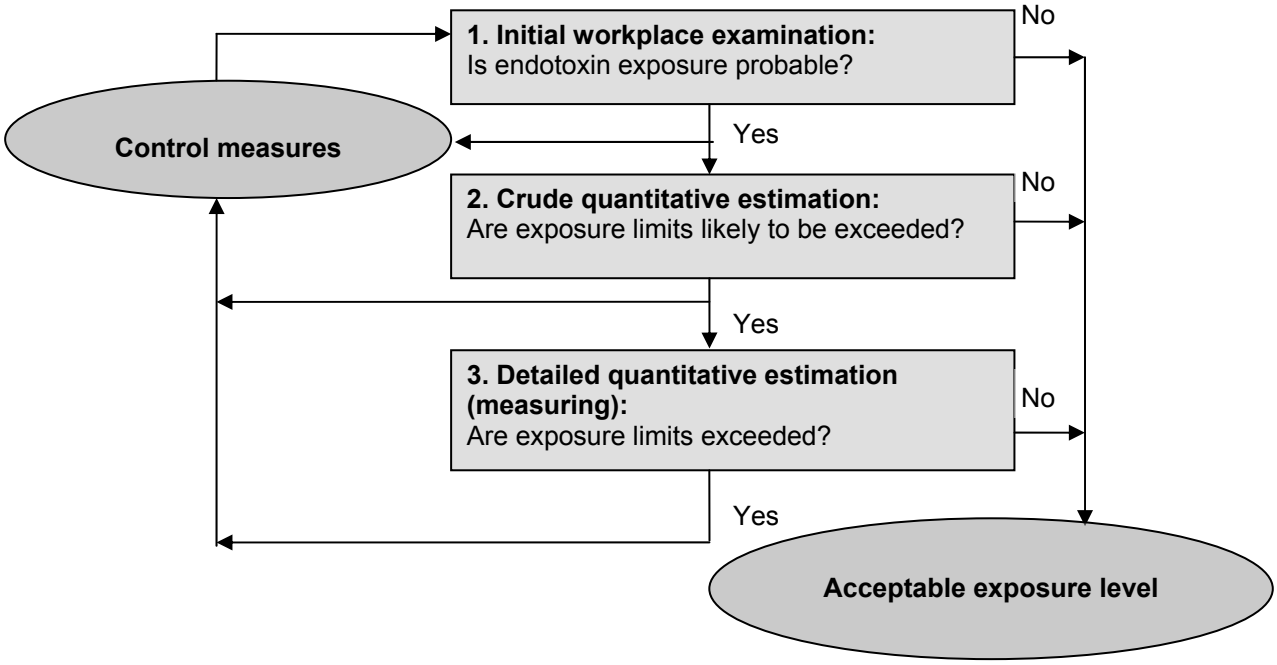

Figure 2: Three-phased structure of the measurement strategy for endotoxin exposure

Phase 1: Initial workplace examination.
In this phase possible sources of exposure are identified. Moreover, activities that have a high probability of (causing) endotoxin exposure are also identified. In contrast to exposure to chemical agents, the focus with biological agents like endotoxin should also be on factors that can cause exponential growth and amplification. Factors associated with endotoxin exposure are presence of organic material or substrate (e.g. manure, paper, plant material, meal, flour, compost and animals), presence of circumstances favorable for growth (e.g. optimum temperature, humidity, pH, and oxygen) and spreading or distribution (e.g. through other particles like moist, dust, aerosols and dirt, and movement) should be assessed. These factors can be transformed into a checklist for each worker with potential exposure, when information about determinants of exposure is available. One may decide to perform measurements when one or more of the factors are present. This approach may be improved on the basis of a priori prediction of overexposure or exceedance based on exposure models. According to this simplistic model endotoxin exposure is assumed to occur when organic material is present, and the circumstances are in favor of growth and distribution.

Phase 2: Crude quantitative estimation of exposure.
A first and crude estimation of endotoxin exposure can be obtained in several ways, namely on the basis of a comparison with previously performed measurements (from open literature or measurements in the company in the past), estimated endotoxin content of bulk or product samples (the source of exposure), stationary measurements (can also be used for identification of sources of exposure or evaluation of control measures), or by performing some indicative personal measurements. Measurements need to be performed according to EN 13098 and EN 14031.[2,3]

On the basis of the available information, decisions about further actions needed have to be made. The above-mentioned options to estimate endotoxin exposure in a crude fashion give insight in the level of endotoxin exposure. However, the way a given exposure situation is assessed should be taken into account. When the GM is estimated on the basis of

measurement data from literature, product samples, inhalable dust concentrations or a very small number of personal measurements, the calculations are less accurate than when for instance ten personal measurements are available. Statistical analysis of the exposure data of for instance ten indicative measurements gives insight into the overall, day-to-day and between-worker variance in exposure.

Table 2: Probability of overexposure and exceedance for different mean exposures (GM) and variance components. The variance components are based on the 25-, 50- and 75-percentile of the variance components of the exposure groups (jobs within industries).

Variance components of exposure groups	GM in EU/m^3	Variance components		Probability of	
		σ_b^2	σ_w^2	overexposure	exceedance
25-percentile	25	0.1	0.3	0.00	0.00
	50	0.1	0.3	0.00	0.01
	100	0.1	0.3	0.04	0.21
Median	25	0.6	0.7	0.01	0.03
	50	0.6	0.7	0.09	0.11
	100	0.6	0.7	0.33	0.27
75-percentile	25	1.4	1.3	0.11	0.09
	50	1.4	1.3	0.27	0.20
	100	1.4	1.3	0.48	0.34

σ_b^2: between-worker variance component, σ_w^2: within-worker variance component

To assist in making decisions about further actions, a scheme is formulated which takes an OEL of 200 EU/m^3 as starting point and has cut points for decision making. These cut points are derived from calculations of overexposure and exceedance for which the between- and within-worker variance components referring to the 25-, 50- (median) and 75-percentile of these variance components of the exposure groups (jobs within industries) in the endotoxin database and possible GMs are used (Table 2). The table shows that probability of overexposure and exceedance depend on the GM and the within and between variability of exposure. For instance, when the GM is 25 and the 25-percentile variance components are used, the probability of overexposure and exceedance are both extremely low and assumed equal to 0. Under the condition of a similar GM, but a considerably higher variability, the probability of overexposure and exceedance are already both around 10%. For higher GMs with similar low or high exposure variability these probabilities are generally higher. The variance components given for the high variability scenario are likely to occur and have been encountered regularly in the database.

Therefore, assuming the 75-percentile of the between- and within variance components, cut points of 25 and 100 EU/m³ are required to avoid false positive and false negative conclusions about the probability of overexposure and exceedance even at relatively low exposure levels. This leads to the following scheme:

- GM < 25 EU/m^3 → acceptable exposure level, and likelihood overexposure and exceedance is low;
- 25 < GM < 100 EU/m^3 → go to phase 3 (detailed quantitative estimation of exposure);
- GM > 100 EU/m^3 → implement control measures (OEL is very likely to be exceeded).

This scheme leads to the decision that when the GM of 10 measurements is below 25 EU/m^3, the exposure level is acceptable and it is unlikely that exposures above 200 EU/m³ will occur. When the GM is over 100 EU/m^3, the OEL is likely to be exceeded. In this case, control measures should first be implemented and then the exposure situation should be evaluated again. However, when little information is available about determinants of exposure, it could be necessary to perform more measurements to be able to effectively control exposure. When the GM is between 25 en 100 EU/m^3, one can pass on to phase 3 of the measurement strategy, a detailed quantitative estimation of exposure. However, when

the GM is above 25 EU/m^3, but the variance in exposure is very small, it might be concluded that exposure situation is acceptable.
The exposure situation is considered acceptable, when the probability of overexposure is less than 10%, which is a commonly accepted probability of overexposure.[16] The higher the (expected) exposure variance, the higher the probability of exceeding the OEL.

Phase 3: Detailed quantitative exposure estimation
Traditionally, occupational hygienists apply a worst-case exposure assessment scenario, in which the highest exposed workers or circumstances are selected for measurements. The idea is that when exposure is below the exposure limit in such situations, there will be no overexposure or exceedance. However, this method is discouraged because it appears that selection of worst case scenarios is not always straightforward and therefore subject to error. In view of the large variability in endotoxin exposure levels, the formation of an accurate sampling strategy is very important. Therefore, a personal random sampling strategy, as shown in Figure 3, is proposed. Besides estimation of the exposure situation and calculation of the probability of exceeding the OEL, this strategy gives information for implementing control measures [7]. For this, repeated personal measurements are to be performed for randomly selected workers from a group (per job title or work location) on randomly selected days, to be able to take into account differences in exposure between workers and within the same worker on different days. For comparison with exposure limits the measurements need to be personal, preferably during a whole working day (8 hr) but at least 4 hr.

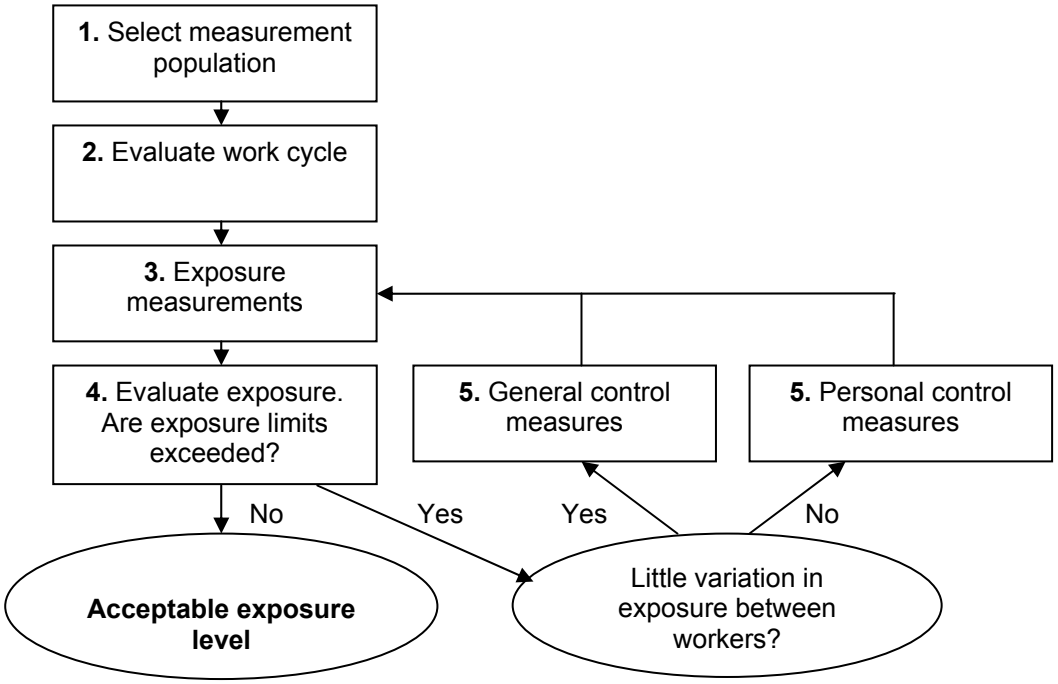

Figure 3: Strategy for quantitative estimation and control of endotoxin exposure

The following steps are suggested (Figure 3):
1. Definition of exposure groups on the basis of the aim of the study, for instance the workers of a certain company, workers with the same job or working with the same substance. If the exposure group is large enough, the workers should be selected randomly. The selected workers should be measured twice.
2. Random selection of days on which the workers are measured, taking into account the work cycle of the activities of workers. Certain tasks are performed on specific moments

of the day or of the week, for instance cleaning activities. The season should also be taken into account, because season (summer or winter) is known to influence endotoxin exposure. When patterns in activities exist, the measurement period should cover the whole work cycle including all activities.

3. To be able to distinguish exposure in two dimensions, namely between- and within-worker variability, repeated measurements within one worker should be performed. The number of persons and the number of measurements per person depend on the expected exposure and exposure variance. As a rule of thumb, two measurements per worker from a certain exposure group should be performed, to give sufficient information.
   The exposure variability in the endotoxin database suggests that 40 repeated measurements (preferably 20 workers with two measurements per worker) are generally necessary to be able to draw conclusions about the probability of exceeding an exposure limit with sufficient certainty. This is an indicative figure since the required number of measurements is dependent on components of variance and ratio of mean exposure and OEL, which varies from situation to situation.
   When assuming that ten indicative measurements have already been performed (phase 2), another 30 measurements should be carried out according to EN 13098 and EN 14031.[2,3]
4. When a dataset of 40 measurements is available, the probability of exceedance of an exposure limit can be calculated. These calculations assume that endotoxin exposure is lognormally distributed and are therefore based on lognormally transformed data.
   In usually applied strategies, like EN 689, the probability of a random selected worker's measurement exceeding the OEL on a randomly selected day (exceedance, γ) is calculated. Because in case of endotoxin exposure chronic health effects are considered, it is important to compare the mean exposure of workers with the OEL by calculating the probability of overexposure (θ). An overexposure of 10% is found acceptable, which means that the probability of a randomly selected worker's mean exposure exceeding the OEL should be 10% or less.
   For the calculations, both the between- and within worker variance component should be available, which can be calculated with most statistical programs. A module for calculations in Excel is being provided by TNO.
   The probability of exceedance (γ) and overexposure (θ) can be calculated with the following formulas[26]:
   $\gamma = P\{X_{ij} > OEL\} = 1 - \Phi \{ (\ln(OEL) - \mu_y) / \sigma_y \}$
   $\theta = P\{\mu_{xi} > OEL\} = 1 - \Phi \{ (\ln(OEL) - \mu_y - (\sigma^2_w / 2)) / \sigma_b \}$
   Φ = the probability that a standard normal variate would fall below the value x
   μ_y = mean value of the lognormal distribution
   σ_y = standard deviation of the lognormal distribution
   σ^2_w = within-worker variance component
   σ_b = between-worker standard deviation
5. There are two dimensions of control measures. General control measures, like spatial ventilation or removal of the source of exposure, have an effect on the whole exposure group. Personal or task-based control measures, like local exhaust ventilation, lower the exposure of an individual worker. When the exposure of a certain exposure group is uniform (small differences in the mean exposure of workers within a group), general control measures will be most effective. When exposure within a group is not uniform, control measures on an individual level are recommended.
   The uniformity of exposure in a certain exposure group can be calculated using the between-worker variance component (σ^2_b). Rappaport et al. have introduced a scale-

independent measure of exposure variability, the ${}_bR_{0.95}$ (= $e^{3.92\sigma_b}$), which is the ratio of the 97.5 and 2.5 percentile of the between-worker exposure distribution ($\sigma^2_{b\ 0.975}$ / $\sigma^2_{b\ 0.025}$).[14,15] In case that ${}_bR_{0.95} < 2$, exposure is arbitrarily considered uniform for that exposure group.

Implications and conclusion

In various sectors and industries, high airborne endotoxin exposure levels have been found. Variability in endotoxin exposure is higher than variability of exposure to inhalable dust or chemical agents. The database with over 2000 endotoxin measurements provided the necessary information to be able to adapt the existing measurement strategy for chemicals (EN 689) to be used for endotoxin exposure assessment for compliance testing and direction for control strategies. This strategy is now based on an OEL of 200 EU/m³. However, the same principles apply for any cut point that is chosen after adapting the calculations.
This new measurement strategy is a first step towards guidance on assessment of the probability of overexposure for agents with chronic health effects, such as endotoxins. With this strategy, occupational hygienists acquire a tool for a structured way to assess exposure, based on modern statistical concepts. Hygienists have to consider that usually more measurements over a longer period of time are required to be able to evaluate endotoxin exposure in a valid way because of the high variability present.
The emphasis of the strategy is on control measures when the circumstances indicate (high) endotoxin exposure, and therefore, needless expensive measurements are avoided. Together with the (being optimized) guidelines for the actual measurement of endotoxin, EN 13089 and EN 14031 [2,3], this measurement strategy can be a practical tool for the assessment of endotoxin exposure in a structural and reproducible way. The structured approach of phase 3 allows the results to be compatible for compliance testing, and should be taken into account in future occupational studies.

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Chapter 4

Optimization of the measurement and analytical procedure

Chapter 4.1

Optimization of airborne endotoxin exposure assessment: effects of filter type, transport conditions, extraction solutions, and storage of samples and extracts

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Published in Applied and Environmental Microbiology, 2007; 73 (19): 6134-6143.

Abstract

Endotoxin exposure occurs in homes and occupational environments and is known to cause adverse health effects. In order to compare results from different studies and establish standards, airborne endotoxin exposures should be assessed using standardized methods. Although the European Committee for Standardization (CEN) developed guidelines for endotoxin exposure assessment, these leave room for individual interpretation. The influence of methods of sampling, extraction and analysis has never been investigated in a full experimental design. Thus, we sought to fully elucidate the importance of all facets of endotoxin assessment.

Inhalable dust samples collected simultaneously were used to investigate the effects on and interactions with airborne endotoxin concentration in two working environments of filter type (glass fiber or Teflon), transport conditions (with/without desiccant), sample storage (-20 or 4˚C), extraction solution (pyrogen-free water (PFW) or PFW plus 0.05% Tween-20), extract storage (-20 or 4˚C) and assay solution (PFW or PFW plus 0.05% Tween-20).

Four hundred samples were collected and randomly distributed over the 20 combinations of treatments. There were no differences found for transport conditions and storage temperature of extracts. Also no interactions between study variables existed. Sampling on glass-fiber filters, storage of samples in the freezer, and extraction in PFW plus 0.05% Tween-20 resulted in a 1.3-, 1.1-, and 2.1-fold-higher estimated endotoxin concentrations, respectively. Use of PFW plus 0.05% Tween-20 in the assay solution had an additive effect.

Thus, this study investigated gaps in the CEN protocol and provides data with which to fully specify a protocol for standardization of endotoxin exposure assessment.

Introduction

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

The European Committee for Standardization (CEN) developed guidelines for the assessment of workplace exposure to airborne bacterial endotoxins, using the knowledge available at that time.[2,3] These guidelines provide methods for sampling, transportation and storage of samples, and determination of endotoxins. However, the NEN-EN 14031 protocol "Workplace atmosphere–determination of airborne endotoxin" fails to clearly delineate aspects that might affect the outcome, for example, what extraction solution or storage conditions to use. There are few empirical data to support some of the assumptions in the protocol. This leaves room for individual interpretation and nonuniform methodology.
Differences exist in laboratory methods for collection of samples (filter type), transport conditions and storage of samples, processing and analysis of samples (extraction medium, rocking, sonication, temperature, type of assay, and control standards) and reporting of results (units).[29] Previous investigations of interlaboratory differences in endotoxin analyses showed that results could differ by a factor of 10 to 1000 between the minimum and maximum concentration of cotton dust samples, a factor which was reduced to a 5- to 12-fold difference when the extraction protocol and assay were standardized.[5] Another study showed that when further restrictions were applied (e.g. same assay supplier, same dilutions, and inclusion of results with valid spike results only), interlaboratory differences could become even smaller (two to three-fold difference), suggesting that interlaboratory differences might be explained to a large extent by the effects of varying procedures.[17]

Several studies investigated how changes in procedures affect the endotoxin concentration in occupational settings[8,9,12,15,19-21,23,31,33,35,38] and in house dust.[11,13,14,22,24] Most of these studies investigated only one or two of the factors possibly influencing the measured endotoxin concentration and in a limited number of samples, although the high variability in endotoxin content of dust calls for experiments with a large number of samples. Therefore, the combined influence of different factors and their interaction is still unknown. In most of the studies only one type of dust was investigated. Recent studies showed that variability between labs also depended on the source of dust that was analyzed.[28,29] Thus, the environment sampled needs to be taken into account when effects of different procedures are investigated.

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

measurement and analysis of endotoxin so that exposure levels can be compared between studies and with established exposure limits.

