Respiratory health

effects of indoor

microbial exposure

A contribution to the

development of exposure

assessment methods

Jeroen Douwes

Stellingen

1. Procedures voor monstername, extractie en analyse van endotoxinen moeten internationaal gestandaardiseerd worden om een valide vergelijking van resultaten van verschillende laboratoria mogelijk te maken.
    Dit proefschrift

2. Extracellulaire polysacchariden van *Penicillium* en *Aspergillus* in huisstof zijn goede markers voor het optreden van schimmelgroei in woningen.
    Dit proefschrift

3. ß(1→3)-glucanen in huisstof zijn geassocieerd met negatieve gezondheids-effecten op de luchtwegen.
    Dit proefschrift

4. Methoden ter bepaling van concentraties levende èn dode micro-organis-men of hun componenten geven een betere schatting van de microbiële blootstelling dan het tellen van alleen levensvatbare micro-organismen.
    Eduard W. and Heederik D., Am Ind Hyg Assoc J. 1998;59:113-127

5. Allergische ontstekingsreacties zijn, in tegenstelling tot wat meestal wordt verondersteld, niet de enige en waarschijnlijk ook niet de belangrijkste mechanismen in de ontwikkeling van schimmel-geïnduceerde luchtweg-symptomen.

6. Of composteren van GFT-afval beter is voor 'het milieu' hangt sterk af van hoe 'het milieu' gedefinieerd wordt.

7. Endotoxins are read by our tissues as the very worst of bad news.
    Thomas I., Notes of a biology watcher. The Viking Press, NY, 1974:78

8. De recent door de gezondheidsraad geadviseerde grenswaarde van 50 EU/m^3 voor endotoxinen is zeker niet te streng getuige de studie van Zock *et al* waarin acute longfunctiedalingen gemeten werden bij een beroeps-populatie die was blootgesteld aan gemiddelde endotoxinenconcentraties tussen de 53 en 60 EU/m^3
    Gezondheidsraadrapport 'health-based recommended exposure limit for endotoxins 1998
    Zock J.P., et al., Am J Ind Med. 1998;33:384-391

9. Indien een woning overmatige schimmelgroei vertoont dient bestaande groei te worden verwijderd en nieuwe groei te worden voorkomen, zelfs als de woning hiervoor grondig gerenoveerd of zelfs afgebroken moet worden.
    Bijbel, Leviticus 14, vers 34-45

10. Arbodiensten in Nederland zijn onvoldoende bekend met de gezondheids-problematiek die geassocieerd is met de blootstelling aan biologische agentia op de werkplek.

11. Het rijden van een klassieke auto met een hoog benzineverbruik is nog altijd minder schadelijk voor het milieu dan het rijden van een nieuwe zuiniger auto die na tien tot vijftien jaar op de schroothoop beland.

12. Wijsheid is niet een kritische stelling bedenken, maar wijsheid is hier ook naar te handelen.

13. Je kunt in dit land zeggen wat je wilt, het is alleen de vraag of er iemand luistert.
    Jules Deelder

14. Humor gaat van 'haha', wetenschap van 'aha' en kunst van 'ah'
    Arthur Koestler

Stellingen behorende bij het proefschrift:
Respiratory health effects of indoor microbial exposure: A contribution to the development of exposure assessment methods.

Jeroen Douwes, Wageningen, 28 oktober 1998.

Respiratory Health Effects of Indoor Microbial Exposure

A Contribution to the Development of Exposure Assessment Methods

Jeroen Douwes

Jeroen Douwes

Respiratory Health Effects of Indoor Microbial Exposure

A Contribution to the Development of Exposure

Assessment Methods

Als één woord niet volstaat,

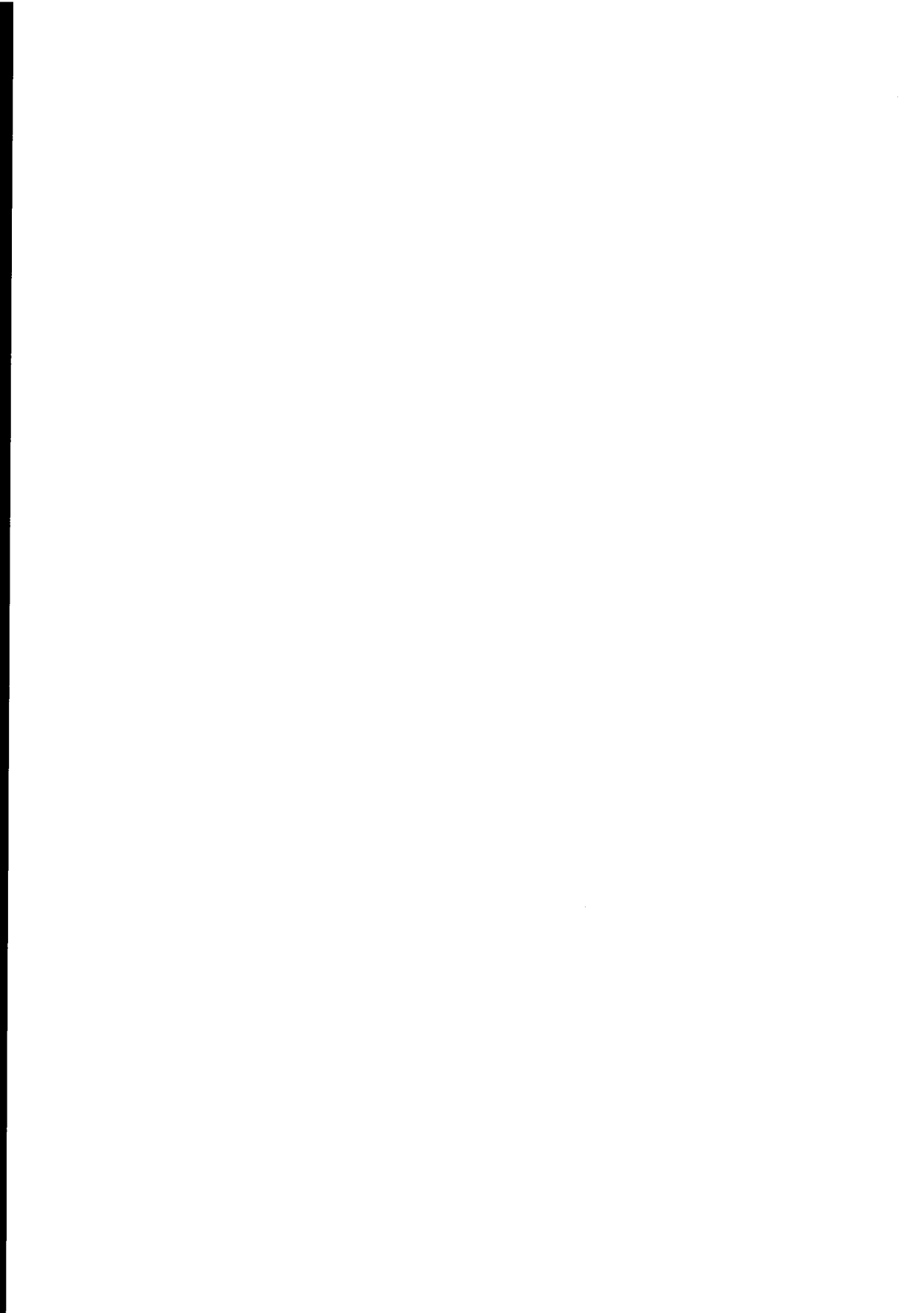zijn duizend woorden een verspilling.

Een chinese wijsheid
(Dit proefschrift bevat 48.682 woorden)

'Je zou er gek van worden', zei de

ééndagsvlinder en rustte even;

en toen hij dat zichzelf had horen zeggen

vloog hij weer stukken lichter verder,

als had hij het eeuwige leven

Ergens in New Mexico, 1977
Sjirk Gerritsma, schrijvend kunstenaar
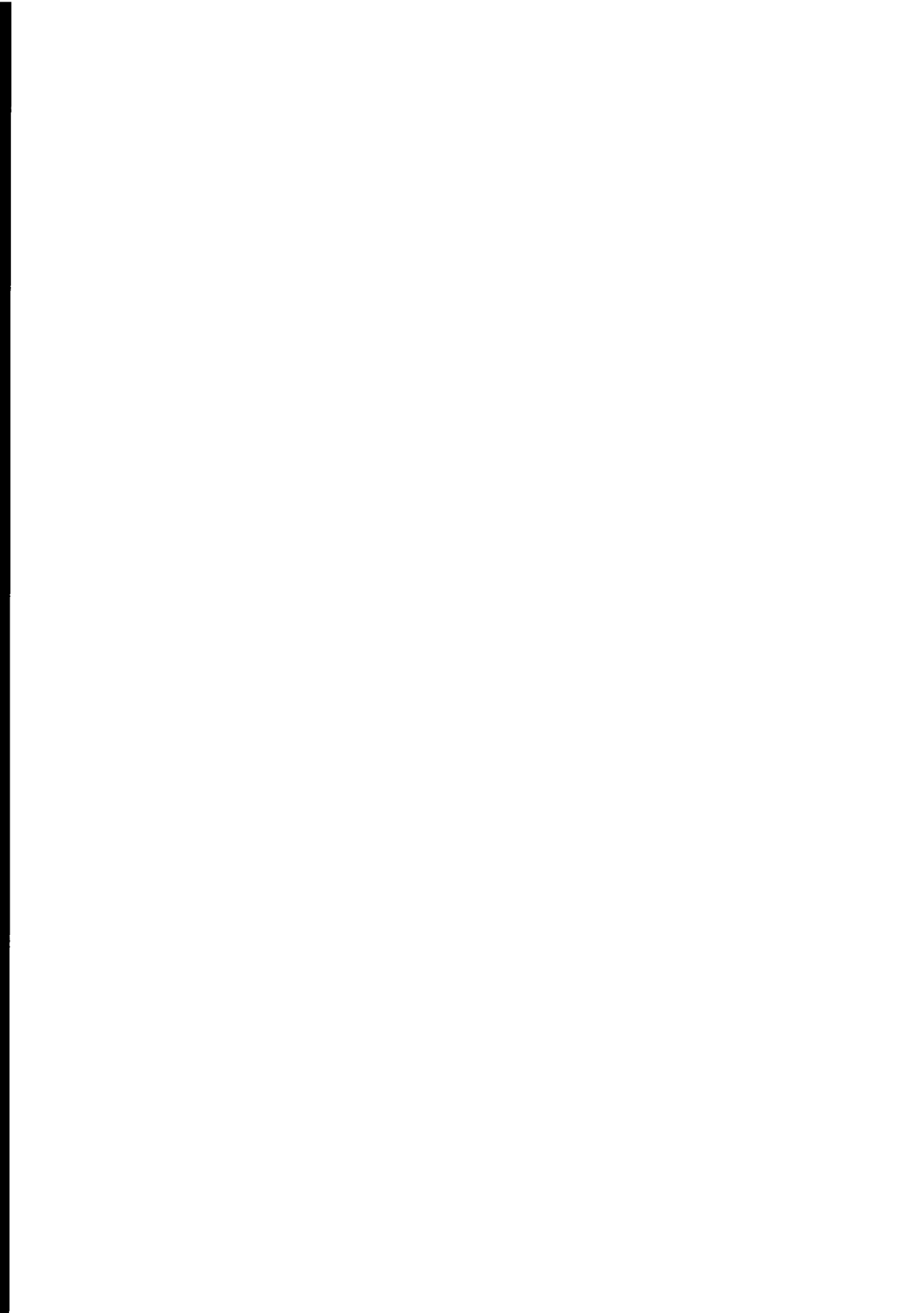

Voor mijn ouders

Abstract

The studies described in this thesis validated an existing method to measure bacterial endotoxin and explored new methods for measuring mold components in the environment, particularly ß(1→3)-glucans and extracellular polysaccharides (EPS). These assays were used to study levels of microbial components in the home and occupational environment, and to investigate their relationships with respiratory health in children or workers, respectively.
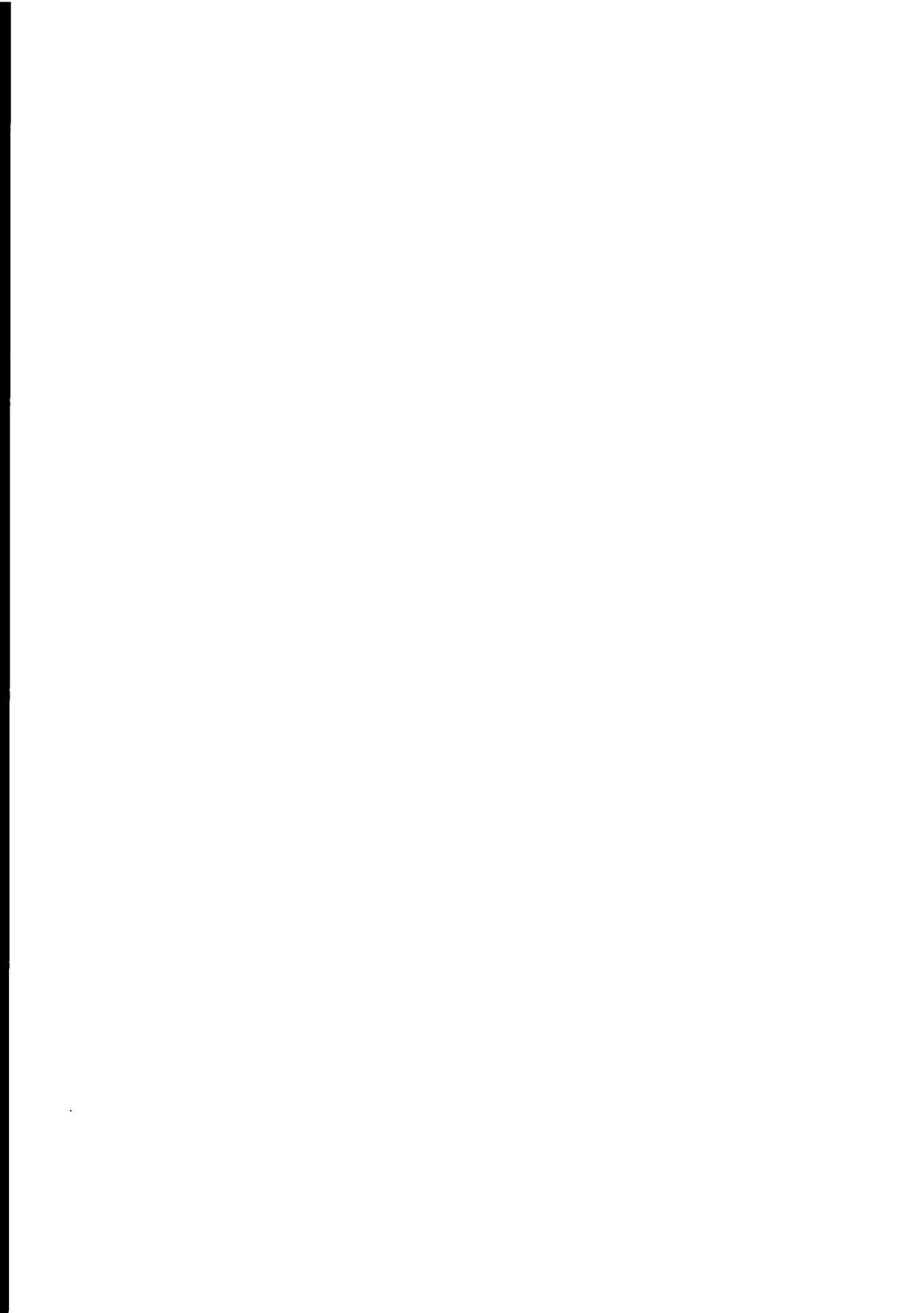
It was shown that results of endotoxin measurements may differ considerably between laboratories when different sampling, extraction, and storage procedures are employed. Highest yields were found when samples were extracted with 0.05% Tween-20 in water. Repeated freezing and thawing resulted in a considerable endotoxin loss. ß(1→3)-glucan, endotoxin and EPS from *Aspergillus* and *Penicillium* spp. (EPS-*Asp/Pen*) were readily detectable in house dust samples. Endotoxin and ß(1→3)-glucan levels were, at least for living room floors, significantly associated with certain home characteristics, particularly heating system and/or age of the home. Both ß(1→3)-glucan and EPS-*Asp/Pen* were significantly but only weakly correlated with culturable fungi in house dust. Living room floor concentrations of EPS-*Asp/Pen* were positively associated with occupant-reported home dampness and respiratory symptoms in children, while no such associations were found for bedroom and mattress concentrations. In another study among children it was shown that ß(1→3)-glucan in house dust from living room floors was strongly associated with peak flow (PEF) variability, particularly in atopic and/or asthmatic children. No associations were found for ß(1→3)-glucan levels on bedroom floors and mattresses. Unadjusted regression models showed that endotoxin levels were also significantly associated with PEF variability, while after adjusting for other known risk factors no significant association was found. A study among compost workers showed that microbial exposure induces acute and possibly chronic inflammation in the upper airways with a mechanism that suggests non-immune or possibly type III but not type I allergic inflammation.

It is concluded that the studies reported in this thesis contribute to new methods for microbial exposure assessment that offer advantages for use in epidemiological studies compared to traditionally employed culture-based methods.

Contents

Chapter 1

General introduction

1.1 background

Microorganisms are ubiquitous in the environment and may be found in elevated concentrations in the air in damp and moldy homes, as well as in industrial environments where microbial contaminated products are handled or processed. People living or working in these environments may become exposed to these microorganisms through inhalation. Indoor exposure to airborne microorganisms and microbial agents, both in the residential and occupational environment, is widely recognized as a plausible cause of acute and chronic non-infectious respiratory diseases such as asthma, rhinitis, bronchitis, organic dust toxic syndrome, and hypersensitivity pneumonitis. It is, however, not clear which pathological mechanisms may be primarily involved. Although various microorganisms, particularly fungi, are known as a source of IgE- and IgG-inducing allergens, non-immune mediated inflammatory reactions to inhaled pro-inflammatory agents or specific microbial toxins may in many cases be more important than IgE or IgG mediated allergy.

Probably the first reference suggesting adverse health effects related to microbial growth in the indoor environment is found in the 3rd bible book, Leviticus, in which home occupants were warned against what was probably mold growth in houses, described as 'green- or red-like spots on, and in the wall that may spread'.[1,2] First health problems related to microbial exposure were already reported in the 18th and 19th century.[3-6] An eighteenth century physician from Iceland described 'heysott', a respiratory disease of those who worked with badly harvested and moldy hay in winter,[7] which was in 1932 given the name 'farmers lung'.[8] Despite this early recognition, the role of non-infectious microbial agents in the development of respiratory symptoms and diseases is currently still poorly understood. More recent studies have suggested a relationship between allergic and non-allergic respiratory symptoms and fungal exposure in both the residential[9-19] and occupational[20-25] indoor environment. However, in only a limited number of these studies quantitative exposure data were collected. Exposure to bacteria and bacterial cell wall components (i.e.

endotoxins) in the home and work environment have also been suggested to be causally related to respiratory health effects.[26-41]

It is not clear which microbial components primarily account for the presumed respiratory health effects. Endotoxins are potent pro-inflammatory agents from the cell wall of gram-negative bacteria and capable of inducing non-immune mediated respiratory diseases such as organic dust toxic syndrome and chronic bronchitis and have long been recognized as an important causal factor, particularly in occupational environments (reviewed in chapter 2). ß(1→3)-glucans, major cell wall components of molds, can elicit, like endotoxins, a wide range of inflammatory reactions in vitro, and have therefore been suggested to be of importance as well.[42] Results from a few small population studies also suggest a role for ß(1→3)-glucan exposure in mold induced respiratory health effects (reviewed in chapter 2), but the evidence for a causal relationship is at present still weak. Methods to measure ß(1→3)-glucans in population studies have only been developed very recently. Mycotoxins have frequently been suggested to contribute to the development of various health problems, including respiratory symptoms.[43-49] Microbial antigens/allergens, particularly mold enzymes and proteins from thermophilic actinomycetes, may cause respiratory diseases such as allergic asthma and hypersensitivity pneumonitis.[50-52] Other microbial components and/or products may also play a role, including mold volatile organic components (mVOCs), peptidoglycans from bacteria and various exotoxins from both bacteria and molds. However, relatively little is known about these components with respect to their actual potential to induce respiratory and other symptoms.

The relative lack of knowledge regarding the role of non-infectious microorganisms in the development and aggravation of respiratory symptoms is mainly due to the lack of valid quantitative exposure assessment methods. Traditionally used culture methods have proven to be of limited use for quantitative exposure assessment (reviewed in chapter 2). Measurement of microbial agents with known or presumed pathogenic effects (e.g. endotoxins and ß(1→3)-glucans) or stable microbial components that may serve as markers of exposure (e.g. fungal extracellular polysaccharides) seems a more promising approach, particularly in epidemiological studies. For bacterial endotoxin, consistent dose-response relations with symptoms and lung function have been reported. Methods to

measure environmental endotoxin have, however, not been well validated. Only very few methods, to measure fungal exposure in the environment are currently available. There is thus a clear need for improved specific and sensitive methods for quantitative assessment of exposure to non-infectious microorganisms.

1.2 Aim and structure of the thesis

The studies described in this thesis had two main objectives: 1) to validate and improve methods to assess indoor exposure to non-infectious microorganisms and their components, and 2) to study the role of this exposure in the development of respiratory symptoms in residentially and occupationally exposed populations.

In order to achieve these aims the following studies were performed:

1. The influence of various dust sampling and extraction methods on the quantitation of environmental bacterial endotoxin with the LAL test was studied using airborne dust samples taken in a potato processing plant. In addition, the influence of storage conditions on the endotoxin concentration was investigated by using commercially available LPS and endotoxin-containing house dust extracts. Results are described in **chapter 3**.

2. An inhibition enzyme immunoassay was developed for the quantitation of ß(1→3)-glucans in dust samples from occupational and residential environments. ß(1→3)-glucan-specific rabbit antibodies were produced. Specificity and sensitivity of the assay were investigated and the assay was applied to dust from i) compost facilities, ii) swine confinement buildings, and iii) homes. Results are described in **chapter 4**.

3. Endotoxin and ß(1→3)-glucans in house dust were studied in 25 German homes that were included in a pilot study on the role of indoor allergens and microbial exposure in the development of asthma in the occupants. The association between concentrations measured in living room floor, bed room floor and mattress dust and home characteristics was investigated, as well as the relation between concentrations of ß(1→3)-glucan and culturable molds in house dust. Results are described in **chapter 5**.

4. A sandwich enzyme immunoassay for measurement of extracellular polysaccharides (EPS) of *Aspergillus* an *Penicillium*, originally developed for the quantitation of molds in food products and plasma, was used to assess

fungal biomass in house dust of 60 homes. Samples were originally taken for measurement of mite allergen and culturable mold propagules. The relation with culturable molds was investigated and, in addition, relations between EPS concentrations and signs of mold and dampness in the home were studied. Moreover the relation between EPS-*Asp/Pen* and respiratory symptoms in children living in these homes was studied. Results are described in **chapter 6**.

5. The relation between endotoxin and ß(1→3)-glucan levels in house dust and peak flow variability was investigated in a group of 150 children. The study population was part of a larger study on outdoor air pollution. Results are described in **chapter 7**.

6. Upper airway inflammation was studied in 14 compost workers who were exposed to high levels of microorganisms and microbial agents by using nasal lavages (NAL). Repeated pre- and post-shift nasal lavages were performed during a three-week period. A group of 10 non-exposed controls underwent pre- and post-shift NAL once. In the nasal lavage total cells, cell differentials, and various cytokines and exudation markers were determined. The association between acute and (sub-)chronic inflammatory responses and personally monitored endotoxin, ß(1→3)-glucan and dust exposure was investigated. Results are described in **chapter 8**.

In **chapter 2** an overview is given of the literature on exposure assessment to non infectious microorganisms, and health effects of endotoxins and ß(1→3)-glucans.

In **chapter 9** the results presented in this thesis are discussed. The focus is on the new methods applied and their potential use in epidemiologic studies to further explore the relation between microbial exposure and respiratory health of populations in the general and occupational environment.

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Chapter 2

Assessment of microbial exposure

2.1 measurement of microorganisms and microbial agents

Airborne exposure to microorganisms in the environment can be studied by counting culturable propagules in air samples or in settled dust samples. Counting of culturable micro-organisms has some serious drawbacks including poor reproducibility, selection towards certain species due to chosen culture media, temperature etc., and the fact that dead microorganisms, cell debris and microbial components are not detected, while they too may have toxic and/or allergenic properties. In addition, methods for personal air sampling of culturable microorganisms are not available, and air sampling during a period of more than 15 minutes is not possible, whereas air concentrations usually vary largely in time. On the other hand, counting of culturable microorganisms is potentially a very sensitive technique and many different species can be identified. Culture-based techniques thus usually provide qualitative rather than quantitative data that can, however, be important in risk assessment, since not all fungal and bacterial species pose the same hazard. An extensive review on techniques for sampling and culturing microorganisms has recently been published.[1]

Alternatively, non-culture-based methods can be used to assess microbial exposure. These methods do not depend on the culturability of microorganisms and are generally believed to give better and more reproducible estimates of the actual microbial concentrations. Only few of these methods, however, are available for use in large scale epidemiological studies. The various techniques that have been described in the literature can be divided in (1) microscopy and flow cytometry for counting individual spores or cells, and (2) chemical/biochemical methods for the measurement of specific microbial components.

2.1.1 Microscopy and flow cytometry

Microorganisms collected on capillary pore membrane filters can be resuspended, stained with a fluorochrome, e.g. acridine orange, and counted with an epifluorescence microscope.[2] Possibilities to classify microorganisms taxanomically are limited because little structure can be observed in the microorganisms. Electron microscopy (EM) or Scanning EM can also be used and allow better determination of microorganisms.[3] The latter technique is, however, more laborious and requires expensive equipment. Simple light microscopy may be used to count microorganisms, but counting is laborious and based only on morphological recognition, which may result in severe measurement errors. Bacteria collected with impingers or filters can be counted by flow cytometry after staining with 4',6-diamino-2-phenylindole (DAPI) or by applying fluorescent in situ hybridization (FISH).[4] FISH involves the use of fluorochrome-labeled nucleic acid probes to target rRNA within morphologically intact cells, allowing taxonomic determination from kingdom to species.[4]

The main advantage of microscopy or flow cytometry is that both dead and living microorganisms are quantified, selection effects are limited, personal air sampling is possible, and sampling time can be varied over a large range. Disadvantages include laborious and complicated procedures, high costs per sample, unknown validity, no detection of possibly relevant toxic or allergenic components or cell debris, while possibilities for determination of microorganisms for most of these techniques are limited. A more extensive review on microscopy and flow cytometry methods for counting non-culturable microorganism has recently been published.[1]

2.1.2 Chemical/biochemical methods

In addition to counting culturable and non-culturable microbial propagules in air or settled dust, molecular constituents or metabolites of microorganisms can be measured as an estimate of microbial exposure. Toxic and/or allergenic components can be determined but also non-toxic molecules that may serve as markers of either large groups of microorganisms or of specific microbial genera or species. General markers for assessment of mold concentrations include ergosterol measured with gas chromatography - mass spectrometry (GC/MS),[5,6] chitin with colorimetric assays,[7] and ß(1→3)-glucan with a glucan specific *Limulus* amebocyte lysate (LAL)-assay.[8] Genus- or species-specific markers

include mold extracellular polysaccharides measured with specific enzyme immunoassays,[9,10] allowing partial identification of the mold genera present, and specific mold allergens measured with the same technique. However, only a limited number of IgE-binding allergens from several fungal spp. has been identified, and for only a few of these immunoassays have been described.[11] One reason may be that immunochemical properties of allergens produced by fungi are usually highly variable.[11-13] General markers for bacteria include muramic acid, measured with GC/MS, as a chemical marker for bacterial peptidoglycans and thus as a marker for bacterial biomass[14,15] and endotoxin, measured with an endotoxin-specific LAL test, as a marker of gram negative bacteria.