Materials and methods

Design of the study

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

These four primary factors of interest and their interactions resulted in 16 combinations that were studied using glass fiber filters, the preferred filter type in the CEN-protocol. Since samples were collected with parallel samplers that had the capacity for 20 parallel samples to be collected simultaneously, an additional four combinations of factors could be investigated. We choose to study selected factors (storage of samples and extraction solution) with another filter type (Teflon), since Teflon filters are used regularly when allergens and endotoxins are measured simultaneously. Teflon filters were transported without desiccant and extracts were stored at -20°C. Furthermore, since there has been debate on the use of Tween-20 when measuring endotoxin, we decided to analyze part of the samples both with and without use of Tween-20 in the assay solution to investigate its influence on the outcome in combination with the other parameters.

Thus, two secondary parameters were also included in the experiment: filter type (glass-fiber or Teflon filters) and assay solution (PFW with or without 0.05% Tween-20).

An overview of the distribution of samples over the combinations is given in Table 1 and Figure 1. The 20 combinations of the above factors were assigned randomly to the 20 parallel sampling positions available per run.

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

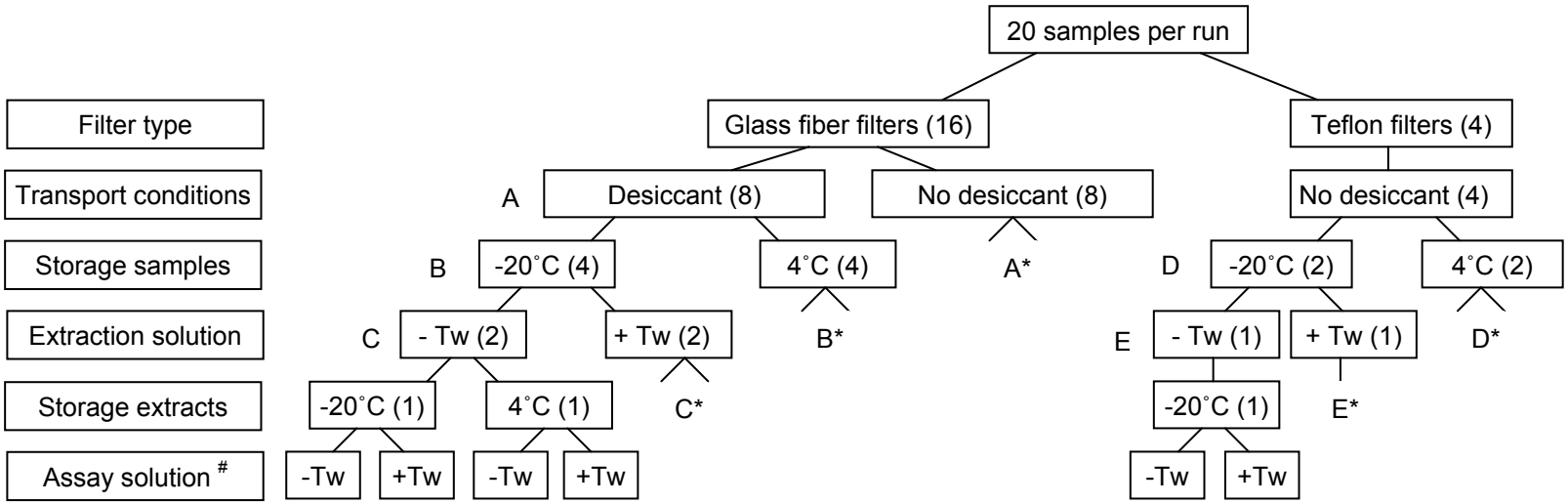

Figure 1: Schematic overview of the design of the experiment. Asteriks marks places from which the scheme follows the same route as written out from the stage with the corresponding letter besides the design step. The number sign (#) indicates that the influence of assay solution was investigated in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

Collection of inhalable dust samples

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

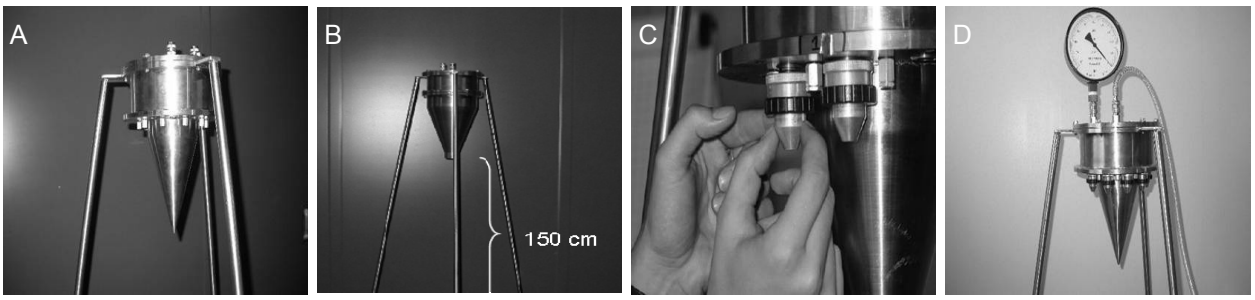

Figure 2: Pictures of the parallel sampler, which contains 10 sampling heads positioned in an annular chamber in between the inner and outer cone. (a) Parallel sampler without outer cone; (b) parallel sampler with outer cone; (c) placement of sampling heads in parallel sampler; (d) parallel sampler with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

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

Extraction and analysis

Samples were stored 12 to 14 days prior to extraction. Extraction of endotoxin was done as described previously, under pyrogen-free conditions.[8] Briefly, filters were immersed in 5 ml extraction solution (being either PFW or PFW plus 0.05% Tween-20) and rocked vigorously for 1 h at room temperature on a horizontal shaker (160 reciprocations/min, deflection 15 cm). After 15 min of centrifugation at 1000xG, 1 ml supernatant per sample was collected and vortexed, and four aliquots of 0.1 ml and the remaining 0.6 ml were stored until analysis. Storage temperature was either 4°C or -20°C, depending on the assigned treatment.

The endotoxin concentration in extracts was assayed using a kinetic chromogenic *Limulus* amoebocyte lysate (LAL) method (Cambrex, Verviers, Belgium; lysate lot no. 3L433E, standard lot no. 3L2950 [reference standard endotoxin/control standard endotoxin ratio 10 ng/0.90 ml = 100 endotoxin units (EU)/ml]).[8] One of every eight samples was randomly selected for analysis in duplicate to assess the coefficient of variance (CV%). The lower LOD

ranged from 0.043 to 0.064 EU/ml depending on the particular assay run. Duplicate analyses took place in the same week.
All sample extracts were analyzed with 0.05% Tween-20 in the assay solution (PFW). In addition, a randomly chosen subset of the samples was also analyzed in PFW without Tween-20 at the same dilutions.

Statistical analysis
The variation in dust levels within a sampling run and between sampling runs was investigated by means of descriptive statistics (SAS version 8e; SAS Institute, Cary, NC, USA). Concentrations of endotoxin (EU/m³ and EU/mg dust) fitted a log-normal distribution; therefore, data were logtransformed prior to analyses. Descriptive statistics (geometric mean, geometric standard deviation and range) of endotoxin concentrations were calculated for every combination of factors of interest. The influence of and interaction between the different variables were determined by applying mixed effects models, with run as a random factor in order to correct for possible correlation between measurements in the same run. Assuming that two repeated measurements of the same run have equal correlation (a compound symmetric covariance structure), between- and within-run components of variance were estimated by using a restricted maximum likelihood method. Determinants influencing endotoxin concentration were explored by introducing them as fixed effects.[25,27] Separate models were constructed for endotoxin and endotoxin per mg dust exposure.
Finally, the influence of the measured dust concentration on the filters and thus the homogeneity of the samples was evaluated by adding the lognormally transformed dust concentration to the various mixed effects models as a fixed effect.

Results

Overview of samples
Of the 400 samples collected in this experiment, 18 dust and 14 endotoxin samples were compromised during weighing, extraction or analysis, leaving 382 dust and 386 endotoxin samples for statistical analysis. Of these samples, 37 were below the LOD (0.05 mg) for dust weight. All samples were detectable for endotoxin. The lost samples originated from different runs and different combinations of variables (Table 1). The mean endotoxin level on the field filter blanks (n=24) was 0.78 EU/ml (range 0.06 to 3.83). Since the minimum of the samples was 9 EU/ml, contamination during assembling of the sampling heads and parallel samplers was unlikely. A random subset of the endotoxin samples (n=56) was analyzed in duplicate, which resulted in an average CV% of 21.5 (range 0.2 to 71.3). Endotoxin levels per combination of variables are summarized in Table 1. The geometric mean concentration varied from 1000 to 3200 EU/m³, and showed relatively little variation in endotoxin levels per combination (geometric standard deviation range 1.6 to 2.0).

Table 1: Overview of combinations of factors of interest, the number of samples per combination, and their geometric mean (GM), geometric standard deviations (GSD), and range in endotoxin concentration

Combination number	Filter type	Transport conditions (desiccant) [b]	Sample storage temp before extraction	Extraction solution [c] (Tween)	Extract storage temp before analysis	No of samples	Endotoxin concn (EU/m³)		
							GM [d]	GSD [e]	Range (min-max)
1	Glass fibre	+	4 °C	+	4 °C	16	2569	1.57	930-7104
2	Glass fibre	+	4 °C	-	4 °C	19	1466	1.88	507-5284
3	Glass fibre	+	4 °C	+	-20 °C	19	2840	1.74	939-9705
4	Glass fibre	+	4 °C	-	-20 °C	18	1427	1.85	572-4795
5	Glass fibre	+	-20 °C	+	4 °C	19	3236	1.72	1346-7235
6	Glass fibre	+	-20 °C	-	4 °C	19	1558	1.86	690-5868
7	Glass fibre	+	-20 °C	+	-20 °C	18	3266	1.74	1137-6649
8 (CEN) [a]	Glass fibre	+	-20 °C	-	-20 °C	20	1334	1.77	518-4745
9	Glass fibre	-	4 °C	+	4 °C	19	2802	1.60	1192 -8938
10	Glass fibre	-	4 °C	-	4 °C	20	1500	1.88	408-5232
11	Glass fibre	-	4 °C	+	-20 °C	20	3060	1.60	1361-7995
12	Glass fibre	-	4 °C	-	-20 °C	20	1241	1.65	495-3345
13	Glass fibre	-	-20 °C	+	4 °C	19	2865	1.67	1206-8050
14	Glass fibre	-	-20 °C	-	4 °C	20	1552	1.98	232-5277
15	Glass fibre	-	-20 °C	+	-20 °C	20	3191	1.56	1257-6821
16	Glass fibre	-	-20 °C	-	-20 °C	20	1571	2.02	515-7277
17	Teflon	-	4 °C	+	-20 °C	20	2285	1.68	1015-6875
18	Teflon	-	4 °C	-	-20 °C	20	1093	1.94	332-3877
19	Teflon	-	-20 °C	+	-20 °C	20	2440	1.86	726-7301
20	Teflon	-	-20 °C	-	-20 °C	20	1046	1.76	404-3309

[a] CEN, Combination of variables which are comparable with the CEN-protocol (reference category).
[b] +, with desiccant; -, without desiccant.
[c] extraction solution is pyrogen-free water, with (+) or without (-) 0.05% Tween-20.
[d] GM, geometric mean.
[e] GSD, geometric standard deviation.

Uniformity of parallel samples

Dust levels were generally higher at the pig farm than at the grass seed plant. Teflon filters yielded slightly higher dust levels than glass fiber filters did (Table 2). The overall difference in measured dust levels (ratio maximum/minimum) within a sampling run was on average a factor of 5 and a factor of 3 and 6 for sampling runs at the pig farm and the grass seed plant, respectively (data not shown). The uniformity in the samples collected by the parallel sampling was investigated further by calculating the CV% between the replicate samples within a sampling run. The overall CV%, reflecting the sampling and analytical error, of dust levels in 20 parallel samples ranged from 11 to 76 (Table 2) and showed a decline with increasing dust levels. This variability is most likely caused by measurement error.

Table 2: Means and ranges of dust levels (mg and mg/m³) and mean CV%s within a run of dust concentrations (based on mg/m³), overall and stratified by filter type and work environment [a]

Stratification	N	Dust levels						CV% per run	
		mg			mg/m³				
		AM	SD	min-max	AM	SD	min-max	mean	min-max
Overall	382	0.54	0.43	ND-1.74	1.41	0.95	ND-3.78	32.7	10.9-76.2
Filter type									
Glass fiber	303	0.53	0.45	ND-1.74	1.37	0.98	ND-3.78	30.7	8.3-77.3
Teflon	79	0.56	0.33	0.08-1.38	1.55	0.80	0.40-3.69	13.2	5.7-28.4
Work environment									
Pig farm	196	0.82	0.40	ND-1.74	2.15	0.67	ND-3.78	17.0	10.9-37.0
Grass seed processing plant	186	0.24	0.22	ND-0.96	0.62	0.45	ND-1.89	48.4	19.9-76.2

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

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

Combination	Description [a]	Endotoxin concn [b]			
		EU/m³ [c]		EU/mg dust [d]	
		e^{β}	95% CI	e^{β}	95% CI
Intercept		1334 *	1024 - 1738	1389 *	847 - 2276
1	D-R-WT-R	2.02 *	1.67 - 2.44	1.92 *	1.40 - 2.65
2	D-R-W-R	1.10	0.92 - 1.31	1.17	0.86 - 1.59
3	D-R-WT-F	2.13 *	1.78 - 2.55	2.00 *	1.47 - 2.73
4	D-R-W-F	1.07	0.89 - 1.29	1.23 #	0.91 - 1.68
5	D-F-WT-R	2.51 *	2.10 - 3.01	2.59 *	1.91 - 3.52
6	D-F-W-R	1.16 #	0.97 - 1.39	1.31 **	0.97 - 1.78
7	D-F-WT-F	2.50 *	2.09 - 3.01	2.45 *	1.80 - 3.34
8	D-F-W-F	Ref		Ref	
9	nD-R-WT-R	2.10 *	1.75 - 2.51	2.07 *	1.53 - 2.81
10	nD-R-W-R	1.12 #	0.94 - 1.34	1.31 **	0.97 - 1.77
11	nD-R-WT-F	2.29 *	1.92 - 2.74	2.55 *	1.89 - 3.44
12	nD-R-W-F	0.93	0.78 - 1.11	1.03	0.76 - 1.39
13	nD-F-WT-R	2.23 *	1.86 - 2.66	2.45 *	1.81 - 3.32
14	nD-F-W-R	1.16 **	0.97 - 1.39	1.23 #	0.91 - 1.67
15	nD-F-WT-F	2.39 *	2.00 - 2.86	2.30 *	1.70 - 3.12
16	nD-F-W-F	1.18 **	0.99 - 1.41	1.27 #	0.94 - 1.71

[a] D, desiccant during transport; nD, no desiccant during transport; R, refrigerator; F, freezer; WT, PFW with 0.05% Tween-20; W, PFW alone.

[b] Symbols and abbreviations: e^{β}, relative effect; 95% CI, 95% confidence interval; *, $p<0.05$; **, $0.05<p<0.10$; #, $0.10<p<0.20$; Ref, reference category (=1.0).

[c] Between-run variability, 0.2375; within-run variability, 0.0810.

[d] Between-run variability, 0.8752; within-run variability, 0.2260.

Influences of transport conditions, storage conditions before and after extraction, extraction solution and filter type

In Table 3 the effect estimates of all possible combinations of variables for the samples collected on glass-fiber filters (n=320) relative to the CEN-protocol (desiccant, samples in freezer, extraction using PFW, extracts in freezer) are presented for both airborne endotoxin concentration (EU/m³) and endotoxin in dust concentration (EU/mg dust). Fourteen of the 15 combinations of variables resulted in a higher exposure level than the reference combination with ratio's ranging from 1.2 to 2.5, although they were not all statistically significant. Combination 5 (desiccant, storage filter at -20˚C, extraction in PFW with 0.05% Tween-20, and storage extracts at 4˚C) resulted in the highest endotoxin concentration levels (for both EU/m³ and EU/mg dust) levels.

Generally, the combinations containing extraction in PFW with the addition of Tween-20 to the solution resulted in significantly higher concentrations. The within-run variability for the endotoxin concentration (0.08) and endotoxin in dust concentration (0.23) was smaller than the between-run variability (0.24 and 0.88 for EU/m³ and EU/mg dust, respectively). Because the estimates of endotoxin and endotoxin per mg dust were in agreement with each other, further analyses in this part of the data set focused only on airborne endotoxin concentrations. The total variability was higher in endotoxin concentrations in dust than airborne endotoxin concentrations, which is largely due to the measurement error that occurs when sampling dust.

In Figure 3 the factors relative to the CEN reference are shown, both for Teflon and glass fiber filters (whole data set). Extraction of Teflon filters with PFW resulted in lower endotoxin levels than when Tween-20 was included, similar as observed for glass fiber filters.

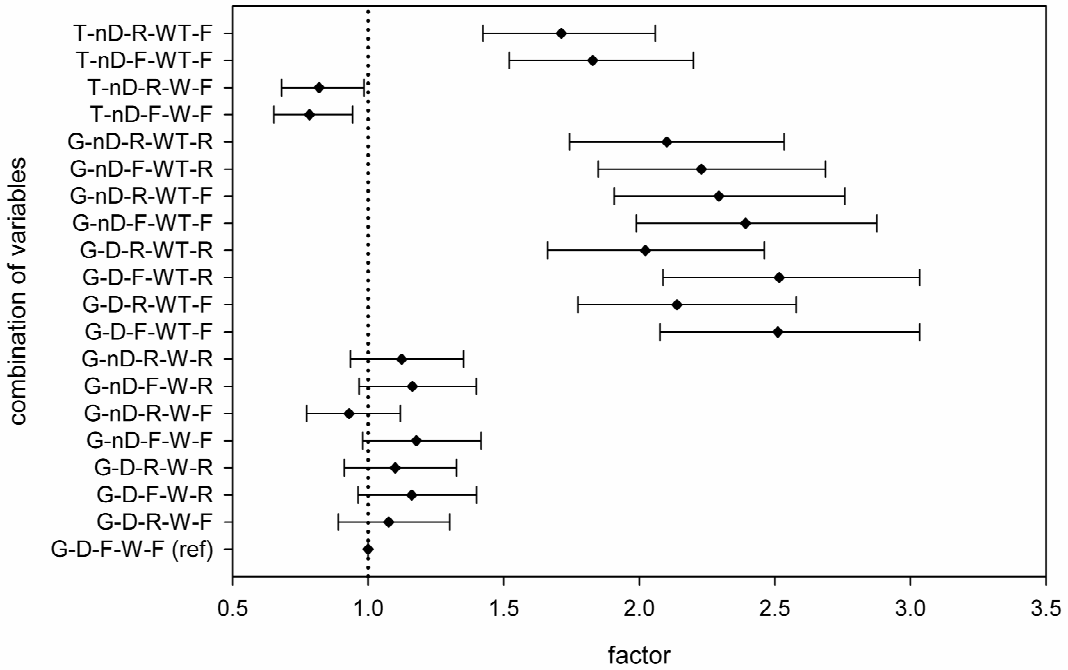

Figure 3: The factor of influence and 95% confidence interval for the effect on endotoxin exposure levels in EU/m³ per combination of variables changed compared to the CEN protocol as a reference. T, Teflon filter; G, glass-fiber filter; nD, no desiccant during transport; D, desiccant during transport;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

Next, the individual effects of the four investigated parameters were studied by applying them as fixed effects in a mixed-effects model for the glass fiber filters, both overall and stratified

for kind of dust (Table 4). Addition of Tween-20 in the extraction solution was the only parameter resulting in significantly higher airborne endotoxin concentrations. Transport conditions and storage of extracts did not have any major impact on endotoxin concentration, although storage of the filters at 4˚C seemed to slightly lower the endotoxin concentration. However, when the data were stratified for work environment, this effect was only seen in pig farm samples. No significant interactions between the parameters were found (data not shown).