In addition to the methods described above DNA probes have been developed for the identification of specific species of bacteria and fungi with the use of polymerase chain reaction (PCR) techniques.[16,17] DNA techniques can, however, currently not be used to quantitatively assess microbial biomass in samples. Specific Mold VOCs (e.g. 3-methyl furan, 1-octene-3-ol, geosmin) can be determined by GC/MS but can also not be used for quantitative assessment of mold concentrations. The isolation and determination of lipids distinctive of fungal species ('lipid signature profiles') with GC/MS, as a new method to assess fungal exposure qualitatively as well as quantitatively is currently under development.[18] Chemical and biochemical assays to assess microbial biomass in the home or occupational environment have been reviewed more extensively by Thorne *et al*[19] and Eduard *et al*.[1]

With the exception of endotoxin measurements most of the non-culture-based techniques described above are in an experimental phase and have as yet not been routinely applied and/or are not commercially available. Some of these methods may have disadvantages such as being (1) too unspecific [chitin, ß(1→3)-glucan], (2) too laborious for use in large field studies [scanning electron microscopy], (3) too species-specific for use as a general marker [DNA probes], and (4) not suitable for quantitative assessment [DNA probes, mVOCs]. Important advantages include (1) the stability of most of the measured components, allowing longer sampling times for airborne measurements, and frozen storage of samples prior to analysis, (2) the use of standards in most of these methods, and (3) the possibility to test for reproducibility. In addition, several of these

agents such as endotoxins, allergens, and possibly ß(1→3)-glucans are known to have etiologic relevance to the health effects that are studied, and their measurement thus may result in a more valid assessment of exposure-effect relationships and accordingly in better defined control options.[20] Moreover, some of these techniques, in particular enzyme immunoassays, are relatively cheap, which make them very useful in large scale epidemiological studies.

2.2 Endotoxin

2.2.1 Physical and chemical properties

Endotoxins are part of the outer membrane of gram-negative bacteria and are composed of proteins, lipids and lipopolysaccharides (LPS). LPS of gram negative bacteria refer to a class of water-soluble lipid carbohydrate molecules, free of protein and other cell wall components, that are responsible for most of the biologic properties characteristic of bacterial endotoxins. The lipid moiety of LPS is termed 'lipid A' and is responsible for its toxic properties. The com-position of Lipid A, an amphipathic and zwitterionic phosphoglycolipid, is remarkably constant among various bacterial species. The hydrophilic polysac-charide moiety is composed of O-specific side chains and core sugars and varies considerably between bacterial species. [21-23]

2.2.2 Monitoring

Environmental monitoring of endotoxins is performed by sampling airborne or settled dust on filters followed by an aqueous extraction. Several types of filters are commonly used for endotoxin sampling; cellulose, polyvinylchloride, glass fibre, teflon and polycarbonate. For extraction no standard method exists. Most laboratories use pyrogen-free water or buffers like Tris, phosphate triethylamine (pH 7,5) with or without detergents such as Tween-20, Tween-80, triton-x-100 and saponin. The most common way of extraction is rocking or sonication of filters in extraction media, or a combination of both. A few studies have been published on optimization of filter choice, filter extraction methods, extraction buffers and choice of glassware,[24-28] but a generally accepted protocol is not yet available. Most laboratories store their extracts at a temperature of -20 °C in either glass or plastic container materials. Little attention has, however, been given to the influence of storage conditions on the stability of endotoxins in dust extracts.

2.2.3 Analytical methods

Electrophoresis techniques, gas chromatography - mass spectrometry (GCMS) and high performance liquid chromatography (HPLC) have been described for the measurement of LPS, and provide concentration estimates in absolute units.[26,-29,30] These methods require elaborate LPS extraction procedures. Few comparative studies have been performed using different analytical methods. Variable results were found comparing GCMS with a *Limulus* amebocyte lysate assay (LAL).[29,31]

The LAL test is at present the most widely used assay for endotoxin measurements. This functional assay, which is highly sensitive, is based on the activation of a clotting enzyme (via factor C) present in the lysate of hemolymph of *Limulus polyphemus* (horseshoe crab). The LAL-assay was adopted in 1980 by the American Food and Drug Administration as the standard assay for endotoxin measurement. The more recent chromogenic kinetic versions of the LAL-assay are very sensitive and have a broad measurement range (0.01 - 100 Endotoxin Units (EU)/ml $\approx$ 1 pg/ml - 10 ng/ml). The LAL-method does not represent an absolute LPS measure but measures the portion of endotoxins that are biologically active in the assay. Therefore endotoxin concentrations should be expressed in Endotoxin Units (EU) rather than on a weight basis. The variation in sensitivity of the LAL-assay for endotoxin from different bacterial species is expected to correspond to the variation in biological response in mammals.[26] This has, however, not been evaluated in experimental studies.

2.2.4 Occurrence

Since endotoxins are normal constituents of all gram negative bacteria, and gram negative bacteria are more or less ubiquitous in nature (e.g. gut microflora), endotoxins can be found in practically every environment.

2.2.5 Human exposure

General population:

The general population may be exposed to airborne endotoxins when living in the vicinity of industries that emit organic dust into the environment. Limited data are, however, available on this subject. Substantial amounts of bacterial endotoxins can be found in house dust. One Belgian study reported mean endotoxin levels in house dust of 2.59 μg/g ($\approx$ 25,900 EU/g).[32] Another study

reported comparable levels of endotoxin in dust from homes in Brasil (10,800 EU/g).[33] At present, information about airborne endotoxin levels in the domestic environment is lacking, and it is not yet possible to make reliable estimates of the dose that can potentially be inhaled by subjects living in homes with elevated levels of endotoxin in house dust.

Occupational population:
For occupational diseases only airborne endotoxins seem relevant. Endotoxins become airborne during manufacturing or handling of organic materials. Animal faeces and bacteria-contaminated plant materials contribute most to organic dust related endotoxin exposure. The role of endotoxin exposure in the development of work related adverse pulmonary effects has been studied intensively the last decades. These studies showed elevated endotoxin exposures in a large variety of occupational environments. Highest endotoxin exposures are found in agriculture and related industries such as pig, chicken, cow and horse farming, grain elevators, cotton industry, potato processing industry, poultry slaughter houses, flax processing industry, animal feed industry, sewage treatment and sewage composting plants, garbage handling facilities, organic waste composting facilities, wood chip composting and timber storing facilities, etc. Air humidifiers in buildings and recycled industrial process water may also be an important source of airborne endotoxin exposure. In addition industrial oils and emulsions may contain substantial amounts of endotoxins as a result of contamination with gram-negative bacteria. In table 1 endotoxin exposure levels are listed from a large number of studies performed the past few decades in various occupational environments. In all studies a LAL assay was used to measure the endotoxin levels.
The occupational population at risk for endotoxin exposure in the Netherlands is estimated to be several hundreds of thousands among which at least 100,000 farmers, their families and other farm workers, and workers in closely related industries.[34]

Table 1: Dust and endotoxin exposures in various occupational environments. Exposures are presented as ranges in mean levels (both arithmic and geometric means have been used) per department or job-title within one industry; or the exposure is given as the mean of all measurements in the industry. EUs were calculated from nanograms by multiplying with a factor 10.

type of industry	dust fraction	n[a]	dust conc (mg/m³)	n[a]	endotoxin conc (EU/m³)
grainelevator and animal feed industry					
DeLucca et al [35]	respirable**	69	<0.3	69	0 - 7.4
Smid et al [36] *	inhalable**	530	0.8 - 9.8	530	12 - 285
	inhalable***	79	0.8	79	19
water sewage treatment plant					
Melbostad et al [37]	?**	24	?	23	300
Westveer et al [38] *	inhalable**	52	<0.3	52	10
	inhalable***	41	<0.3	48	0.8 - 81
air humidifiers in buildings					
Kateman et al [39] *	inhalable**	15	0.5 - 0.6	6	0.18 - 0.64
pig farmers					
Clark et al [40]	total***	18	1.8 - 5.2	18	400 - 2,800
Attwood et al [41] *	total***	170	2.8 - 4.9	166	1,200 - 1,280
	$D_{50}=8.5\ \mu m$***	171	0.9 - 1.5	166	1,050 - 1,150
Donham et al [42]	total**	57	6.8	57	2,400
	respirable**	57	0.34	57	2,300
Preller et al [43] *	inhalable**	360	2.4	350	920
chicken farmers					
Clark et al [40]	total***	7	1.0 - 3.7	7	1,200 - 5,000
Thelin et al [44]	?**	25	5.8 - 28.1	25	1,300 - 10,900
Veld [45] *	$D_{50}\leq 8,5\ \mu m$**	23	0.8 - 4.9	19	1,450 - 8,710
	total***	24	2.5 - 13.1	22	2,250 - 13,400
Jones et al [46]	total***	9	2 - 10	7	240 - 590
	respirable***	9	0.08 - 0.5	7	38 - 98
poultry slaughter houses					
Lenhart et al [47]	inhalable***	17	20.2	17	2,500
	respirable***	19	1.75	19	130
Hagmar et al [48]	total**	24	3.1 - 7.7	24	400 - 7,800
cotton industry					
Rylander & Morey [49]	respirable***	-	-	36	200 - 3,700
Kennedy et al [50]	PM<15 μm***	130	0.59 - 1.17	62	20 - 5,300
potato processing industry					
Zock et al [51] *	inhalable**	211	0.4 - 21.1	195	110 - 980
	inhalable***	81	0.4 - 44.2	68	1 - 46,000
	respirable***	78	<0.1 - 1.6	47	1 - 1,050
Dutkiewicz et al [52]	?***	?	5 -100	?	$100 - 10^7$
garbage handling and composting facilities					
Amelsfoort et al [53] *	inhalable**	25	0.5 - 25.7	28	30 - 1,310
Sigsgaard et al [54]	total***	63	0.62 - 0.74	63	8 - 25
sugar beet processing industry					
Forster et al [55]	?***	?	1.4 - 3.5	?	25 - 320
biotechnology industry					
Palchak et al [56]	?	34	?	34	1,629
breweries					
Carvalheiro et al [57]	?***	?	?	?	600 - 9,270

[a] number of samples   * Dutch situation   ** Personal sampling   *** Area sampling

2.2.6 Effects

Thomas' comment that endotoxins are "read by our tissues as the very worst of bad news" and that in response to these molecules "we are likely to turn on every defense at our disposal,"[58] elaborate beautifully the toxic potential of these macromolecules.[59]

Endotoxins are capable of inducing a wide range of inflammatory reactions in vitro and in vivo in man. The harmful host responses to endotoxins are mediated by endogenous cytokines and metabolites (IL-1, IL-6, IL-8, TNF-*a*, reduced oxygen species, arachidonic acid, PAF etc) which are released by various cells, i.e. macrophages, vascular cells and epithelial cells and polymorphonuclear cells (reviewed by Ulmer 1998).[60] Acute and chronic respiratory effects are most likely induced through these inflammatory responses occurring in the lungs in which the alveolar macrophage plays a key role. Macrophages/monocytes carry specific LPS binding receptors (CD14), that play a crucial role in the activation of these cells and the subsequent inflammatory reactions.[60] Because B and T lymphocytes are known to proliferate in vitro after LPS stimulation it has been hypothesized that chronic inhalatory endotoxin exposure may increase non-specifically the immune response to antigens in man (adjuvant effect). A stimulatory effect on IgE production has been suggested by experimental studies in mice,[61] and LPS is widely used in *in vitro* studies as potent B cell mitogen, being particularly effective as a co-stimulatory signal, in addition to the cytokine IL-4, for IgE production.[62,63] It is, however, at present not confirmed in population studies that endotoxin exposure can stimulate atopic sensitization to allergens.

Clinical symptoms after intravenous administration of LPS in man comprise joint aches and fever, shivering, and other influenza-like symptoms. Symptoms usually disappear after one day. Inhalation experiments with LPS showed the following acute clinical effects in human volunteers: fever, shivering, joint aches, influenza-like symptoms, general malaise, dry cough, dyspnea, chest tightness and leucocytosis. Exposed subjects also showed dose-related acute lung function decrease (FVC, FEV_1 and flow-volume variables), acute decreased lung diffusion capacity and acute bronchial obstruction.[64,65,66] Acute lung function effects and respiratory symptoms found in experimental studies where

subjects were exposed to endotoxin-contaminated cotton dust were confirmed by several field studies, conducted in swine confinement workers,[42] animal feed workers,[67] fiberglass factory workers,[68,69] cotton mill workers,[70] and potato processing workers.[71] Three large epidemiological studies, one conducted in the cotton industry,[50] one in the animal feed industry,[72,73] and one in pig farming[74] reported dose-related chronic effects such as decreased FEV_1 and FVC, and respiratory symptoms. Two other, somewhat smaller studies, also showed endotoxin related chronic respiratory effects in swine confinement workers[75] and cotton mill workers.[76] Occupational epidemiologic studies as well as animal studies suggest that chronic endotoxin exposure may lead to chronic bronchitis. The epidemiology of endotoxin related health effects has recently been reviewed extensively by Douwes and Heederik. [77,78]

Some studies have also suggested that endotoxin in the home environment may play a role in the development of respiratory symptoms. Two cross-sectional studies by Michel *et al*[32,79] showed that in adult asthma patients, the severity of asthma was related to endotoxin levels measured in house dust, whereas in the same study no significant relation was found with mite allergen concentrations. Another study also found a significant association in asthmatic children between clinical symptom scores and endotoxin levels in house dust.[33] These studies suggested that endotoxin exposure in the home environment exacerbates symptoms in asthmatic individuals.

2.2.7 No observed adverse effect levels

In the literature estimated 'No Observed Adverse Effect Levels' (NOAEL) for inhalatory endotoxin exposure have been described ranging from approximately 5 to 170 ng/m^3 (50 to 1,700 EU/m^3).[42,50,68,69,71-73,78,80,81-85] Many of these NOAELS have been based on experimental endotoxin exposure studies. Estimated NOAELs for chronic and acute respiratory effects based on epidemiological studies in occupationally exposed populations were in a much narrower range (approximately 5 - 20 ng/m^3, $\approx$ 50 - 200 EU/m^3).[50,68,69,71-73] On the basis of these findings, a health based recommended occupational exposure limit of 50 EU/m^3 ($\approx$ 5 ng/m^3) has recently been proposed in the Netherlands by the Dutch Expert Committee on Occupational Standards of the Dutch Health Council.[78] At present, there are no data from residential indoor studies from which NOALS can be calculated from the home environment.

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2.3 ß(1→3)-glucans

2.3.1 Physical and chemical properties

ß(1→3)-glucans are glucose polymers with variable molecular weight and degree of branching that may appear in various conformations i.e. triple helix, single helix or random coil structures of which the triple helix appears to be the predominant form.[86] ß(1→3)-glucans originate from a large variety of sources, including most fungi, some bacteria, most higher plants and many lower plants.[87] They are water insoluble structural cell wall components of these organisms, but may also be found in extracellular secretions of microbial origin. A major part of mold ß(1→3)-glucans can be solubilized in alkaline solution or hot water.[87] In the fungal cell wall, ß(1→3)-glucans are linked to proteins, lipids and carbohydrates such as mannan and chitin and they contain ß(1→6)-glucan side-branches which may connect with adjacent ß(1→3)-glucan polymers.[88-90] Glucans may account for up to 60% of the dry weight of the cell wall of molds, of which the major part is ß(1→3)-glucan.[88] The ß(1→3)-glucan content of mold cell walls has been reported to be relatively independent of growth conditions.[91]

2.3.2 Monitoring and analytical methods

Environmental sampling of ß(1→3)-glucans can in principle be performed as described for endotoxins. A different extraction procedure, however, is needed to extract ß(1→3)-glucan from dust samples because of the water-insolubility of these components. Environmental samples can be extracted in alkaline solution (0.3 M NaOH), [92,93] or by hot water extraction (120 - 135°C),[94,95] and a glucan-reactive lysate from the *Limulus polyphemus* can be used to quantify ß(1→3)-glucans. This assay, based on the ability of ß(1→3)-glucans to activate the *Limulus* coagulation system,[8,96] has only recently become commercially available, and has some serious drawbacks including its reactivity with various other polysaccharides and the high cost of the reagents required for the analysis. The glucan specific LAL assay is, like the LAL assay for endotoxin measurements, very sensitive (detection limit: 1 pg/ml). Recently a sensitive (detection limit: 0.1 ng/ml) sandwich enzyme immunoassay has been described, that makes use of specific anti ß(1→3)-glucan antibodies as coating material and peroxidase labeled glucan-binding-protein from the lysate of the *Limulus polyphemus* as the detecting agent.[97] It is not clear whether the two assays give comparable results.

2.3.3 Occurrence and human exposure

Like endotoxin, ß(1→3)-glucans can be found in a large number of environments. Human exposure may occur in the outdoor environment by organic dust emitting industries and indoors as a result of living in damp and moldy homes. In the occupational environment elevated exposures of ß(1→3)-glucan can be expected in agricultural and related industries where elevated organic dust exposures are prevalent but as yet, only few studies have reported actual data. Rylander *et al*[94] measured mean airborne ß(1→3)-glucan concentrations of 0.06 - 0.6 ng/m^3 in 82 samples taken in two offices (n = 48), a day care centre (n = 8), and two school buildings (n = 26). With the exception of one, all were suspected 'sick buildings'. A subsequent study showed mean ß(1→3)-glucan levels of 41.9 ng/m^3 and less than 5 ng/m^3 in a mold infested and non-mold infested house, respectively.[98] Another study showed a mean ß(1→3)-glucan concentration in the air of 11.4 ng/m^3 (SD = 2.3; n = 24) in a day care center that had microbial growth problems.[93] After renovation of the day care center the mean exposure level had dropped to 1.2 ng/m^3 (SD = 0.9, n = 13). Airborne ß(1→3)-glucan concentrations in 75 Swedish rowhouses with previous problems of dampness and water leakage ranged from non detectable to 19 ng/m^3.[99] The concentrations found in these studies probably represent peak concentrations and no average concentrations since samples were taken from airborne dust that was generated by agitating settled dust in the indoor environment with a machine. The ß(1→3)-glucan measurements were performed with a glucan-specific LAL test.

Measurements in which the same LAL test was employed indicated that levels of airborne ß(1→3)-glucan in industry may be much higher: cotton industry, 200-300 µg/m^3; wood pulp handling, 150 µg/m^3; and poultry houses, 4-870 ng/m^3 (unpublished data, Rylander 1998). One study among waste collectors showed concentrations ranging from 2-36 ng/m^3 (n = 14).[100]

2.3.4 Effects

ß(1→3)-glucans can initiate a variety of biological responses in vertebrates such as stimulation of the reticulo-endothelial system,[101] activation of neutrophils,[102] macrophages[103-105] and complement[96] and possibly activation of eosinophils,[106] resulting in enhancement of host-mediated induced resistance to infections[87] and antitumor activity,[107] Furthermore, it was demonstrated that ß(1→3)-glucans can

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induce T lymphocyte activation and proliferation in experimental animals.[87,107] It is believed that macrophages play a key role in most ß(1→3)-glucan mediated biological responses. The presence of a ß(1→3)-glucan-specific receptor on a human macrophage cell line has been demonstrated and it was shown that the glucan receptor possessed higher affinity for branched than for non-branched glucans.[86,108] Another study also showed differences in biological properties between ß(1→3)-glucans of different sources, presumably because of differences in degree of branching and conformational structures (triple helix, single helix and random coil).[109] Thus far, studies on the inflammatory effects of glucans have mainly focused on fungal ß(1→3)(1→6)-glucans, and it is not clear whether plant ß(1→3)(1→4)-glucans have similar properties. These components are abundantly present in the insoluble and indigestible fraction of vegetable food and have been suggested to have benificial health effects such as prevention of colon cancer and infectious diseases, and decreased serum cholesterol levels.[110-,111] Underlying biological mechanisms are not yet completely resolved.

Recently it has been suggested that ß(1→3)-glucans play a role in bio-aerosol induced inflammatory responses and resulting respiratory symptoms.[94,95,112,113] At present, however, almost no studies have been published on ß(1→3)-glucan related lung pathophysiology, and biological mechanisms involved in presumed respiratory health effects therefore remain unclear. Two exposure studies in which guinea pigs were experimentally exposed to an aerosol of curdlan [ß(1→3)-glucan from bacteria] indicated that ß(1→3)-glucan does not cause neutrophilia in the airways 4 and 24 hours after a single exposure.[114,115] A combined aerosol exposure to ß(1→3)-glucan and endotoxin showed that the inflammatory response to the endotoxin exposure was reduced by curdlan (ß(1→3)-glucan from bacteria) in a dose-related fashion. Several other tested ß(1→3)-glucans from other organisms (schizophyllan, pullulan, particulate glucan, barley glucan and grifolan) also did not induce a change in the number of various inflammatory cells in the airways of exposed animals compared to non-exposed animals. Exact exposure levels or doses were not given in these studies. In another study guinea pigs were repeatedly exposed for 5 weeks to an aerosol of curdlan, endotoxin or a combination of both.[113] In this study it was found that both endotoxin and curdlan induced an increase in inflammatory cells in the airways of exposed animals compared to non-exposed control animals. The combined exposure resulted in an even larger increase in inflammatory cells.

It also resulted in more alveolar infiltrates and early granulomas (resembling hypersensitivity pneumonitis histology). Based on these results and changes in enzyme secretion from alveolar macrophages observed after combined exposures, the authors suggested an effect of curdlan on the inflammatory regulation capacity of airway macrophages. Thus, it may be, that in order to mediate symptomatology, glucans need to act in combination with other etiologic agents such as endotoxin, as was suggested recently.[86]

Almost no data from human exposure studies (experimental or environmental) are available. One small population study (n = 39) suggested a relationship between airborne ß(1→3)-glucan levels measured in various environments (a day care center, post office and two school buildings) and questionnaire assessed symptoms i.e. dry cough, throat and eye irritation, and itching skin.[94] The associations reported were not adjusted for other potential risk factors. A subsequent study showed a small increase in the severity of symptoms of nose and throat irritations, in 26 subjects experimentally exposed for 4 hrs to aerosolized ß(1→3)-glucan in a concentration of approximately 210 ng/m^3.[92] Symptoms were assessed by questionnaire directly after exposure. No effects on FEV_1 or airway responsiveness were found for the whole group, nor for subgroups that were defined based on atopic status (n = 12). In the same study it was shown that in 16 non-atopic and non-symptomatic individuals, exposed to particulate ß(1→3)-glucan (appr. 210 ng/m^3 for 4 hrs), a statistically significant but very small decrease of FEV_1 was found directly and 3 days after exposure, whereas no significant association was found with airway responsiveness.[92] Several other small studies have been performed by the same group in the home environment, a day care center and among house hold waste collectors, suggesting a relation with respiratory symptoms and airway inflammation in exposed individuals.[93,98,100,116] Another study in 129 individuals suggested a relation between indoor ß(1→3)-glucan levels and increased serum MPO levels, increased prevalence of atopy and decreased lung function (FEV_1).[99] At present, however, no data from large epidemiologic studies are available that can confirm the data from these relatively small studies.

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2.4 Mold extracellular polysaccharides

2.4.1 Physical and chemical properties

Mold EPS are heat-stable and water-soluble non-branched glycoproteins with variable molecular weight that are an essential part of the mycelial cell wall of practically all molds .[9,117-120] During growth of molds these polysaccharides are released in the environment.[120,121] The carbohydrate part of EPS contains polymeric structures of primarily glucose, mannose and galactose.[9,119,120] Mold EPS possess antigenic properties that are usually specific at the genus level, while EPS from *Aspergillus* and *Penicillium* spp. are cross reactive.[119] The antibody responses in rabbits immunized with EPS from *Aspergillus/Penicillium* are directed to the galactomannans in EPS, of which ß(1→5)-linked D-galactofuranosides residues are immunodominant.[122,123] The quantity of antigens produced by molds is fairly well related to the quantity of mycelium, and antigens are produced under almost all growth conditions.[124] This demonstrates the potential usefulness of EPS as a quantitative marker for mold biomass in the general and occupational environment.

2.4.2 Monitoring and analytical methods

Environmental sampling and subsequent extraction of EPS can be performed similarly as described for endotoxins. A standardized protocol, however, does not exist. A sandwich enzyme immunoassay (EIA) has been described by Notermans *et al*[9] and Kamphuis *et al*[10] with which EPS of *Aspergillus* and *Penicillium* spp. can be specifically measured in food samples as a means for large scale screening for mold contamination. The EPS-*Asp/Pen* rabbit polyclonal IgG antibodies used in the EIA were raised against an EPS preparation of *Penicillium digitatum*. A simple, rapid and sensitive latex-agglutination assay has also been developed using latex beads coated with identical anti EPS-*Asp/Pen* antibodies.[125,126] The anti-EPS-*Asp/Pen* antibodies, were highly specific as demonstrated by the lack of reactivity with extracts of 33 spp. from the genera *Alternaria, Mucor, Rhizopus, Botrytis, Geotrichum, Cladosporium, Fusarium* and *Tricothecium*, whereas all of the 12 tested *Aspergillus* spp. and 41 out of 44 tested *Penicillium* spp. showed a positive reaction.[124] Both methods are relatively sensitive with a detection limit of approximately 0.5-1.0 ng/ml for the EIA and 1-10 ng/ml for the agglutination test. Both *Penicillium* and *Aspergillus* are important airborne molds that appear in large concentrations in many environ-

ments with microbial problems, particularly in damp homes. Measurement of EPS-*Asp/Pen* may therefore be an important tool for exposure assessment in epidemiologic studies on the relationship between mold exposure and respiratory health effects. Other sandwich EIAs have been developed for the specific detection of mold spp. from various other mold genera including *Alternaria, Mucor* and *Cladosporium*. Application of these assays to environmental samples would thus allow partial identification of the fungal genera present.

2.4.3 Occurrence

Like ß(1→3)-glucans and endotoxin, elevated mold EPS levels may be found in the outdoor environment due to industrial emissions of mold containing dusts and in the residential environment, particularly in damp and moldy homes. In the occupational environment elevated EPS-levels may be measured in agricultural and related industries where elevated mold exposures are known to be prevalent (farming, compost facilities, saw mills, paper mills, timber storing etc). At present, however, no EPS levels in indoor or occupational environments have been reported.

2.4.4 Effects

Although EPS can induce an IgG-response both in man and experimental animals, there is at present no evidence for a pathogenic role of EPS in mold induced allergic or inflammatory reactions. EPS may thus as yet be primarily regarded as a potentially good marker for fungal biomass in environmental samples.