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

Model and decription	Endotoxin concn (EU/m^3)					
	Overall (n=306) [b]		Pig farm (n=158) [c]		Grass seed plant (n=148) [d]	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
Intercept	1487 *	1054-2097	1503 *	1120-2017	1492 *	959-2320
Transport conditions						
No desiccant	1.00	0.94-1.07	0.97	0.89-1.07	1.03	0.95-1.12
Desiccant	Ref		Ref		Ref	
Storage before extaction						
Refrigerator (4°C)	0.91 *	0.86-0.98	0.85 *	0.77-0.94	0.99	0.91-1.08
Freezer (-20°C)	Ref		Ref		Ref	
Extraction solution						
Water-Tween	2.09 *	1.96-2.23	2.35 *	2.14-2.58	1.84 *	1.70-2.00
Water	Ref		Ref		Ref	
Storage before analysis						
Refrigerator (4°C)	1.02	0.96-1.09	1.01	0.92-1.12	1.03	0.95-1.12
Freezer (-20°C)	Ref		Ref		Ref	
Work environment						
Pig farm	1.01	0.65-1.58				
Grass seed plant	Ref					

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

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

Subsequently, the individual effect of the three parameters was investigated (Table 6). In the whole subset, sampling with Teflon filters and storage of samples in the refrigerator (4˚C) resulted in significantly lower endotoxin concentrations, whereas extraction in PFW with addition of Tween-20 resulted in significantly higher endotoxin concentrations. After

stratification for work environment, the direction of the effects of the parameters was mostly unchanged, although the positive effect of extraction in PFW with Tween-20 and the negative effect of sampling on Teflon filters on measured endotoxin concentrations were larger in pig farm samples than in grass seed plant samples. Storage conditions of samples before extraction did not affect endotoxin concentrations in the samples from the grass seed plant, although storage of samples at 4˚C significantly lowered endotoxin concentrations in pig farm samples.

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

Combination	Description [a]	Endotoxin concn [b]			
		EU/m³ [c]		EU/mg dust [d]	
		e^{β}	95% CI	e^{β}	95% CI
Intercept		1572 *	1205-2050	1757 *	1133-2723
11	G-nD-R-WT-F	1.95 *	1.60-2.37	2.01 *	1.53-2.65
12	G-nD-R-W-F	0.79 *	0.65-0.96	0.81 #	0.62-1.07
15	G-nD-F-WT-F	2.03 *	1.67-2.47	1.84 *	1.39-2.44
16	G-nD-F-W-F	Ref		Ref	
17	T-nD-R-WT-F	1.45 *	1.20-1.77	1.05	0.80-1.38
18	T-nD-R-W-F	0.70 *	0.57-0.85	0.46 *	0.35-0.61
19	T-nD-F-WT-F	1.55 *	1.28-1.89	1.02	0.77-1.35
20	T-nD-F-W-F	0.67 *	0.55-0.81	0.43 *	0.33-0.57

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

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

Model and description	Endotoxin concn (EU/m³) [a]					
	Overall (n=160) [b]		Pig farm (n=80) [c]		Grass seed plant (n=80) [d]	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
Intercept	1553 *	1103-2185	1403 *	1011-1949	1508 *	994-2289
Filter type						
Teflon	0.76 *	0.69-0.84	0.66 *	0.57-0.76	0.88 *	0.79-0.98
Glass fiber	Ref		Ref		Ref	
Storage before extaction						
Refrigerator (4°C)	0.93 #	0.84-1.02	0.83 *	0.72-0.96	1.03	0.93-1.15
Freezer (-20°C)	Ref		Ref		Ref	
Extraction solution						
Water-Tween	2.22 *	2.02-2.45	2.71 *	2.35-3.12	1.82 *	1.64-2.03
Water	Ref		Ref		Ref	
Work environment						
Pig farm	1.14	0.57-1.36				
Grass seed plant	Ref					

[a] Symbols and abbreviations: e^{β}, relative effect; 95% CI, 95% confidence interval; *, $p<0.05$; #, $0.10<p<0.20$; Ref, reference category (=1.0).
[b] Between-run variability, 0.2383; within-run variability, 0.0975.
[c] Between-run variability, 0.1608; within-run variability, 0.1000.
[d] Between-run variability, 0.3117; within-run variability, 0.0560.

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

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

Model and description	Endotoxin concn (EU/m^3) for model [a]			
	Without assay solution [b]		With assay solution [c]	
	e^{β}	95% CI	e^{β}	95% CI
Intercept	1102 *	863-1408	907*	712-1155
Transport conditions				
No desiccant	0.99	0.90-1.09	0.99	0.91-1.07
Desiccant	Ref		Ref	
Storage before extraction				
Refrigerator (4°C)	0.95	0.86-1.04	0.95 #	0.87-1.03
Freezer (-20°C)	Ref		Ref	
Extraction solution				
Water-Tween	2.55 *	2.31-2.80	2.55 *	2.35-2.76
Water	Ref		Ref	
Storage before analysis				
Refrigerator (4°C)	1.03	0.93-1.13	1.03	0.95-1.11
Freezer (-20°C)	Ref		Ref	
Assay solution				
Water-Tween			1.48 *	1.37-1.60
Water			Ref	

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

Determinants of within- and between-run variability

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

Discussion

In the field of endotoxin exposure assessment almost every institute has its own sampling and analysis protocol. Various filters types, extraction and assay solutions, transport conditions and storage temperatures are used. In Europe, CEN has formulated guidelines for the assessment of airborne endotoxin to standardize exposure assessment. However, these guidelines leave room for individual interpretation and thus varying methodology. This study investigated the influence of various factors using an experimental design to cover all combinations of factors and their possible interactions. Of the five initial variables in our experiment (four primary parameters and filter type), extraction solution influenced the airborne endotoxin concentration the most. Addition of Tween-20 to PFW yielded significantly more endotoxin and thus resulted in an improvement of the extraction efficiency, which has been found before.[8] This may be caused by disruption of hydrophobic interactions between endotoxin and filter material or by disaggregating of endotoxin micelles or dissociation of cell-wall-bound endotoxin.[6,8] Transport conditions, in this case use of a desiccant or not, did not influence the measured endotoxin concentration, suggesting that further microbial growth did not occur during transport. Storage of extracts by different temperatures (-20 or 4˚C) did not influence the estimated endotoxin concentration. Storage of filters in a freezer yielded about 10% higher estimated airborne endotoxin than did storage in a refrigerator. Freeze-thaw cycling of bacteria is known to lyse bacteria, and therefore more lipopolysaccharide (LPS) may be available in the assay after extraction.[4,36] There was no significant effect of storage temperature for the extracts, perhaps because these had been centrifuged and there were no bacterial cells to lyse. Sampling on glass-fiber filters results in a higher endotoxin concentration than does sampling on Teflon filters, as previously reported.[8,12,15,31,35] Interaction between filter type and assay methodology has been reported,[35] as well as inactivation of LPS in solution by a variety of filter media.[20] It is suggested that in the latter study the LPS was absorbed to the surfaces of the filter material and thus not available to the *Limulus* amoebocyte lysate (LAL) reagent and that the extraction procedure was not sufficient.[30]

In addition to the initial 5 factors under investigation, the influence of assay solution was also investigated in a subset of the data. Use of Tween-20 in the assay yielded a higher airborne endotoxin concentration than did the use of only PFW. This seemed to be an additive effect and did not depend on the extraction solution used (no interactions).
The type of dust sampled had a clear but small effect on the effect estimates of the different variables that were studied. The influence on extraction efficiency of adding Tween-20 to the extraction solution was higher in samples from the pig farm compared to grass seed plant samples. Also, the ‘freezing’ effect (higher endotoxin concentration when the sampled filters were stored in the freezer than when they were stored in a refrigerator) occurred only in the pig farm samples. Furthermore, the factor for sampling on glass fiber filters compared to Teflon filters was higher for pig farm samples than for grass seed plant samples. Gordon et al. found that the endotoxin extraction efficiency of different filters types was dependent on the aerosol type.[12] Confirmation of these observations in other environments might be needed. However, the work environments included in this experiment are representative for different types of endotoxin exposure, namely, those originating from animal and from plant material. Since the estimates of the variables studied do not vary much for the different types of dust and the direction of the models remain approximately the same, these results are thought to represent the general underlying effects of the studied variables on the measured endotoxin concentration.

Several studies showed differences between laboratories when analyzing endotoxin samples.[5,17,28] One of these showed that the generally high variations between laboratories were reduced by using a common extraction protocol and endotoxin assay kit, although differences remained.[5] When further limitations were dictated, interlaboratory differences became even smaller,[17] suggesting that differences in endotoxin exposure estimation are caused mostly by procedural differences. Further standardization (training, use of identical equipment, tubes, etc.) may thus lead to comparable interlaboratory analysis of samples.
This experiment is a vigorous attempt to come to an optimized protocol for airborne endotoxin measurements in occupational settings, which may be expanded to other settings. The experiment was designed to look at key variables and their interactions established a priori as opposed to consideration of one or a few variables at a time as done in most previous studies.[9,11-15,19-24,31,33,35,38] Furthermore, some of these studies used commercial LPS[8,15,19,20,23] or house dust[8,11,13,14,24,34] instead of rather homogeneous parallel occupational dust samples for (part of) their research, which reduces the applicability of their results for work environments. Nevertheless, to a large extent this full design appeared to confirm and extend earlier findings.

Several gaps in the CEN-protocol[3] have been evaluated. With full knowledge of assay parameters that have an effect on the exposure estimate, one can clearly specify these in an agreed-upon international protocol. A fully standardized international protocol would support the establishment of an occupational exposure limit for endotoxin. Based on the outcomes of this experiment and earlier research, the following procedural steps are preferred: inhalable dust sampling on glass-fiber filters, transport with desiccant, storage of samples at -20˚C, extraction in PFW with 0.05% Tween-20 and rocking/shaking during the procedure for maximal extraction efficiency, storage of extracts at -20˚C and analysis using PFW without Tween-20 in the assay solution. No evidence was found that transport with or without desiccant and storage of extracts in a refrigerator or freezer results in different endotoxin concentrations. However, it is preferred that possible growth of biological material be prevented by use of desiccant and storage in a freezer. Although the exact effect of repeated freezing and thawing has not been established,[8,23] storage of extracts in multiple aliquots is strongly encouraged. The 25% decline in endotoxin activity in house dust extracts after one freeze-thaw cycle that has been found elsewhere did not occur in this experiment.[8] In this study all extracts were analyzed within 24 h after extraction, and thus no statements can be made about the influence of long-term storage. However, other studies have shown that long-term storage of extracts did not affect on the endotoxin concentration.[8,11,22] One study found higher endotoxin concentrations in extracts stored at 4˚C than in extracts stored at -20˚C for up to 20 to 30 days and in samples stored at 4˚C with immediate extraction after sampling than in sampled stored without extraction.[15] This was possibly due to the growth of Gram-negative bacteria during storage.

Douwes et al. previously showed that the endotoxin extraction efficiency of PFW with 0.05% Tween-20 was seven times higher than of only PFW and that 0.05% Tween-20 in the assay solution did not influence the slope of the standard curve.[8] We also saw an increased extraction efficiency from the addition of Tween-20, although the effect was lower. Wouters et al. found that addition of 0.05% Tween-20 to the assay mixture suppressed the assay reactivity but did not alter the slope of the standard curve. The assay reactivity was affected to a larger extend for the standard curve than for the samples for at least some of the dust types (I.M. Wouters, S. Spaan, D. Heederik, and G. Doekes, data presented at the International Conference of the American Thoracic Society, 2007), which might explain the

smaller effect of the addition of Tween-20 during extraction in the current study. Therefore, it is concluded that Tween-20 enhances the extraction efficiency but should not be used during analysis because of possible interference with the assay.

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

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

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

Acknowledgements

The authors would like to acknowledge the following individuals for their contribution to this work: The company owners who cooperated and made this study possible, Isabella Oosting-van Schothorst and Karel van der Heijden for conducting the fieldwork and Hans Jongsma for his analytical assistance. This research was granted by the Ministry of Social Affairs and Employment, the Netherlands by means of the long term research program on biological agents in the work environment of TNO Quality of Life.

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Chapter 4.2

Effect of extraction and assay media in the analysis of airborne endotoxin

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Published in Applied and Environmental Microbiology, 2008; 74 (12): 3804-3811.

Abstract

The measurement of airborne endotoxin is thus far not standardized. Earlier studies reported higher endotoxin yields when Tween-20 was added to media used for filter extraction and in the *Limulus* amebocyte lysate (LAL) assay. This study compared four common media and assessed the effects of Tween during extraction and analysis separately.
Parallel airborne dust samples from five work environments (n=250) were used to compare four media (pyrogen-free water (PFW), PFW-Tween-20, PFW-Tris and PFW-triethylamine-phosphate (TAP)) and an extraction time of 10 or 60 minutes. A subset of the extracts in PFW or PFW-Tween (n=40) were analyzed in parallel LAL assays with PFW or PFW-Tween as the assay medium.
The results produced by a shorter extraction time or the presence of Tris were similar to the results for the reference procedure (PFW and 60 min of shaking). The use of PFW-TAP showed overall lower yields and a deviant calibration curve. The presence of Tween in the extraction medium resulted in significantly ($p<0.05$) higher endotoxin yields from all dust types, independent of the effect of Tween in the assay. Tween in the LAL assay, however, also strongly inhibited the reactivity of the lipopolysaccharide (LPS) standard, thus shifting the calibration curve to higher values. The inhibition of LPS in test samples was less pronounced and varied between dust sources, resulting in enhanced calculated concentrations. This assay effect could be circumvented by diluting extracts at least 50-fold before the LAL assay.
In conclusion, of the media tested, only Tween enhances the efficiency of endotoxin extraction from airborne samples in a consistent manner. We recommend extraction in PFW-Tween combined with dilution and LAL analysis in PFW.

Introduction

Endotoxins are lipopolysaccharide components (LPS) of the cell wall of gram-negative bacteria with a high proinflammatory potency. Exposure to airborne endotoxins has been associated with the development of nonallergic asthma, bronchitis, organic dust toxic syndrome and accelerated lung function decline in a variety of agricultural and industrial environments.[22] However, endotoxin exposure in early childhood is associated with a lower prevalence of atopy and allergic disease, especially in farm children,[11,29] and studies of adult working populations suggest that it may also protect against atopic sensitization at a later age.[11,18]

In order to compare results from studies investigating endotoxin exposure, related health effects, and compliance with possible exposure limits, the exposure assessments should be comparable. Although the *Limulus* amebocyte lysate (LAL) assay is part of most common procedures for endotoxin exposure assessment, the procedure is not completely standardized. Guidelines for exposure assessment, like those published by the European Committee for Standardization (CEN),[3,4] are in fact only partially based on systematically collected empirical data, which leaves room for variation in interpretation of the procedure.
The effects of variations in extraction protocol and/or extraction medium [7,9,12-15,26,28] or modifications of the LAL assay conditions [12,13,16,28,30] on the measured endotoxin concentration have been investigated. However, most of these studies investigated only some options in a limited number of samples from a few different types of dust, whereas a previous study showed dust type to be of importance to the outcome.[21] We recently studied the influence of and interactions between transport conditions, storage of samples, extraction medium, storage of extracts, filter type, and assay medium on the measured endotoxin concentration in parallel samples from two work environments.[23] The extraction medium appeared to be the most important determinant, with higher measured endotoxin concentrations when extraction was done in presence of 0.05% Tween-20 than in pyrogen-free water (PFW). Although a more efficient extraction of endotoxin from filters was the most likely explanation, the use of Tween in the medium of the LAL assay also appeared to result in higher endotoxin concentrations. This seemed to be an additive effect, but was only studied in a subset of the samples.

Besides PFW with or without addition of 0.05% Tween-20, other media have also been used as extraction and assay media.[7,10,12,25] Therefore, the current study investigated the effect of several extraction media that are used regularly, as well as the effect of the duration of shaking on the measured endotoxin concentrations in parallel samples from five representative work environments. In addition, experiments were performed to further elucidate the effect of Tween during extraction and/or analysis separately.

Material and methods

Collection of inhalable dust samples
A parallel sampler, developed within the European MOCALEX project,[2,8] was used to collect air samples in five work environments (grass seed production, pig farm, household-waste composing, potato processing, and sewage treatment) representing different sources of endotoxin exposure. As previously described, the parallel sampler enabled the simultaneous collection of 10 close to identical airborne dust samples per run using PAS-6 sampling heads

for inhalable dust [24] equipped with 25-mm glass fiber filters (Whatman GF/A, United Kingdom).[23] The loaded filters were stored 56 to 90 days at -20˚C prior to extraction.
A total of 250 samples were collected by performing five runs of 10 parallel samples per work environment. Air samples were collected during 15 days in 2003, with 3 or 4 sampling days per location. The sampling time varied from 1 to 8 h to obtain a sufficient range in dust and endotoxin loads on the filters. On every sampling day, a control filter (field blank) was included which was handled like the test filters except for the actual exposure in the sampler.

Comparison of different media used in both extraction and the LAL assay and of extraction time
In the first experiment, as schematically shown in Figure 1, the effect of four commonly reported extraction media on the measured endotoxin concentration were evaluated: (I) PFW (Braun, Germany); (II) PFW-Tween (PFW with 0.05% (vol/vol) Tween-20 (polysorbate 20; Merck, Germany)); (III) triethylamine-phosphate (TAP) (PFW with 0.05M K_2HPO_4 (Merck, Germany) and 0.01% triethylamine (Fisher, United Kingdom), pH=7.5); and (IV) Tris (PFW with 1mM Tris HCl (Gibco, UK), pH=7.4). To assess the effect of the shaking period, the extraction in PFW was done for both 10 and 60 minutes. Of each series of 10 parallel-collected filters, 2 filters were extracted with each of the five extraction methods. The different treatments were randomly assigned to the 10 parallel sampling positions available per run. Each filter was immersed in 5 ml of the extraction medium in a glass tube and rocked vigorously for 1 h at room temperature on a horizontal shaker (160 reciprocations/min and 15 cm deflection), except for the filters assigned to the procedure of shaking for 10 min in PFW. After 15 minutes of centrifugation at 1000xG, 1 ml supernatant per sample was collected and vortexed, and four aliquots of 0.1 ml and the remaining 0.6 ml were stored in pyrogen-free glass tubes at -20˚C until analysis.

Effect of Tween in the LAL assay.
To assess the effect of Tween on the reactivity of the calibration standard in the LAL assay, two experiments were performed. First, two vials of the LPS standard were dissolved in either PFW or PFW-Tween, serially diluted (12 dilution steps) to the usual concentration range (0.05-100 endotoxin units (EU)/ml) in either PFW or PFW-Tween, and tested with the LAL reagent dissolved in either PFW or PFW-Tween, all tested in the same microplate. In a second experiment, eight parallel dilution series of standard LPS were tested in PFW with Tween concentrations varying from zero to 0.15%.

Crossover analysis of the effects of Tween in extraction or assay medium
A crossover experiment was performed with unused replicate aliquots from a selection of the sample extracts from the comparison study (see Figure 1). For each of the five work environments, two runs were selected, and from each run were selected four samples which had been extracted for 60 minutes in either PFW or PFW-Tween, resulting in 40 sample extracts. Each extract was tested in parallel in the same microplate, either in PFW as test medium, i.e. with the sample dilution, LAL reagent, and calibration standard series in PFW, or with the samples, standard, and reagent diluted or dissolved in PFW-Tween.