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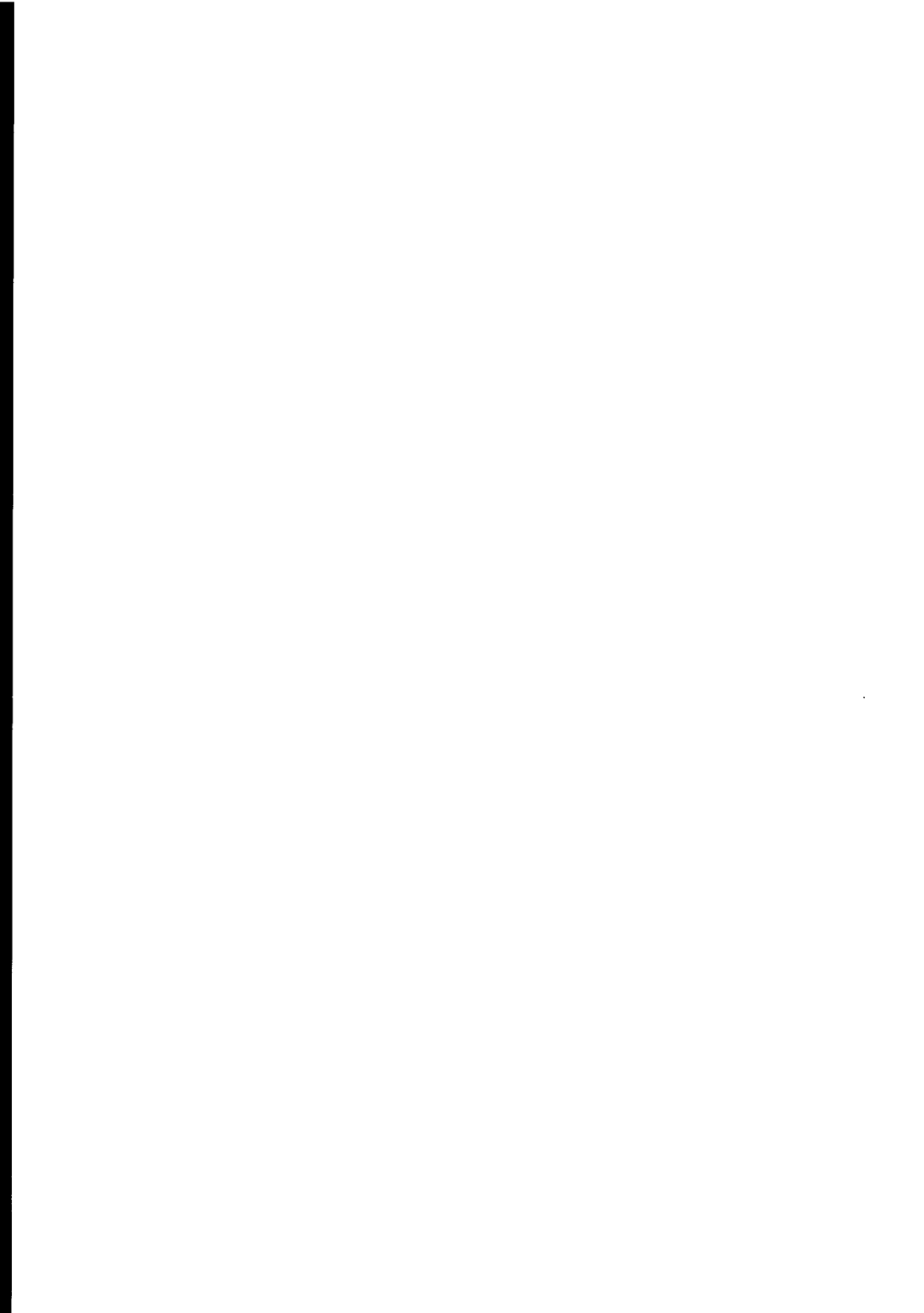
Chapter 3

The influence of various dust sampling and extraction methods on the measurement of airborne endotoxin

Abstract

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

Introduction

Because of their ubiquitous nature, bacterial endotoxins are commonly found in various environments. Several epidemiological and experimental studies have focused on the health effects of airborne endotoxin exposure in both occupational and non-occupational environments. Endotoxins are believed to play an important role in the development of organic dust related diseases in exposed workers. Exposure to airborne endotoxins can cause acute fever and lung function alterations accompanied by respiratory complaints such as chest tightness, cough, shortness of breath and wheezing.[1-4] Chronic endotoxin exposure may lead to chronically decreased pulmonary function, byssinosis and chronic bronchitis in workers exposed to cotton and grain dust.[5-7]

Discrepancies in the dose-response relations between endotoxin exposure and health effects observed by different groups may partly be explained by the use of different techniques to measure environmental endotoxin and different sampling media, extraction methods, and storage conditions. In most studies the *Limulus* amebocyte Lysate (LAL) assay is commonly used to quantify environmental endotoxin. Few comparative studies using different analytical methods and different LAL assays for endotoxin analysis have been performed. Variable results were found in comparisons of a gas chromatography-mass spectrometry method with a LAL-assay.[8,9] A recent study comparing two LAL methods showed variable results.[10] While in general there appeared to be agreement between the two LAL-assays, discrepancies were found in some specific environments.

In regard to sampling media, extraction media and methods, and storage conditions for endotoxin, there are no generally accepted procedures, and thus different practices continue to exist. Few studies on the optimization of filter choice, filter extraction methods, extraction buffers, and choice of glassware have been published.[11-14] Generalization of these studies is difficult, since the interaction of endotoxin with the above-mentioned variables often depends on the aerosol type. Thus, analytical procedures as well as sampling, extraction and storage methods need further validation in order to establish a standard protocol for measuring airborne endotoxin, which is necessary in order to obtain results that are comparable between studies.

This study was conducted to determine the influence of various sampling media and different extraction and storage conditions on the endotoxin analysis of

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

Materials and methods

Parallel samples

(i) Air sampling. Sampling of inhalable dust was carried out in a fibre dehydration department of a potato processing plant. A previous study showed that the personal endotoxin exposure in this department was relatively high (24.5 - 489 ng/m^3).[15]

Series of 16 parallel samples were collected by using four 25 mm filter types commonly used in endotoxin exposure studies:[7,10,16,17] glass fibre (Whatman GF/A), teflon (Millipore, pore size 1 μm), polycarbonate, (Millipore, pore size 1.2 μm) and cellulose mixed ester (Millipore, pore size 1.2 μm). Sample collection was performed using PAS-6 filter holders at a flow rate of approximately 2 l/min.[18] The filter holders were mounted in an apparatus (fig. 1), described by Eduard *et al.*,[19] that was designed to provide parallel samples. The parallel-sampling apparatus was constructed by the technical service of the Agricultural University Wageningen. A vacuum pump was used to provide a sufficient airflow through the filters. The airflow through each filter was controlled by critical orifices and was measured before and after the sampling period. The sampling time was 8 hours.

The experiment was set up by using an incomplete factorial design[20] in which 32 possible combinations of sampling and extraction variables were investigated. Table 1 shows the sample and extraction variables studied. Four filters per type were randomly allocated to the 16 available places in the parallel-sampling device. The samples were then equally distributed per filter type over a preselected combination of extraction procedures in such a way that each different combination occurred only once. Only 16 combinations could be covered per measurement series, while 32 combinations were investigated. Thus 12 series were run in order to get 6 repeated measurements per sampling-
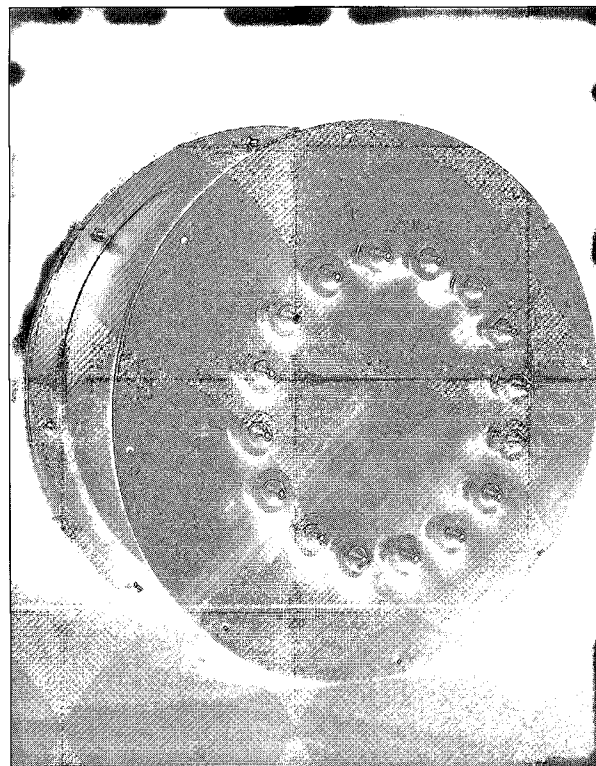
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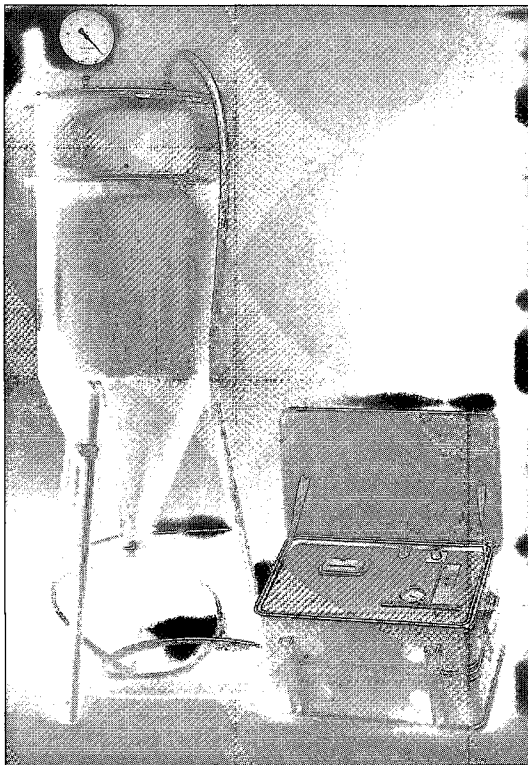
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Figure 1.   *1A: Apparatus and pump for collection of parallel samples (height, 170 cm; diameter, 50 cm; inlet 37 cm above floor level; inlet diameter, 10 cm) 1B : Detached upper section of parallel-sampling apparatus with 16 mounted PAS-6 filter holders (height, 22 cm; diameter, 50 cm).*

extraction combination, resulting in a total of 192 samples. Each series was sampled under similar conditions on 12 sampling days. After the sampling, the filters were stored at - 20°C until analysis.

Table 1. Sample and extraction variables studied[a]

Filter type..................................	glass fibre, teflon, polycarbonate, cellulose mixed ester
Extraction medium.....................	pyrogen-free water, 0.05% Tween-20 in pyrogen-free water
Temperature;...........................	room temperature, 68°C
Rocking conditions....................	vigorous, quiet

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

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

Field samples
Dust was sampled from floors of living rooms and bedrooms, from rugs (if present), and from mattresses in 147 homes as part of an epidemiological study on childhood respiratory disease. Sampling was performed according to an internationally standardized protocol[21] with vacuum cleaners (Philips Topomatic T518, 1000 W) equipped with a special nozzle (ALK, Horsholm, Denmark) to collect dust on paper filters (589 black ribbon 70 mm, Schleicher & Schuell). An

area of 1 m^2 per floor was sampled for 2 minutes. The entire upper mattress surface, with an area of approximately 1.6 m^2, was vacuum cleaned for 2 minutes after the bedding had been removed.

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

Endotoxin determination

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

Endotoxin was assayed with a quantitative kinetic chromogenic LAL method (Kinetic-QCL no. 50-650U; BioWhittaker, LAL lot no. OL1920) at 37°C with an automated microtiter plate reader. *Escherichia coli* O55:B5 endotoxin (BioWhittaker; Lot no. OL1460) was used as standard endotoxin. The endotoxin potency of this standard was 17 Endotoxin Units (EU)/ng. The data processing part of the assay is a modification of the supplier's version described earlier by Hollander *et al.*[22] All samples were vortexed for 30 seconds prior to dilution or analysis. Data from the samples were compared with the standard curve, which ranged from 0.01 - 100 EU/ml. Parallel samples were analyzed in duplicate on different days, with one portion of each sample per analysis, and were frozen and thawed only once. The concentration was calculated as the mean of the two values and was expressed in EU/ml. The house dust samples were analyzed only once per freeze-thaw cycle. Parallel and house dust samples did not show inhibition or enhancement of the LAL assay tested as described by Hollander *et al.*[22]

Storage conditions for commercial LPS

The following container materials were compared in the storage experiment; Duran borosilicate glass culture tubes (Schott; cat. no. 530K12), 'soft' glass culture tubes (Rofa-mavi; cat. no. 773119), and polypropylene tubes (Greiner; cat. no. 121261). The polypropylene tubes were sterile and new, and testing showed that they were endotoxin free. Glassware was rendered pyrogen-free before use. Commercial LPS (*E. coli* O55:B5; BioWhittaker; Lot no. OL1460) was reconstituted and diluted in pyrogen-free water to obtain 5-, 0.5-, and 0.05-EU/ml solutions. These three endotoxin dilutions were made separately in the three preselected container materials. A total of 53 test tubes per type were filled with 250 μl of one of the three possible endotoxin dilutions, and 6 test tubes per type were filled with 2 ml of one of the three endotoxin dilutions. Half of the test tubes, equally representing all other variables, were stored frozen at -20°C, while the other half were stored in the refrigerator at a temperature of 7°C. The endotoxin analyses were performed at days 0, 1, 3, 7, 14, 28, 90, 180 and 364. The test tubes filled with 2 ml were re-used during the 9 analyses; thus, these samples underwent 8 repeated freeze-thaw cycles. The other samples (250 μl per test tube) were discarded after the endotoxin analysis. The dendrogram in figure 2 explains the distribution of the different test tubes over the variables used in this study.

Statistical analysis

To evaluate the efficiency of the sampling and extraction conditions for the endotoxin concentration obtained, data from the parallel-sampling experiment were statistically analyzed by an analysis of variance (ANOVA). Statistical analyses were carried out with SAS statistical software.[23] Like most air pollution data, endotoxin measurements were characterized by a right skewed distribution. For this reason the data were ln-transformed. The analysis of variance was then performed with the normally distributed ln-transformed data by using SAS PROC GLM. The analysis was started with a full model as expressed in the following equation, which contains filter and extraction variables and all interactions between these variables: $LEC = Int + \beta_1 * Day + \beta_2 * Filter\ holder + \beta_3 * F + \beta_4 * E + \beta_5 * T + \beta_6 * R + \beta_7 * F|E|T|R$, where LEC = ln-transformed endotoxin concentration; Int = intercept; $F, E, T,$ and R = main effects (Filter type, Extraction media, Temperature, Rocking conditions); $\beta_{1,2,3,4,5,6}$ = regression coef-

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ficients for main effects including day (of sampling) and (location of) filter holder; $F|E|T|R$ = all possible interactions between the main effects F, E, T, and R; and $ß_r$ = regression coefficients for the interaction effects $(ß_7 - ß_{17})$. The sampling day $(ß_1*Day)$ was brought in the model to adjust for differences in endotoxin concentration caused by daily changes in airborne endotoxin levels in the sampling area. The model also adjusted for differences in endotoxin con-centrations due to variation in dust collection by the 16 filter holders mounted on fixed places inside the parallel sampling apparatus $(ß_2*Filter\ holder)$. Variables and interactions that were not statistically significant $(p > 0.1)$ in the full model were omitted and were no longer considered in the further statistical analysis.

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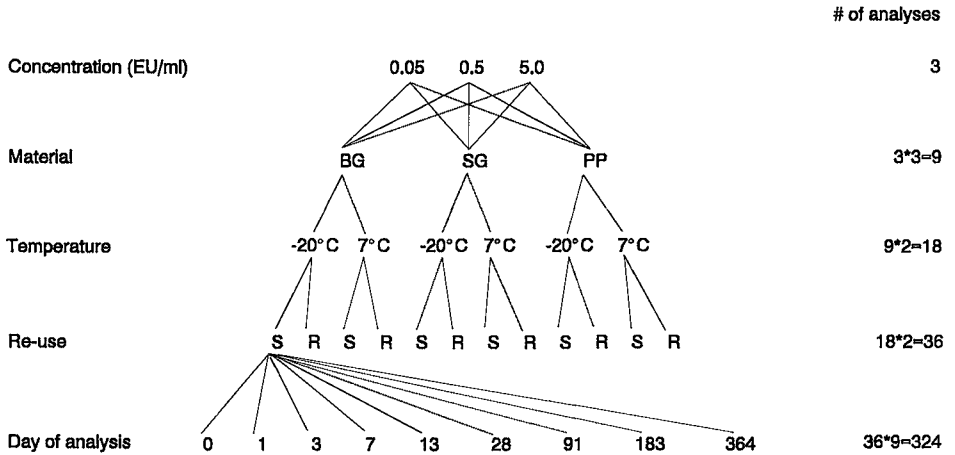

Fig 2. Dendrogram of storage condition variables studied. BG, borosilicate glass; SG, 'soft' glass; PP, polypropylene; S, single use; R, reused.

Results

Parallel samples

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

Because of analytical errors, 2 of the 192 endotoxin measurements had to be deleted from the data set. A full model, including all main and interaction effects, could explain 94% (p<0.01) of the variation in ln-transformed endotoxin concentrations. More than 50% (p<0.01) of the variance in endotoxin concentration was explained by the extraction medium. The filter type explained approximately 6% (p<0.01), and only 2.8% of the variance was explained by statistically significant interaction effects (p<0.01). The variables of temperature and rocking conditions did not significantly contribute to the explained variance in endotoxin concentration. The day of sampling explained 30.9% (p<0.01), while only 0.7% (not significant) was explained by the location of the filter holder inside the apparatus. Thus, good parallel samples were obtained independent of location of the filter holder inside the parallel-sampling device. A reduced model in which only the significant main variables of extraction medium and filter type were considered, adjusted for sampling day, was then applied. In table 2 the antilogs of the regression coefficients (e^{β}) are given for each main effect; these represent differences in geometric mean levels, since ln-transformed concentrations were used in the model. Significant interaction effects (p< 0.01) were no longer considered in the reduced model because of their small contribution to the extraction efficiency compared with that of the main effects. It is concluded that extraction medium and filter type are the most important variables in determining the endotoxin extraction efficiency. The addition of 0.05% Tween-20 resulted in an extraction efficiency approximately 7 times better than that with pyrogen-free water only (p<0.01). The use of glass fibre, teflon and polycarbonate filters resulted in a significant (p<0.01) approximately two-fold increase in detectable endotoxin compared with the use of cellulose filters. There were no significant differences in the extraction efficiencies of glass fibre, teflon and polycarbonate filters.

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The endotoxin concentration in the fibre dehydration department where sampling took place, ranged from 71.5 to 1770 EU/m^3 (4.2 to 104.1 ng/m^3) during the 12 sampling days. This was calculated using geometric mean concentrations (n = 8) determined for each sampling day and only for samples that were extracted in pyrogen-free water. A range of 681.3 to 13,279 EU/m^3 (40.1 to 781.1 ng/m^3) was calculated when endotoxin concentrations in the Tween-20 (0.05%) extracts were used.

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

Main effects	Partial R^2(%)[c]	Condition	Ratios (e$^\beta$)	95% confidence interval
Medium	52.6*	0.05% Tween-20	7.24*	6.34 - 8.28
		Pyrogen-free water	1.00[b]	-
Filter type	5.6*	Glass fibre	2.34*	1.94 - 2.82
		Teflon	2.05*	1.70 - 2.48
		Polycarbonate	1.84*	1.52 - 2.22
		cellulose ester	1.00[b]	-
Sampling day	30.9*	-	-	-

[a] Analysis of variance; degrees of freedom (df) for model = 15; df for error = 174; sum of squares for model = 315.10; sum of squares for error = 38.25; mean square for model = 21.01; mean square for error = 0.21; F = 95.56; P = 0.0001; R^2 = 89%; and n = 190;

[b] Arbitrarily chosen as a reference level; [c] Explained variance in %; * p < 0.01;

storage conditions

Re-used samples that were repeatedly frozen (-20°C) and thawed showed a continuous decline in endotoxin concentration, up to 90% and more after 8 freeze-thaw cycles. The semi-logarithmic relationship between the observed endotoxin concentration and the number of freeze-thaw cycles (fig 3; shown only for 5 EU/ml) indicates that each freeze-thaw cycle results in a loss of endotoxin activity of approximately 25%. The regression coefficients thus estimated for the starting concentration of 5 EU/ml stored in three different container materials (borosilicate glass, 'soft' glass, and polypropylene) differed significantly from 0 and were, respectively, -0.20 (standard error = 0.019), -0.25

(SE = 0.018) and -0.29 (SE = 0.025). A half-life for endotoxin activity was calculated to be approximately 3 freeze-thaw cycles. The steady decrease in endotoxin activity after repeated freeze-thaw cycles was observed for all endotoxin concentrations and container materials used.
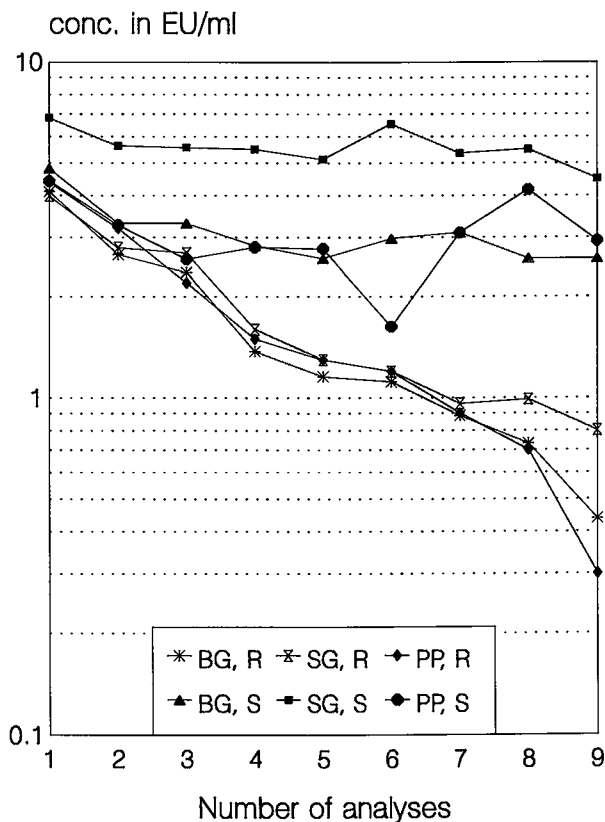

Fig 3. *Effect of repeated freeze-thaw cycles (-20°C) and duration of frozen storage (-20°C) on the recovery of commercial LPS in pyrogen-free water with a stock solution of 5 EU/ml. BG, borosilicate glass; SG, 'soft' glass; PP, polypropylene; R, re-used; S, single-use.*

The estimated regression coefficients for the starting concentration of 0.5 EU/ml were all statistically significant (p<0.05) at -0.22, -0.21 and -0.33. The regression coefficients for the starting concentration of 0.05 EU/ml showed a similar trend but did not reach statistical significance because of detection limitations in this concentration area (<0.05 EU/ml). Single-use samples stored at -20 °C did not show a significant decrease in endotoxin concentration during the year of the experiment compared with the concentration measured on day 1 (fig. 3). An approximately 25% decrease, however, was observed for single-use samples analyzed on day 1 to day 364 compared with day 0 (not ever frozen), which was in accordance with the effect observed in the re-used samples after one freeze-thaw cycle. The endotoxin concentration measured in the 'soft' glass single-use samples of 5 EU/ml appeared to be slightly elevated (fig 3). This might be due to the container material, but this explanation seems improbable since this phenomenon was not observed for samples in 'soft' glass containing 0.5 and 0.05 EU/ml. No significant deviations in endotoxin concentration were detected in both the single-use and re-used samples that were stored for one year at 7°C (data not shown). This was the case for all container materials and endotoxin concentrations used.

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

Freeze-thaw effects were also demonstrated with endotoxin activity measured in house dust extracts. The endotoxin concentration in the house dust extracts ranged from 0 to 10,000 EU/ml. Linear regression showed a significant (p<0.01) decrease in endotoxin concentration of approximately 20% when duplicate analyses of 487 samples were compared after the samples had been frozen and thawed a second time (Y = 0.77X + 107, where X = once frozen and thawed and Y = twice frozen and thawed, figure 4). The correlation (R^2) between the two analyses was 84%. The Omission of seven outliers of this dataset (Cook's distance, > 0.1) resulted in a slightly higher R^2 (0.88) and a regression coefficient and intercept of 0.83 and 48 respectively. The intercept deviates from zero but is relatively small compared with the range of measured endotoxin concentrations and is therefore not important in the interpretation.
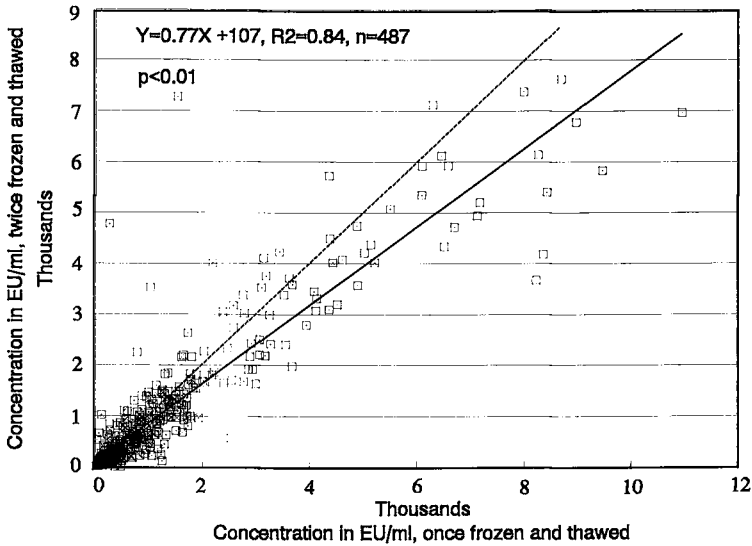
Fig 4. Effect of freezing and thawing on the recovery of endotoxin in house dust extracts. The solid line represents the regression line Y = 0.77X + 107. The dashed line represents the identity line Y = X.

Discussion

Several types of filter materials are commonly used for endotoxin sampling: cellulose, polyvinylchloride, glass fibre, teflon, and polycarbonate. Using purified (no airborne) endotoxin in buffered solutions, Milton and coworkers[12] demonstrated that different filter types could inactivate LPS to different degrees, with recoveries ranging from 6 to 25%. Gordon *et al.*[11] examined the influence of filter type on endotoxin extraction for a variety of laboratory-generated aerosols contaminated with endotoxin. They concluded that the endotoxin extraction efficiency for different filters was dependent on the aerosol type. For extraction, no standard method exists. Most laboratories use pyrogen-free water or buffers such as Tris[24] and phosphate triethylamine (pH 7.5)[12] with or without endotoxin-dispersing agents such as Tween-20, Tween-80, Triton-X100 or saponin.[14] The

use of buffers and dispersing agents may be beneficial in the LAL assay in the case of deviation from the optimal pH (6.5) or increased ionic strength of the extract.[25,26] The most common method of extraction is rocking or sonication of filters in extraction medium, or a combination of both. The duration of this extraction and the temperature during the extraction may differ considerably between research groups. Olenchock and coworkers[14] demonstrated that there is a peak extractable endotoxin concentration after rocking of airborne grain dust in water for 2 hours, while measured levels declined rapidly after that time. At present, however, there is still limited insight into the influence on efficiency of most of these variations in extraction procedures.

Little attention has been given to the influence of storage conditions on the yielded endotoxin level in dust extracts. Most laboratories store extracts frozen at a temperature of -20°C in either glass or plastic container materials. In experiments with grain dust extracts, the detectable endotoxin level was not significantly affected by repeated freeze (-85°C)-and-thaw procedures.[14] Another study showed that the use of polypropylene may introduce recovery problems because of its capacity to irreversibly adsorb LPS.[13]

In this study the addition of 0.05% Tween-20 to the regularly used extraction media resulted in a considerable improvement in the extraction efficiency. Olenchock and coworkers[14] studied the use of 1% Tween-20 in pyrogen-free water as extraction solution for endotoxins in grain dust. They demonstrated a substantial increase in the slopes of the standard curves with the use of 1% Tween-20 compared with pyrogen-free water to reconstitute and dilute the endotoxin standard. Obviously a 1% Tween-20 solution affected the kinetics of the chromogenic LAL assay used, which came from the same source and was probably essentially identical to the test used in our study. Olenchock *et al*[14] were not able to show improved extraction with a 1% Tween-20 solution when sample extracts were referenced to standards reconstituted and diluted in the same Tween-20 solution. In our study the use of a 0.05% Tween-20 solution did not significantly change the slope of the standard curve. Only a slight parallel decrease in the level of the standard curve was observed, which had no consequences for the outcomes of the assays. We thus conclude that endotoxin extraction with 0.05% Tween-20 yields better results than extraction with pyrogen-free water. Disruption of hydrophobic interactions between LPS and filter material, because of the surface-active properties of Tween, is one of the

possible explanations of the increased extraction efficiency. Since formation of micelles by endotoxin and cell wall-bound endotoxin lead to an underestimation of the actual endotoxin concentration,[25] disaggregation of endotoxin micelles or dissociation of cell wall bound endotoxin may be another explanation for an increased extraction efficiency.

The endotoxin extraction was significantly better for glass fibre, teflon and polycarbonate filters than for cellulose mixed ester filters. Apparently cellulose mixed ester irreversibly binds more endotoxin than the other filter materials used. In this study, the glass fibre filter yielded the highest extractable endotoxin concentrations. Gordon and coworkers[11] reached the same conclusion in their study. They demonstrated, however, that the effects of different filter types and extraction media on the endotoxin analysis were strongly dependent on the matrix in which endotoxin was sampled. They concluded that differences in extraction recoveries were caused by specific interactions between endotoxin and filter type, which in turn are influenced by the sample matrix. Therefore, the results found in our study may not be directly generalizable to other organic dusts.

Temperature and rocking conditions did not significantly contribute to the explained variance determined by the analysis of variance. Differences in detectable endotoxin concentration, if any, due to these factors would therefore be very small and in this context would not be relevant compared with the relatively high coefficient-of-variation values (17.5%)[22] of the LAL assay itself. Storage of commercially available LPS dissolved in pyrogen-free water at -20°C resulted in a reduction of approximately 25% in activity in the LAL test compared with non-frozen samples. Each additional freeze-thaw cycle reduced the endotoxin activity by another 25%. The duration of storage at -20°C, up to one year, did not influence the endotoxin activity. This indicates that only the number of freeze-thaw cycles, rather than the duration of storage, explains the considerable loss in endotoxin activity observed during the year of the experiment. The observed 20% decrease in endotoxin content after freezing and thawing of the house dust extracts for a second time indicates that the endotoxin activity in environmental samples may also be negatively influenced as a result of freezing-and-thawing procedures. Olenchock and coworkers[14] showed that there was no influence on the endotoxin level in two water extracts of airborne grain dusts (spring wheat dust and oat dust) that were frozen (-85°C)

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and thawed 13 times during a 30-day period. The endotoxin concentrations used in their experiments were relatively high (50 mg of dust/ml, $\approx$ 1,100 EU/mg dust; $\approx$ 55,000 EU/ml). The endotoxin concentration in almost 90% of our house dust extracts ranged from 0 to 2,000 EU/ml, and it was below 10,000 EU/ml in the remaining samples. It is therefore not clear from our data whether these findings can be extrapolated to higher concentrations as used in the study by Olenchock *et al*. Relative decreases in the endotoxin level after freezing and thawing may be negligible at higher concentrations. Figure 4, however, does not indicate any improvement in endotoxin recovery at higher concentrations. The differences in the results of the two studies may also be explained by the temperature difference itself (-20°C vs. -85°C). However, a good comparison of our results with those of Olenchock *et al* is hampered by the fact that in their study only two samples from different origins were tested, with a relatively large day-to-day variation, ranging from 800 to 1,250 EU/mg ($\approx$ 40,000 - 62,500 EU/ml). The decreased endotoxin activity observed in our study, after repeated freezing and thawing of aqueous endotoxin solutions could be caused by denaturation of the functional structure of endotoxin or by irreversible binding of endotoxin to the container material. The first explanation seems more likely, since no differences in effect were observed for the different container materials used.

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

None of the container materials used showed irreversible adsorption of aqueous LPS during storage for 1 year at 7°C, while the observed recovery problems at -20°C were similar for all container materials used. Thus, recovery problems seem to be associated with freezing and thawing of samples rather than with the material in which aqueous LPS is stored. Novitsky and coworkers showed that the use of polypropylene leads to a higher degree of non-recoverable, adsorbed endotoxin than the use of other materials, such as borosilicate glass,

flint glass, and polystyrene.[13] Polypropylene exhibited an LPS recovery of less than 1%.[13] In that study, however, the endotoxin solution was dried to apply endotoxin to the container surface by either lyophilizing or air drying, after which it was reconstituted in pyrogen-free water. In the same paper it was reported (as unpublished results) that polystyrene was found to adsorb negligible amounts of endotoxin from aqueous solutions. Thus, the use of different container materials to store aqueous LPS is not likely to influence the LPS recovery dramatically.

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

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

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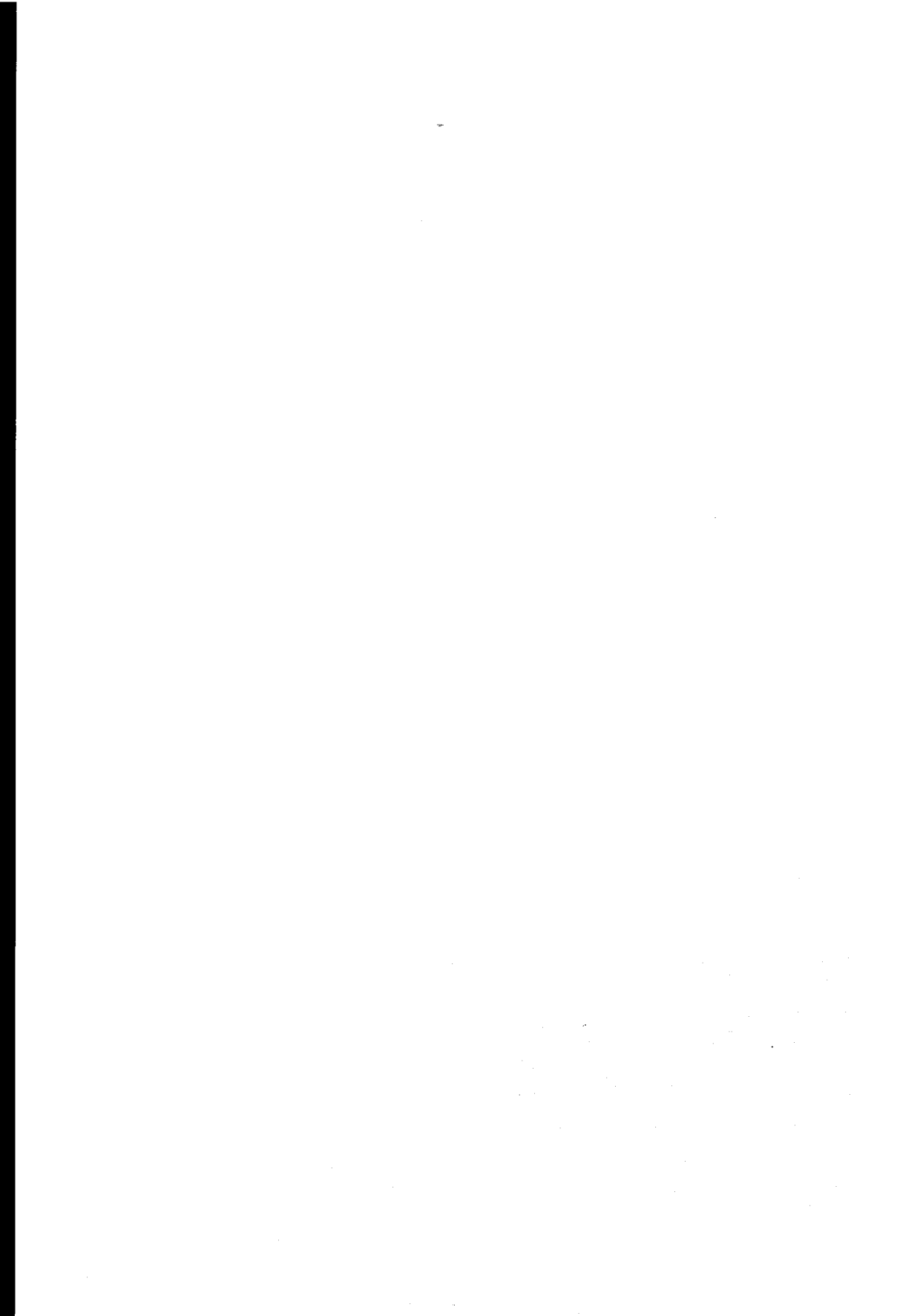
Chapter 4

Measurement of ß(1→3)-glucans in the occupational and home environment with an inhibition enzyme immunoassay

Abstract

ß(1→3)-glucans are known for their potency to induce non-specific inflammatory reactions and are believed to play a role in bioaerosol-induced respiratory symptoms. An inhibition enzyme immunoassay (EIA) was developed for the quantitation of ß(1→3)-glucans in dust samples from the occupational and residential environment. Immunospecific rabbit antibodies were produced by immunization with BSA-conjugated laminarin (ß(1→3)-glucan) and affinity chromatography on epoxy-Sepharose coupled ß(1→3)-glucans. The laminarin-based calibration curve in the inhibition EIA ranged from ≈ 40 - 3,000 ng/ml (15 - 85% inhibition). Another ß(1→3)-glucan (curdlan) showed a similar inhibition curve, but was three to five times less reactive on a weight basis. Pustulan, presumed to be a ß(1→6)-glucan, showed a parallel dose-response curve at concentrations 10 times higher than that of laminarin. Control experiments with $NaIO_4$ and ß(1→3)-glucanase treatment, to destroy ß(1→6)- and ß(1→3)-glucan structures, respectively, indicated that the immunoreactivity of pustulan in the assay was due to ß(1→3)-glucan- and not to ß(1→6)-glucan structures. Other polysaccharides such as mannan and α(1→6)-glucan did not react in the inhibition EIA. ß(1→3)-glucan extraction of dust samples in water (with mild detergent) was performed by heat treatment (120 °C) because aqueous extracts obtained at room temperature did not contain detectable ß(1→3)-glucan levels. The assay was shown to detect heat extractable ß(1→3)-glucan in dust samples collected in a variety of occupational and environmental settings. On the basis of duplicate analyses of dust samples, a coefficient of variation of approximately 25% was calculated. It was concluded that the new inhibition EIA offers a useful method for indoor ß(1→3)-glucan exposure assessment.

Introduction

Exposure to bio-aerosols in indoor environments, both residential and occupational, can induce allergic, toxic and inflammatory reactions resulting in acute and chronic respiratory symptoms. At present, however, it is not clear which components primarily account for the observed effects. Endotoxin, a cell wall component of gram-negative bacteria and potent pro-inflammatory agent, has been recognized as an important causal factor, particularly in occupational environments. Recently a similar role has been suggested for ß(1→3)-glucans in bio-aerosol induced inflammatory responses and resulting respiratory symptoms and complaints.[1-4] ß(1→3)-glucans are glucose polymers with variable molecular weight and degree of branching, and may originate from a large variety of sources, including most fungi and yeasts, some bacteria, most higher plants and many lower plants. They are water insoluble structural cell wall components of these organisms, but may also be found in extracellular secretions of microbial origin. ß(1→3)-glucans can initiate a variety of biological responses in vertebrates such as host-mediated antitumor activity,[5] stimulation of the reticulo-endothelial system,[6] activation of neutrophils,[7] macrophages[8-10] and complement[11] and possibly activation of eosinophils.[12]

At present, knowledge about airborne ß(1→3)-glucan exposure as a potential respiratory health hazard is limited due to a lack of generally available methods to measure environmental ß(1→3)-glucan. One relatively small population study has been described in which a relationship was suggested between levels of airborne ß(1→3)-glucans in office buildings and the occurrence of eye and throat irritation, dry cough and itching skin.[3] In this study a glucan-reactive lysate from *Limulus polyphemus* was used to quantify ß(1→3)-glucans in airborne dust samples. This assay, based on the ability of ß(1→3)-glucans to activate the *Limulus* coagulation system,[11,13] has only recently become commercially available, and has some serious drawbacks including its reactivity with various other polysaccharides and the high costs of the reagents required for the analyses.

There is a clear need for an improved specific, sensitive and cost efficient method to quantify ß(1→3)-glucans which can be used in large scale environmental hygiene and epidemiological studies. In this paper we describe a new assay which meets these requirements. An inhibition immunoassay was developed with which ß(1→3)-glucans can be quantified with the use of affinity-

purified anti ß(1→3)-glucan rabbit antibodies. The new assay was applied on organic dust samples from various environments and preliminary results indicated high ß(1→3)-glucan levels in house dust samples, personal dust samples from swine confinement workers and waste composting workers. We also investigated ß(1→3)-glucan content of various plant materials and culture fluid of in vitro yeast cultures.

Materials and methods

Materials and Reagents

Flat-bottom 96-wells polystyrene microtitre plates with high binding capacity (Greiner, Nuertingen, Germany; no. 655061), bovine serum albumin (BSA) and o-phenylenediamine (no. P 1526) (Sigma Chemicals, St. Louis, MO), epoxy-activated Sepharose 6B (no. 17-0480-01) and Sephadex G-25 (Pharmacia LKB, Upssala, Sweden), Centricon-30 micro-concentrators (Amicon Inc, Beverly, MA, USA), Microsep-100 Centrifugal Microconcentrators (Filtron Technology Corp, Northborough, MA), peroxidase labelled horse-anti-rabbit Ig (Central Laboratory of the Red Cross Bloodtransfusion Service (CLB), Amsterdam, The Netherlands, no. M1234), Tween-20 (polyoxyethylenesorbitan monolaurate), H_2O_2 and gelatin (Merck, Darmstadt, Germany), zymolyase 100 T (Seikagaku Corp., Tokyo, Japan, lot no. 109303) and BCA-protein assay reagent (Pierce, Rockford, IL; no. 23235) were obtained as indicated. All other chemicals were of analytical grade. Glucans and other polysaccharides used in this study are described in Table 1 and were purchased as indicated; dextran (no. D 5251) and mannan (no. M 7504) (Sigma chemicals, St. Louis, MO), laminarin (Fluka AG, Buchs SG Switzerland), pustulan (Calbiochem, La Jolla, CA), CM-curdlan (Wako Pure Chemicals Ind, LTD, Osaka, Japan).

BSA-laminarin conjugate for immunization

Since isolated carbohydrates are weak immunogens, conjugates of ß(1→3)-glucan coupled to bovine serum albumin (BSA) were prepared for immunization purposes. Laminarin was dissolved (20 mg/ml) in twice-distilled water by autoclaving for 20 min at 120 °C. Immunoreactivity of ß(1→6)-glucans was abolished by oxidation with 0.25 M $NaIO_4$ at 20°C for 60 min.[14] The reaction was stopped by desalting the solution on a Sephadex G-25 column. Conjugation of oxidized laminarin to BSA was performed by reductive amination.[18] Briefly,

Table 1. ß(1→3)-glucans and related saccharides used in this study

Saccharides	Linkage	Organisation[a]	Source	used extraction procedure	Reference
laminarin	ß(1→3)(1→6)-glucan	LSB	algae	H_2O, 120 °C	(15, 16)
carboxymethyl-curdlan	ß(1→3)-glucan	L	bacteria	0.05 M NaOH	(15, 16)
pustulan	ß(1→6)-glucan ß(1→6)(1→3)-glucan	L	lichen	H_2O, 120 °C	(15) (17)
dextran	α(1→6)(1→3)(1→4)(1→2)-glucan	B	bacteria	H_2O, room temp	(15)
mannan	α(1→2)(1→3)(1→6)-mannan	B	fungi	H_2O, room temp	(16)

[a] L, Linear; B, Branched; LSB, Linear with Side Branches.

boric acid and borax were added to obtain a borate (0.2 M) buffered glucan solution of pH 9.0. Cyanoborohydride (500 mg) and BSA (340 mg) were dissolved in 25 ml of this glucan solution. The mixture was shaken for 24 hrs at 50 °C in a thermostated incubator. Precipitated material was removed and discarded by centrifuging at 3,000 x g.

Buffer exchange of the glyco-BSA-conjugate in PBS (pH 7.0) - removing cyanoborohydride and unreacted glucans - was performed using a microsep-100 centrifugal microconcentrator (3,000 x g). Conjugation of glucan with BSA was confirmed by gel electrophoresis. The glucan-BSA conjugate contained approximately 8 % carbohydrate on weight basis.

Rabbit anti ß(1→3)-glucan antibodies

Male New Zealand White rabbits (Broekman Institute, Someren, The Netherlands), were immunized by subcutaneous injection of one ml of the glucan conjugate solution (0.1 mg protein/ml) mixed 1:1 with Freund's complete adjuvant. Booster injections were administered at 4-8 week intervals with the same amount of antigen mixed 1:1 with incomplete Freund's adjuvant. Serum was collected at 1 and 2 week intervals after each booster and stored at -20 °C. The antibody titre of the serum which amounted to 1×10^6 was determined using a direct Enzyme Immuno Assay (EIA) in which laminarin (2 μg/ml, PBS pH 7.0) was directly coated onto the microtitre plate.

Specific antibodies were isolated by affinity chromatography using epoxy-activated Sepharose 6B as described by Hutchins *et al.*[19] Briefly, 360 mg ß(1→3)-glucan (laminarin) was dissolved in 21 ml twice-distilled water by autoclaving for 20 min at 120 °C. After heating NaOH was added to yield a 0.1 M solution with pH 12-13. Then 9 ml preswollen gel was added and the mixture was shaken at 37 °C for 20 hr. Excess ligand was washed away and any remaining active groups were blocked by incubation of the gel overnight in 1 M ethanolamine at 42 °C. The gel was stored at 7 °C in PBS pH 7.0 containing 0.05 % NaN$_3$, and washed extensively with PBS pH 7.0 before use.

Immunoglobulins were precipitated from pooled antiserum (100 ml) with ammonium sulphate (0.2 g/ml serum), and redissolved in PBS (pH 7.0) in one fifth of the original volume, filtered through a 0.45 μm filter (Millipore) and applied at a flow rate of 0.25 ml/min on the packed affinity column (1.0 x 9.5 cm). The column was washed using PBS (pH 7.0) with 0.5 M NaCl and eluted

with 0.1 M glycine HCl (pH 2.5) at a flow rate of 0.5 ml per min. Fractions of 0.5 ml were collected and neutralized by addition of 0.2 ml of 0.5 M Na_2HPO_4. All fractions with OD_{280} > 0.1 were pooled, dialyzed against twice-distilled water for 16 hrs and concentrated using centricon-30 micro-concentrators by centrifuging at 3,000 x g. The affinity purified antibodies were stored at -20 °C in aliquots in PBS pH 7.0 at a concentration of 1.5 mg protein/ml (total volume: 11 ml).

Rabbit anti ß(1→6)-glucan antibodies
Anti-ß(1→6)-glucan antibodies were raised in rabbits as described by Montijn *et al.*[14]

Inhibition EIA
Laminarin (2 μg/ml) in PBS (pH 7.0) was coated overnight onto each well (200 μl/well) of a microtitre plate at 4 °C. After extensive washing during 3 cycles on an automatic plate washer (LKB-Pharmacia) with PBS containing 0.05% (v/v) Tween-20 (PBT), 300 μl of 0.5 % gelatine in PBT (PBTG) was applied and incubated at 37 °C and discarded after 30 min. Test sample or laminarin standard (100 μl diluted in PBTG) was then added in a microwell and subsequently mixed with an equal volume of affinity-purified anti ß(1→3)-glucan antibodies diluted 1/75,000 in PBTG. The microtitre plate was then shaken at 37 °C for one and a half hr. After extensive washing, 200 μl of peroxidase-labelled horse anti-rabbit Ig antibodies diluted 1/5,000 in PBTG was added and shaken for one hr at 37 °C. Then after extensive washing, 200 μl *o*-phenylene-diamine (OPD; 2mg/ml) in 0.05 M citrate/phosphate buffer, pH 5.5, containing 0.015% H_2O_2 was added and incubated for 30 min at 20 °C. The enzyme reaction was terminated by the addition of 50 μl 2 N HCl, and the optical density was read at 492 nm.
On each microtitre plate 12 dilutions of the reference laminarin preparation (9.8 ng/ml - 20 μg/ml) were included and also 4 control wells, in which only antibodies mixed with PBTG were incubated. Samples were tested in 4 different dilutions and ß(1→3)-glucan concentrations were determined by interpolating the OD_{492} of each test sample dilution on a semi-log calibration curve obtained with the 12 dilutions of the reference preparation, and calculated with the 4-parameter curve fitting program of the SOFTmax software package (Molecular

Devices Corporation; Menlo Park, Ca USA). Laminarin stock solutions of 1 mg per ml twice-distilled water were prepared by autoclaving for 15 min at 120 °C (1 bar). Stock solutions were stored at 7 °C and prior to analysis autoclaved again.

ß(1→3)-glucanase and NaIO₄ treatment

ß(1→3)-glucanase and NaIO$_4$ treatment
Specific destruction of ß(1→3)- or ß(1→6)-glucan conformational structures was accomplished by treatment with specific ß(1→3)-glucanase (zymolyase 100T)[16] or NaIO$_4$, respectively.[14] Solutions of pustulan and laminarin (0.5 mg/ml in PBS, pH 6.5) were pre-incubated overnight at 45 °C with zymolyase (5000 U/ml), which was dissolved in 50% glycerol in PBS pH 6.5. The reaction was stopped by heating at 100 °C for 5 min. Periodate oxidation of pustulan and laminarin was performed by incubation for two hr with 0.25 M NaIO$_4$ in twice-distilled water at 20 °C.[14] The reaction was stopped by adding 1 M ethanolamine, which also blocked the newly formed aldehyde groups. Specific anti-ß(1→6)-glucan rabbit antibodies were used to confirm the efficacy of NaIO$_4$ treatment and the specificity of the ß(1→3)-glucanase activity of zymolyase.

Plant samples

Plant samples
Extracts were made of cereals (wheat, barley, corn flour), soy (soy beans and soy flour), tapioca, potato and potato starch. Potato starch was obtained from a potato processing plant in the Netherlands, the other products were purchased from a local grocery shop. Because of the insolubility of most ß(1→3)-glucans under neutral conditions, alternative extraction procedures i.e. heat and alkaline treatment were explored. Each product was suspended (1% w/v) and homogenized in twice-distilled water with addition of 0.05 % Tween-20 and in 0.05 M NaOH by using an Ultrathurax (Polytron, Switzerland). Samples suspended in twice-distilled water with 0.05% Tween-20 were rocked vigorously for 15 min, subsequently autoclaved at 120 °C (1 bar) for one hr and then rocked once more for 15 min. Samples suspended in 0.05 M NaOH were rocked vigorously for two hr. Sample suspensions from both extraction procedures were centrifuged at 1,000 x g for 15 min, and the supernatant was collected and stored at -20 °C.

Yeast samples

Cell-free yeast culture media were tested in which wild-type and various mutants of *Saccharomyces cerevisiae* FY384 (*MATα his3△300 ura3-52 leu2△1 lys2△202 trp1△63*)[20] had been cultured. Yeasts were grown at 28 °C to the early exponential phase in standard minimal medium [0.17% (wt/vol) yeast nitrogen base without amino acids and $(NH_4)_2SO_4$] with addition of 0.5% (wt/vol) $(NH_4)_2SO_4$, buffered to pH 6.0 with 50 mM 2-(N-morpholino)-ethanesulphonic acid, with the appropriate amino acids and uracil. Cell-free medium was obtained by centrifugation for 10 min at 2,000 x g, and subsequent filtration through a 0.22-μm-pore-size filter (Millipore). Culture medium in which no yeasts had been cultured was used as control.

Environmental dust samples

Inhalable airborne dust sampling, both area and personal, was carried out in two waste composting facilities, during two days in the summer of 1994 (plant 1) and during 11 days in the winter of 1994 (plant 2). Personal inhalable dust samples were collected from 10 pig farmers during spring, autumn and winter of 1991. Personal and area sampling of inhalable dust was performed on glassfibre filters (Whatman GF/A 25 mm) using PAS-6 sampling heads at a flow rate of 2 l/min.[21] Personal sampling was performed in the workers' breathing zone during full-shift periods of 6 - 8 hr. Area samples were collected also during full-shift periods at ± 1.5 m above the working floor. Filter samples were stored at -20 °C until extraction. Settled dust samples from floors of living rooms, bedrooms and kitchens, and from mattresses were collected in a series of 25 German homes on paper filters (589 black ribbon, 70 mm; Schleicher & Schuell) according to an internationally standardized protocol,[22] as described earlier.[23] As a negative control also non organic dust samples were collected at a construction site. Both area and personal respirable dust sampling was performed, using casella cyclones at a flow rate of 2 l/min during 2 - 4 hr periods.

Gravimetric measurements were performed in a preconditioned room of 18-22 °C and 45-55% humidity using a Mettler AT261 analytical balance. The detection limit for this measurement was approximately 0.3 mg.

All dust samples were initially extracted for endotoxin analyses as described by Douwes *et al.*[23] A subsequent heat extraction to dissolve ß(1→3)-glucans was

performed on pellets after the first extraction resuspended in twice-distilled water with 0.05% Tween-20. Airborne dust samples were extracted in 5 ml and settled dust samples in 5 - 20 ml depending on the dust weight (<0.5 g; 5.0 ml, 0.5 - 1.0 g; 10.0 ml, >1.0 g; 20.0 ml). The applied extraction procedure was the same as described for heat extraction of plant samples. After extraction, samples were centrifuged at 1,000 x g for 15 min and supernatant was stored in several small portions (for duplicate analyses) at -20 °C and analyzed within three months.

Analytical Methods

Protein concentrations were determined with BCA-protein assay reagents from Pierce (no. 23223 and 23224) with bovine serum albumin as a reference protein. Carbohydrate was measured with phenol-sulphuric acid with mannose as a reference.[24]

Results

Specificity of the inhibition EIA

In Figure 1 dose-response curves are depicted for two ß(1→3)-glucan preparations and various other polysaccharides described in Table 1. The inhibition curve for laminarin, the ß(1→3)-glucan used for the production and isolation of the antibodies, ranged from approximately 40 to 3,000 ng/ml (15 - 85% inhibition). The other ß(1→3)-glucan (curdlan) showed a parallel inhibition curve but was approximately 3 to 5 times less reactive on a weight basis (200 - 10,000 ng/ml for 15 - 85% inhibition). Of the other tested polysaccharides only pustulan, presumed to contain exclusively ß(1→6)-glucan,[15] showed a parallel dose-response curve, at concentrations approximately 10 times higher than laminarin. The other polysaccharides were all incapable of inhibiting the anti ß(1→3)-glucan antibodies, even at concentrations up to 50 - 100 µg/ml.