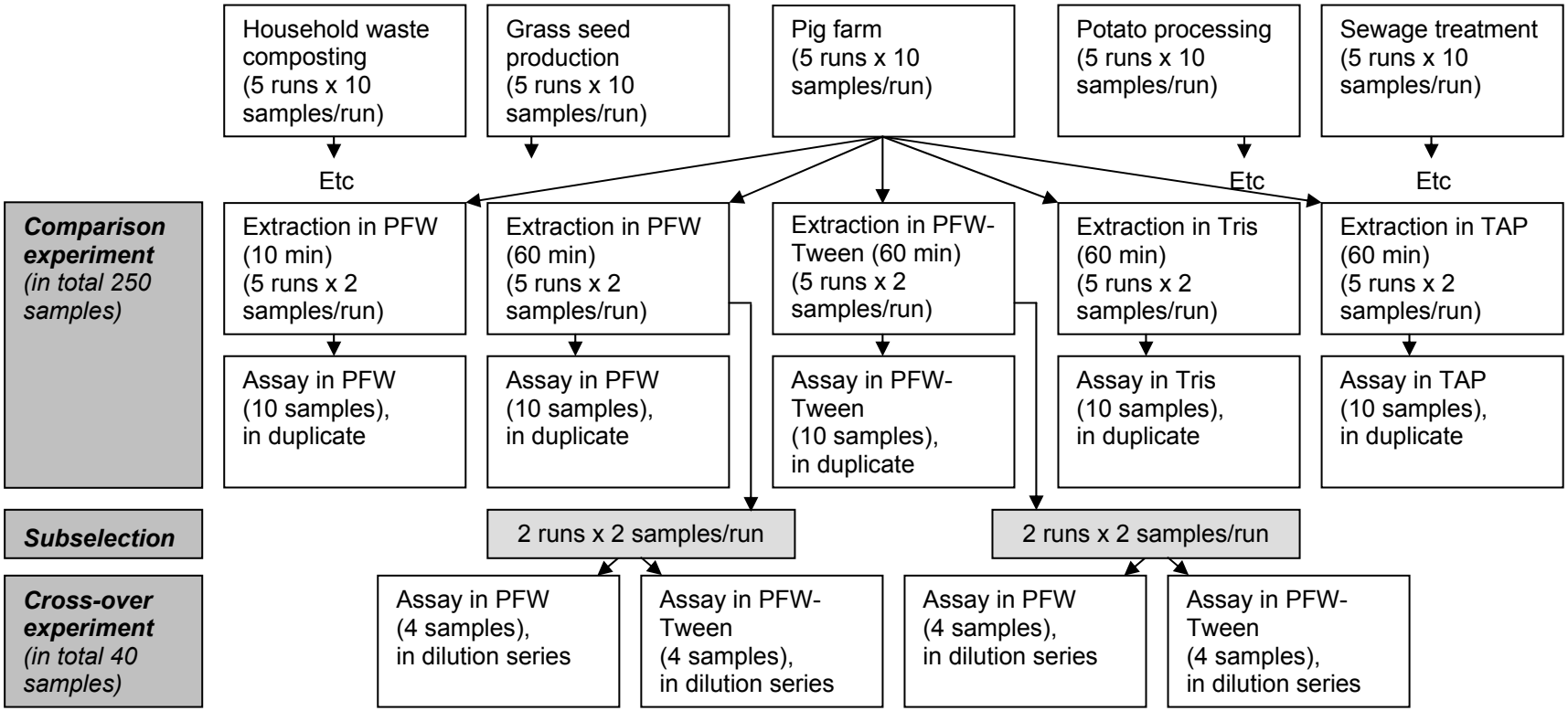

Figure 1: Schematic overview of the design of the experiments with filter samples

Analysis

The endotoxin concentrations were measured in pyrogen-free microplates (Costar, Corning, NY) with the kinetic, chromogenic LAL method (Cambrex, Verviers, Belgium; lysate lot no. 3L433E and standard lot no. 3L2950 (reference standard endotoxin/control standard endotoxin ratio 10 ng/0.90 ml=100 EU/ml)) with the maximal reaction rate (V_{max}) as derived from kinetic readings with 30-s intervals (V_{max} in milli-optimal density units (mOD)/min) as the primary outcome parameter for each test well. The endotoxin concentrations in the extracts were determined by comparing the V_{max} values in the test wells with the V_{max} calibration curve obtained with serial dilutions of the LPS standard in the same microplate.

In the first experiment, all extracts were analyzed in duplicate at various dilutions (1:2 to 1:400, depending on the type of dust and the medium used), and the assay, including dilution of the samples, the standard, and the LAL reagent, was performed in the same medium as was used for extraction of the filters. Samples with nondetectable endotoxin levels were assigned a value of two-thirds of the limit of detection (LOD) of the particular assay run (range 0.01 to 0.06 EU/ml).

In the crossover experiment, the selected sample extracts were analyzed in three dilutions (1:90, 1:270 and 1:810). The mean of the results that were greater than the LOD was used in further statistical analyses. When all results were less than the LOD, a value of two-thirds of the LOD for that particular test, depending on the assay medium, was assigned.

Statistical analysis

Data were analyzed with SAS statistical software (version 9e; SAS Institute, Cary, NC, USA). The endotoxin concentrations were log-normally distributed. Therefore, all calculations were performed with natural log-transformed concentrations. The influence of, differences between, and possible interactions between extraction and assay media were determined by applying mixed-effects analysis of variance with sampling run as the random effect, in order to correct for possible correlation between measurements in the same run. Determinants influencing the endotoxin concentration, i.e. the extraction and assay media, were explored by introducing them into the model as fixed effects.[17,19]

Results

Of the 250 parallel airborne dust samples collected, endotoxin levels below the LOD were found in duplicate tests of 14 samples and for 5 samples, one of the two values was below the LOD. The endotoxin concentration of 14 out of 15 field blanks was below the LOD, and in one was 0.8 EU/ml. Three of the samples from the crossover experiment, all extracted and analyzed in PFW, showed values below the LOD. The average coefficients of variation of the results of duplicate analyses and intratest coefficients of variation of the results of serial dilutions of the same extract were 15% and 18%, respectively.

Comparison of different media used in both extraction and the LAL assay and of extraction time

Extraction in PFW with 10 minutes of shaking did not significantly change the endotoxin yield in comparison to the yield of extraction in PFW with 60 minutes of shaking, and this was the case for samples from all five work environments (Table 1). This confirms that there was no additional release of endotoxin during the longer period of shaking. The presence of Tris in the extraction medium and during analysis had also no significant effect on the overall measured endotoxin concentration, although the results suggested some heterogeneity in

endotoxin release among workplaces, reflected by relative effects that were <1 except for household-waste composting. The use of TAP instead of PFW showed on average a lower endotoxin yield ($p<0.05$). The data suggested an even more pronounced dependence on workplace, with decreased yields in samples from three workplaces (pig farm, potato processing, and sewage treatment), no significant effect in samples from one workplace (household-waste composting) and a significant and substantial increased yield in samples from the grass seed production. The addition of Tween-20 to PFW increased the endotoxin yield both overall and for the different work environments, with factors ranging from 2.1 to 6.6 (Table 1).

Effects of assay media on the reactivity of the standard in the LAL assay

As shown in Figure 2, the application of the four different dilution media to the LAL assay resulted in marked differences in the shapes of the four calibration curves (based on three curves per medium) and their positions relative to the *x*-axis. Compared to the performance of the assay in PFW, the use of Tris resulted in a very similar curve, while for TAP a change in the dose response was observed, with decreased reactivity at low and increased V_{max} values at high concentrations. Thus, the assay in TAP appeared to have a lower sensitivity, with an approximately 10-fold increased LOD compared to the LOD of the assay in PFW. The addition of Tween to PFW showed a consistent effect on the reactivity of the standard, with a downward shift of approximately 35 to 50 V_{max} units (mOD/min) over the whole concentration range, resulting in a fivefold increased LOD.

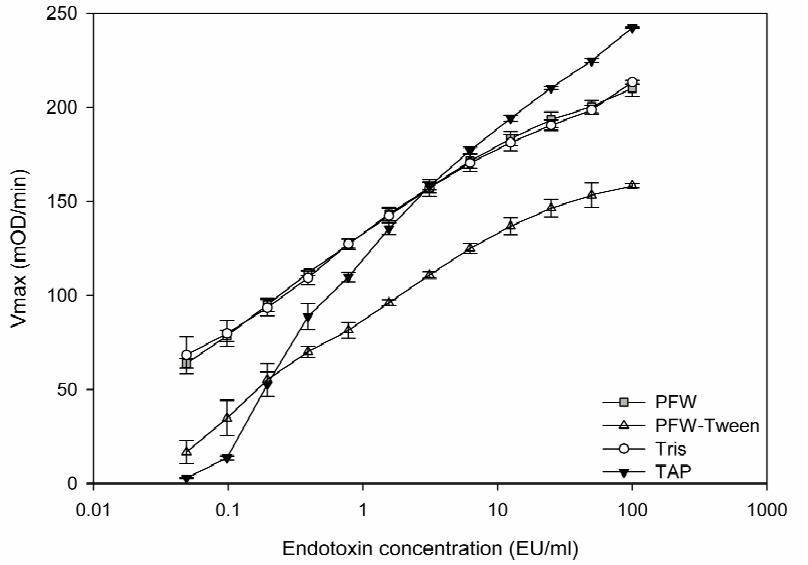

Figure 2: Mean (and 95% confidence interval) standard curves of the results of the LAL assay performed using different dilution media, based on 3 standard curves per medium.

Effect of Tween on the reactivity of the calibration standard

As PFW-Tween showed the most consistent results and because of previously reported effects,[23] we further focused specifically on the effects of PFW-Tween in the extraction and/or assay medium. The shift of the standard curve was always observed when the standard was diluted in PFW-Tween and/or when the LAL reagent was prepared in PFW-Tween, irrespective of the Tween content of the primary standard solution (Figure 3). The standard curve with the use of PFW-Tween for dissolving and further dilution of the standard and for preparation of the lysate was parallel to the calibration curve for the assay without Tween in each step over nine twofold dilutions but showed reduced reactivity. Preparation of the

standard in PFW-Tween followed by dilution in PFW and preparation of the LAL reagent in PFW yielded a nonparallel curve that was kinetically invariant over the first six dilutions. At these first six dilutions, the V_{max} was markedly lower than in the complete absence of Tween. Though, from a dilution of approximately 50 to 100 and higher, V_{max} values were practically identical to those for the Tween-free tests (Figure 3). Thus, the apparent inhibitory effect of Tween on the LAL assay was dose dependent and could be resolved if extracts containing 0.05% Tween-20 were diluted at least 1:50.

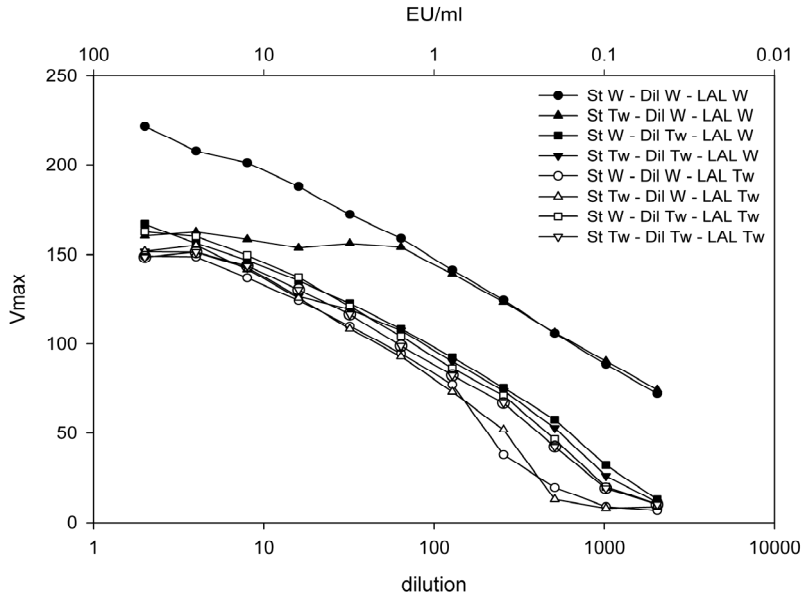

Figure 3: Effect of Tween-20 on the reactivity of the LPS standard during analysis investigated in a full crossover experiment in which the standard was dissolved in PFW with or without 0.05% Tween-20, serially diluted in either PFW or PFW-Tween, and then tested with the LAL lysate reagent dissolved in either PFW or PFW-Tween. St, medium for dilution of LPS standard; Dil, diluting medium; LAL, LAL assay medium; W, PFW; Tw, PFW-Tween

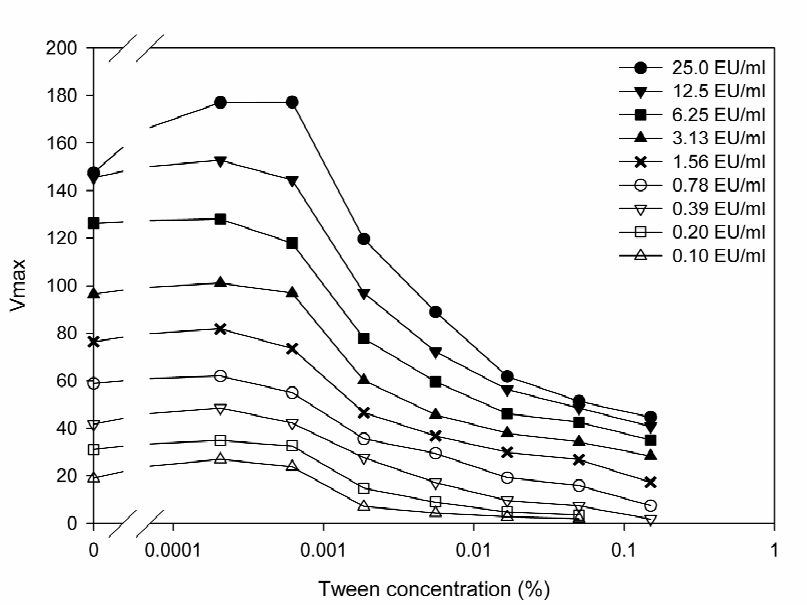

Figure 4: Dilution series of LPS standard in PFW with various Tween concentrations

Testing of the LPS standard in PFW with various Tween concentrations confirmed the dose dependency of the inhibitory effect of Tween (Figure 4). Tween concentrations of approximately 0.002% and higher caused a dose-dependent inhibition of reactivity in the LAL assay at all concentrations of the LPS standard.

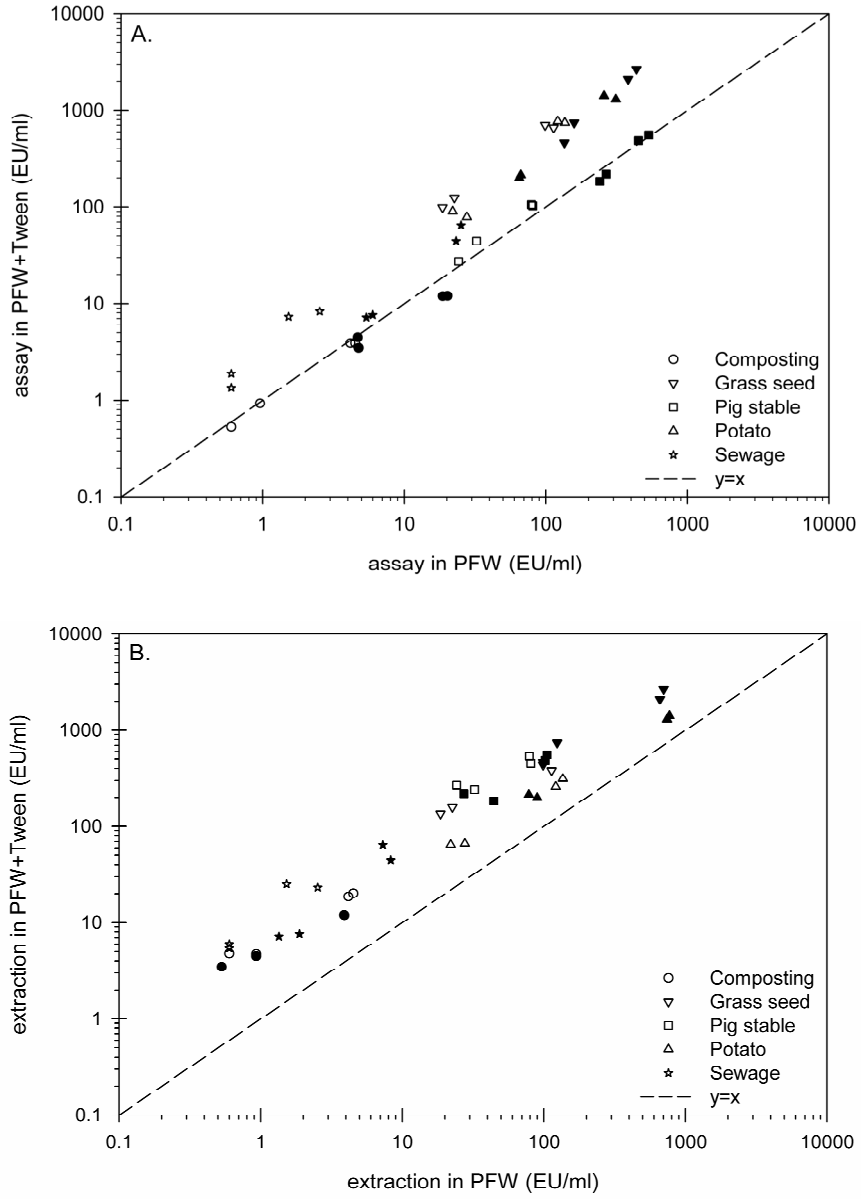

Figure 5: Plots of the assay effect, stratified for extraction solution, of assays performed in PFW-Tween versus PFW on measured endotoxin concentration (EU/ml) for samples extracted in PFW (open symbols) or PFW-Tween (closed symbols) (A) and the extraction effect, stratified for assay solution, of extraction in PFW-Tween versus PFW on measured endotoxin concentration for extracts analyzed in PFW (open symbols) or PFW-Tween (closed symbols) (B). Key indicates type of production or processing facility sampled.

Tween effect in airborne dust samples

To test whether Tween applied during extraction might result in an increased yield irrespective of the effect of Tween in the analysis phase, a crossover study was performed in a subset of the airborne dust samples. Figure 5A shows a high correlation (r=0.93) between the results from analyses in PFW and PFW-Tween. Very similar values were found in both

tests for samples from the pig stable and composting industry, because the inhibitory effect of Tween on their LPS reactivity in the assay was nearly identical to that of Tween on the standard LPS, thus with the V_{max} values in PFW-Tween approximately 40 to 45 mOD/min lower than in the absence of Tween. However, for samples from the other sources, the effect of Tween on the observed V_{max} values was markedly less, with a downward shift of only 25 to 30 mOD/min in the presence of Tween. As a result, the concentrations in samples from sewage treatment, potato industry, and grass seed processing were significantly higher when the LAL assay was performed in PFW-Tween and sample results were calculated by comparison with the corresponding standard curves made in PFW-Tween. Irrespective of this test artifact, however, Figure 5B (r=0.95) shows a pronounced enhancing effect of Tween during extraction for all dust types, with a yield that was on average 5 to 10 times higher with the presence of Tween during extraction. Enhanced release of endotoxins from airborne dust filters was observed over a wide range of endotoxin levels, with some indications that this effect might be relatively stronger in samples with a lower endotoxin concentration.

Mixed regression analysis confirmed that extraction in PFW-Tween resulted in a significantly increased measured endotoxin concentration in the extracts of airborne dust samples from all work environments, both for assays in PFW (on average fivefold higher) and for assays in PFW-Tween (on average fourfold higher) (Table 2). The presence of Tween in the assay medium led to more heterogeneous results. For all airborne dust samples except those taken during composting, the addition of Tween to the assay medium resulted in higher calculated endotoxin concentrations for samples extracted in PFW. For samples extracted in PFW-Tween, the enhancing effect of Tween in the assay medium on calculated endotoxin concentrations was on average 1.8 and varied between 0.7 and 4.8 depending on the location of sampling (Table 2).