The results shown in Figure 1 suggested that the antibodies were not completely ß(1→3)-glucan-specific, but also partially reactive with ß(1→6)-glucans. Alternatively, the inhibitory activity of pustulan could be due to the presence of ß(1→3)-glucosidic structures in that preparation. Therefore control experiments were performed with laminarin and pustulan after pre-treatment with either periodate or zymolyase, which specifically destroys ß(1→6)- or ß(1→3)-glucan structures respectively.[14,16] Zymolyase almost completely

abolished the inhibitory activity of both glucans (Fig 2a), whereas periodate treatment did not abolish and even slightly enhanced the inhibitory capacity of the preparations (Fig 2b). NalO$_4$ and glycerol did not influence the reaction in the control wells without inhibitor. Additional control experiments using an inhibition EIA with pustulan (inhibitor and coating) and anti ß(1→6)-glucan antibodies confirmed that the periodate pre-treatment abolished the immunoreactivity of ß(1→6)-glucan structures. The pustulan based inhibition curve ranged from 100 - 9,000 ng/ml (15 - 85% inhibition), whereas NalO$_4$ treated pustulan showed ≥ 15% inhibition only at concentrations higher than 10 μg/ml. After zymolyase treatment, 15% and 85% inhibition was observed at pustulan concentrations of 110 and 9,000 ng/ml respectively, indicating that zymolyase had no effect on the immunoreactivity of ß(1→6)-glucan structures.

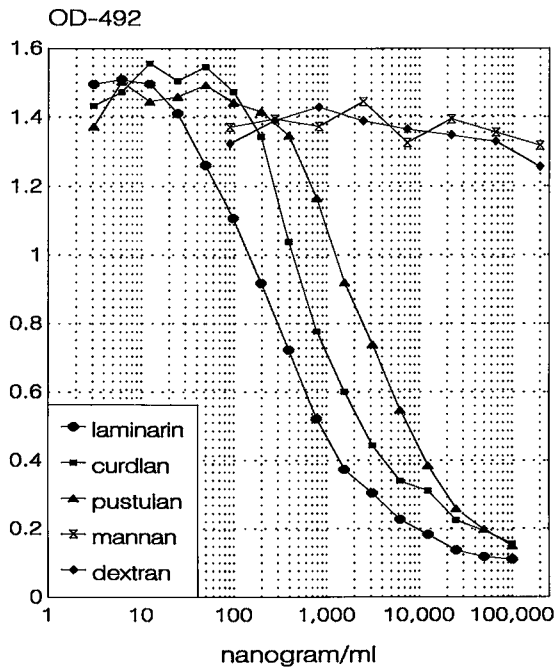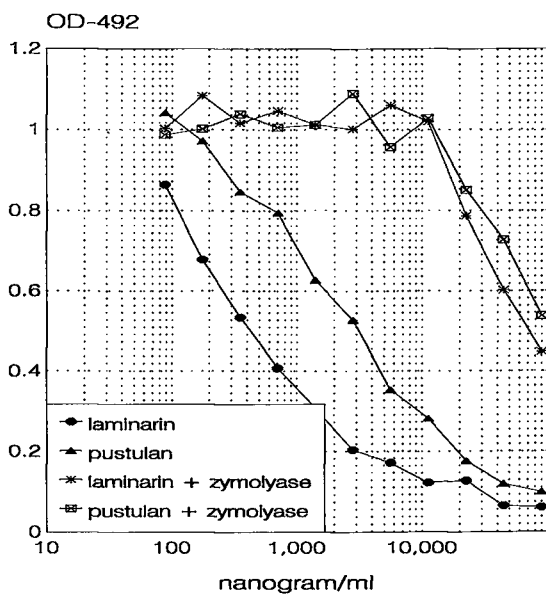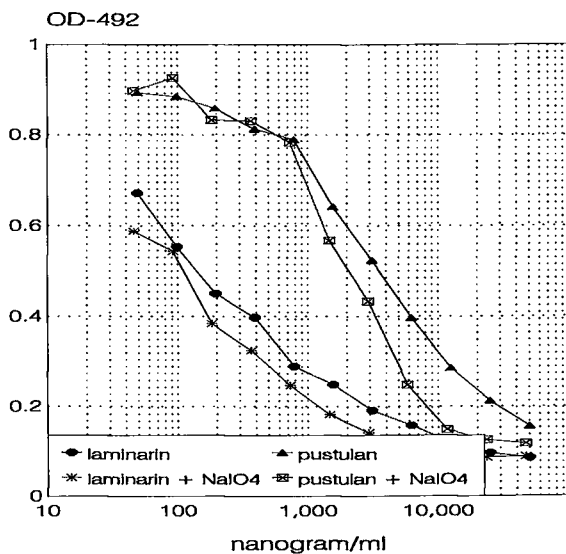Fig 1. *Inhibition-dilution curves of four different glucans and mannan; Laminarin and CM curdlan [both ß(1→3)-glucans], Pustulan [ß(1→6)-glucan contaminated with ß(1→3)-glucan], Dextran [α(1→6)-glucan] and Mannan (polymannose).*

2a

2b

Fig 2. Inhibition-dilution curves for 2a) untreated- and zymolyase treated laminarin and pustulan, 2b) untreated- and NaIO₄ treated laminarin and pustulan.

Thus the cross-reactivity observed with pustulan in the ß(1→3)-glucan EIA was most probably due to the presence of ß(1→3)-glucan structures in the preparation, and not to a lack of specificity of the anti ß(1→3)-glucan antibodies. The specificity of the assay was also confirmed by the lack of reactivity of aqueous extracts of environmental dust samples obtained at room temperature, while subsequent heat extraction resulted in detectable immunoreactivity in the majority of samples. This indicates that the ß(1→3)-glucan antibodies do not react with water-extractable (at room temperature) polysaccharides which can be present in appreciable amounts in most organic dust samples (approximately 10% in dust from the pigfarm environment; unpublished results).

Sensitivity and reproducibility of the inhibition EIA
The detection limit of the inhibition EIA was determined using the mean of 12 individually calculated detection limits based on the laminarin calibration curves of 12 assays each performed on an individual microtitre plate on different occasions. The detection limit for each individual microtitre plate was determined by calculating the minimum % of inhibition that significantly deviated (> 3 times standard deviation) from the four 0 % inhibition controls included on each plate. The detection limit ranged from 4 - 20 % with a mean of 10%. However, to minimize the chance of obtaining false positive results the detection limit was set at 15 % inhibition which corresponded with a mean detection limit of 42.6 ng/ml (SD 10.3). Since airborne inhalable dust was sampled at a flow rate of 2 l/min for 8 hrs ($\approx$ 1 m^3) and filters were extracted in 5 ml extraction solution, the resulting detection limit for airborne inhalable dust samples was approximately 200 ng/m^3. For settled dust 5% - 10% (w/v) suspensions were made for extraction; thus the detection limit for settled dust samples was approximately 0.5 μg/g dust.
To evaluate the use of the new assay to measure ß(1→3)-glucans in different environments, extracts of dust samples of various origin were tested. Dilution curves obtained with autoclaved (120 °C) extracts of environmental samples (house dust, waste composting dust and swine confinement building dust) were essentially parallel to the calibration curve, as shown in Figure 3 for five dust samples from swine confinement workers. This indicates that the reference curve can be applied for different types of samples at various dilutions. The reproducibility of the inhibition EIA was determined on detectable (> 40 ng/ml)

duplicate analyses of the environmental dust extracts (duplicate extract aliquots from the same dust sample) and expressed as a Coefficient of Variation (CV). Duplicate analyses were performed on different microtitre plates at different days. Based on the waste composting facility samples (plant 2), samples from swine confinement workers and the house dust samples, mean CV values for the inhibition assay of, respectively, 20% (n = 26), 20% (n = 59) and 27% (n = 100) were calculated.
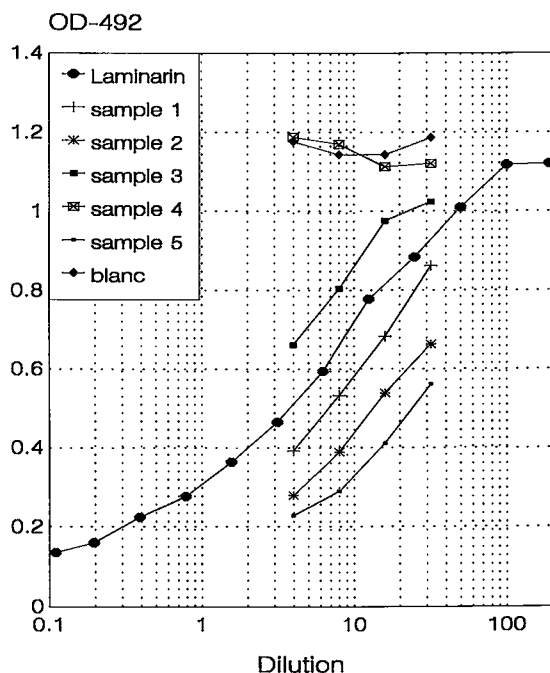
Fig 3.   *Inhibition-dilution curves of heat extracts of personal dust samples from 5 swine confinement workers. Calibration was done with laminarin.*

ß(1→3)-glucan-levels in plant extracts

As shown in Table 2, all tested extracts of cereals, soy, tapioca and potato contained measurable amounts of ß(1→3)-glucan. Dilution curves of all extracts were essentially parallel to the calibration curve in the ß(1→3)-glucan assay and calculated concentrations ranged from approximately 0.01% (w/w) for potato to

approximately 0.7% (w/w) for barley. Differences between the results obtained with two extraction methods (autoclaving at 120 °C versus 0.05 M NaOH extraction) were small and not systematic.

Table 2.   *ß(1→3)-glucans in cereal and other plant materials extracted by heat or alkaline treatment.*

	ß(1→3)-glucan concentration in µg/mg	
Source	Heat treated [a]	Alkaline treated [b]
potato	0.13	0.19
potato starch	0.16	0.90
barley	6.17	7.21
corn flour	1.38	1.63
soy beans	1.75	2.41
soy flour	3.51	1.49
tapioca	4.87	3.78
wheat	4.19	2.96

[a]   Autoclaved at 120 °C, 1 bar for one hr.
[b]   Shaken in 0.05 M NaOH for two hr.

ß(1→3)-glucan in yeast culture media

The ß(1→3)-glucan levels in yeast culture media were moderate, ranging from approximately 0.2 - 1 µg/ml, while in the control medium (without yeast) no immunoreactivity was detected (data not shown).

Exposure levels in various environments

In two small pilot studies appreciable levels of ß(1→3)-glucan could be measured in compost plant and swine confinement building environments (Table 3). These environments - which are known for their high bio-aerosol exposures[25,26] - were also characterised by relatively high mean dust exposures. House dust sampled from mattresses and from floors of living rooms, bed rooms and kitchens did also contain substantial amounts of ß(1→3)-glucan (Table 4). The non-organic dust samples collected at the construction site did not contain detectable ß(1→3)-glucan levels (Table 3). In Table 3 and 4, ß(1→3)-glucan- and dust levels are expressed as Geometric Means (GM) with Geometric Standard Deviations

(GSD) since exposure data in occupational and home environments are in general best described by a lognormal distribution. Concentrations below the detection limit were considered to have a value of two-thirds of this limit.[27]

Table 3. ß(1→3)-Glucan (µg/m³) and dust (mg/m³) exposure measurements in three occupational environments. (glucan detectable/total samples), Geometric Mean (GM), Geometric Standard Deviation (GSD).

waste composting facility	dust conc.	ß(1→3)-glucan concentration			
Plant 1, July '94	*GM*[‡]	*GM*[‡]	*GSD*	*Min*	*Max*
Offices, control rooms (0/2)[a]	<0.3[†]	<0.2[†]	-	-	-
Compost ripening (4/4)[a]	0.43	1.02	2.1	0.53	2.95
Process hall (12/12)[a, #]	4.6	19.35	1.6	11.00	47.03
Plant 2, Dec '94					
Offices, control rooms (0/5)[a]	<0.3[†]	<0.2[†]	-	-	-
Compost ripening (1/6)[a]	0.77	0.39	11.0	<0.2[†]	28.61
Composting hall (0/8)[a]	0.46	<0.2[†]	-	-	-
Process hall (2/16)[a, §]	0.38	<0.2[†]	1.6	<0.2[†]	0.51
Unloading inorganic waste (8/8)[a]	2.87	3.80	2.1	2.02	15.97
Personal (19/21)[b]	2.67	6.57	7.2	<0.2[†]	210.11
swine confinement workers	**dust conc.**	**ß(1→3)-glucan concentration**			
	GM[‡]	*GM*[‡]	*GSD*	*Min*	*Max*
Personal (55/59)[b]	2.1	4.34	3.4	<0.2[†]	38.49
construction workers	**dust conc.**	**ß(1→3)-glucan concentration**			
	GM[‡]	*GM*[‡]	*GSD*	*Min*	*Max*
Personal and stationary (0/15)[a,b]	2.7	<0.2[†]	-	-	-

[a] Ambient air sampling, [b] Personal air sampling.

[#] Unloading and sieving of organic waste and transfer of compost.

[§] Unloading and sieving of organic waste.

[†] Below detection limit (dust conc.: 0.3 mg/m³, glucan conc. airborne: 0.2 µg/m³).

[‡] GM were calculated including non detectable results (non detectable values were set on 2/3 of the detection limit.[27])

Blank glass fibre filters like those used for sampling of airborne dust did not contain detectable levels of ß(1→3)-glucan. Heat extracts (5 ml, 122 °C, 1 bar) of blank paper filters like those used for house dust sampling, however, contained significant amounts of ß(1→3)-glucans. In 6 blank filters, a mean concentration of 188 μg (SD, 39.3) ß(1→3)-glucan per filter was detected, which was, on average, 21% (min; 2%, max; 53%) of the total amount of ß(1→3)-glucan detected in each dust sample. To calculate the ß(1→3)-glucan level per gram of house dust with correction for the ß(1→3)-glucan contribution of the filter, the filter background level was subtracted from the absolute ß(1→3)-glucan level measured for the whole sample (including the dust and the filter). After this correction, ß(1→3)-glucans were still detected in all house dust samples at concentrations ranging from 180 up to 6,500 μg/g.

Table 4. ß(1→3)-glucan exposure (μg/g) measurements in house dust. (glucan detectable/total samples), Geometric Mean (GM), Geometric Standard Deviation (GSD).

Sampling location	ß(1→3)-glucan concentration			
	GM	*GSD*	*Min*	*Max*
Living room (25/25)	1,293	1.4	627	2,915
Bedroom (25/25)	1,286	1.7	408	3,507
Kitchen (25/25)	1,168	2.0	376	6,540
Mattress (25/25)	757	1.7	182	1,654

Discussion

In this paper we describe a ß(1→3)-glucan inhibition EIA that can be used to assess ß(1→3)-glucan levels in airborne and settled dust. Application of the new inhibition EIA showed that ß(1→3)-glucans could be measured with high specificity and reproducibility in dust samples from various environments.

We immunized rabbits with BSA conjugated glucan and obtained polyclonal antibodies with high affinity for ß(1→3)-glucans. Affinity chromatography with epoxy-Sepharose bound ß(1→3)-glucan was used to remove antibody activity against BSA and, if relevant, newly formed (during conjugation) neo-antigens of BSA. The resulting affinity purified antibodies were indeed completely devoid of

activity against BSA but showed strong reactions against the ß(1→3)-glucan used for immunization, as well as another ß(1→3)-glucan (curdlan). Thus direct coupling of ß(1→3)-glucan to epoxy-Sepharose was proven to be suitable for antibody isolation. Direct coupling of ß(1→3)-glucan onto the microtitre plate - which is not common practice for polysaccharides - could also successfully be applied in our inhibition assay. This also precludes false positive results due to antibodies directed against BSA or BSA-associated neo-antigens. Moreover this procedure avoids the use of the often limited amount of BSA-conjugate for coating.

In our study we immunized with linear ß(1→3)-glucan in which ß(1→6) branches were destroyed by $NaIO_4$ to avoid reactivity of the antibodies with ß(1→6)-glucans. This was in contrast with other studies involved in the production of anti ß(1→3)-glucan antibodies, that used branched ß(1→3)(1→6)-glucans for immunization.[28-30] Two different linear ß(1→3)-glucans, curdlan and laminarin, were demonstrated to react in the inhibition assay. Control experiments showed that the antibodies also reacted with pustulan which is presumed to contain only ß(1→6)-glucan (Fig 1).[15] The reactivity of pustulan, however, was, like that of laminarin, retained after $NaIO_4$ treatment, whereas it was completely lost after treatment with ß(1→3)-glucanase (Fig 2a,b). Similar experiments with a polyclonal anti-ß(1→6)-glucan antiserum confirmed the specific ß(1→3)-glucanase activity of zymolyase and the complete destruction of ß(1→6)-glucan structures by $NaIO_4$ treatment. Consequently, we conclude that pustulan also contains ß(1→3)-glucan structures. One other study has also suggested ß(1→3)-glucan presence in pustulan.[17] Since all other tested polysaccharides were not reactive in our assay we conclude that the inhibition EIA recognizes specifically ß(1→3)-glucan epitopes. Preliminary results indicating reactivity with yeast and plant glucans (see below), mainly consisting of ß(1→3)(1→4)- and ß(1→3)(1→6) linkages respectively, suggest that both linear and branched ß(1→3)-glucans are being recognised in the inhibition assay.

Our inhibition EIA was less sensitive (40 ng/ml) than a sandwich EIA for ß(1→3)-glucans described by Hirata *et al*[30] (1 ng/ml) but more sensitive than an inhibition EIA described by Adachi *et al*[28] (>1 μg/ml). Comparison on a weight basis, however, using different reference glucans (schizophyllan and grifolan respectively, versus laminarin) may not be justified since purity and possibly chain length and conformation of the glucans may significantly influence the

detection limit expressed on a weight basis (see below). An example might be the 3 - 5 fold difference in detection limit and reactivity on weight basis for the two ß(1→3)-glucans shown in Figure 1.

Another method to measure ß(1→3)-glucans with the use of a glucan reactive preparation of *Limulus* amebocyte lysate (LAL) has recently been described by Rylander *et al*[3] and Obayashi *et al*.[31] The reactivity of several ß(1→3)-glucans in this assay was reported earlier in several other studies.[13,16,32-34] The glucan reactive LAL test is very sensitive (1 - 10 pg ß(1→3)-glucan/ml) but probably not highly specific since it also reacts with other glucans as gyrophoran (ß(1→6)-glucan) at ng/ml concentrations, and with very high concentrations (1-100 μg/ml) of D-mannans and dextran[16,32] but not with bacterial lipopolysaccharides (endotoxin). A possibly more serious problem may be that activation of LAL depends on chain length and conformation of the glucans.[13,16,33] ß(1→3)-glucan conformers with triple helix structures are severely underestimated compared to single helix and random coil ß(1→3)-glucan structures,[13,33] while all three conformers appear to be biologically active.[33] A similar problem may exist with our inhibition assay due to steric hindrance of ß(1→3)-glucan epitopes, especially in large triple helix conformations. Our data, however, did not support this, since alkaline and heat extracts of plant samples showed similar results in the ß(1→3)-glucan EIA (Table 2). Heat treatment increases the triple helix ratio compared to single helixes while alkaline treatment transforms triple helix to single helix or random coil formation.[33]

The use of a specific immuno-assay that is also less expensive and possibly less dependent on structural conformation of ß(1→3)-glucans, may be advantageous in hygiene and epidemiological effect studies, compared to the modified LAL-test that has recently become commercially available.

Thus far, discussions on the immunobiological effects of glucans have mainly focused on fungal ß(1→3)(1→6)-glucans. However, it is not clear whether plant ß(1→3)(1→4)-glucans have similar properties. Plant glucans like laminarin and barley ß-D-glucan also interact with the Limulus coagulation factor G.[16] This suggests that plant glucans are indeed also biologically active and detection of both plant and fungal ß(1→3)-glucans in a ß(1→3)-glucan assay - as described in this paper - may therefore be relevant.

Very high ß(1→3)-glucan levels were found in the studied occupational and residential dust samples. Mean ß(1→3)-glucan concentrations in the waste

composting facilities ranged from <0.2 to 19 μg/m^3. The mean personal exposure was 7 μg/m^3 for waste composting workers and 4 μg/m^3 for pig farmers. Comparable ß(1→3)-glucan concentrations (1 μg/m^3) have been measured in an experimental cotton cardroom by Rylander *et al*[35] using a glucan reactive LAL-assay. The detection limit of 40 ng/ml allows quantification of the exposure in most samples from these environments, but airborne measurements in 'low' exposure environments such as office buildings (0.00 - 0.55 ng/m^3)[3] and dwellings probably require a more sensitive assay. The high ß(1→3)-glucan content of organic dust from both occupational and residential origin (0.5 - 20 ‰) suggests a large contribution of plant and/or fungal material in that dust. We therefore reconsidered the possibility of non-specific false positive reactions and analysed aqueous dust extracts obtained at room temperature, and heat extracts of (non organic) dust, sampled in construction workers with comparable gravimetric dust exposure. None of these extracts contained detectable ß(1→3)-glucan levels.

High ß(1→3)-glucan content of organic dust - as measured in our study - seems plausible, considering that ß(1→3)-glucans occur as major structural cell wall or storage components of many plants and microorganisms, which are known to contribute largely to the content of most organic dust. Moreover ß(1→3)-glucans (as well as other polysaccharides) are likely to be relatively degradation resistant in the environment, which may result in a high carbohydrate content of organic dust. Organic dust contains a substantial proportion of extractable carbohydrate of approximately 10 % (w/w) when extracted at room temperature and 20 % for heat extraction. (determined for pigfarm dust; unpublished results). Thus, ß(1→3)-glucans would in fact contribute only approximately 1 - 2 % to the total carbohydrate concentration.

In conclusion our ß(1→3)-glucan inhibition assay offers a specific and sensitive method for exposure assessment of airborne glucans in at least high exposure environments and for the assessment of ß(1→3)-glucan levels in settled dust from the home environment. The new assay is therefore expected to be useful for epidemiological studies to investigate the relationship between ß(1→3)-glucan exposure and respiratory health.

Acknowledgments

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Chapter 5

Endotoxin and ß(1→3)-glucan in house dust and the relation with home characteristics: a pilot study in 25 German houses

Abstract

Residential microbial exposure has been suggested to be involved in the development of asthma. This paper describes bacterial endotoxin and mold ß(1→3)-glucan levels in house dust and the relations with home characteristics. Dust was sampled from mattresses and living room and bedroom floors of 25 houses. Endotoxin and ß(1→3)-glucan levels ranged from 200 - 48,600 EU/g dust (100 - 32,900 EU/m^2 sampled surface) and 182 - 3,507 μg/g (157 - 3,652 μg/m^2), respectively. Biocontaminant levels were highest on living room floors and lowest in mattresses. Dust, endotoxin and ß(1→3)-glucan levels were 2-3 times higher on living room floors of centrally heated houses built after 1970 compared to older individually heated houses. This was not found for mattresses and bedroom floors. No associations between biocontaminant levels and other home characteristics were found. ß(1→3)-glucan levels were associated with total culturable fungi (per m^2) in house dust, as well as with the fungal genus *Alternaria* (per g dust and per m^2). In conclusion endotoxin and ß(1→3)-glucan were readily detectable in house dust and significantly associated with type of home, most likely characterized by heating system and/or age of the home. A weak association between ß(1→3)-glucan and culturable fungi in settled dust existed.

Introduction

There is growing evidence that the incidence of respiratory allergies and asthma in the western world is increasing.[1] Bio aerosol exposure in residential indoor environments is believed to play an important role. Type I allergy to indoor allergens from mites and pets may contribute, but non-immune inflammatory reactions towards other biological agents present in the house dust (e.g. micro-organisms and microbial components such as bacterial endotoxins and mold glucans) may also be important.[2-6] The role of microorganisms in house dust-induced respiratory symptoms is poorly understood. Several population-based studies have suggested that allergic or non-allergic inflammatory reactions to inhaled mold components, together with reactions to house dust mites, might account for the frequently reported association between living in damp homes and respiratory symptoms.[7-10] Other studies have suggested a role for bacteria and bacterial components. Michel *et al*[3,6] showed that bacterial endotoxin levels in settled house dust were associated with the clinical severity of symptoms in asthmatic patients. Another recent case-control study showed a significant association between airborne bacteria levels in houses and asthma related symptoms.[5]

In this study two microbial components (bacterial endotoxin and mold ß(1→3)-glucans) in house dust were studied. Bacterial endotoxins are cell wall components of gram-negative bacteria that can exert a wide range of biologic effects on a variety of inflammatory cells.[11-15] Several epidemiological and experimental studies have shown dose-response relationships between endotoxin exposure and lung function alterations accompanied by respiratory symptoms, particularly in the occupational environment.[3,16-18] ß(1→3)-glucans are important cell wall constituents of most fungi but also of some bacteria and many higher and lower plants which can elicit a wide range of inflammatory reactions.[19-24] One relatively small population study suggested a dose-dependent relationship between ß(1→3)-glucan levels in dust, and dry cough, throat and eye irritation, and itching skin in office workers.[25] A subsequent study showed an increase in the severity of symptoms of nose and throat irritations in subjects experimentally exposed to ß(1→3)-glucan, while after exposure a relationship was found between intensity of subjective throat irritation and increase in airway responsiveness.[26]

Recently a large German field study has started to investigate the potential role

of house dust allergen and microbial exposure (mite and pet allergens, endotoxin and ß(1→3)-glucan) in the development of asthma. This paper describes one part of the pilot phase of this study in which endotoxin and ß(1→3)-glucan levels were measured in house dust from 25 German houses. The association between these exposure levels and home characteristics was investigated, as well as the relation between ß(1→3)-glucan and culturable molds in house dust.

Materials and methods

Dust sampling and extraction

Settled dust was sampled during three weeks in November/December 1994 in 25 houses in two cities of Germany (Erfurt; n = 20, Hamburg; n = 5) from floors of living rooms and bedrooms and from mattresses. All 25 houses had wall-to-wall carpets as floor cover in the living room and bedroom. The 5 houses in Hamburg consisted of 2 houses built in the period 1945 - 1970 and three houses built between 1971 - 1990. In Erfurt 11 out of the 20 houses were constructed in the period 1945 - 1970 while 9 were built in the period 1971 - 1990. The homes in both cities were not selected as a representative sample but were selected based on practical considerations. Samples were collected according to an internationally recognized protocol,[27] using a vacuum cleaner (Philips Flüsterjet Vitall 371, 1000W) equipped with a special nozzle (ALK, Horsholm, Denmark) to collect dust on a paper filter (ALK, 70 mm). Floor dust samples were sampled from four square meters with a sampling time of 2 minutes per square meter. One square meter of the upper mattress surface of the adult occupant(s) was vacuum cleaned for 2 minutes after removal of sheets and blankets. Mattress covers were not removed prior to sampling. Filter samples were stored at -20 °C until extraction two weeks later.

Gravimetric measurements and endotoxin and ß(1→3)-glucan extractions were performed as described earlier.[28,29] Extracts were stored in four portions at -20 °C and analyzed for endotoxin and ß(1→3)-glucan content within three months.

Endotoxin analysis

New glassware (sterile and pyrogen free), pipette tips (Gilson; type Tipac), and microtitre plates (96 well, flat bottomed, sterile polystyrene; Costar 3596) were used.

Endotoxin was assayed with a quantitative kinetic chromogenic *Limulus*

Amebocyte Lysate (LAL) method (Kinetic-QCL no.50-650 U; Bio Whittaker; LAL lot no 3L3020) at 37 °C with an automated microtitre plate reader as described earlier.[28] *Escherichia coli* O55:B5 endotoxin (Bio Whittaker; lot no 3L2300) was used as standard endotoxin. The endotoxin potency of this standard was 12 endotoxin units (EU)/ng. The data processing part of the assay was a modification of the supplier's version described earlier by Hollander *et al.*[30] Diluted samples (1:500) were analyzed in duplicate in two independent test runs. Samples did not show inhibition or enhancement of the LAL assay at the 1:500 dilution, tested as described earlier.[30] Endotoxin concentrations were expressed per g house dust and per square meter.