Discussion

Previous studies have shown that the addition of Tween-20 to the extraction medium (PFW) results in higher measured endotoxin concentrations in the LAL assay.[7,23] This effect has usually been ascribed to enhanced extraction efficiency in the presence of Tween. However, we had preliminary results showing an additional effect of Tween in the actual LAL assay (unpublished data). The previously reported apparently higher yields in presence of Tween could thus be partially due to a change in reactivity of LPS in the LAL assay, especially if the reactivity of LPS in extracts of airborne dust sample and that of the calibration standard would be differentially affected by Tween in the assay medium. Therefore, this study looked at the effect of the medium during extraction and analysis separately.

The assay medium with Tween showed markedly lower V_{max} values for the standard LPS and therefore led to a decreased assay sensitivity. This inhibitory effect of Tween on the reactivity of LPS was similar for airborne dust samples from two work environments (pig farming and composting) but less for samples from three other sources (sewage treatment, grass seed, and potato processing). This resulted in higher measured endotoxin concentrations in the latter samples when analyzed in LAL assays in the presence of Tween, while the concentrations in the former were independent of assay medium. Furthermore, this inhibitory effect of Tween seemed to be stronger when the samples had also been extracted in PFW-Tween.

Table 1: Components of between- and within-run variance and effects of extraction medium on measured endotoxin levels (in EU/ml) in parallel airborne dust samples from five work environments relative to extraction (60 min) and analysis in PFW as the reference procedure [a]

Sources (no.) of samples	BR [b] variance	WR [b] variance	GM [b] with PFW, 60 min	PFW, 10 min		PFW-Tween, 60 min		TAP, 60 min		Tris, 60 min	
				e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
All sources (250)	4.36	0.19	10.80	1.05	0.89-1.25	3.47 *	2.92-4.12	0.67 *	0.56-0.79	0.96	0.80-1.14
Household waste composting (50)	0.39	0.08	2.31	1.03	0.80-1.32	2.09 *	1.63-2.69	0.98	0.76-1.26	1.30 *	1.02-1.67
Grass seed prodution (50)	0.94	0.03	57.80	1.11	0.94-1.31	3.59 *	3.04-4.24	1.64 *	1.39-1.93	0.98	0.83-1.16
Pig stable (50)	0.55	0.06	55.87	0.99	0.80-1.24	2.89 *	2.32-3.60	0.26 *	0.20-0.32	0.83	0.67-1.03
Potato processing (50)	1.30	0.02	34.45	1.01	0.90-1.14	3.48 *	3.09-3.92	0.82 *	0.73-0.93	0.98	0.87-1.10
Sewage treatment (50)	0.78	0.23	0.57	1.12	0.72-1.73	6.62 *	4.28-10.24	0.40 *	0.26-0.61	0.77	0.50-1.19

[a] e^{β}, effect of the procedure relative to the reference procedure of PFW and 60 min of shaking; CI, confidence interval; *, $p<0.05$.
[b] BR, Between-run; WR, Within-run; GM, geometric mean

Table 2: Effect of PFW-Tween (compared to PFW) in the extraction and assay media on endotoxin yield (in EU/ml), stratified for assay medium and extraction medium, respectively [a]

Source (no.) of samples	Extraction effect (PFW-Tween vs. PFW)				Assay effect (PFW-Tween vs. PFW)			
	Assay in PFW		Assay in PFW-Tween		Extraction in PFW		Extraction in PFW-Tween	
	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI	e^{β}	95% CI
All sources (40)	5.58 *	4.50-6.92	4.08 *	3.40-4.90	2.51 *	1.86-3.38	1.83 *	1.35-2.49
Household waste composting (8)	5.28 *	3.77-7.39	4.16 *	2.53-6.83	0.91	0.60 - 1.39	0.72 *	0.57-0.90
Grass seed production (8)	5.24 *	3.52-7.80	4.29 *	2.84-6.49	5.89 *	4.74 - 7.32	4.82 *	3.27-7.13
Pig stable (8)	7.45 *	5.51-10.07	5.36 *	3.91-7.36	1.27	0.92 - 1.76	0.91	0.72-1.16
Potato processing (8)	2.43 *	1.96-3.00	2.09 *	1.70-2.58	4.47 *	3.13 - 6.39	3.86 *	2.94-5.07
Sewage treatment (8)	10.80 *	7.72-15.11	5.66 *	3.89-8.23	3.24 *	2.12 - 4.96	1.70 *	1.16-2.49

[a] e^{β}, effect of the procedure relative to the reference procedure of PFW and 60 min of shaking; CI, confidence interval; *, $p<0.05$.

An explanation for the Tween-related assay inhibition is not directly available. The effect of Tween in the assay medium appeared to be reversible, and interference of Tween from the extraction medium in the LAL assay can thus be avoided if extracts are sufficiently diluted. Tween might change the tertiary structure of LPS molecules or interfere with one or more of the (pro-)enzymes of the LAL reagent. As a surfactant, Tween might reduce the availability of LPS by partially capturing it in micelles, or it might affect the molecular interactions with and between the LAL factors. Since the effect differed between the (semi)purified LPS standard and LPS in the dust samples from various work environments, an effect on extracted or dissolved LPS is the most likely explanation. However, little is yet known about the precise physical-chemical properties and appearance of LPS in organic dust extracts (level of aggregation and linkage to other macromolecular complexes) and their impact on its in vivo and in vitro proinflammatory effects and LAL assay reactivity.

We found a higher endotoxin yield when samples were extracted in PFW-Tween than in PFW that was independent of the assay medium and showed that the effect of extraction and analysis in the presence of Tween is not only due to the change in assay reactivity but results from increased extraction efficiency. The results found in earlier studies [7,23] thus were likely caused by an enhanced extraction efficiency. Disruption of hydrophobic interactions between LPS and filter material caused by the surface-active properties of Tween or disaggregation of endotoxin-containing molecular complexes from the cell wall of whole bacteria or from cell walls have been given as possible explanations.[6,7] Furthermore, Tween might reduce the sticking of LPS to the walls of tubes and vials used for extraction, storage, or dilution. However, the Tween effects in the earlier studies appeared to be independent on the use of different types of tubes and vials, which argues against this explanation.[7]
The presence of Tween during extraction significantly increased the endotoxin yield for all dust types tested. In an analogous experiment with bulk dust samples, we found a similar but less pronounced effect (mean relative effect of 2.9 for bulk dust versus 5.6 for airborne dust samples, data not shown). This suggests that addition of Tween during the extraction procedure enhances the release of LPS from its natural matrices as well as from filters. However, the effect of addition of Tween to the extraction and/or assay medium showed some heterogeneity with the type of dust or work environment, although the small number of samples this observation is based on precludes firm conclusions. Differences between sampled environments have been reported before.[21,23] The fact that the effect of Tween on the extraction efficiency seemed somewhat higher at lower endotoxin concentrations might partly explain the now-observed differences.

Other buffers and dispersing agents have also been proposed to increase extraction efficiency and/or to stabilize the pH and ionic strength of the extract in the LAL assay.[6] In this study, the use of Tris for extraction (and during analysis) resulted in endotoxin concentrations which were comparable with those found in PFW, and the use of TAP lowered the measured endotoxin concentration compared to that obtained in PFW, with relatively large variations depending on the kind of dust investigated. Furthermore, the use of TAP showed a deviant calibration curve, with a lower sensitivity. Our findings for Tris and TAP are consistent with findings reported earlier.[9,28] Based on the consistent results with Tween, we decided to only further investigate the separate effect of Tween during extraction and analysis, although similar experiments could have been done with TAP or Tris. For instance, Laitinen mentioned an average 17% decrease in endotoxin concentration when a Trizma-buffer was present in the assay compared to PFW, and a 25% increase when a KH_2PO_4-buffer was used, but details were not reported.[9]

Rocking, sonication, or a combination of both are the most commonly used methods for extraction of filters in an extraction medium. Additionally, the temperature during the extraction may be altered. We found no differences in endotoxin yield after extraction in PFW with 10 or 60 minutes of shaking, which indicates that longer extraction duration, at least after a certain time of vigorous rocking, does not result in increased endotoxin yields. Likewise, others found no difference between results with gentle or vigorous rocking for 1 h at either room temperature or 60˚C [7] or in endotoxin activity between 120 min of vigorous shaking at 22˚C or 30 min of gentle rocking at 68˚C.[28]

Tween in the extraction medium thus on average clearly increases the efficiency of extraction and the availability of LPS in the assay. However, the actual extraction efficiency of airborne endotoxin from filters after sampling is still unknown and remains to be investigated. In the case of allergen extraction, 20 to 25% of allergens could be additionally released and measured in extracts after a second extraction of filters.[1] In the study of Laitinen, spiking standard endotoxin on several filter types revealed recovery rates of 70 to 100% from filters that were placed in PFW directly after the spiking and highly variable recovery rates of 5 to 90% from filters that were dried first, with the percentage of recovery depending on the filter type. The highest recovery rates were from glass fiber filters.[9] Spiking of electrostatic wiping cloths with house dust of defined endotoxin content resulted in 37 to 96% recovery rates.[27]

Differences between laboratories in measured endotoxin concentrations of parallel samples have been reported,[20,21] which poses a serious problem when an exposure limit has to be estimated or compliance with an exposure limit is required. It has been suggested that harmonization of protocols can lead to more comparable results.[5,10] Although the CEN-14031 protocol is meant to provide a protocol for measurement and analysis of airborne endotoxin concentrations,[4] some parameters are left unspecified, and in practice, many different protocols are used. This is also the case for the American Society of Testing and Materials' method for analysis of endotoxin in metal-working fluids.[26]

Based on the results of this study and previously reported results,[23] it is recommended that airborne endotoxin samples should be extracted in PFW plus 0.05% Tween-20 to obtain optimal endotoxin yields. In case of airborne (and bulk) dust samples in which dilution factors of at least 50 can be applied to fully rule out a possible effect of the presence of Tween in the extract on the LAL assay, we propose analysis of the extracts in PFW. The sensitivity of the LAL assay in PFW is higher, which allows for the determination of relatively low endotoxin concentrations (0.05 EU/ml) and thus also the application of relatively high dilution factors. The suggested dilution factor of 50 might even be too conservative, as in the application of airborne house dust samples, it was found that dilution factors of 25 led to essentially the same results as analyses with extracts diluted 1:50 (I. Noss, I.M. Wouters, M. Visser, D.J.J. Heederik, P.S. Thorne, B. Brunekreef, and G. Doekes, submitted for publication). Our results have also revealed a better reproducibility of the standard curve in PFW than in PFW-Tween, especially when switching over to a new batch of LAL assay reagent (data not shown). This might be due to practical difficulties when handling Tween-containing medium, which may cause the actual amount to vary during pipetting. The presence of Tween in the assay may also be a source of other inaccuracies, and we therefore prefer PFW as the assay medium. At this moment, however, there is too little information available to extrapolate these findings to samples with another origin or constitution or samples with a very low endotoxin content that does not allow much dilution to be detectable, like medical fluids, cell culture media, or

pharmaceutical samples. Neither is it possible to introduce the relative effects for the different dust types observed in this study as conversion factors for a certain environment or kind of dust until they show to be reproducible. Finally, the physicochemical manner by which Tween enhances endotoxin extraction and the LAL assay should be further investigated, which would require studies on a molecular level.

Acknowledgements

The authors would like to acknowledge the following individuals for their contribution to this work: The company owners who cooperated and made this study possible, Marc Lurvink and Roel Engel (TNO) for conducting the fieldwork, and Hans Jongsma and Jack Spithoven (IRAS) for their analytical assistance. This research was funded by the Ministry of Social Affairs and Employment, the Netherlands, through the long-term research program on biological agents in the work environment of TNO Quality of Life.

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Chapter 5

General discussion

Main findings

The main objectives of this thesis were to give insight into levels and variability in endotoxin exposure and to optimize the measurement and analysis of airborne endotoxin. Endotoxin and inhalable dust exposures were studied in a broad range of agricultural industries. Overall endotoxin exposure was high, but varied considerably between sectors and industries. In all three sectors (grains, seeds and legumes, horticulture, and animal production), exposure was higher in primary production compared to the further (industrial) processing. Several determinants of exposure could be identified; dustiness of the process and contact with animals/feces were associated with higher exposures, and ”wet” processes were associated with lower measured exposures. In a large survey of sewage treatment workers, measured endotoxin exposures were moderate, although clear differences between jobs could be distinguished and sources of exposure could be identified. Several analytical techniques were compared (measuring viable microorganisms, *Limulus* amoebocyte lysate (LAL) assay, gas chromatography-mass spectrometry (GC-MS), and fluorescence microscopy), as well as filter and impinger measurements. Measured concentrations from filter sampling were higher and more consistent than from impinger samples. The LAL assay and GC-MS showed comparable endotoxin levels, and fungi and Gram-positive bacteria were found in higher levels than Gram-negative bacteria. These two studies were combined with several other studies in a database comprising 46 industries in 4 broadly defined sectors, in which exposure variability and determinants of exposure were investigated. The between-worker variability exceeded the within-worker variability, overall and within sectors and subsectors. Variance components were larger for endotoxin than for inhalable dust exposure. After grouping measurements in job categories within industries, the between-worker variability also exceeded the day-to-day variability in almost half of the exposure groups for endotoxin exposure and in 10% of the groups for dust exposure,. Determinants of exposure like dustiness of the process, contact with animals, bulk production, presence of plant material or a cyclic process largely explained between-worker variability. Exposure groups were considerably less homogeneous for endotoxin exposure than for dust exposure. Large exposure variability is inherent to endotoxin exposure, which is in part probably caused by growth of microorganisms. This is distinctly different than for exposure to chemicals. Information from the database about exposure levels and exposure variability was used to adapt the existing CEN 689 guideline for measurement of chemical components to a strategy suitable for assessment of endotoxin exposure. The strategy is tiered, with emphasis on control measures when circumstances indicate (elevated) endotoxin exposure. The database and measurement strategy give input for compliance testing, implementation of control measures, and design of future endotoxin exposure studies. Furthermore, two studies with series of experiments with parallel collected samples in several work environments were performed to fill gaps in the existing CEN protocols 13098 and 14031 for the assessment of workplace exposure to airborne bacterial endotoxins. One study investigated the effects on and interactions with airborne endotoxin concentration of filter type (glass fiber or Teflon), transport conditions (with or without desiccant), sample storage (-20 or 4 ˚C), extraction medium (pyrogen-free water (PFW) or PFW + 0.05% Tween-20), extract storage (-20 or 4 ˚C), and assay medium (PFW or PFW + 0.05% Tween-20). No interactions and no differences were found for transport conditions and storage temperature of extracts, but sampling on glass fiber filters, frozen sample storage, and extraction in PFW-Tween resulted in higher estimated endotoxin concentrations. The other study first compared four media (PFW, PFW-Tween, PFW-Tris, and PFW-triethylamine-phosphate (TAP)) and an extraction time of 10 and 60 min. As only use of PFW-Tween resulted in higher endotoxin yields and

consistent results, the effect of PFW-Tween compared to PFW during extraction and analysis was analyzed separately. The presence of Tween in the extraction medium resulted in higher endotoxin yields, independent of the effect of Tween in the assay. Tween in the LAL assay, however, also strongly inhibited the reactivity of the lipopolysaccharide (LPS) standard, thus shifting the calibration curve to higher values. Based on these two studies we recommend use of glass fiber filters, transport with desiccation, frozen sample storage, extraction in PFW with 0.05% Tween-20, frozen storage of extracts and analysis in PFW (with dilution of extracts at least 50-fold before the LAL assay) for an optimal assessment of airborne endotoxin exposure.

Consequences of the current Dutch occupational exposure limit system

Although a substantial part of the working population is potentially exposed to endotoxins, there is as yet no occupational exposure limit (OEL), which leaves it unclear how endotoxin exposure should be regulated. Introducing an OEL for endotoxin exposure has been under debate in the Netherlands during the last decade. In the past few years, after proposing a health-based recommended OEL (HBROEL) of 50 EU/m³ in 1998, more studies on endotoxin exposure, health effects, and measurement methods have been performed, and currently the national Health Council is evaluating the recent available information in order to propose an adjusted HBROEL for endotoxin exposure. The study of Castellan et al.[9] is probably still a key study in the decision-making process for an exposure standard. However, more information has become available on disease mechanisms and inter- and intra-individual variation in response to exposure.[36,71] This may change the need for some of the earlier applied safety factors, which may lead to a higher exposure limit.

In the meantime, however, the Dutch OEL system has been modified. From 1976 to 2007, statutory OELs were established in a three-stage procedure: 1) a group of independent experts (the Dutch Health Council Expert Committee on Occupational Standards) made a recommendation for an OEL for a given substance; 2) a tripartite committee (the SER OEL Subcommittee) considered the socio-economic and technical feasibility of introducing it as an OEL; and 3) on the basis of both recommendations, the Ministry of Social Affairs and Employment decided whether to set the proposed limit as the statutory OEL for the substance.[52] Since January 2007 a modified OEL system is introduced, that is based on a distinction between ‘public’ and ‘private’ OELs. The Ministry sets public (statutory) OELs for 1) substances for which the EU requires limit values (Binding Limit Values and Indicative Limit Values), and 2) substances for which it is not expected that the EU will require a limit. The set of public OELs comprises substances ‘without owner’ and with a high chance of causing damage to health (high-risk substances), including those for which the government deems it necessary to establish a public limit. All other OELs are private, i.e. OELs that are set by employers. Employers and employees share responsibility for dealing safely with substances in the workplace, which means that together they must now establish OELs to prevent damage to the health of workers as a result of exposure to particular substances. In principle, all OELs within the new system are health-based OELs, with the exception of carcinogenic and mutagenic substances for which no safe health-based OEL can be established.[53]
This new OEL system implies that, in theory, many different OELs for endotoxin exposure may exist in parallel, different ones for different branches of trade or industry. The government will no longer take the initiative in risk assessment and standard setting for

hazardous substances. The regular update of OELs to include recent developments has for many agents become the responsibility of industry. Companies must assess the extent to which they comply with the health-based OELs for all substances, and if they do not comply they must draw up a plan of approach to meet the OEL. Employers and employees indeed have responsibilities to ensure a safe workplace, but it remains to be seen if this type of self-regulation works. At this time it is unknown how compliance will be monitored; perhaps a stronger statutory framework is needed to guarantee a safe work environment.

In the discussion about working conditions, employers have the option to select best practices instead of the use of OELs for prevention of health effects in workers. However, it is unclear how employers should judge if best practices are sufficiently protective when OELs are nonexistent. These details have not been described under the new legislation. Apart from the new Dutch OEL system, another example is the Control of Substances Hazardous to Health (COSHH) Essentials in the United Kingdom. The British law requires employers to control exposure to hazardous substances to prevent ill health. They have to protect both employees and others who may be exposed. COSHH is a tool for good management with eight basic measures: 1) assess the risk, 2) decide what precautions are needed, 3) prevent or adequately control exposure, 4) ensure that control measures are used and maintained, 5) monitor the exposure, 6) carry out appropriate health surveillance, 7) prepare plans and procedures to deal with accidents, incidents and emergencies, and 8) ensure employees are properly informed, trained, and supervised.[22] The Dutch agricultural industry was earlier encouraged and supported in gaining better control over exposure to hazardous substances like endotoxins as part of an earlier *ad hoc* program (VASt, Versterking Arbeidsomstandighedenbeleid Stoffen). This program, preceding EU-initiated REACH (Registration, Evaluation and Authorization of Chemicals) regulations, focused on improving the awareness of risks when handling hazardous substances and improving communication about risks along the chain from producer to end-user.[62] Agricultural employer organizations joined in an action plan to minimize exposure to agricultural dust and particularly endotoxins in an integrated approach to control exposure to agricultural dust. This action plan mainly focused on implementation of control measures and dissemination, with suggestions for control measures for specific activities within industries and sectors.[3] These control measures primarily focused on the reduction of dust exposure, because dust exposure was well known to occur and more visible than endotoxin exposure. However, the effectiveness of these control measures, certainly in case of endotoxin exposure, is mostly unknown and likely to be low considering exposure levels encountered in earlier surveys. Furthermore, the investments needed to reduce dust and endotoxin exposure to a safe level, for instance by adapting the production process, would put a considerable pressure on the economic situation in the sector, which should also be taken into account.

How to deal with endotoxin exposure?

At the moment it is unclear how the Dutch government will deal with endotoxins in the near future. Will endotoxin be seen as an agent that requires a public standard, as the agent is ubiquitous in the environment, has no ‘owner’, and the risk of health effects is potentially even considered high? Or will industries and employers have to set private standards? And are best practices an option for industries for controlling exposure as alternative for a standard? Either way, both government and industries can and probably have to use the intended HBROEL of the Health Council. And in both cases, industry has to make an effort to

control endotoxin exposure to protect their workers. Within this framework, an action plan and research agenda should be formulated by the industries involved, with a focus on reduction of endotoxin exposure. Some topics that ideally should be incorporated are discussed below.

1. Improvement of awareness of the possible health risks associated with endotoxin exposure

Becoming aware of the possible health risks associated with endotoxin exposure is an important step on the way of reduction of exposure. However, in practice employers and employees often do not recognize the risk, do not relate occurring health effects to endotoxin exposure. This might be caused by the fact that most of the effects are non-specific, and could thus be caused by other factors. Therefore, informing and instructing employers and employees about possible risks would be important to raise awareness and reduce endotoxin exposure.

Both negative and protective effects of endotoxin exposure have been found. Endotoxin exposure in early childhood is associated with a lower prevalence of atopy and allergic disease, especially in farm children,[34,67] although some studies suggested that exposure during adulthood continues to protect against atopy.[17,19,41] However, at the same time endotoxins from both house dust and organic dust from occupational settings are associated with adverse health effects.[8,31,49,65] These two sides of endotoxin exposure are illustrated in studies among farmers and agricultural workers, showing an increased risk of reduced lung function, non-atopic asthma, wheeze, and airway responsiveness (bronchial hyper-responsiveness) with airborne endotoxin exposure, as well as an inverse association with hay fever, atopy or atopic asthma.[17,41,54,56] The reduction in atopy may lead to a reduction of symptoms. However, since atopy prevalence is low in farming communities and most symptoms have a non-atopic background, the net association between endotoxins and symptoms or bronchial hyper-responsiveness is still positive. Thus, occupational endotoxin exposure needs to be lowered to protect workers against acute and chronic respiratory health effects. Furthermore, the paradoxal findings with regard to endotoxin exposure and health effects should be carefully communicated to prevent inaccurate interpretation.

2. Further investigation of relation between endotoxin exposure and health effects and underlying mechanisms.

Variation in response to endotoxin exposure is another topic of importance. There is a large variation in inflammatory response caused by inhaled endotoxin between people.[27,37,50] This is determined in part by the atopic status of the person, as atopy seems to be inversely related to the systemic inflammation.[37] Furthermore, differences in individual sensitivity (hypo- and hyper-responders) might be explained by mutations in genes involved in the development of receptors associated with LPS signaling.[27,50] It is also known that repeated exposure to LPS will alter the cellular reactions, which is called adaptation or tolerance.[70] Invasion of neutrophils was significantly reduced after daily LPS exposure up to 90 days in animals,[57] as were lung lavage cytokines IL-6 and TNF-α.[39] The relation with the response to other proinflammatory agents is variable, since both higher sensitivity to an additional response after inhalation of other inflammatory agents in the adaptation stage,[18] and a reduced inflammatory response to other proinflammatory agents after inducing endotoxin tolerance [51] have been found.

Although many cross-sectional studies investigating health effects have been performed in occupational populations, not much information is available from longitudinal studies

investigating effects from chronic exposure. Exceptions are a few follow-up studies in the cotton industry and among pig farmers, which suggest that long-term endotoxin exposure is associated with accelerated lung function decline.[43,69] However, this should be investigated in more occupational environments, also to exclude possible selection effects (for instance the healthy worker effect) and take into account factors like variability in response and tolerance. Furthermore, there is a lack of experimental studies in working populations. For instance, the study of Castellan et al. is performed in healthy subjects with demonstrated moderate and consistent reactivity to cotton dust exposure containing endotoxins,[9] who might react differently from previously exposed workers. However, the fact that workplaces almost always generate a heterogeneous exposure makes it difficult to relate observed effects solely to endotoxin exposure. Endotoxin is frequently regarded as the most important agent in organic dust. Strong evidence for this observation comes from animal inhalation studies that used grain dust and endotoxin,[14,23,51] but similar results were also found in humans.[63] However, the dose of pure endotoxin needed to observe effects comparable to those observed in workers exposed to organic dust containing endotoxin was much higher [16,63] Furthermore, respiratory health effects were regularly associated with other agents (like dust, bacteria, fungi, glucans and ammonia) besides endotoxins in epidemiologic studies, which makes it probable that the observed health effects are (also) caused by others agents. However, endotoxin is at least an informative marker of risk, both by being a true causative agent and by serving as a surrogate for microbial exposure.

Exploring the precise physical-chemical properties of endotoxin, its appearance in organic dust and the impact on its proinflammatory effects may result in better understanding of the effects of endotoxin exposure. For instance, free endotoxins might be more potent in causing health effects, but it is known that cell-bound endotoxins also induce effects.[48] Furthermore, reactivity of LPS in the LAL assay does not necessarily predict the reaction in the human body.

3. Development of improved protocols for the analysis of endotoxin

The development of new protocols for standardized exposure assessment is one of the responsibilities of employers under the new exposure limit system. At the moment, the extremely sensitive and reliable kinetic quantitative chromogenic *Limulus* amebocye lysate (LAL) assay is most commonly used for analysis of endotoxin. This method does not represent an absolute LPS measure, but measures the portion of endotoxins that is biologically available to the assay, which is expected to represent a relative toxicity measure. Other methodologic types of the LAL assay for quantitative measurement of endotoxin are the turbidimetric, endpoint and endpoint chromogenic assays, but these are not used very often.[49,66] However, the LAL assay is sensitive to interferences, among which the principal sources are contamination, temperature, suboptimal pH conditions, aggregation or adsorption of endotoxin, unsuitable cation concentrations, enzyme or protein modifications, non-specific LAL activation and effects of sterilization.[13] Currently also portable (direct reading) test systems are available, but it is unknown whether these systems are applicable for environmental endotoxin samples.[1,2] It is known that free and cell-bound endotoxins react differently [35] and possibly predominantly free endotoxins are measured in this assay,[25] although both free and cell-bound endotoxin are usually present.

Recently a modification of the LAL assay was developed, recombinant Factor C (rFC) using genetically engineered rFC, which should be less sensitive to interference and less potential for lot-to-lot variation compared to the 'original' LAL assay, and also measures only biologically available endotoxin. A preliminary study showed comparable results from LAL

and rFC.[5] However, the method appears to be 10-fold less sensitive because it lacks the amplifying enzyme cascade present in the 'original' LAL assay, which makes replacement implausible for the time being.
Mainly in Germany the whole blood assay (WBA) is suggested as a substitute for the LAL assay. Diseases inflicted by organic dust are mainly caused by inflammation reactions and the pyrogenic activity of the dust is a characteristic feature, which is imitated using WBA by measuring the amount of released cytokines from activated blood cells as a measure for the pyrogenic activity of the sample.[32] It is suggested that the WBA can be useful as a biomarker of inhalation exposure to inflammatory agents and the assessment of susceptibility to organic dust-induced lung inflammation.[39,55] However, the test is difficult to standardize due to individual variation in response and release of cytokines,[6,72] and the test detects endotoxins and other biological agents, reflecting the total inflammatory capacity of a sample.[26] This makes the applicability of WBA for a quantitative estimation of endotoxin exposure as improvement over the LAL assay impossible at the moment.
Chemical methods based on GC-MS, determining the concentrations of 3-hydroxy fatty acids from lipid A, are also used for the quantification of endotoxin.[4,60] These measurements do not reflect the biological activity of a sample, nor the absolute amount of LPS since different species of bacteria reveal different 3-hydroxy fatty acids. Furthermore, the method has a low sensitivity, which makes it difficult to use when analyzing airborne samples unless large volumes are collected. Advantageous is that GC-MS can measure both free and extractable cell-bound endotoxins, but disadvantageous are the high equipment and labor costs and the fact that mammalian body fluids also contain these 3-hydroxy fatty acids. However, in combination with the LAL assay, GC-MS enables estimation of the potency of samples (EU measured with LAL assay / amount of LPS measured with GC-MS), which is known to vary depending on the type of dust.[45]
Immunoassays have also been developed for the determination of LPS,[7] but those have not been widely adopted. The use of these antibodies to quantify environmental endotoxin has not been successful, which might be related to the availability of the lipid A moiety for reactivity with the antibody.[47]

A detailed evaluation of the CEN guidelines 13098 [10] and 14031 [11] for the measurement and analysis of airborne endotoxin revealed that these guidelines still leave room for individual interpretation. The fact that a small round-robin study with three laboratories working according to the CEN 14031 resulted in different measured endotoxin concentrations has been brought up as a reason for not introducing an OEL for endotoxin exposure, since testing compliance with an OEL is not possible when measured results are not comparable.[24] Endotoxin evaluation methods are known to vary in collection of samples, sample handling and storage, extraction of samples, analysis of samples, reporting of results, and dust type.[46] Furthermore, standardization of procedures have been shown to reduce interlaboratory differences in measured endotoxin concentrations.[12,33] Of the studies performed, most investigated only one or two of the possible influencing factors and in a limited number of samples.[15,20,21,29,38,40,44,59,64,66,68] Therefore, the effects and interactions of the gaps in the current guidelines were investigated, with special attention for extraction and assay media. This led to the following recommendations: use of glass fiber filters, transport with desiccation, frozen sample storage, extraction in PFW with 0.05% Tween-20 with rocking/shaking, frozen storage of extracts, and analysis in PFW (with dilution of extracts at least 50-fold before the quantitative kinetic chromogenic LAL assay). The existing guidelines should be updated and adapted to form a truly standardized protocol for airborne occupational endotoxin exposure assessment, followed by the implementation of those

guidelines in everyday practice, which will lead to more comparable results and possibilities for compliance testing.

4. Reduction of variability in exposure assessments with tailor-made measurement strategies.

Endotoxins come from Gram-negative bacteria, a source that can grow and amplify. Gram-negative bacteria are ubiquitous in the environment, have a heterogeneous source, and their growth is dependent upon many factors, for instance presence of a substrate for nutrients, favorable water activity and temperature. Furthermore, aerosolization and distribution of particles are necessary conditions for exposure. Consequently, environmental exposure to endotoxin is highly variable. Possible growth of organisms on or in the sampling medium or in the extract after sampling, reaction of organisms or their products with the assay, or agglomeration in the solution may cause even more variability in the results from analysis of biological agents and thus the measured exposure. This is in contrast with exposure to chemical agents, which are less subject to these factors. Although the total population exposed to biological agents is substantial, variability in biological exposure has rarely been investigated.

Studies conducted as part of this thesis showed that there is large variability in endotoxin exposure. This variability is larger than for inhalable dust exposure or exposure to chemical agents,[28] and on a general level mostly consists of between-worker variability. This information about exposure levels and variability in endotoxin exposure was used to adapt the existing CEN guideline for the measurement of chemical substances to fit the situation concerning endotoxin exposure. The large variability in endotoxin exposure creates the need for taking a large number of measurements in order to make accurate decisions in compliance testing,[30,42,58] which is a drawback in practice. A next step might be a more tiered approach, with the addition of instruments and exposure models [61] to help employers manage their risks.

For some exposures, such as crystalline silica or asbestos, a certain variability in measured concentrations is accepted, and methods taking this variability into account are generally applied. This acceptance of variability in results is probably caused, at least in part, by the fact that those exposures can produce severe health effects (pneumoconiosis and lung cancer instead of ODTS and asthma for endotoxin exposure) and the familiarity of the topics among the general public.

5. Expansion of investigations of determinants of exposure and control measures.

Knowledge about determinants of exposure is very important in the search for effective control measures to reduce endotoxin exposure. In the past, most studies were not designed to explore determinants next to studying exposure levels and possible health effects. Therefore, as is seen in the exposure database, determinants of endotoxin exposure are often on a more general level. To be able to find detailed determinants of exposure, both between workers and over time, repeatedly measuring exposure over a longer period of time and gathering detailed information about tasks, products and environmental circumstances is required. Information about determinants of exposure can serve as input when searching for control measures to reduce exposure.

With respect to control measures, their effectiveness and level of exposure reduction needs to be investigated, to make sure that implementing them is useful. For instance, current exposure control programs in the Netherlands are now aimed at reducing organic dust exposure. However, dust and endotoxin exposure are not always well correlated, and those measures will thus not necessary reduce endotoxin exposure.

Conclusions

Endotoxins are ubiquitous in the work environment and are known to have proinflammatory properties that can cause adverse health effects. Variability in endotoxin exposure will always occur, both in actual exposure of workers and in analysis results, since it originates from of living material, for which growth and amplification is influenced by many factors. Also, standardization of the measurement and analysis protocol will not result in a low variability in analytical results and measured concentrations, which has to be accepted and dealt with in an appropriate manner. In the past few years, the discussion about endotoxin exposure and how to deal with it has mainly been a political discussion. Uncertainty about the measurement and analysis method, disease mechanisms and possible protective effects has delayed the implementation of an OEL. In turn, this also minimized the willingness of industries involved to actively reduce endotoxin exposure, by adapting their processes or purchasing effective control measures.

Our present understanding is that endotoxin exposure is known to be high in a variety of industries. Apart from a possible protective effect, endotoxin exposure, either by itself or as a proxy for microbial exposure, is known to cause several adverse health effects. In the long run, endotoxins may cause disability from chronic lung function impairment. Thus, endotoxin exposure is surely a hazard which has to be taken into account. This thesis gives an overview of endotoxin exposure and exposure variability in several industries, which was used to set up a measurement strategy to help occupational hygienists in the field in their decision-making process. Furthermore, several experiments were performed to optimize the measuring and analytical procedure for endotoxin exposure assessment. And although optimized (European) guidelines for exposure assessment and a practical measurement strategy are useful tools, these are not the sole solution for dealing with endotoxin exposure. Generally, one should be aware that endotoxin exposure poses a potential threat to the working population, which should be rendered into monitoring and controlling this exposure. Even in the case of private OELs, employers should pay active attention to this matter, and set an OEL based on available information from the Health Council and similar bodies. And of course, many other issues next to endotoxin exposure need attention for employers to meet their obligation to ensure a healthy work environment for their workers.

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Summary

In **chapter 1** background and aim of this thesis are introduced. Organic dust, a generic term used to refer to plant, animal and microbial matter, forms a complex mixture or infectious and non-infectious agents. Organic dust exposure is associated with several health effects, caused by specific agents in the dust, like bacterial endotoxins, fungal (1→3)-β-D-glucans, urinary proteins from animals, aflatoxin, and bacterial enzymes. This thesis focuses exclusively on endotoxin exposure. Endotoxins, components of the cell walls of Gram-negative bacteria, are ubiquitous in the environment. They are an integral part of the outer layer of the cell wall, composed of proteins, lipids and lipopolysaccharides (LPS), and are released into the environment by lysis, which occurs during cell growth and after cell death. Gram-negative bacteria are present on for instance the surfaces of plants and in animal feces, and their occurrence, growth and amplification is influenced by many factors. As a consequence, endotoxin levels may be highly variable in soil, water and air, and exposure to endotoxin occurs in various industries. Inhalation exposure is thought to be the major route of exposure.
Both experimental and (epidemiological) field studies have shown several negative health effects induced by endotoxin exposure, which can be divided into 1) inflammatory reactions in the airways, which may lead to respiratory symptoms like dry cough, shortness of breath and wheeze, clinical conditions such as non-allergic asthma, accelerated lung function decline, and byssinosis, and 2) systematic reactions, resulting in a range of symptoms including fever, shivering, joint aches, malaise and other influenza-like symptoms. Protective effects of endotoxin exposure have mainly been described for the development of asthma, and recently with regard to (lung) cancer risk.
In 1998, a health-based occupational exposure limit of 50 EU/m^3 for endotoxin exposure was suggested in the Netherlands. A temporary legal limit of 200 EU/m^3 was introduced, which should eventually be lowered to 50 EU/m^3 within two years. However, this legal limit was withdrawn because its enforcement was not feasible from a technical as well as a social-economical point of view. However, the industries involved had to formulate an approach for dealing with endotoxin exposure, which should consist of:
1. A plan to minimize endotoxin exposure, including application and development of control measures and investigations of the state of the art with regard to exposure control and instruction;
2. A schedule for introducing and applying the measurement method described in CEN guidance 14031 in combination with CEN 13098 in a proper way, since a standardized method is needed to be able to compare measured endotoxin concentrations to an exposure limit; and
3. An adapted measurement strategy for assessing endotoxin exposure.

This thesis aimed to provide information on some of the major issues around introducing an occupational exposure limit (OEL) for endotoxin. The main objectives of this thesis were to give insight in levels and variability in endotoxin exposure and to optimize the measurement and analysis of airborne endotoxin. This was divided in three subjects, namely:

- To investigate levels of inhalable dust and endotoxin exposure in several industries, namely agricultural industries and sewage treatment, as well as exploring determinants of exposure and comparing techniques for the measurement of microbial load;
- To study inhalable dust and endotoxin exposure variability in endotoxin exposure, in order to adapt the existing measurement strategy for chemical agents (CEN 689) for endotoxin exposure assessment;

- To investigate the gaps present in the CEN guidance for endotoxin measurement (CEN 13098 and CEN 14031) to give input for a standardized protocol for the measurement and analysis of endotoxin exposure.

In **chapter 2,** two endotoxin exposure studies are presented. In **chapter 2.1**, personal endotoxin exposure in a broad spectrum of agricultural industries was investigated, and possible determinants of exposure were explored. This study was performed because although high exposure to organic dust and endotoxins has been described regularly in agricultural industries, a detailed overview of levels of airborne exposure to endotoxins in the agricultural industry, as well as a systematic comparison between several specific branches using the same exposure assessment protocols, were lacking. Over 600 personal inhalable dust samples were performed in 46 companies of three agricultural sectors: Grains, seeds & legumes, Horticulture and Animal production. Dust and endotoxin levels were determined, and basic descriptive analysis and elaborate analysis of variance were performed. Mean exposure levels were high, with large differences between sectors and between companies within the sectors. Furthermore, differences in exposure between workers were larger than the day-to-day variability. Highest dust and endotoxin exposures were found in companies of the Grains, seeds & legumes sector. In all three sectors exposure was higher in the primary production part compared to the (industrial) products processing part of the sector. The Dutch proposed health-based occupational exposure limit (50 EU/m^3) and temporary legal limit (200 EU/m³) for endotoxin were often exceeded. Identified determinants increasing exposure levels were among others type of company, dustiness of the product and contact with animals/feces. 'Wet' processes resulted in less dusty working environments and thus lowered endotoxin exposure. Overall can be concluded that exposure to endotoxins over the whole range of agricultural industries is high. A 10-1000 fold reduction in exposure is needed to reduce endotoxin related health risks.
The objectives of **chapter 2.2** were to give an overview of endotoxin exposure and its determinants in sewage treatment workers. Furthermore, exposure to culturable and non-culturable microorganisms and the applicability of the *Limulus* amebocyte lysate (LAL) assay for measuring endotoxin exposure in this work environment were studied. In 43 Dutch sewage treatment plants 470 full-shift, 123 task-based personal and 54 stationary inhalable dust samples were collected. The endotoxin concentration in these inhalable dust samples was determined with the LAL-assay. Mixed effects models were used to investigate possible determinants of exposure. Simultaneous parallel filters samples, impinger samples and viable total bacteria and Gram-negative bacterial samples were taken to compare analysis techniques. Filter and impinger samples were analyzed with the LAL-assay, gas chromatography-mass spectrometry (GC-MS) and fluorescence microscopy. Endotoxin exposure levels were moderate, yet distinct differences between jobs and sources and some determinants of exposure were identified. The measured concentrations in filter samples were higher and more consistent than in impinger samples. Fungi and Gram-positive bacteria were found in higher levels than Gram-negative bacteria. Furthermore, the LAL assay and GC-MS showed comparable endotoxin levels. The study showed that exposure to inhalable dust and endotoxin in Dutch sewage treatment workers was relatively low, and that sewage treatment workers are exposed to a mixture of microbial agents. In contrast with what was thought before, comparison of analytical techniques suggested that the LAL-assay did not result in much exposure misclassification.