ß-(1→3)-analysis
ß-(1→3)-glucan was assayed with a specific inhibition enzyme immunoassay (EIA) which was recently developed and described by Douwes *et al.*[29] The sensitivity of this assay was 40 ng/ml and the reproducibility expressed as a coefficient of variance was 27% for house dust samples.[29] Samples were analyzed in duplicate in two independent test runs. Since blank paper filters used for dust sampling contained ß(1→3)-glucan (mean concentration 188 μg, SD 39.3) a correction was applied as described earlier.[29] ß(1→3)-glucan concentrations were expressed per g house dust and per square meter.

Culturable mold spore counts
Separate dust samples from living room floors of the same homes were collected for the measurement of culturable molds. Sampling was similar as described for dust collection for endotoxin and ß(1→3)-glucan measurements, except that only one square meter was vacuum cleaned for two minutes. Samples were sieved through a 500 μm-mesh sieve and stored at room temperature until analysis, which was performed one day after sampling. A mean loss of dust weight due to sieving amounted to 71% (range: 39% - 90%). Culturable mold counts in house dust were performed as described by Koch *et al.*[31] Briefly, 0.1% dust suspensions in 0.9% NaCl were made, vortexed for 5 minutes, diluted 10 and 100 times and transferred (100 μl) to Kimig agar culture media. Ten replicates were cultured for each dilution. Molds were incubated at 26°C for more than 10 days and colony forming units (CFU) were counted. Colonies of the following genera were counted: *Alternaria*, *Aspergillus*,

Cladosporium and *Penicillium*. Determination of the genus was performed by using light microscopy.

Home characteristics

On the basis of available questionnaire information, several home characteristics were selected for further evaluation of their relationship to endotoxin and ß(1→3)-glucan levels found in the house dust: period when the house was built (old buildings; 1945-1970 vs new buildings; 1971-1990), type of heating (central heating vs individually heated rooms), insulation of windows (single vs double glazing), the presence of a pet (yes vs no), self reported damp spots on walls, ceilings etc. (yes vs no) in the house, and age of the mattress. Possible relationships between indoor temperature and air humidity (recorded for one week during the period that dust sampling was performed in dwellings in Erfurt [n = 20]) and biocontaminant levels in the home was also investigated. Temperature and relative humidity were recorded by using Tinytalk temperature and relative humidity data loggers (Gemini data loggers LTD, Chisester, West Sussex, UK) with a range of respectively -40 °C to + 75 °C and 0% to 90% RH. Temperature and humidity measurements were performed every 12 minutes at 2 m above the floor in the living room and bedroom. Mean values of measurement series were used in the statistical analysis to study the relationship with biocontaminant and dust levels in the home.

Statistical analysis

Statistical analyses were performed using SAS statistical software (version 6.11; SAS institute, Cary, N.C.). Because data were best described by a lognormal distribution, endotoxin, ß(1→3)-glucan and culturable mold concentrations (expressed per gram house dust and per square meter) were ln transformed. Mean concentrations were therefore expressed as geometric means (GM) with a geometric standard deviation (GSD). Pearson correlation was calculated using SAS PROC CORR. Statistical testing comparing endotoxin, ß(1→3)-glucan and dust levels in homes with and without specific characteristics, was performed by using a t-test (SAS PROC TTEST) on ln-transformed data. The continuous variables 'temperature' and 'relative humidity' were dichotomized using the median as the cutpoint to compare 'low' versus 'high' temperature or relative humidity. Linear regression (SAS PROC REG) was

performed on ln transformed data to study the relationship among culturable mold, glucan and endotoxin concentrations. When no colonies of a certain mold genus were counted in a dust sample, a value of 2/3 of the lowest observed CFU concentration of that genus per gram dust or per m^2 was given for that sample.

Results

Endotoxin and ß(1→3)-glucan were readily detectable in all tested samples. Highest endotoxin concentrations were detected on living room floors both when expressed per gram dust and per m^2, while lowest concentrations were found for mattresses (table 1). ß(1→3)-glucan concentrations expressed per gram dust were similar for living and bedroom floor samples and approximately two times higher than for mattress samples. ß(1→3)-glucan concentrations expressed per m^2 were similar for mattress and floor samples.

Table 1. Geometric mean and Geometric Standard Deviation (GSD) of endotoxin and ß(1→3)-glucan concentrations expressed per m^2 and per gram dust, and dust concentration per m^2 in 25 homes.

	Mean (GSD)	Range	Mean (GSD)	Range
Endotoxin	*EU/m^2*		*EU/g*	
Living room floor	7,400 (2.5)	900 - 32,900	17,300 (1.9)	4,200 - 48,600
Bedroom floor	4,000 (2.3)	700 - 19,300	7,300 (1.9)	1,200 - 19,500
Mattress	2,600 (4.6)	100 - 26,300	2,700 (3.0)	200 - 15,700
ß(1→3)-glucan	*µg/m^2*		*µg/g*	
Living room floor	556 (2.0)	157 - 2,461	1,293 (1.4)	627 - 2,915
Bedroom floor	698 (1.7)	272 - 3,652	1,286 (1.7)	408 - 3,507
Mattress	707 (2.2)	165 - 2,954	757 (1.7)	182 - 1,654
Dust	*mg/m^2*			
Living room floor	430 (1.6)	174 - 1,106	-	-
Bedroom floor	543 (1.6)	238 - 1,294	-	-
Mattress	934 (1.9)	204 - 3,384	-	-

While there were strong correlations between levels of endotoxin, ß(1→3)-glucan and dust per m^2 (R = 0.45-0.86; p < 0.05; stratified per dust reservoir), no correlations between endotoxin and ß(1→3)-glucan concentrations were found when expressed per gram dust. In fact, dust weight explained 44% - 61% (R^2) of the variance in endotoxin concentration per m^2 and 23% - 74% of the variance in ß(1→3)-glucan concentration per m^2. No significant correlations were found among the three dust reservoirs (studied within the same house) with respect to their endotoxin and ß(1→3)-glucan levels per g dust (R^2 < 0.05). There was, however, a strong correlation between living room floor and bedroom floor samples when levels were expressed per m^2 (dust; R = 0.67, endotoxin; R = 0.54, ß(1→3)-glucan; R = 0.46, p < 0.01).

Endotoxin and ß(1→3)-glucan levels on living room floors did not significantly differ between samples from Erfurt and Hamburg (table 2). Endotoxin, ß(1→3)-glucan and dust levels per square meter sampled in the living room were approximately two times higher (p < 0.05) in homes built between 1971 and 1990 compared to the older homes built between 1945 and 1970. Mean (GM) endotoxin concentration per gram dust was also almost twice as high in the 'new' buildings (not significant; NS). Glucan concentrations (per g dust) were only slightly elevated in the 'new' buildings (NS). Excluding homes from Hamburg did not alter the results. Endotoxin, ß(1→3)-glucan and dust levels on living room floors expressed per m^2 were 2 to 3 times higher (p < 0.05) in homes with a central heating system compared to homes with individual heaters (mainly oil and gas heaters). Mean (GM) endotoxin concentration per gram dust was almost two-fold higher (p < 0.1) in the centrally heated homes, while ß(1→3)-glucan concentration per g dust was only marginally higher (NS). Excluding homes from Hamburg (that all had central heating) did not alter the results. Important to acknowledge is that central heating was closely associated with 'new' buildings and individual heaters with 'old' buildings. Insulation by means of double glazed windows and reported presence of damp spots in the home had no correlation with endotoxin, glucan or dust levels. Higher temperatures (≥ 22.7 °C) were associated (p < 0.05) with lower ß(1→3)-glucan levels (per gram dust and per square meter; table 2). No other associations existed between biocontaminant levels and temperature and relative humidity data. The effects of pets in the home could not be evaluated since in Hamburg all occupants had pets while in Erfurt none of the occupants had pets in the home.

Table 2. Geometric mean of living room endotoxin, ß(1→3)-glucan (per m² and per gram dust) and dust (per m²) concentrations in relation to various home characteristics.

Characteristics		N	Endotoxin EU/m²	Endotoxin EU/g	Glucan µg/m²	Glucan µg/g	Dust mg/m²
City[a]	Hamburg	5	11,200	18,600	863	1,437	596
	Erfurt	20	6,700	17,000	497	1,261	395[*]
Home built in	1945-1970	13	5,200	14,500	424	1,187	358
	1971-1990	12	10,900[**]	21,000	735[**]	1,408	523[**]
Home built in[b]	1945-1970	11	4,300	13,800	369	1,176	314
	1971-1990	9	11,500[**]	22,000	713[**]	1,366	523[**]
Heating	individual	10	4,000	12,800	369	1,187	311
	central	14	11,300[**]	21,200[*]	713[**]	1,339	534[**]
	central[c]	9	11,300[**]	22,700[*]	645[*]	1,300	498
Windows	single glazing[d]	8	5,900	16,000	503	1,353	372
	double-glazing	17	8,300	17,900	584	1,261	459
	double-glazing[b]	12	7,300	17,700	498	1,200	412
Damp spots	yes	5	7,100	12,500	804	1,422	567
	no	20	7,600	18,800	508	1,261	399
Temperature[b]	<22.7°C	9	8,700	19,300	699	1,556	450
	≥22.7°C	10	5,600	15,700	384[**]	1,075[**]	354
Relative humidity[b]	<42.6%	10	5,500	14,500	455	1,200	380
	≥42.6%	9	8,800	21,200	578	1,380	416

[*] p<0.1, [**] p<0.05

[a] Homes have been selected based on practical choices and do not allow a valid comparison of both cities.

[b] Only houses from Erfurt.

[c] Only houses from Erfurt with central heating system.

[d] double single-glazed windows

No consistent associations were found between home characteristics and biocontaminant and dust levels in bedroom floor samples (data not shown). Biocontaminant levels in mattress samples from Erfurt were considerably higher than those from Hamburg as shown in table 3. Dust levels also differed but not

as much. Because of these differences, relations with home characteristics were analysed only within the group of homes in Erfurt, indicating no significant associations between biocontaminant levels or dust levels and any of the studied home characteristics (data not shown).

Table 3. Geometric mean of mattress endotoxin, ß(1→3)-glucan (per m² and per gram dust) and dust (per m²) concentrations in samples from Hamburg and Erfurt.

| Characteristic | N | Endotoxin | | Glucan | | Dust |
		EU/m²	EU/g	µg/m²	µg/g	mg/m²
Hamburg[a]	5	300	700	317	596	528
Erfurt[a]	20	4,200**	3,900**	862**	804	1,075**

** p<0.05

[a] Homes have been selected based on practical choices and do not allow a valid comparison of both cities.

Figure 1a shows lack of association between total culturable mold counts and ß(1→3)-glucan levels per g dust while a statistically significant positive association was observed when concentrations were expressed per m² and one outlier was omitted (fig 1b). Total mold CFU measurements (per m²) were also positively associated with endotoxin and dust concentrations expressed per square meter (R=0.53 and 0.66 respectively; p<0.01). A significant positive association was found between culturable molds from the genus *Alternaria* and ß(1→3)-glucan levels both per m² (fig 1c) and per gram dust (fig 1d). Colony counts of *Alternaria* were not associated with endotoxin levels and only weakly with dust levels (R=0.35, p<0.1). The other determined mold genera (*Aspergillus* and *Penicillium*) were not associated with ß(1→3)-glucan levels nor with measured endotoxin or dust levels, while *Cladosporium* CFU counts were only weakly associated with ß(1→3)-glucan when expressed per gram dust (R=0.37; p<0.1).

Discussion

Few studies have been published describing endotoxin levels in house dust. A previous study showed average endotoxin levels in 487 dust samples from Dutch houses of 10,000 - 20,000 EU/g dust.[28] In that study endotoxin levels of

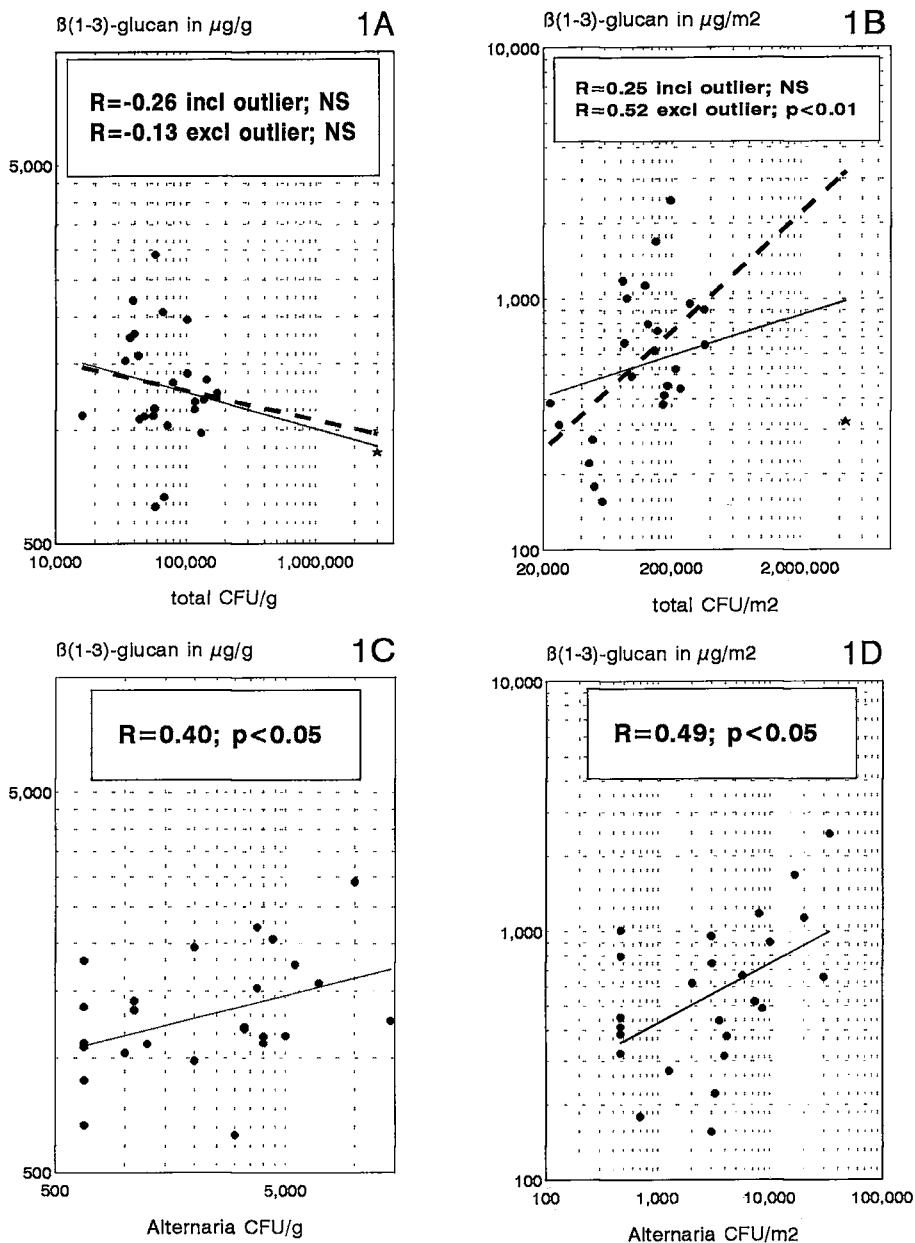Fig 1. Regression between total CFU and ß(1→3)-glucan (n = 25) per gram dust (1A) and per m² (1B) and between Alternaria CFU and ß(1→3)-glucan per gram dust (1C) and per m² (1D). Dotted lines represent regression when one outlier (*) was excluded.

mattress dust were substantially lower than for floor dust samples (unpublished results) similar to what is described in this paper. Michel *et al*[3] found a mean level of 2,590 ng/g house dust (approximately 25,900 EU/g) in bedroom floor and mattress dust from 28 Belgian homes of asthmatic patients, which is also in the same order of magnitude as found in our study. Cole *et al*[32] found a mean endotoxin level of 74,000 ng/g (approximately 740*10³ EU/g) for floor dust collected in a carpeted university building in the United States, which is 40 times higher than found in our study. This large difference may be explained by differences in dust composition or to differences in analytical procedures.

At present, it is not possible to make reliable estimates of the dose that can potentially be inhaled by subjects living in homes with elevated levels of endotoxin in settled dust, and therefore the health significance of our findings is not yet clear. Some studies however, showed that endotoxin levels in house dust, which were comparable to the levels reported here, were associated with severity of symptoms in asthmatic patients.[3,6,33]

No data is available on ß(1→3)-glucan levels in house dust. Rylander *et al*[25] measured mean airborne ß(1→3)-glucan concentrations of 0.2 - 0.6 ng/m³ in office and school buildings using a ß(1→3)-glucan reactive LAL assay. A subsequent study showed mean ß(1→3)-glucan levels of 41.9 ng/m³ and less than 5 ng/m³ in a mold infested and non-mold infested house, respectively.[4] Again, it is difficult to give an estimate of airborne levels of ß(1→3)-glucan based on levels measured in house dust as was done in our study. However, assuming that composition of settled and airborne dust is similar, it can be calculated that approximately 5 μg/m³ house dust should be airborne (based on a mean ß(1→3)-glucan level of 1 mg/g dust as found in our study) to explain a concentration of 5 ng/m³ in a non-mold infested house as described by Rylander *et al*.[4] This estimated dust concentration of 5 μg/m³ does not seem unrealistic and thus (assuming that both ß(1→3)-glucan assays give comparable results) 'normal' airborne ß(1→3)-glucan concentrations in non mold infested houses may be around 5 ng/m³.

Strong correlations among absolute levels (per m²) of endotoxin, ß(1→3)-glucan and dust, and the lack of correlation between endotoxin and ß(1→3)-glucan levels when expressed per gram dust, indicated that variance in biocontaminant levels per m² (within the same sampling location) was largely determined by the amount of dust sampled. Currently, it is not clear which measure (per gram dust

or per square meter) may better reflect the actual exposure of the occupants and therefore both measures should be explored in further epidemiologic investigations.

Michel *et al*[3] were not able to identify home characteristics associated with the level of endotoxin per gram house dust. Our study suggests that endotoxin and ß(1→3)-glucan levels were associated with type of house, most likely characterized by heating system and/or age of the home. Living room floor samples collected in newer homes with central heating system contained more endotoxin and ß(1→3)-glucan (per m² and gram dust). Wood *et al*[34] found a similar difference in culturable mold concentration (per gram dust) between 'new' (built after 1975; n = 12) and 'old' homes (built between 1945 - 1975; n = 30) in the Unites States of America. No explanations were given. Possibly, climatological differences in the indoor environment as well as differences in ventilation between 'old' and 'new' houses contributed to the observed differences. A direct relation with measured temperature and relative humidity was, however, not found in our study and no differences existed with these variables between living rooms in 'old' and 'new' houses. Interestingly, endotoxin and ß(1→3)-glucan levels were not associated or showed an inverse association (age of home) with home characteristics that have been suggested to determine mite allergen levels in house dust (damp spots, relative humidity, age of mattress).[35] In addition, microbial component levels were lowest in mattresses, while mite allergen levels are usually highest in mattresses.[35] Moreover absence of correlation between endotoxin and mite allergen has been reported by others.[3,6] Thus endotoxin and ß(1→3)-glucan levels in house dust seem to be, at least partially, determined by other characteristics.

Much lower endotoxin and ß(1→3)-glucan concentrations in mattress samples from Hamburg could not be explained by difference in sampling protocol (identical for both cities) nor by material or age of the mattress (data not shown). A sound comparison between both cities can, however, not be made since selection of houses may not have been representative and only a small number of samples were taken.

Only weak associations between culturable mold levels and ß(1→3)-glucan concentrations in house dust were found. The reason may be that only culturable molds were counted, while ß(1→3)-glucan most likely represents total mold biomass, including non-culturable propagules. Also, CFU and ß(1→3)-

glucan measurements were performed on two different dust samples, one of which was sieved, while the other was extracted without prior sieving. In addition ß(1→3)-glucan is not completely specific as marker for molds since it may also originate from plants and some bacteria. Detection of ß(1→3)-glucan from other sources is, however, relevant when its relation with health effects is studied, since some evidence exists that plant ß(1→3)-glucans may also be biologically active.[36] It is not clear why the best association was found with *Alternaria* spore counts.

In conclusion, this study shows that endotoxin and ß(1→3)-glucan can readily be detected in floor and mattress dust collected in German houses and that the levels are similar as reported previously for endotoxin levels in Belgium[3] and the Netherlands.[28] This study further suggests that endotoxin and ß(1→3)-glucan levels in house dust are determined by type of home (most likely characterized by heating system and/or age of the home) and not so much by home characteristics that have been suggested to be associated with mite allergen levels in house dust.

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Chapter 6

Fungal extracellular polysaccharides in house dust as a marker for exposure to fungi: relations with culturable fungi, reported home dampness and respiratory symptoms

Abstract

Epidemiologic studies have demonstrated an association between indoor fungal growth and respiratory symptoms. In only a few studies fungal exposure was actually measured, however. Main objectives of this study were to evaluate the measurement by enzyme immunoassay (EIA) of extracellular polysaccharides (EPS) of *Aspergillus* and *Penicillium* species (EPS-*Asp/Pen*) in house dust, as a marker for fungal exposure, and to study the relations between EPS-*Asp/Pen* levels and home dampness, and respiratory symptoms in children. Extracts of house dust samples from bedroom and living room floors and mattresses from homes of 31 children with, and 29 children without chronic respiratory symptoms were analysed for EPS-*Asp/Pen*. EPS-*Asp/Pen* were readily detectable (40-46,513 ng eq/g dust) in 161 house dust extracts, with highest concentrations in living room floor dust. EPS-*Asp/Pen* levels were 2-3 times higher on carpeted floors than on smooth floors. EPS-*Asp/Pen* were significantly correlated with total culturable fungi (r = 0.3-0.5) and with house dust mite allergens (*Der p* I; r = 0.3-0.5). EPS-*Asp/Pen* levels in living room floor dust were positively associated with occupant-reported home dampness. This was not observed for EPS-*Asp/Pen* in bedroom floor and mattress dust. EPS-*Asp/Pen* levels in living room floor dust were positively associated with respiratory symptoms. EPS-*Asp/Pen* in bedroom floor and mattress dust showed a reversed association, possibly due to allergen-avoidance measures taken in the bedroom. It was concluded that the EIA for fungal EPS-*Asp/Pen* may be a very useful method for exposure assessment of indoor fungi.

Introduction

Exposure to mite and pet allergens in the home environment is an important risk factor for the development of respiratory allergy and asthma. The role of fungal exposure is less well understood. Several population-based studies have suggested that fungal exposure, together with house dust mite exposure, might account for the frequently reported association between living in damp housing conditions and respiratory disorders.[1-5] Exposure to fungi and damp housing conditions in these studies has invariably been assessed by questionnaire, however, and although several authors have attempted to relate reports of dampness and exposure to fungi, it is currently not clear to what extent questionnaire reports of damp and mold spots correlate with exposure to relevant fungal components. It is also not clear which fungal components and which inflammatory and/or allergic mechanisms primarily account for the presumed pathogenic effects of indoor fungal exposure. Atopic sensitization to fungal allergens may play a role[5, 6] but other inflammatory mechanisms might also be of importance, including type III allergy to fungal-specific antigens, or non-immune inflammatory reactions to fungal components such as e.g. $\beta(1\rightarrow3)$-glucans, a major cell wall component of fungi.[7-9]

Limited data from field studies is available on indoor fungi and/or fungal allergen levels. The traditional counting of culturable propagules in house dust has been applied in some studies[10-12], but has serious drawbacks including poor reproducibility and limitation to culturable propagules only. Quantitative assessment of major fungal allergens in house dust or indoor air have been reported only incidentally. Few major fungal allergens have been characterized and allergen stability and production may be highly variable.[13-15] General markers for fungal biomass in house dust or indoor air such as ergosterol and $\beta(1\rightarrow3)$-glucans have been described[16-18], but have not yet routinely been applied in indoor studies and do not allow identification of fungal species or genera.

There is thus a need for specific, sensitive and cost-efficient methods to assess fungal exposure quantitatively in the indoor environment, that would allow at least partial identification of fungal species present. In this study a sandwich enzyme immunoassay (EIA) was evaluated with which extracellular polysaccharides (EPS) of *Aspergillus* and *Penicillium* species (EPS-*Asp/Pen*) can be specifically measured. EPS are stable carbohydrates secreted or shed during fungal growth, with an antigenic specificity usually at the genus level, while EPS

from *Aspergillus* and *Penicillium* spp. are cross reactive.[19-22] Although at present there is no evidence for a pathogenic role of EPS in allergic or inflammatory reactions to fungal components, EPS may be a good quantitative marker for fungal biomass in house dust. In addition, genus-specific EPS assays may provide important qualitative information allowing differentiation between indoor and outdoor fungal genera. We report here EPS-*Asp/Pen* levels in house dust in a sample of 60 homes in which in a previous study dust samples had been taken for mite allergen measurements and for the determination of culturable fungi.[11, 23] The relation with culturable fungi was investigated and, in addition, relations between EPS concentrations and signs of mold and dampness in the home were studied. Moreover the relation between EPS-*Asp/Pen* and respiratory symptoms in children living in these homes was studied.