Chapter 3 describes an analysis of combined data from several exposure studies. Variability in exposure has rarely been investigated for biological agents, and more specifically

endotoxins. Since information about exposure variability is essential in exposure assessment, variance components and determinants of exposure were studied in a large database with >2000 personal inhalable dust and endotoxin measurements in **chapter 3.1**. The database was created by combining the data from 10 individual studies conducted over the last decade. In this database, exposure groups were defined based on job codes within industries. Inhalable dust and endotoxin exposure levels were summarized for 46 industries in 4 broadly defined sectors. The between-worker variability exceeded the within-worker (day-to-day) variability overall and within sectors and subsectors, and variance components were larger for endotoxin than for dust. Between-worker variability also exceeded within-worker variability in nearly half of the exposure groups based upon industries or job code within industries for endotoxin exposure, and in 10% of the groups for dust exposure. Determinants of exposure like dustiness of the process, contact with animals, bulk production, presence of plant material or a cyclic process largely explained the between-worker variability. Exposure groups were much less homogeneous for endotoxin exposure than for dust exposure. Large variability in measured exposure levels is inherent to endotoxin exposure, which is caused in part by determinants that influence growth of microorganisms. This is distinctly different than for exposure to chemical agents, which demonstrate lower exposure variability. These findings have major consequences for the design of future occupational intervention and epidemiological studies. For instance, the measurement effort needs to be greater than when exposure to chemical agents is investigated, especially when evaluating endotoxin exposure for compliance testing. The established determinants of exposure give direction for potential exposure control, although more information about determinants of day-to-day variability in exposure is still needed to be able to effectively control endotoxin exposure.

Based on the information about exposure levels and variance components studied in the endotoxin database, in **chapter 3.2** a proposal was made for the existing measurement strategy for chemical agents, the European standard EN 689, to adapt the strategy for endotoxin exposure assessment. The measurement strategy has three phases, and every phase will result in the conclusion that either the exposure situation is acceptable, advice on control measures, or a more precise estimation of the exposure situation. The phases are:

1. Initial workplace examination – Is endotoxin exposure probable? (presence of substrate, circumstances favourable of growth, and spreading or distribution);
2. Crude quantitative estimation – Are exposure limits likely to be exceeded? (previously performed measurements, literature search, endotoxin content of product samples, stationary measurements, indicative personal measurements);
3. Detailed quantitative estimation (measuring) – Are exposure limits exceeded? (repeated personal measurements for randomly selected workers from a group on randomly selected days).

This measurement strategy for endotoxin exposure should be seen as a novel approach taking the higher exposure variability usually encountered with endotoxin exposure into account. It can be a useful guide for occupational hygienists to assess endotoxin exposure and the probability of exceedance and overexposure in a certain working environment. The emphasis of the strategy is on control measures when the circumstances indicate (elevated) endotoxin exposure.

In **chapter 4** the procedure for measurement and analysis of airborne endotoxins is evaluated and proposals are made for further optimization. In order to compare results from different studies and establish standards, airborne endotoxin exposures should be assessed using standardized methods. Although the European Committee for Standardization (CEN)

developed guidelines for endotoxin exposure assessment, these leave room for interpretation. To fully elucidate the importance of all facets of endotoxin assessment, the gaps in CEN 14031 (determination of airborne endotoxine) were explored and are investigated in this chapter, by means of full experimental designs and the use of parallel collected inhalable dust samples.

In **chapter 4.1,** the effects on and interactions with airborne endotoxin concentrations of filter type (glass fiber or Teflon), transport conditions (with/without desiccant), sample storage (-20 or 4˚C), extraction solution (pyrogen-free water (PFW) or PFW + 0.05% Tween-20), extract storage (-20 or 4˚C) and assay solution (PFW or PFW + 0.05% Tween-20) were investigated in two working environments. Four hundred samples were collected and randomly distributed over the 20 combinations of treatments. There were no differences found for transport conditions and storage temperature of extracts. Also no interactions between study variables existed. Sampling on glass-fiber filters, storage of samples in the freezer, and extraction in PFW + 0.05% Tween-20 resulted in a 1.3-, 1.1-, and 2.1-fold-higher estimated endotoxin concentrations, respectively. Use of PFW + 0.05% Tween-20 in the assay solution had an additive effect.

In addition, in **chapter 4.2** the effect of four common media during extraction and analysis were compared. Because this and earlier studies reported higher endotoxin yields when Tween-20 was added to the media used for filter extraction and in the LAL assay, the effects of Tween during extraction and analysis were investigated separately. 250 dust samples from five work environments were used to compare four media (pyrogen-free water (PFW), PFW-Tween-20, PFW-Tris and PFW-triethylamine-phosphate (TAP)) and extraction for 10 or 60 minutes. A subset of the extracts in PFW or PFW-Tween (n=40) were analyzed in parallel LAL assays with PFW or PFW-Tween as the assay medium. Compared to the reference procedure (PFW with 60 min shaking), shorter extraction or presence of Tris produced similar results. The use of PFW-TAP showed overall lower yields and a deviant calibration curve. Tween in the extraction medium resulted in significantly higher endotoxin yields from all dust types, independent of the effect of Tween in the assay. Tween in the LAL assay, however, strongly inhibited the reactivity of the lipopolysaccharide (LPS) standard, shifting the calibration curve to higher values. Inhibition of LPS in test samples was less pronounced and varied between dust sources, resulting in enhanced calculated concentrations. This assay effect could be circumvented by diluting extracts at least 50-fold before the LAL assay. In conclusion, of the media tested, only Tween enhances the endotoxin extraction efficiency from airborne samples in a consistent manner.

Based on the results of these two studies, we recommend use of glass-fiber filters, transport with desiccation, frozen sample storage, extraction in PFW with 0.05% Tween-20 with rocking/shaking, frozen storage of extracts, and dilution and LAL analysis in PFW. These studies provide data with which a protocol for standardization of endotoxin exposure assessment can be fully specified.

In **chapter 5** main findings are summarized. Furthermore, consequences of the current (new) occupational exposure limit system for the control of occupational endotoxin exposure are considered. The system shifted from only statutory OELs to a combination of public or statutory and private OELs. At this time it is unknown how this new system will be established and its function monitored. Also, it is as yet unclear whether employers will be active in the process of setting private OELs. In the discussion about working conditions, employers have the option to select best practices for prevention of health effects in workers, in which control measures are very important to limit and control exposure to hazardous substances. For this, the effectiveness of suggested control measures to reduce dust and

endotoxin exposure should be investigated. At this moment it is unknown to which category of the OEL system endotoxin exposure will belong. In case of a private OEL, in theory many different OELs for endotoxin may exist in parallel. Either way, both the government and industries can use the intended health-based recommended OEL (HBROEL) of the Health Council, which may be higher than the earlier HBROEL of 50 EU/m^3. Anyhow, industry has to make an effort in controlling endotoxin exposure to protect its workers. This effort could be translated into an action plan and research agenda, consisting of:

1. Improvement of awareness of the possible health risks associated with endotoxin exposure, which is an important first step towards exposure reduction;
2. Some issues which refer to relation between exposure and health effects still require some additional study. Specific issues are variation in response to endotoxin exposure between individuals and tolerance, as well as investigating effects due to chronic exposure;
3. Development of improved protocols for the analysis of endotoxin, on the basis of our present insights;
4. Development of tailor-made exposure assessment strategies which take the highly variable exposure levels into account;
5. Investigation of determinants of exposure and control measures. Determinants of exposure, both between workers and over time, can serve a staring point for control measures. Furthermore, the effectiveness of control measures should be investigated.

It can be concluded that endotoxins are ubiquitous in the work environment and that various groups of workers are exposed to high amounts of endotoxin. Variability in endotoxin exposure is eminent, and thus has to be accepted and dealt with in an appropriate way. Instead of a political discussion resulting in stagnation with regard to controlling endotoxin exposure, endotoxin exposure should be recognized as a potential threat to the working population and be treated that way by actively monitoring and controlling this exposure to ensure a healthy working environment.

Samenvatting

In **hoofdstuk 1** worden de achtergrond en het doel van dit proefschrift geïntroduceerd. Organisch stof, een algemene term die wordt gebruikt om te verwijzen naar plantaardig, dierlijk en microbiologisch materiaal, bestaat uit een complex mengsel van infectieuze en niet-infectieuze agentia. Blootstelling aan organisch stof is geassocieerd met verschillende gezondheidseffecten die worden veroorzaakt door specifieke agentia in het stof, zoals endotoxinen afkomstig van bacteriën, (1→3)-β-D-glucanen afkomstig van schimmels, eiwitten afkomstig uit dierlijke urine, aflatoxinen en bacteriële enzymen. Dit proefschrift richt zich uitsluitend op endotoxinen. Endotoxinen zijn onderdeel van de celwand van Gram-negatieve bacteriën en alomtegenwoordig in het milieu. Gram-negatieve bacteriën zijn bijvoorbeeld aanwezig op het bladoppervlak van planten en in dierlijke uitwerpselen. Endotoxinen zijn een integraal deel van het buitenste laag van de celwand en opgebouwd uit eiwitten, lipiden en lipopolysacchariden (LPS). Endotoxinen komen vrij in het milieu door lysis, wat plaatsvindt tijdens celgroei en nadat de cel sterft. Het voorkomen, de groei en het vermenigvuldigen van deze bacteriën wordt beïnvloed door vele factoren. Hierdoor zijn de endotoxinenniveaus in bodem, water en lucht zeer variabel, en vindt blootstelling aan endotoxinen in verschillende industrieën plaats. Inhalatie van endotoxinen wordt gezien als de belangrijkste blootstellingsroute.
Zowel experimentele als (epidemiologische) veldstudies hebben verschillende negatieve effecten van blootstelling aan endotoxinen aangetoond, welke kunnen worden verdeeld in 1) inflammatoire reacties in de luchtwegen, die kunnen leiden tot respiratoire klachten zoals droge hoest, kortademigheid en piepen op de borst, klinische symptomen zoals niet-allergisch astma, versnelde longfunctiedaling, en byssinose, en 2) systemische reacties die resulteren in een verscheidenheid aan symptomen zoals koorts, rillingen, gewrichtspijn, malaise en andere griepachtige verschijnselen. De beschermende effecten van endotoxinenblootstelling worden voornamelijk beschreven in verband met de ontwikkeling van astma, en meer recent in verband met het risico op (long)kanker.
In 1998 werd in Nederland een gezondheidskundige grenswaarde van 50 EU/m^3 geadviseerd. Er werd een tijdelijke grenswaarde van 200 EU/m^3 geïntroduceerd, welke uiteindelijk binnen twee jaar tot 50 EU/m^3 zou moeten worden teruggebracht. Deze grenswaarde werd echter ingetrokken, omdat handhaving vanuit een technisch en sociaal-economisch oogpunt niet haalbaar was. De betrokken industrieën moesten echter wel een plan van aanpak opstellen voor het omgaan met blootstelling aan endotoxinen, dat moest bestaan uit:

1. Een plan van aanpak over de wijze waarop de problematiek van blootstelling aan endotoxinen zal worden aangepakt, waarin naast het toepassen en eventueel ontwikkelen van (aangepaste) beheersmaatregelen tevens moet worden voorzien in onderzoek naar de stand der techniek en voorlichting;
2. Een programma voor het op de juiste manier introduceren en toepassen van de meetmethode zoals beschreven in de CEN-norm 14031 in combinatie met CEN 13098, aangezien een gestandaardiseerde methode noodzakelijk is voor het kunnen vergelijken van gemeten endotoxinenconcentraties met een grenswaarde; en
3. Het aanpassen van de huidige meetstrategie, die is afgestemd op chemische stoffen, aan de problematiek van blootstelling aan biologische agentia, en in het bijzonder endotoxinen.

Het doel van dit proefschrift is het geven van informatie over enkele van de belangrijkste kwesties rondom het introduceren van een grenswaarde (Maximaal Aanvaarde Concentratie, MAC) voor endotoxinen. De belangrijkste doelstellingen van dit proefschrift waren een overzicht geven van niveaus en variabiliteit in endotoxinenblootstelling en het optimaliseren

van de meetmethode en de analyse van endotoxinen in de lucht. Deze doelstellingen werden onderverdeeld in de volgende onderdelen:

- Het bestuderen van inhaleerbaar stof en endotoxinenniveaus in verschillende industrieën, met name in de agrarische industrie en waterzuivering, het onderzoeken van determinanten van blootstelling, en het vergelijken van verschillende technieken voor het meten van microbiële blootstelling;
- Het bestuderen van variabiliteit in inhaleerbaar stof- en endotoxinenblootstelling om de bestaande meetstrategie voor chemische agentia (CEN 689) aan te passen en zo geschikt te maken voor de beoordeling van endotoxinenblootstelling;
- Het bestuderen van de aanwezige hiaten in de CEN richtlijnen voor het meten van endotoxin (CEN 13098 en CEN 14031) om zo bij te dragen aan het tot stand brengen van een gestandaardiseerd protocol voor het meten en analyseren van endotoxinenblootstelling.

In **hoofdstuk 2** worden twee blootstellingonderzoeken beschreven. In **hoofdstuk 2.1** zijn persoonlijke endotoxinenblootstelling in een verscheidenheid van agrarische industrieën en mogelijke determinanten van blootstelling onderzocht. Ondanks dat hoge blootstellingen aan organisch stof en endotoxinen regelmatig waren beschreven in deze industrieën, onbrak zowel een gedetailleerd overzicht van niveaus in blootstelling aan endotoxinen in de lucht, als een systematische vergelijking tussen verschillende specifieke branches met gebruikmaking van hetzelfde protocol. Meer dan 600 persoonlijke inhaleerbaar stofmetingen werden uitgevoerd in 46 bedrijven uit 3 sectoren: Granen, zaden & peulvruchten, Tuinbouw, en Vee, vlees en eieren. De stof- en endotoxinenniveaus werden bepaald, en beschrijvende statistiek en uitgebreide variantieanalyse werden uitgevoerd. De gemiddelde blootstellingniveaus waren hoog, met grote verschillen tussen sectoren en bedrijven binnen sectoren. Er was meer verschil in blootstelling tussen werknemers dan variabiliteit van dag tot dag. De hoogste stof- en endotoxinenblootstelling werd in de sector Granen, zaden & peulvruchten gevonden. In alle drie de sectoren was de blootstelling hoger tijdens de primaire productie dan tijdens de verdere (industriële) verwerking van de producten. De Nederlandse voorgestelde gezondheidskundige grenswaarde (50 EU/m^3) en tijdelijke grenswaarde (200 EU/m^3) voor endotoxinen werden regelmatig overschreden. Determinanten als soort bedrijf, stoffigheid van het product en contact met dieren/uitwerpselen waren onder andere geassocieerd met een verhoogde blootstelling. ‘Natte’ processen resulteerden in een minder stoffige werkomgeving en verlaagden dus de endotoxinenblootstelling. Over het algemeen kan worden geconcludeerd dat blootstelling aan endotoxinen in de hele agrarische industrie hoog is. Om aan endotoxinen gerelateerde gezondheidsrisico’s te verminderen is een 10-1000-voudige verlaging van blootstelling noodzakelijk.

Het doel van **hoofdstuk 2.2** was het geven van een overzicht van blootstelling aan endotoxinen en zijn determinanten voor werknemers van rioolwaterzuiveringsinstallaties. Verder werd blootstelling aan levensvatbare en niet-levensvatbare micro-organismen en de toepasbaarheid van de *Limulus* amebocyte lysaat (LAL) assay voor het meten van blootstelling aan endotoxinen in deze werkomgeving onderzocht. In 43 Nederlandse rioolwaterzuiveringsinstallaties werden 470 persoonlijke, 123 taakgerichte persoonlijke en 54 stationaire inhaleerbaar stofmonsters verzameld. De concentratie endotoxinen in deze stofmonsters werd bepaald met behulp van de LAL-assay. Mogelijke determinanten van blootstelling werden onderzocht door middel van mixed effects modellen. Simultaan genomen monsters met parallelle monsternameapparatuur (filters), impingers en de totale hoeveelheid levensvatbare bacteriën en Gram-negatieve bacteriën werden gebruikt om

analyse-technieken te vergelijken. De filter- en impingermonsters werden geanalyseerd met de LAL-assay, gas chromatografie-massa spectometrie (GC-MS) en fluorescentie microscopie. De blootstellingniveaus van endotoxinen waren matig, hoewel duidelijke verschillen tussen functies en bronnen van blootstelling en een aantal determinanten van blootstelling konden worden aangetoond. De op filters gemeten concentraties waren hoger en meer consistent dan die gemeten in impingermonsters. Er werden meer schimmels en Gram-positieve bacteriën dan Gram-negatieve bacteriën gemeten. De endotoxinenniveaus gemeten met de LAL-assay en GC-MS waren vergelijkbaar. Het onderzoek heeft aangetoond dat de blootstelling aan inhaleerbaar stof en endotoxinen van werknemers van rioolwaterzuiveringsinstallaties vrij laag is, en dat deze werknemers worden blootgesteld aan een verzameling van microbiële agentia. In tegenstelling tot wat eerder werd gedacht suggereert de vergelijking van analysetechnieken dat gebruik van de LAL-assay niet resulteert in veel misclassificatie van blootstelling.

In **hoofdstuk 3** wordt een analyse van de gecombineerde gegevens van verschillende blootstellingonderzoeken beschreven. Variabiliteit in blootstelling aan biologische agentia, en meer specifiek endotoxinen, is zelden onderzocht. Omdat informatie over variabiliteit in blootstelling noodzakelijk is voor het beoordelen van blootstelling, is in **hoofdstuk 3.1** onderzoek gedaan naar variantiecomponenten en determinanten van blootstelling in een grote database met meer dan 2000 persoonlijke inhaleerbaar stof- en endotoxinenmetingen. De database werd opgesteld door middel van het samenvoegen van gegevens van 10 individuele studies die de laatste 10 jaar zijn uitgevoerd. In deze database werden blootstellinggroepen gedefinieerd op basis van functiecategorieën binnen industrieën. Er werd een overzicht gegeven van de blootstellingniveaus aan inhaleerbaar stof en endotoxinen in 46 industrieën binnen vier breed gedefinieerde sectoren. Voor zowel het totaal als wanneer de metingen werden gegroepeerd in sectoren en subsectoren was de tussenpersoonsvariabiliteit groter dan de binnenpersoons- (dag tot dag) variabiliteit. Verder waren de variantiecomponenten voor endotoxinen groter dan die voor stof. Ook wanneer de metingen werden gegroepeerd op basis van industrie of functiecategorie binnen industrie was de tussenpersoonsvariabiliteit in bijna de helft van de groepen groter dan de binnenpersoonsvariabiliteit voor endotoxinenblootstelling, en voor stofblootstelling in 10% van de groepen. Determinanten van blootstelling, zoals stoffigheid van het proces, contact met dieren, bulk productie, aanwezigheid van plantaardig materiaal of een cyclisch proces, verklaarden grotendeels de tussenpersoonsvariabiliteit. Blootstelling aan endotoxin was minder homogeen verdeeld binnen blootstellinggroepen dan blootstelling aan stof. Een grote variabiliteit in gemeten endotoxinenniveaus is inherent aan blootstelling aan endotoxinen en wordt deels veroorzaakt door determinanten die samenhangen met de groei van micro-organismen. Dit verschilt van blootstelling aan chemische stoffen, waar de variabiliteit in blootstelling over het algemeen dan ook lager is. Deze bevindingen hebben belangrijke gevolgen voor de opzet van toekomstige interventiestudies en epidemiologische onderzoeken. Zo zal bijvoorbeeld de meetinspanning groter moeten zijn dan bij beoordeling van blootstelling aan chemische stoffen, zeker wanneer de beoordeling van blootstelling aan endotoxinen is gericht op het toetsen aan een grenswaarde. De gevonden determinanten van blootstelling geven een indicatie voor het beheersen en controleren van deze blootstelling, hoewel meer kennis over de oorzaken van variabiliteit in blootstelling van dag tot dag noodzakelijk is om blootstelling een endotoxinen effectief te kunnen controleren.
Op basis van de informatie over blootstellingniveaus en variantiecomponenten uit de database met endotoxinengegevens wordt in **hoofdstuk 3.2** een voorstel gedaan om de bestaande meetstrategie voor chemische stoffen, de Europese standaard CEN 689, aan te

passen voor de beoordeling van blootstelling aan endotoxinen. De meetstrategie heeft 3 fases, en elke fase kan resulteren in de conclusie dat de blootstellingsituatie acceptabel is, advies over beheersmaatregelen, of verder gaan naar een meer precieze schatting van de blootstellingsituatie. De drie fases zijn:

1. Oriënterend werkplekonderzoek – is endotoxinenblootstelling waarschijnlijk? (Is er een voedingsbodem voor bacteriën aanwezig, zijn er goede groeiomstandigheden om te kunnen overleven, groeien en vermeerderen, en vindt er verspreiding plaats);
2. Globale kwantitatieve schatting – Is grenswaardenoverschrijding waarschijnlijk? (aan de hand van vergelijking met eerder uitgevoerde metingen, literatuuronderzoek, de hoeveelheid endotoxinen in productmonsters, stationaire metingen en/of indicatieve persoonlijke metingen);
3. Gedetailleerde kwantitatieve schatting – Is er sprake van grenswaardenoverschrijding? (herhaalde persoonlijke metingen bij aselect gekozen werknemers uit een groep op willekeurige dagen)

Deze meetstrategie voor de beoordeling van blootstelling aan endotoxinen moet worden gezien als een manier om rekening te houden met de grote variabiliteit in blootstelling die gewoonlijk wordt gevonden. Voor arbeidshygiënisten kan het een nuttig instrument zijn voor de beoordeling van blootstelling aan endotoxinen en de waarschijnlijkheid van 'exceedance' en 'overexposure' in een bepaalde werkomgeving. Bij het protocol ligt de nadruk op beheersmaatregelen wanneer de omstandigheden wijzen op (verhoogde) blootstelling aan endotoxinen.

In **hoofdstuk 4** wordt de procedure voor het meten en analyseren van endotoxinen in de lucht geëvalueerd en worden voorstellen gedaan voor de optimalisatie van deze procedure. Om het vergelijken van resultaten van verschillende studies en het instellen van standaarden mogelijk te maken zou de blootstelling van endotoxinen in de lucht moeten worden beoordeeld met gebruikmaking van gestandaardiseerde methoden. Hoewel de Europese Commissie voor Standaardisatie (CEN) richtlijnen heeft opgesteld voor de beoordeling van blootstelling aan endotoxinen, laten deze richtlijnen ruimte voor eigen interpretatie. Om het belang van alle facetten van het meten en analyseren van endotoxinen aan het licht te brengen is uitgezocht welke onduidelijkheden er nog zaten in CEN-richtlijn 14031 (beoordeling van endotoxinen in de lucht). Deze zijn in dit hoofdstuk onderzocht met behulp van een volledig experimenteel design en met gebruikmaking van parallel verzamelde inhaleerbaar stofmonsters. In **hoofdstuk 4.1** zijn de effecten van en interacties tussen soort filter (glasvezel of Teflon), transportomstandigheden (met of zonder dehydratie), opslag van monsters (bij -20 of 4 °C), extractievloeistof (pyrogeen-vrij water (PFW) of PFW + 0,05% Tween-20), opslag van extracten (bij -20 of 4 °C) en analysevloeistof (PFW of PFW + 0,05% Tween-20) op de gemeten endotoxinenconcentratie in de lucht in twee werkomgevingen onderzocht. Er werden 400 monsters verzameld en willekeurig verdeeld over de 20 verschillende combinaties van behandelingen. Er werden geen verschillen gevonden voor transportomstandigheden en de temperatuur waarbij extracten werden opgeslagen. Ook werden er geen interacties tussen de onderzochte variabelen gevonden. Monstername met glasvezelfilters, opslag van monsters in de vriezer en extractie in PFW + 0,05% Tween-20 leverde respectievelijk een 1,3x, 1,1x en 2,1x hogere gemeten endotoxinenconcentratie. Het gebruik van PFW + 0,05% Tween-20 had een additioneel effect.

Verder werden in **hoofdstuk 4.2** de effecten van vier gangbare media tijdens de extractie en analyse met elkaar vergeleken. Omdat dit onderzoek en eerdere onderzoeken een hogere endotoxinenopbrengst hebben aangetoond bij toevoeging van Tween-20 aan de media die worden gebruikt voor de extractie van filters en tijdens de LAL-assay, werden de effecten

van Tween tijdens extractie en analyse ook apart onderzocht. Voor de vergelijking van vier media (PFW, PFW-Tween-20, PFW-Tris en PFW-triethylamine-fosfaat (TAP) en extractie gedurende 10 of 60 minuten werden 250 stofmonsters uit vijf werkomgevingen gebruikt. Een deel van de extracten van monsters die waren geëxtraheerd in PFW of PFW-Tween-20 (n=40) werden geanalyseerd in parallelle LAL-assays met PFW of PFW-Tween-20 als het analyse-medium. In vergelijking met de referentie-procedure (gebruik van PFW met 60 minuten schudden tijdens de extractie) gaven een kortere extractietijd of gebruik van PFW-Tris vergelijkbare resultaten. Het gebruik van PFW-TAP resulteerde in het algemeen in lagere opbrengsten en een afwijkende calibratiecurve. De aanwezigheid van Tween in het extractiemedium resulteerde in significant hogere endotoxinenopbrengsten voor alle soorten stof, onafhankelijk van het effect van Tween in de assay. Gebruik van Tween in de LAL-assay verminderde echter ook sterk de reactiviteit van de lipopolysaccharide (LPS) standaard, waardoor de calibratiecurve naar hogere waarden werd verschoven. De inhibitie van LPS in de feitelijke stofmonsters was minder uitgesproken en varieerde tussen de soorten stof, wat resulteerde in verhoogde berekende concentraties. Dit assay-effect kon worden voorkomen door de extracten minimaal 50x te verdunnen voor te analyseren in de LAL-assay. Er kan worden geconcludeerd dat van de geteste media alleen het gebruik van Tween de efficiëntie van de extractie van endotoxinen op een consistente manier verhoogd.
Op basis van de resultaten van deze twee studies wordt het gebruik van glasvezelfilters in combinatie met transport met dehydratie, bevroren opslag van monsters, extractie in PFW + 0,05% Tween-20 met schudden, bevroren opslag van extracten, en verdunnen en LAL analyse in PFW aanbevolen. Deze studies voorzien in gegevens waarmee een protocol voor het standaardiseren van de beoordeling van blootstelling aan endotoxinen kan worden gespecificeerd.

In **hoofdstuk 5** worden de belangrijkste resultaten samengevat. Ook worden de consequenties van het huidige (nieuwe) grenswaardenstelsel voor de beheersing van werkgerelateerde blootstelling aan endotoxinen besproken. Dit stelsel is verschoven van alleen wettelijke grenswaarden naar een combinatie van publieke (wettelijke) en private grenswaarden. Op dit moment is het nog onbekend hoe dit nieuwe stelsel vorm zal krijgen en hoe het functioneren ervan in de gaten zal worden gehouden. Het is ook onduidelijk of werkgevers actief aan de gang zullen gaan met het vaststellen van private grenswaarden. In de hele discussie rond werkomstandigheden kunnen werkgevers ook kiezen voor ‘goede praktijken’ om gezondheidseffecten bij werknemers te voorkomen, waarbij beheersmaatregelen zeer belangrijk zijn voor het controleren/beheersen van blootstelling aan gevaarlijke stoffen. Daarvoor moet de effectiviteit van de voorgestelde beheersmaatregelen voor de reductie van blootstelling aan stof en endotoxinen worden onderzocht. Op dit moment is het nog onbekend tot welke categorie van het grenswaardenstelsel endotoxinen zal gaan behoren. In het geval van een private grenswaarde zouden in theorie vele verschillende grenswaarden voor endotoxinen naast elkaar kunnen bestaan. Echter, zowel de regering als de industrie kunnen de verwachte gezondheidskundige grenswaarde van de Gezondheidsraad gebruiken, welke hoger zou kunnen zijn dan de eerdere gezondheidskundige grenswaarde van 50 EU/m^3. De industrie moet zich hoe dan ook inspannen om blootstelling aan endotoxinen te beheersen om zo zijn werknemers te beschermen. Deze inspanning kan worden vertaald in een actieplan en een onderzoeksagenda, bestaande uit:

1. Verhoging van het bewustzijn van de mogelijke gezondheidsrisico’s die zijn verbonden met blootstelling aan endotoxinen, wat een belangrijke eerste stap is naar vermindering van de blootstelling;

2. Sommige onderwerpen die te maken hebben met de relatie tussen blootstelling en gezondheidseffecten vereisen extra onderzoek. Het gaat hierbij om specifieke onderwerpen, zoals variatie in reactie op blootstelling aan endotoxinen tussen mensen en tolerantie, en het onderzoeken van effecten veroorzaakt door chronische blootstelling;
3. Ontwikkeling van verbeterde protocollen voor de analyse van endotoxinen op basis van onze huidige inzichten;
4. Ontwikkeling van op maat gemaakte strategieën voor de beoordeling van blootstelling, waarbij rekening wordt gehouden met de hoge variabiliteit in blootstelling;
5. Onderzoek naar determinanten van blootstelling en beheersmaatregelen. Determinanten van blootstelling, zowel tussen werknemers als over de tijd, kunnen dienen als startpunt voor beheersmaatregelen. Verder zou ook de effectiviteit van beheersmaatregelen moeten worden onderzocht.

Er kan worden geconcludeerd dat endotoxinen alomtegenwoordig zijn in de (werk)omgeving, en verschillende groepen werknemers worden blootgesteld aan hoge concentraties endotoxinen. Er is sprake van een hoge mate van variabiliteit in blootstelling, wat moet worden geaccepteerd en waar op de juiste manier mee moet worden omgegaan. In plaats van een politieke discussie die resulteert in stagnatie van de beheersing van blootstelling aan endotoxinen zou blootstelling aan endotoxinen moeten worden gezien als een potentieel risico voor de werkende bevolking en zou ook als zodanig moeten worden behandeld door deze blootstelling actief te monitoren en te beheersen en zo een gezonde werkomgeving te garanderen.

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Dankwoord

Zo, en dan is het nu tijd voor het meest gelezen hoofdstuk van een proefschrift! En niet voor niets, aangezien veel mensen direct dan wel indirect betrokken zijn bij de totstandkoming ervan. Het is prettig dat ik nu iedereen, en een paar mensen in het bijzonder, kan bedanken.
Allereerst natuurlijk mijn promotoren en copromotor. Dick, jouw optimisme, enthousiasme en overtuigingskracht hebben er toe geleid dat ik me überhaupt aan het schrijven van dit proefschrift heb gewaagd, en ik niet bij de pakken neer ging zitten. Onze aanpak komt niet altijd overeen, maar ik heb veel van je geleerd. Peter, you joined the 'team' in a later phase of this thesis, but your contribution is highly appreciated. I enjoyed working with you while your were in Utrecht, and having conversations by email as well. Thanks! Inge, eerst als mijn stagebegeleider en later als mijn dagelijks begeleider was jij van groot belang. Altijd even binnen kunnen lopen met een vraag of een praatje, we zaten aardig op één lijn. Tijdens de afronding van mijn scriptie werd Tara geboren, en tijdens de afronding van mijn proefschrift Kalle, ik zie een verband!

Graag wil ik ook alle instanties en bedrijven bedanken voor hun medewerking en (financiële) bijdrage aan de onderzoeken die staan beschreven in dit proefschrift. Mijn dank gaat met name uit naar alle werknemers die met pompjes en filters voor me hebben rondgelopen, vragenlijsten hebben ingevuld en me van allerlei informatie hebben voorzien, en maakten dat het veldwerk zeker geen straf was. Verder wil ik Isabella Oosting, Lidwien Smit, Jack Spithoven, Sigfried de Wind, Maaike Visser, Griet Terpstra, Karel van der Heijden, Marc Lurvink, Olaf Welting en Anouk Besselink bedanken voor hun inzet tijdens het veldwerk dan wel in het lab. Ook wil ik de co-auteurs van de verschillende hoofdstukken bedanken voor hun bijdrage: Gert Doekes, Erik Tielemans, Jody Schinkel, Liesbeth Preller, Evelyn Tjoe Nij, Huib Arts, Lennart Larsson en Wijnand Eduard. Wijnand, de paar dagen die ik bij STAMI in Oslo heb doorgebracht waren erg leerzaam en gezellig.

Zonder de broodnodige afleiding (weekendjes weg, naar de film, naar theater, de Parade, Lowlands, Oerol, naar de kroeg, dansen, samen (uit) eten, spelletjes spelen, kletsen, etc.), interesse in mijn promotie-doen-en-laten, een luisterend oor, en af een toe een schouder was dit hele traject een stuk minder leuk geweest, en daarom wil ik graag mijn familie en vrienden bedanken. Fijn dat jullie er zijn!

Natuurlijk wil ik ook mijn (oud-)collega's op IRAS bedanken voor de vele leuke momenten, gezellig koffiepauzes, borrels, bbq's en feestjes, de sfeer was altijd goed. En in het bijzonder de groep aio's/junioronderzoekers waarmee ik het hele traject min of meer gelijk heb afgelegd. Ondanks de heel verschillende onderwerpen heeft een brainstorm hier en wat SAS-input daar veel geholpen en was het bovenal erg gezellig. Dus Jeroen de H., Wouter, Frank, Lidwien, Saskia, Jeroen van M., Bas, José, Ulrike en Lutzen, bedankt! Sofie, gastvrouw pur sang en verrijker van mijn Vlaamse woordenschat, je bent de afgelopen jaren een dierbare vriendin geworden en ik kom snel Antwerpen onveilig maken om bij te kletsen. En niet te vergeten mijn kamergenoten Anjoeka, Rob en Tim: radio aan, dropjes, rondvliegende elastiekjes, kletsen maar ook hard werken, eetdates, terrasjes, lunchwandelen, en ga zo maar door… Helemaal goed.
Sinds november ben ik opgenomen in het team exposure assessment and management van TNO, waar ik me zeker op mijn plaats voel. Bedankt voor het aanhoren van mijn gezucht en gesteun de afgelopen tijd, en voor de prettige werkomgeving waarin ook ruimte was voor het afronden van mijn proefschrift.

Tim en Marjolijn, heel fijn dat jullie mijn paranimfen willen zijn! Tim, eerst bij het IRAS en nu bij TNO ben jij mijn kamergenoot. Je fungeert zo'n beetje als mijn geweten en klets je me overal doorheen als het moet. Gevraagd of ongevraagd ventileer je jouw mening, die soms pijnlijk raak is, maar altijd oprecht. Je bent een fijne collega en ondertussen ook een goede vriend. Marjolijn, ik heb je leren kennen tijdens het invoeren van de composteer-vragenlijsten tijdens mijn afstuderen (lang leve Social Talk voor de broodnodige afleiding), en dat is uitgegroeid tot een fijne vriendschap. Je bent zelfs mee geweest op veldwerk, hebt vragenlijsten ingevoerd, bent altijd geïnteresseerd in wat ik aan het doen ben, en altijd in voor een filmpje, spelletje etc. En nu dus mijn paranimf :-)

Mijn familie, mama, Marlies en Joep, Femke en Marc, bedankt dat jullie er altijd voor me zijn. Ik heb jullie niet altijd even veel betrokken bij mijn onderzoek, maar jullie waren wel altijd benieuwd naar de voortgang en hebben zeker geholpen met de laatste loodjes. We hebben al heel wat moeilijke tijden doorstaan. Papa is er niet meer bij, maar hij was vast apetrots geweest. Marlies, tweeling zijn is toch wel heel erg bijzonder, we hebben vaak aan een half woord genoeg. Bij deze benoem ik je (officieus) tot mijn derde paranimf en hoop ik dat je de commissie een beetje van de wijs kunt brengen vanuit de zaal :-)
Nout, misschien is het afronden van een proefschrift en het opbouwen van een nieuwe relatie op het eerste gezicht niet de meest optimale combinatie, maar in ons geval is dat prima samen gegaan :-) Bedankt voor het zo goed bent omgegaan met mijn proefschrift-nukken en voor het zorgen voor de nodige relativering en afleiding. Klaar voor de rest? :-)

Curriculum Vitae

Suzanne Spaan was born in Hoorn (the Netherlands) on February 23, 1979. In 1997 she graduated secondary school at the Atlas College locatie Openbare Scholengemeenschap West-Friesland in Hoorn, and started the study Health Sciences at Maastricht University (the Netherlands). After her internship at the Institute for Risk Assessment Sciences (IRAS) of Utrecht University (the Netherlands), in which she conducted a study investigating exposure to organic dust and microbial agents and health effects in the composting industry, she received her MSc degree in health sciences in 2001, with a specialization in occupational hygiene. From November 2001 she started working for IRAS on several projects, described in this thesis. Furthermore, she participated in a study investigating exposure to trichloramine and respiratory symptoms in indoor swimming pool workers and other exposure studies. In 2006 she also worked on several projects at TNO Quality of Life in Zeist (the Netherlands). Since November 2007 she is working as a researcher/occupational hygienist at TNO Quality of Life.

Suzanne Spaan werd geboren op 23 februari 1979 in Hoorn. In 1997 behaalde zij op het Atlas College locatie Openbare Scholengemeenschap West-Friesland te Hoorn het VWO diploma, en begon daarna met de studie Gezondheidswetenschappen aan de Universiteit van Maastricht. Na een afstudeerstage bij the Institute for Risk Assessment Sciences (IRAS) van de Universiteit van Utrecht, waarin zij onderzoek deed naar blootstelling aan organisch stof en microbiële agentia en gezondheidseffecten in de composteerindustrie, rondde zij in 2001 de studie Gezondheidswetenschappen af met een specialisatie in arbeidshygiëne. Daarna kwam zij in november 2001 in dienst van het IRAS en werkte aan verschillende onderzoeken die zijn beschreven in dit proefschrift. Daarnaast nam zij deel aan een onderzoek waarin blootstelling aan chloramines en luchtwegklachten bij werknemers van binnenzwembaden werd onderzocht en andere blootstellingstudies. Verder werkte zij in 2006 aan verschillende projecten bij TNO Kwaliteit van Leven in Zeist. Sinds november 2007 is ze werkzaam als onderzoeker/arbeidshygiënist bij TNO Kwaliteit van Leven.