Materials and methods
Study population
Samples of settled house dust were obtained in October and November 1990 from 60 homes in The Netherlands. The homes were selected from a group of 516 homes that were included in a case-control study on home dampness, house dust mite allergen exposure and respiratory symptoms in children.[24] The selection of children (aged 6-12 years) and their homes have been described previously.[11] Briefly, homes were selected on the presence or absence of: 1) mold or damp spots anywhere in the home of the child, observed by the investigators during a home visit; and 2) reported respiratory symptoms (chronic wheeze, chronic cough, attacks of shortness of breath with wheezing, or doctor diagnosed asthma or bronchitis) of children living in those homes. Thus, four groups of homes with approximately the same group size were selected: 1) homes of children without reported respiratory symptoms and without observed dampness (n = 13), 2) homes of children without respiratory symptoms but with observed dampness anywhere inside the house (n = 16), 3) homes of children with reported respiratory symptoms, without observed dampness (n = 12), and 4) homes of children with respiratory symptoms and with observed signs of dampness (n = 19). Two subgroups within the group of symptomatics were defined i.e. a group with doctor-diagnosed asthma ('asthma'; n = 9), and a group with doctor-diagnosed bronchitis ('bronchitis'; n = 9). Characteristics of the study population have been described elsewhere.[11] Respiratory symptoms as

well as resident reported dampness were assessed by questionnaire.[11, 23]

Dust sampling and extraction
Dust was sampled from floors of living rooms and bedrooms (2 m^2 in 4 minutes) and from the childrens' mattresses (approximately 2 m^2 in 2 minutes) according to an internationally standardized protocol[25], using vacuum cleaners (Philips Topomatic T518, 1000W) equipped with a special nozzle (ALK, Horsholm, Denmark) to collect dust on paper filters (ALK, 70 mm) as described before.[23] When rooms had a smooth floor with a rug, 1 m^2 of the rug and 1 adjacent m^2 of the smooth floor cover were sampled. Dust samples were weighed and stored at -20 °C until extraction. Of each dust sample, 200 mg was extracted by shaking in 2 ml ammoniumbicarbonate buffer (0.125 M) with NaN$_3$ (0.1 % w/v) for two hours.[23] Extracts were stored in aliquots at -20 °C before analyses on *Der p* I and EPS-*Asp/Pen* content. Methods for *Der p* I analyses have previously been reported.[23] In total 19 extracts were not available for EPS-*Asp/Pen* analysis because the entire volume of the extracts was used for previous dust mite allergen analyses. From these extracts 18 came from living room floor dust samples.
Separate dust samples were collected in the same time period from bedroom floors and mattresses of the same homes to determine the concentration of culturable fungi in dust.[11] Methods used for fungal counts have been described previously.[11]

EPS-Asp/Pen production
EPS from *Penicillium digitatum* was prepared as previously described.[19] Briefly, spores of *Penicillium digitatum* were inoculated in dialyzed malt extract broth and incubated at 25°C for 7 days with orbital shaking at 90 rpm. NaN$_3$ was added (0.1% w/v) and incubation continued for another 16 hrs at 25 °C. Mycelia and proteins were removed by filtration and ammonium sulfate precipitation, respectively. The supernatant was subsequently dialyzed against distilled water and applied to a PBS equilibrated Sepharose CL-4B gel filtration column (2.5 x 95 cm). Column fractions were tested by an EPS-*Asp/Pen* EIA (see below) and peak fractions were pooled, concentrated with Amicon concentrators, dialyzed against distilled water and freeze dried. The antigenic responses against EPS from *Penicillium digitatum* are directed to the

galactomanans in EPS, of which ß(1→5)-D-galactofuranosides are immuno-dominant.[26] The antigenic properties of EPS are identical for all species within the *Penicillium* and *Aspergillus* genera. Anti EPS-*Asp/Pen* IgG antibodies were raised in rabbits of which a part was subsequently conjugated with peroxidase.[19]

EPS-Asp/Pen analysis

EPS-*Asp/Pen* were measured in the dust extracts almost 4 years after sample collection and extraction, with a specific sandwich enzyme immunoassay (EIA). An essentially identical assay was described earlier, by Notermans *et al*[19] and Kamphuis *et al*[27] as a means for large scale screening for fungal contamination of food products. Polystyrene high capacity microtitre plates (no 655061, Greiner) were coated overnight at 4°C with polyclonal rabbit anti EPS-*Asp/Pen* IgG antibodies (100 μl of 10 μg/ml antibody in PBS, pH 7.0). After washing with PBS containing 0.05% (v/v) Tween-20 (PBT), 100 μl of five serial dilutions (diluted in 1% w/v BSA in PBT; PBTB) of test samples (routinely tested at 1/10 - 1/160) and 10 serial dilutions of EPS standard (0.05 - 25 ng/ml) were added to the wells. For calibration, EPS of *Penicillium digitatum* was used, which was an identical EPS preparation as was used for immunization for the production of specific IgG antibodies. On each plate 10 negative controls (blanks; PBTB) were tested. The microtitre plate was incubated at 22°C for 1.5 hours. After washing, 100 μl of peroxidase-labelled polyclonal rabbit anti EPS-*Asp/Pen* IgG antibodies diluted 1/2,000 in PBTG was added and incubated for 1.5 hour at 22°C. After washing, 200 μl tetramethylbenzidine (TMB; 0.1 mg/ml) in 0.11 M citric acid/acetate buffer, pH 5.5, containing 0.006% H_2O_2 was added and incubated for 20 minutes at 20°C. The enzyme reaction was terminated by the addition of 50 μl 2 N H_2SO_4, and the optical density was read at 450 nm.

Since at lower dilutions dose-response curves of the samples were often not parallel to that of the standard, the EPS-*Asp/Pen* concentrations in the house dust samples were estimated by end-point titration, comparing the titre of each extract with the titre of the standard. The titre was defined as the dilution at which an optical density at 450 nm (OD-450) of 0.1 above the background of the reagent blank was found. To calculate the titre a regression was performed between log dilution and logit transformed OD-450 values of the multiple diluted standard or samples. The EPS-*Asp/Pen* concentration in a test sample was calculated as the ratio between the titre of the sample and the titre of the

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standard, multiplied by the EPS concentration of the standard. Since not all EPS standard preparations show, on a weight basis, equal reactivity in the EIA, sample concentrations were expressed in ng-equivalents/g dust (ng eq/g), thus referring to the concentration of the EPS standard preparation used in this particular study. All samples were analyzed in duplicate in two independent test runs and concentrations were presented as the mean of the duplicate analyses.

Crude fungal extracts
Mixtures of crude extracts of 4 *Aspergillus* spp.; *A. versicolor, A. fumigatus, A. amstelodami, A. nidulans* (ALK; prod no 22.00), of 4 *Penicillium* spp.; *P. brevicompactum, P. expansum, P. notatum, P. commune* (ALK; prod no 25.00), and of 3 *Cladosporium* spp.; *C. cladosporiodius, C. elatum, C. herbarum* (ALK; prod no 23.00), respectively, were used to confirm the specificity of the EPS-*Asp/Pen* EIA.

Measurement of indoor relative humidity
In the child's bedroom of 19 dwellings, relative humidity and temperature were measured with thermohygrometers for periods of 22-42 days in the same time period as dust sampling was performed, as previously described.[23] The average relative humidity for the whole measurement period was used in further analyses.

Statistical analysis
Statistical analyses were performed using SAS statistical software (version 6.12; SAS institute, Cary, N.C.). Because data were best described by a lognormal distribution, EPS-*Asp/Pen* concentrations were ln transformed, and mean concentrations were expressed as geometric means (GM) with a geometric standard deviation (GSD). Pearson correlations were calculated using SAS PROC CORR. Multivariate analyses to assess the association between EPS-*Asp/Pen* levels and CFU-counts were performed by using multiple regression analysis on ln-transformed data (SAS PROC REG). Statistical testing comparing EPS-*Asp/Pen* levels in homes with and without specific characteristics (type of floor, damp and mold spots), and comparing EPS-*Asp/Pen* levels between non-symptomatic and symptomatic subjects (and subgroups of symptomatics; 'asthma' and 'bronchitis') was performed by using a t-test (SAS PROC TTEST)

on ln-transformed data. When differences between floors with different covers (smooth vs carpet) were considered, smooth floors with a rug were grouped together with carpet floor covers. Odds ratios (ORs) were calculated by means of logistic regression using SAS PROC LOGISTIC. The continuous EPS-*Asp/Pen* exposure variables (for living room, bedroom and mattress) were dichotomized using median values as cutpoints (living room; 2495 ng eq/g, bedroom; 957 ng eq/g; mattress 1530 ng eq/g) to define 'high' and 'low' exposure groups and calculate ORs for various health outcome variables. All presented odds ratios were adjusted for house dust mite allergen levels by using a multiple logistic regression model. *Der* p I exposure variables were therefore also dichotomized based on their median values (living room; 7.2 μg/g, bedroom; 2.4 μg/g; mattress 4.1 μg/g).

Results

The detection limit of the assay was approximately 0.2 ng eq/ml. Reproducibility expressed as a coefficient of variation (CV) was 16% based on duplicate analyses of the house dust extracts. Commercial crude fungal extracts of *Aspergillus* and *Penicillium* spp. showed dose response curves parallel to that of the EPS-*Penicillium digitatum* standard at concentrations ranging from 50 - 5,000 ng/ml. In contrast, the extract of the *Cladosporium* spp. mixture did not show any response at these concentrations (figure 1). The same *Cladosporium* preparation contained comparable amounts of *Cladosporium*-specific EPS, as determined with a similar EIA with specific rabbit polyclonal anti *Cladosporium*-EPS antibodies (data not shown).

EPS-*Asp/Pen* concentrations were readily detectable in the 161 available house dust extracts, with concentrations ranging from 40 to 46,537 ng eq/g house dust (table 1). EPS-*Asp/Pen* concentrations were approximately 2-3 times higher in house dust from textile floor covers compared to completely smooth floors. In living room floor dust EPS-*Asp/Pen* concentrations were highest, being almost 4 times higher than EPS-*Asp/Pen* concentrations in bedroom floor dust samples (paired comparison of data from textile floor covers only; P < 0.01; n = 37).

EPS-*Asp/Pen* concentrations in bedroom floor dust and mattress dust were significantly but not very strongly correlated with total culturable fungi and fungi of various specific genera (table 2). All of the individual genus-specific fungal counts were significantly correlated with total CFU counts (r = 0.4-0.8;

p<0.01), with the exception of *Ulocladium* spp in floor dust samples and *Aspergillus* spp in mattress dust samples (data not shown). In bivariate analyses with both total culturable fungi and individual genus-specific fungal counts in one regression model, EPS-*Asp/Pen* levels appeared to correlate in all cases more strongly with counts of total fungi than with counts of any of the individual genera, including *Aspergillus + Penicillium* (data not shown).

EPS-*Asp/Pen* levels were significantly correlated with *Der p* I levels (expressed per g dust) in living room floor, bedroom floor and mattress dust, with Pearson correlation coefficients of 0.39, 0.47 and 0.32, respectively (p<0.05). Similar to what was observed for EPS-*Asp/Pen* levels, highest *Der p* I levels were measured in living room floor dust (GM = 4,228 ng/g) compared to bedroom floor dust (GM = 2,099 ng/g).
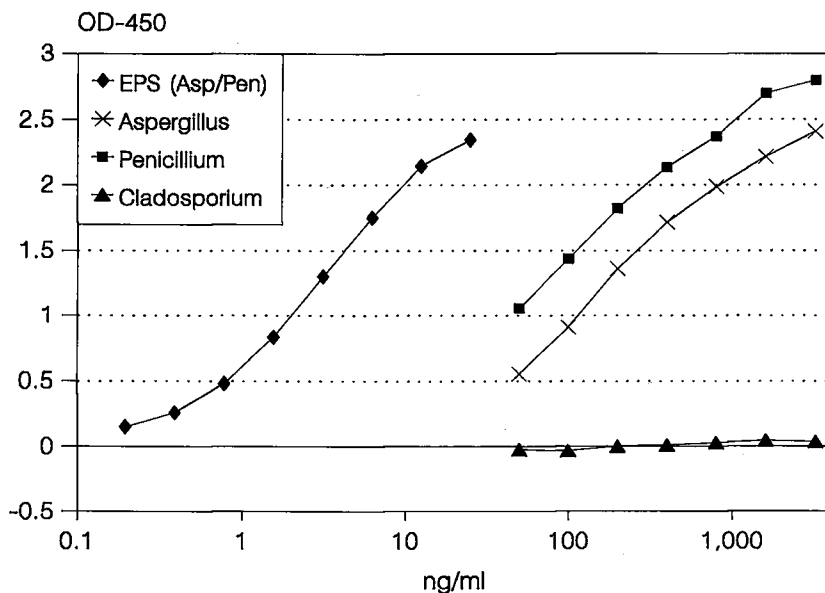
Fig 1. Dose-response curves for three fungal mixtures (Aspergillus spp., Penicillium spp., Cladosporium spp.) and the EPS reference preparation (Penicillium digitatum).

Table 1. Geometric mean (GM) and geometric standard deviation (GSD) of EPS-Asp/Pen concentrations in ng eq/g house dust from living and bedroom floors and mattresses. Textile floor cover was compared with smooth floor cover.

Location	n	GM	GSD	range (min-max)
Living room	42	2,919	4.2	131 - 46,513
smooth	5	952	3.6	253 - 3,630
textile	37	3,395*	4.0	131 - 46,513
Bedroom	60	987	3.9	40 - 21,917
smooth	18	530	2.8	78 - 3,496
textile	42	1,289**	4.1	40 - 21,918
Mattress	59	1,281	2.8	93 - 27,806

* $p < 0.1$, ** $p < 0.05$; compared to smooth floor covers

Table 2. Pearson correlation coefficients for the relation between EPS-Asp/Pen concentrations (ng eq/g dust) and CFU concentrations (CFU/g dust) of the most frequently isolated fungal species as well as total number of culturable fungi (in parentheses: number with detectable CFU-counts/total number of observations).

Fungal species	Bedroom	Mattress
Aspergillus spp	0.22 (26/36)	0.02 (30/37)
Penicillium spp	0.36 (36/36)**	0.14 (34/37)
Eurotium spp	0.41 (21/36)**	0.20 (29/37)
Cladosporium spp	0.39 (36/36)**	0.28 (34/37)*
Alternaria spp	0.46 (31/34)***	0.25 (37/37)
Ulocladium spp	0.26 (23/35)	0.06 (32/37)
Aspergillus + *Penicillium* spp	0.37 (36/36)**	0.20 (37/37)
Total CFU	0.45 (53/53)***	0.34 (55/55)**

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$

Table 3. *Association of EPS-Asp/Pen concentrations (ng eq/g) and self reported mold and damp spots in the living room, bedroom or anywhere in the house. Samples from smooth floor covers were excluded from the analyses.*

	Living room		Bedroom		Mattress	
mold and damp patches (yes/no)	**n**	**GM (GSD)**	**n**	**GM (GSD)**	**n**	**GM (GSD)**
no mold and/or damp spots in the home	14	2,077 (2.9)	17	1,150 (3.3)	21	1,080 (2.9)
mold spots in the room	4	12,875 (1.5)***	2	568 (-)	3	590 (3.1)
damp spots in the room	10	8,594 (3.7)***	4	810 (1.8)	7	1,016 (2.3)
mold spots in the home	20	4,235 (4.4)	17	2,038 (3.9)	28	1,478 (2.9)
damp spots in the home	20	5,843 (4.0)**	21	1,251 (4.3)	31	1,496 (2.7)
mold or damp spots in the home	23	4,580 (4.4)*	23	1,491 (4.6)	35	1,430 (2.7)

* $p<0.1$, ** $p<0.05$, *** $p<0.01$; compared to homes with no mold and/or damp spots

Table 3 compares EPS-*Asp/Pen* levels in house dust between homes with occupant-reported damp and/or mold spots in the living room, bedroom or anywhere in the house, and homes with no damp spots or mold spots in the home. The analyses with floor dust EPS-*Asp/Pen* concentrations only included observations from textile floor covers, to avoid confounding by differences in EPS-*Asp/Pen* concentrations between textile and smooth floor covers. EPS-*Asp/Pen* levels were approximately 6 and 4 times higher (p < 0.01) in living rooms with occupant-reported mold and damp spots, respectively, compared to living rooms in homes where no mold or damp spots were reported. No clear associations were found for bedroom and mattress EPS-*Asp/Pen* concentrations between bedrooms with reported damp and mold spots, and bedrooms from homes without damp or mold spots. EPS-*Asp/Pen* concentrations were 2-3 times higher in living rooms and only slightly higher in bedrooms and mattresses in homes with reported damp and mold spots (mold or damp spots anywhere in the house) compared to homes with no damp and mold spots. No relations between EPS-*Asp/Pen* concentrations and investigator-observed damp and mold spots were found (data not shown). Mattress EPS-*Asp/Pen* concentrations were correlated with relative humidity of the air in the bedroom (R = 0.54; P < 0.05), whereas no correlation with EPS-*Asp/Pen* concentrations in bedroom floor dust was found.

EPS-*Asp/Pen* levels in the living room were higher in homes of symptomatic children compared to non-symptomatic children (controls), whereas they were lower in bedroom floor and mattress dust samples (table 4). Consequently, odds ratios (ORs) showed positive associations between living room floor EPS-*Asp/Pen* concentration ('high' vs 'low') and respiratory symptoms, with highest OR for doctor diagnosed asthma (OR = 9.5; p < 0.1), and negative associations for bedroom and mattress EPS-*Asp/Pen* concentrations. All ORs were adjusted for house dust mite allergen levels. Confidence intervals for ORs associated with living room EPS-*Asp/Pen* concentrations were wide for the asthma and bronchitis group due to small number of children with these symptoms, and the relatively small number of control children for whom living room exposure data were available. A reversed trend for EPS-*Asp/Pen* levels of bedroom floors and mattresses was possibly due to allergen avoidance measures taken particularly in the childs' bedroom. Thirty percent of the parents of symptomatic children reported to have purchased a new floor cover in the childs' bedroom because of

Table 4: EPS-Asp/Pen concentrations (GM), and odds ratios for high and low EPS-Asp/Pen concentrations, comparing the non symptomatic subjects with subjects with general respiratory symptoms, doctor diagnosed asthma and doctor diagnosed bronchitis (Geometric Standard Deviation and 95% confidence intervals in parentheses). Analyses were controlled for house dust mite concentrations.

	No resp. symptoms	Resp. symptoms	Asthma	Bronchitis
Living room	n = 18	n = 24	n = 7	n = 8
EPS-*Asp/Pen* in ng eq/g (GSD)	2,568 (3.8)	3,214 (4.5)	7,709 (3.5)[*]	3,815 (3.2)
Odds Ratio (95% CI)	1.0	2.5 (0.7-9.3)	9.5 (0.9-103.5)[*]	3.3 (0.5-20.9)
Bedroom	n = 29	n = 31	n = 9	n = 9
EPS-*Asp/Pen* in ng eq/g (GSD)	1,549 (4.1)	647 (3.3)[**]	781 (4.1)	386 (3.7)[**]
Odds Ratio (95% CI)	1.0	0.2 (0.0-0.6)[***]	0.1 (0.0-0.7)[**]	0.2 (0.0-1.1)[*]
Mattress	n = 28	n = 31	N = 9	n = 9
EPS-*Asp/Pen* in ng eq/g (GSD)	1,771 (2.3)	956 (2.9)[**]	1,765 (2.4)	521 (3.3) [***]
Odds Ratio (95% CI)	1.0	0.3 (0.1-0.9)[**]	0.8 (0.2-3.9)	0.2 (0.0-0.9)[**]

[*] $p < 0.1$, [**] $p < 0.05$, [***] $p < 0.01$; compared to the non symptomatic group

respiratory symptoms of their children, whereas none of the controls reported to have done so. These differences in allergen avoidance measures between cases and controls were much smaller when new floor covers in the living room were considered (16% in cases vs 7% in controls). Moreover 10% of the cases had changed mattresses because of respiratory complaints compared to 0% in the controls. Restricting the analyses to bedrooms where no allergen avoidance measures had been taken in the past and no smooth floor covers were present resulted in marginally higher and less significant odds ratios for general respiratory symptoms. For bedroom floors the odds ratio shifted from 0.2 (p < 0.01) to 0.3 (95% CI 0.1-1.1, p < 0.1, n = 39 of which 18 symptomatics) and for mattresses the odds ratio shifted from 0.3 (p < 0.05) to 0.5 (95% CI 0.4-5.0, p > 0.1, n = 38 of which 18 symptomatics). A similar statistical analysis, adjusting for smooth floor covers and allergen avoidance measures, could not be performed for the individual asthma and bronchitis groups due to low number of subjects with these symptoms.

Discussion

In this study we evaluated a sandwich EIA to measure EPS-*Asp/Pen* as a marker for indoor fungal exposure. Application of the EIA showed that EPS-*Asp/Pen* could readily be measured, with adequate reproducibility and sensitivity, in settled house dust. Main advantages of EPS tests are the ability to at least partially identify specific fungal genera in house dust, store samples frozen prior to analysis and low costs per sample. These tests do not discriminate at species level, which may both be an advantage and disadvantage depending on the goal of the study.

EPS-*Asp/Pen* levels were significantly but not very strongly correlated with counts of culturable fungi. Reasons may be 1) that only culturable fungi were counted, while EPS-*Asp/Pen* most likely represents total fungal biomass of *Aspergillus* and *Penicillium* spp., including non-culturable spores and mycelia, and 2) that CFU and EPS-*Asp/Pen* measurements were performed on two different dust samples. We previously reported that in the same study sample a poor reproducibility of total CFU counts in duplicate house dust samples (taken within a 6-week interval) from the same location was found.[28] Variability in isolated genera and species between duplicate samples was even more substantial. Measurement error for genus specific culturable counts was thus

larger than for total culturable counts. Considering that *Aspergillus* and *Penicillium* spp. constitute a large and relatively constant fraction of total culturable fungi, it is likely that the difference in measurement error may explain why EPS-*Asp/Pen* levels in house dust were more strongly associated with total culturable counts than with genus specific counts of *Penicillium* and *Aspergillus*. High correlation between the sum of *Aspergillus* and *Penicillium* spp and total culturable fungi (r = 0.8 for bedroom floor and 0.5 for mattresses; p < 0.01) indicated that the fraction of *Aspergillus* and *Penicillium* spp, was indeed more or less constant. A consistent significant association with total culturable fungi suggests that at least in the present study EPS-*Asp/Pen* was a good marker for total fungal biomass. Whether this would be generalizable to other indoor studies is unclear and probably depends on the variation and rank order of the genera present in the indoor environment of those particular studies.

Significant associations between EPS-*Asp/Pen* and the other culturable fungal genera were most likely due to correlation between total CFU and CFU counts of specific genera. Cross reacting epitopes of EPS from other genera with the anti-EPS-*Asp/Pen* antibodies do not seem likely, since a crude extract of 3 *Cladosporium* spp. was not reactive in our assay (fig 1). Moreover, the specificity of the anti-EPS-*Asp/Pen* antibodies used in the present study has previously been confirmed in a sandwich assay with 32 fungal species, in which none of the following genera showed positive reactions; *Mucor, Rhizopus, Botrytis, Geotrichum, Cladosporium, Fusarium* and *Tricothecium*.[20]

Smooth floor covers contained 2-3 times less EPS-*Asp/Pen* per gram dust compared to textile floor covers. Fungal CFU counts in bedroom floor samples showed a similar difference.[11] Wickman *et al*[12] reported only slightly higher numbers of CFU in dust from wall-to-wall carpets. A substantial difference in concentration (6-14 times) between smooth and textile floor covers is generally also observed for house dust mite allergens in The Netherlands.[23] Levels of EPS-*Asp/Pen* were higher in living room floor dust than in bedroom floor or mattress dust, similar to what was observed for *Der p* I levels in dust. EPS-*Asp/Pen* levels were moderately correlated with levels of house dust mite allergen (*Der p* I). Wood *et al*[29] also demonstrated an association between fungal CFU counts and house dust mite allergens. The correlations are probably due to dependence of both mites and fungi on high humidity levels.

EPS-*Asp/Pen* levels in living room floor dust were higher when occupants

reported mold or damp spots in the living room or anywhere in the home, whereas bedroom floor and mattress EPS-*Asp/Pen* levels did not show associations with a report of mold or damp spots in the bedroom or anywhere in the home. Mattress EPS-*Asp/Pen* concentrations were, however, correlated with relative air humidity in the bedroom. Lack of a clear association between EPS-*Asp/Pen* levels and damp characteristics with bedroom dust samples might have been due to allergen avoidance measures, taken particularly in the bedroom. This may also explain why we observed much lower levels of EPS-*Asp/Pen* in bedroom floor dust compared to living room floor dust. Several studies have investigated the relation between signs of fungal growth and dampness in the home and fungal CFU counts in house dust or in the air, and have found at best only weak associations.[1, 11, 12, 17, 30, 31] A reason for this lack of a stronger association may be found in the limitations of the traditional methods to assess fungal exposure (CFU counts) in these studies, as discussed earlier in this paper, and by Dales *et al.*[17] The relatively strong association found in our study for living room concentrations may indicate that the EPS-*Asp/Pen* assay provides a better method to assess indoor fungal exposure.

In contrast to occupant-reported mold and/or damp spots, no relations between investigator-observed damp and/or mold spots and EPS-*Asp/Pen* concentrations were found. We previously showed a similar difference when the relation between observer and occupant-reported dampness and *Der p* I and culturable fungi were studied[11, 23]. In these studies weak positive associations were found between occupant-reported dampness and culturable fungi and *Der p* I in house dust, while no associations were found with observer-reported dampness. Apparently, occupant reports are more reliable estimates for dampness than observer reports.

The present study suggests a positive association between EPS-*Asp/Pen* levels in living room floor dust and respiratory symptoms and a reversed association with EPS-*Asp/Pen* levels in bedroom floor and mattress dust. This reversed association may also be due to allergen-avoidance measures. We previously showed that in the larger population, from which the present sub-population was recruited [24], allergen avoidance measures biased the relation between *Der p* I concentrations and respiratory health parameters. In that study ORs increased and p-values decreased substantially after excluding subjects where allergen avoidance measures had been taken in the past in their homes. This was

particularly the case for relations with *Der p* I levels in bedroom floor and mattress dust. Allergen avoidance measures were mainly taken in the bedroom, because carpeted bedroom floors and mattresses are generally assumed to contribute most to house dust mite allergen exposure. Floor covers in living rooms were much less frequently changed. A similar pattern was also noted in the present study: allergen avoidance measures in bedrooms were taken in 30% of symptomatic and 0% of non-symptomatic children, while for the living room these prevalences were 16% and 7%, respectively. Allergen avoidance measures as an explanation for the reversed associations could, however, not be confirmed in the present study. A restriction of the analyses to bedrooms where no allergen avoidance measures had been taken in the past resulted in only marginally increased odds ratios. Larger, and preferably prospective studies are required to allow proper adjustment for allergen avoidance measures and further elucidate the relationship between EPS-*Asp/Pen* and respiratory symptoms.

In the present study, EPS-*Asp/Pen* levels in living room floor dust were associated with reported signs of mold growth and home dampness, and with respiratory symptoms. In spite of the interpretation problems, our study thus seems to confirm various other epidemiologic studies that have consistently demonstrated an association between reported mold growth and home dampness and respiratory symptoms.[2-5, 32-34] Of these studies only few, however, have used actual exposure measurements.[12, 29, 30]

We conclude that the EIA for fungal EPS-*Asp/Pen* may be a useful method for indoor fungal exposure assessment, with adequate sensitivity and reproducibility. The new assay may thus provide a useful epidemiological tool to further investigate the relationship between damp homes, fungal exposure and respiratory health.

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Chapter 7

ß(1→3)-glucan and endotoxin in house dust and peak flow variability in asthmatic children

Abstract

The role of indoor microbial exposure in the development of respiratory symptoms is poorly understood. Bacterial endotoxin and fungal ß(1→3)-glucan in house dust have been suggested to be associated with respiratory symptoms and disease severity in asthmatic subjects. In this study we investigated the relation between endotoxin and ß(1→3)-glucan levels in house dust and peak flow variability in 159 school children aged 7-11 years. Children were selected based on respiratory symptoms, such that 50% had respiratory symptoms (chronic wheeze, shortness of breath with wheeze, chronic cough and/or doctor diagnosed asthma), and 50% had no symptoms. Children self-monitored twice daily their peak flow (PEF) for a period of 16 weeks. Endotoxin and ß(1→3)-glucan were measured in house dust samples from the homes of the children. Samples were collected from living room and bedroom floors and mattresses. The relations between mean daily PEF-variability (Ampl%mean) and endotoxin and ß(1→3)-glucan levels in the home were investigated by linear regression analysis, adjusting for dust mite allergen levels, presence of pets in the home and type of floor cover (carpet vs. smooth). Unadjusted regression analyses showed that living room exposure to endotoxin and ß(1→3)-glucan (expressed per m^2 sampled surface) were significantly associated with PEF-variability in symptomatic children, and particularly in atopic children with asthma symptoms. Adjusted regression analyses showed the same association for ß(1→3)-glucan but not for endotoxin. No relations were found between PEF-variability and levels of microbial agents in bedroom floor or mattress dust. We conclude that high levels of ß(1→3)-glucan in house dust may increase peak flow variability in asthmatic children.

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Introduction

Probably the first reference suggesting adverse health effects related to microbial exposure in the indoor environment is found in the bible.[1] In more recent times, reports of health effects related to microbial exposure already appeared in the 17th and 18th century.[2-4] Despite this early recognition, however, the role of microbial exposure in the development of respiratory symptoms is, apart from infectious diseases, still poorly understood. Indoor house dust mite and pet allergens have been recognized as major causes of asthma in many areas of the world, particularly in children. In the past decade, however, there has been an increased interest in a possibly similar role of indoor microorganisms. Epidemiological studies have suggested that allergic and non-allergic inflammatory reactions to fungal components in the indoor environment may account for the frequently observed association between home dampness and prevalence and severity of respiratory symptoms.[5-15] Other studies have suggested a role for bacteria and particularly for bacterial endotoxins.[16-19] Endotoxins, pro-inflammatory components of gram negative bacteria[20], have long been recognized as causal agents in non-immune mediated airway inflammation and associated respiratory disorders, particularly in the occupational environment.[21,22] Recently a similar role for ß(1→3)-glucans has been suggested.[23,24] ß(1→3)-glucans originate from most fungi, some bacteria and most plants and have the capacity to initiate a variety of inflammatory reactions in vertebrates.[25]

At present no epidemiologic data have been reported that show a relation between objectively assessed indoor microbial exposure and lung function variability. In a previous study peak flow variability in asthmatic children was significantly associated with dust mite allergen levels in the home environment.[26] In the present study we investigated the relation between endotoxin and ß(1→3)-glucan levels in house dust and peak flow variability in 159 school children, 50% of whom had pre-existing chronic respiratory symptoms.

Methods

Study design

The data were collected in the winter of 1993/1994 as part of a study on acute respiratory effects of air pollution in panels of children with or without chronic

respiratory symptoms.[27] 159 children, aged 7-11 yr and living in Amsterdam were selected based on a parent-administered screening questionnaire, such that approximately 50% (n = 78) had chronic respiratory symptoms (chronic wheeze, shortness of breath with wheeze, chronic cough and/or doctor-diagnosed asthma), while the other 81 had no reported respiratory symptoms. Children performed peak flow measurements in the morning after waking up and in the evening before going to bed, for a period of 16 weeks as described previously.[13] Skin-prick tests (SPT) were performed to identify atopic subjects, using a panel of 6 common allergens: birch, timothy grass, cat fur, dog dander, house dust mite and *Cladosporium herbarum*, all from ALK laboratories (Denmark).[13] Subgroups of symptomatic children were defined based on symptoms ('asthma symptoms'; shortness of breath with wheeze or doctor-diagnosed asthma, 'cough symptoms'; chronic cough but no asthma symptoms) and atopy ($\geq$ 1 positive SPT). Written consent was given by the parents of all children that participated in this study.

House dust samples

House dust samples were collected in 148 homes from floors of living rooms and bedrooms and from mattresses.[28,29] Endotoxin, ß(1→3)-glucan and *Der p*1 (house dust mite allergen) were determined as previously described[28-30] by using a *Limulus* Amoebocyte Lysate (LAL) method, a glucan specific enzyme immunoassay (EIA) and a *Der p*1 specific EIA, respectively. Concentrations were expressed per square meter of sampled area.

Statistical analysis

Statistical analyses were performed using SAS (version 6.12; SAS institute, Cary, N.C.). Because exposure data followed a lognormal distribution, endotoxin, ß(1→3)-glucan and *Der p*1 concentrations were ln-transformed. A chi-square test was used for comparing prevalences, and a Student's t-test for all other comparisons.

To calculate PEF-variability (Ampl%mean), the absolute difference between AM and PM PEF of each day was divided by the mean PEF of that day, and subsequently this daily PEF-variability was averaged over the whole period.[31] PEF data from the first two days of each subject were excluded from the analysis, because of a possible learning effect. Ampl%mean followed a

Table 1. Characteristics of study population, including peak flow and peak flow variability (AM; arithmetic mean, SD; standard deviation, GM; geometric mean, GSD; geometric standard deviation).

	No respiratory symptoms (n = 81)	Respiratory symptoms (n = 78)	Asthmatic symptoms (n = 34)	Chronic cough (n = 37)
Girls (%)	51.9	47.4	38.2	56.8
Age in years (AM, SD)	9.4 (1.0)	9.3 (1.0)	9.3 (1.0)	9.3 (1.1)
Smokers in the home (%)	59.3	55.1	47.1	67.6
Pets in the home (%)	64.4	48.7*	41.2**	52.9
SPT + to common allergens[¶] (%)	19.2	49.3***	61.8***	41.2**
SPT + to dust mite allergens (%)	7.7	37.3***	44.1***	32.4***
SPT + to dog and/or cat allergens (%)	9.9	34.6***	50.0***	21.6*
PEF-morning (AM, SD)	341 (43)	310 (50)[###]	307 (43)[###]	316 (44)[###]
PEF-evening (AM, SD)	345 (42)	319 (50)[###]	316 (53)[###]	325 (48)[##]
PEF-mean (AM, SD)	344 (42)	314 (50)[###]	311 (54)[###]	320 (46)[###]
PEF-Ampl%mean (GM, GSD)	5.2 (1.7)	6.3 (1.6)[##]	6.4 (1.7)[##]	6.3 (1.6)[#]

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$; Chi-square test (symptomatics compared to non-symptomatics)

[#] $p < 0.1$, [##] $p < 0.05$, [###] $p < 0.01$; Student's t-test (symptomatics compared to non-symptomatics)

[¶] positive to one or more common allergens; grass, birch, cat, dog, house dust mite, *Cladosporium herbarum*

lognormal distribution. Regression analysis of the relation between exposure and Ampl%mean, was therefore performed on ln-transformed data to normalize residuals. Results are presented as relative increases in Ampl%mean associated with an increase in exposure of two geometric standard deviations (GSDs). Stratified analyses were performed for non-symptomatic and symptomatic children and for subgroups of symptomatic children. After unadjusted analyses, multiple regression models were used to relate PEF variability to either endotoxin or ß(1→3)-glucan, adjusting for *Der p*1, pets in the home and type of floor cover (carpet vs smooth). Due to the high correlation between endotoxin and ß(1→3)-glucan these were analyzed separately in the multiple regression analysis.

Results

There were no differences with respect to gender and age between subgroups (table 1). Parental smoking and pets were less prevalent in the asthmatics compared to the non-symptomatic children. PEF was lower in symptomatic children and their PEF-variability was higher.

Endotoxin and ß(1→3)-glucan levels did not differ between non-symptomatic and symptomatic subjects, with the exception of mattress endotoxin which was higher in subjects with chronic cough (table 2). Mite allergen levels were relatively low - compared to previous studies in Dutch homes - with lowest concentrations in homes of symptomatic children. Highest *Der p*1 levels were measured in mattresses, with 43% of the samples being higher than 2 μg/g. Endotoxin, ß(1→3)-glucan and *Der p*1 levels were approximately 10 fold higher on carpets than on smooth floors (data not shown).

Unadjusted regression analyses showed that Ampl%mean in symptomatic children was significantly associated with endotoxin, ß(1→3)-glucan and *Der p*1 levels on living room floors, and also with pets and type of carpet (table 3). The associations were strongest in atopic and/or asthmatic children. Environmental tobacco smoke (ETS) was not associated with PEF variability. PEF-variability in non-symptomatic children and in children with chronic cough showed no significant relations with any of the investigated exposure parameters. Also, there was no association between Ampl%mean and endotoxin, ß(1→3)-glucan and mite allergen levels in bedroom floors and mattresses (data not shown).

Table 2: Geometric mean (GM) and geometric standard deviation (GSD) of endotoxin, ß(1→3)-glucan and Der p1 concentrations (per m^2 sampled surface) in house dust from living and bedroom floors and mattresses, stratified for non-symptomatic and symptomatic children.

	living room			bedroom			mattress		
	n^1	GM	GSD	n^1	GM	GSD	n^1	GM	GSD
Endotoxin (EU/m^2)									
non-symptomatics	68/68	2,082	(4.4)	70/70	2,683	(6.7)	69/69	1,820	(3.6)
symptomatics	71/71	2,443	(6.1)	71/71	2,655	(5.3)	72/72	2,082	(4.3)
asthmatics	31/31	2,493	(6.5)	28/31	2,128	(3.2)	31/31	1,202	(3.3)
cough	34/34	2,550	(6.3)	34/34	2,850	(6.8)	35/35	3,402**	(4.7)
ß(1→3)-glucan (μg/m^2)									
non-symptomatics	65/69	126	(5.6)	62/69	184	(7.9)	71/71	276	(2.4)
symptomatics	72/74	169	(4.2)	68/69	208	(3.9)	73/73	293	(2.3)
asthmatics	33/34	167	(5.0)	31/31	234	(2.6)	32/32	283	(1.9)
cough	33/34	170	(3.7)	31/32	168	(5.2)	35/35	303	(2.6)
Der p I (ng/m^2)									
non-symptomatics	26/69	35	(7.6)	39/70	117	(14.6)	64/70	537	(6.1)
symptomatics	27/74	29	(5.5)	37/67	83	(11.8)	67/73	432	(7.5)
asthmatics	12/34	32	(7.5)	15/30	62	(11.3)	28/32	318	(9.0)
cough	12/34	27	(4.3)	19/33	101	(13.5)	33/35	646	(5.9)

** $p<0.05$; Student's t-test, comparing symptomatics with non-symptomatics

[1] no. with detectable concentrations/total no. of samples

Table 3. Unadjusted regression results describing the relation between ln transformed Ampl%mean of symptomatic and non-symptomatic children and ln transformed living room floor endotoxin, ß(1→3)-glucan and Der p1 concentrations (expressed per m². Results are presented as relative increases (ratio) in Ampl%mean associated, either with a difference of two times GSD in exposure, or the presence of wall-to-wall carpet, pets or ETS in the home.

	No symptoms	Symptoms		Asthmatic symptoms	
	all (n=81)	all (n=78)	atopic[¶] (n=37)	all (n=34)	atopic[¶] (n=21)
Endotoxin	1.08 (0.91-1.43)	1.20 (0.97-1.48)*	1.60(1.12-2.28)***	1.43 (1.00-2.05)*	1.66 (1.08-2.54)**
ß(1→3)-glucan	0.94 (0.75-1.18)	1.33 (1.06-1.67)**	1.58 (1.26-1.98)***	1.45 (1.10-1.92)**	1.63 (1.23-2.16)***
*Der p*1	1.03 (0.85-1.26)	1.23 (1.00-1.50)**	1.31 (1.00-1.72)*	1.56 (1.19-2.04)***	1.55 (1.12-2.18)**
carpet[§] (y/n)	1.08 (0.82-1.43)	1.22 (0.93-1.61)	1.54 (1.06-2.23)**	1.90 (1.31-2.75)***	1.73 (1.10-2.72)**
pets (y/n)	0.96 (0.73-1.26)	1.34 (1.08-1.66)**	1.54 (1.15-2.06)***	1.54 (1.08-2.19)**	1.72 (1.14-2.59)**
ETS (y/n)	0.86 (0.68-1.09)	1.07 (0.85-1.36)	1.07 (0.78-1.47)	1.02 (0.70-1.48)	1.07 (0.67-1.72)

* $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$; Student's t-test.
[¶] atopy: positive SPT to one or more common allergens; grass, birch, cat, dog, house dust mite and *Cladosporium* spp.
[§] wall-to-wall carpet versus smooth floor cover.

To determine whether these associations were independent of other known risk factors, two multiple regression models were applied; one with ß(1→3)-glucan (figure 1a) and one with endotoxin (figure 1b), both adjusted for mite allergen exposure, type of floor cover and presence of pets. As shown in Fig 1a, the association between ß(1→3)-glucan and Ampl%mean remained in symptomatic subjects, particularly in atopic asthmatics. The associations with pets, and to a lesser extent, mite allergens remained as well. In contrast, the association with endotoxin (fig 1b) did not remain after adjustment for pets, mite allergen and floor cover.

Discussion

In this study, PEF variability in children with chronic respiratory symptoms was shown to be strongly associated with ß(1→3)-glucan levels in dust from living room floors. These associations were strongest for atopic children with asthma symptoms. In non-symptomatic children and children with only chronic cough no significant associations were found.

Reporting bias seems unlikely since assessment of exposure and PEF-variability were based on objective measurements. Confounding by mite allergen and pets was demonstrated for endotoxin but not for ß(1→3)-glucan. ETS was not associated with PEF-variability and could thus not have biased the observed associations.

Animal and *in-vitro* studies have shown that ß(1→3)-glucans have profound pro-inflammatory effects including activation of neutrophils, macrophages, complement, and possibly eosinophils.[25] Furthermore, ß(1→3)-glucans have been suggested to be involved in bio-aerosol induced inflammatory responses and resulting respiratory symptoms.[23,32] Our study is the first to show a relation between indoor ß(1→3)-glucan exposure and PEF variability as an objective marker of respiratory health. This association was found primarily in atopic, asthmatic children who thus seem more susceptible to react to ß(1→3)-glucan. The relation is probably not based on type I allergic reactions since glucans are considered non-immunogenic in man.[25] Presumably, ß(1→3)-glucan exposure may increase PEF-variability in atopic asthmatics by enhancing pre-existing allergic or non-allergic inflammation. Alternatively, ß(1→3)-glucan may activate an independent pathway of airway inflammation that is most strongly expressed in hyper-responsive atopic asthmatics.

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Fig 1a. Multiple regression between In transformed Ampl%mean and In transformed living room floor ß(1→3)-glucan concentration, adjusted for Der p1, pets, and type of floor cover. Results are presented as relative increases (ratio) in Ampl%mean associated with an increase of two GSDs in exposure or with the presence of wall-to-wall carpet or pets in the home.

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Sub-groups:

Non-symptomatic
(n = 81)

Symptomatic
(n = 78)

Symptomatic and atopic
(n = 37)

Asthmatic
(n = 34)

Asthmatic and atopic
(n = 21)

●	endotoxin
○	*Der* p1
▼	pets
▽	carpet
*	p < 0.1
* *	p < 0.05
* * *	p < 0.01

0.0   0.5   1.0   1.5   2.0   2.5   3.0   3.5

Relative increase (ratio) in PEF-variability with 95% C.I.
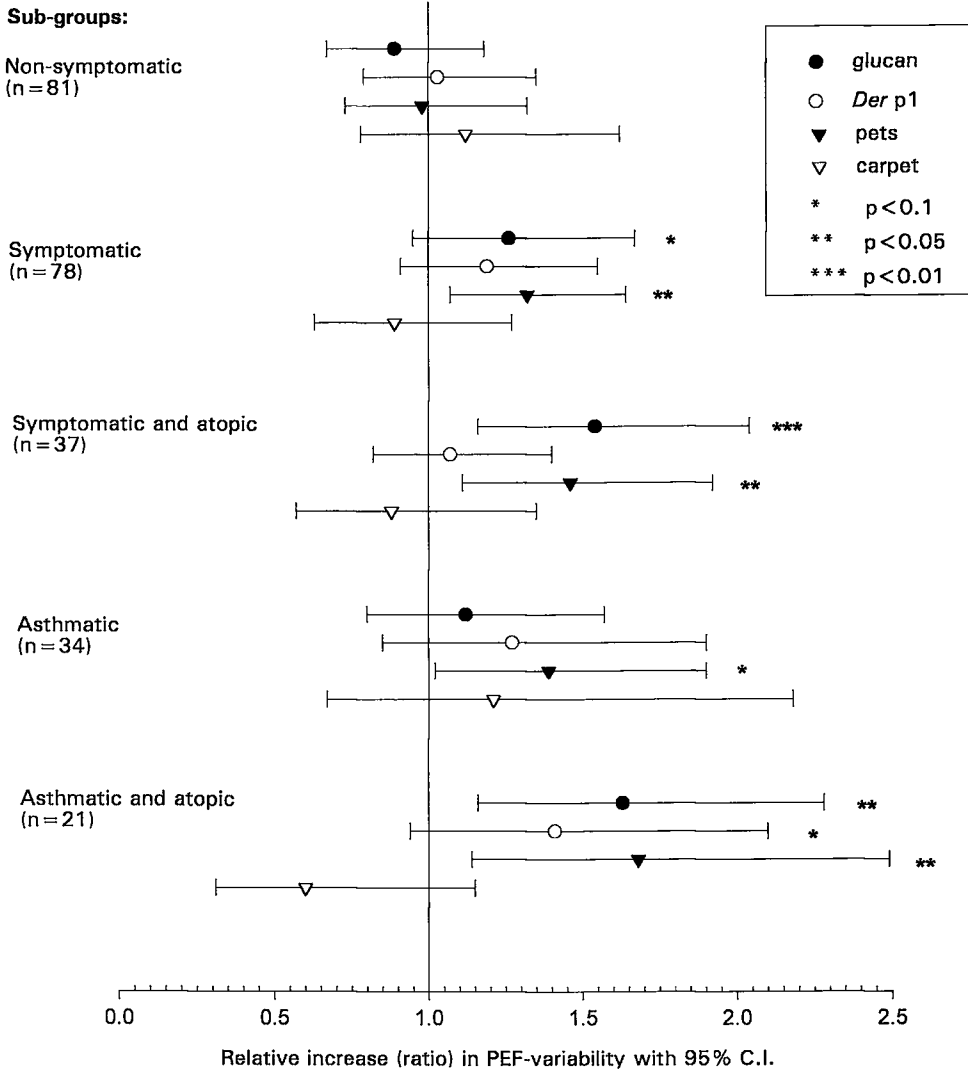
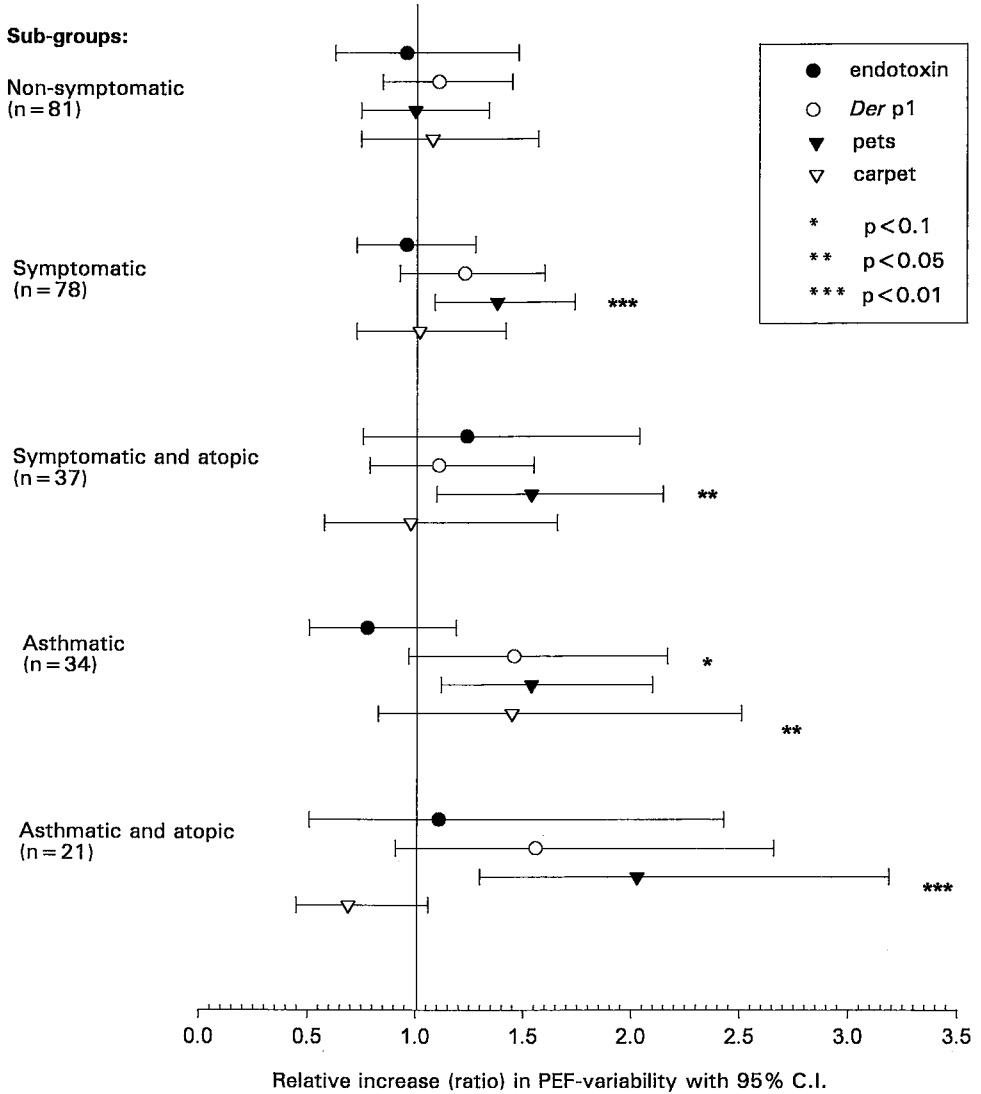*Fig 1b. Multiple regression between ln transformed Ampl%mean and ln transformed living room floor endotoxin concentration, adjusted for Der p1, pets, and type of floor cover. Results are presented as relative increases (ratio) in Ampl%mean associated with an increase of two GSDs in exposure or with the presence of wall-to-wall carpet or pets in the home.*

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In the atopic asthmatics an increase in living room floor ß(1→3)-glucan levels by 2 standard deviations - in our data a 25 fold increase - was associated with a 1.6 fold increase in PEF-variability. PEF-variability was also approximately 1.7 times higher in subjects with pets in their homes. This is a substantial increase, considering the fact that the Ampl%mean in the asthmatic children was only 20% higher than in the non-symptomatic children. A previous study among European children with chronic respiratory symptoms showed that in atopic children fungal growth in the home was associated with a 15% increase in PEF-variability.[13]

Other studies have shown an association between endotoxin in house dust and clinical severity of asthma in asthmatic adults and children.[17-19] In those studies relationships were not adjusted for the presence of pets in the home. In our study, presence of pets was associated with higher endotoxin levels in living room floor samples (3074 vs 1569 EU/m^2; $p < 0.05$), and after adjustment for mite allergen and pets there was no association between endotoxin and PEF-variability. Pets are well known indoor sources of allergens, and may thus have contributed to PEF-variability through allergic mechanisms. This would be plausible considering the high prevalence of dog and cat positive skin prick tests among the symptomatic children (up to 50% in the asthmatics).

No relationships were found between PEF-variability and endotoxin and ß(1→3)-glucan levels in the bedroom. This was also true for *Der p*1. Allergen-avoidance measures, taken particularly in bedrooms of children with more severe asthma, may have biased these results. This has previously been shown in another Dutch study for the relationship between *Der p*1 levels and chronic respiratory health parameters.[33] In our study it was not possible to adjust for allergen avoidance measures, due to the relatively small number of atopic asthmatic children.

In conclusion, ß(1→3)-glucan in house dust may increase peak flow variability in asthmatic children.

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Chapter 8

Airway inflammation assessed by nasal lavage in compost workers: a relation with bio-aerosol exposure

Abstract

Upper airway inflammation and the relation with bio-aerosol exposure, were studied in 14 compost workers by repeated pre and post-shift nasal lavages (NAL) during 3 weeks on Mondays and Fridays. Results were compared with those of pre- and post-shift NALs in ten controls on Monday. In NAL total cells, cell differentials, MPO, IL8, ECP, NO, uric acid, urea and albumin were determined. Job title-based mean endotoxin and ß(1→3)-glucan exposure levels ranged from 75-527 EU/m^3 and 0.5-4.9 μg/m^3, respectively. A cross-shift 1.4-3.2 fold increase in total cells, MPO, IL8, NO and urea was observed in NAL of compost workers compared to controls. In controls, parameters showed a cross-shift decrease, presumably due to a wash out effect. Most cells in NAL were neutrophils, whereas eosinophils were only incidentally observed. Within the group of compost workers, no association between exposure level and cross-shift inflammatory responses was found. Total cell, MPO, IL8, NO and albumin levels in pre-shift NAL of workers were elevated (1.1-4.8 fold) compared to controls and weakly associated with levels of bio-aerosol exposure. These results indicate work related acute and possibly (sub-)chronic non-immune or type lil allergic inflammation in the upper airways of compost workers, most likely related to bio-aerosol exposure.

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Introduction

The introduction of industrial composting of source separated organic household waste in recent years in many industrialized countries, has resulted in new and poorly described health risks for increasing numbers of workers. High exposures to microorganisms, organic dust and bacterial endotoxins in the waste composting industry, have recently been described.[1] Workers involved in this rapidly developing industry have been reported to experience respiratory and systemic influenza like symptoms (shivering, fever, joint pain, malaise),[2,3] most likely due to high bio-aerosol exposures. Some case reports exist of the occurrence of allergic diseases in waste and compost workers such as hypersensitivity pneumonitis, allergic bronchopulmonary aspergillosis and asthma due to excessively high exposures to micro-organisms and microbial components.[2,4] Non allergic responses such as airway inflammation may occur as well, but have hardly been studied.

To study mechanisms of airway inflammation, bronchial alveolar lavages and lung biopsies have been applied extensively in clinical or poli-clinical populations. These techniques, however, are in general too invasive and laborious for use in population studies. Nasal lavage is a relatively new, non invasive, safe and simple technique, which has proven its feasibility mainly in experimental exposure studies[5-9] but also in a limited number of population studies.[10-13] The inflammatory responses in the upper airways detected by NAL may be indicative for similar inflammatory responses in the lower airways - provided that the causal agents can be deeply inhaled - since mucosal histology and infiltrating cells during inflammation are similar.[5,6] In addition, the nasal mucosa usually is the site of first contact of inhaled agents with the respiratory tract, and an inflammatory response in the nasal mucosa would demonstrate their pro-inflammatory capacity.

In this study upper airway inflammation was assessed in compost workers, by performing twice weekly, and for a period of three weeks, pre- and post-shift nasal lavages on Monday after an exposure free weekend and at Friday after a week of work. A group of non-exposed control subjects underwent NAL prior and after work only on Monday. In the nasal lavage total cells, cell differentials, and various cytokines and exudation markers were determined. The association between inflammatory responses and personally monitored bacterial endotoxin, mold ß(1→3)-glucan and dust exposure was investigated.

Materials and methods

Compost plant description

In the compost plant where this study was performed, source separated organic waste of several big cities in the Netherlands -mainly consisting of food and yard waste- was stored indoors in a large processing hall for several days to several weeks. Composting was performed in closed aerated concrete tunnels. Storage, composting and transport of waste and compost all took place in the same processing hall. Crude waste and compost was shredded and sieved several times prior to and after composting. Waste and compost were transferred by bulldozers and open conveyer belts. Warm and humid conditions existed most of the time in the process hall. Employees worked in two shifts, consisting of approximately 7 people per shift. Main tasks of the workers were performed in the processing hall, consisting of operating bulldozers, process control, cleaning, maintenance and controlling technical failures. Most tasks were performed without any respiratory protection. Bulldozers, however, had overpressure cabins to protect the operator from exposure.

Dust sampling and extraction

During three weeks on Mondays and Fridays in the autumn of 1995, repeated full-shift personal inhalable dust exposure was monitored in 14 compost workers. Inhalable dust was sampled on 25 mm glass fibre filters (Whatmann GF/A) mounted in a PAS-6 sampling head at a flow rate of 2 l/min using portable pumps (Gilian Gil-Air). In addition full-shift ambient inhalable dust sampling was performed at various locations in the facility. Gravimetric measurements and endotoxin and ß(1→3)-glucan extractions were performed as described earlier.[14,15]

Endotoxin analysis

New glassware (rendered pyrogen free), pipette tips (Gilson; type Tipac), and microtitre plates (96 well, flat bottom, sterile polystyrene; Costar 3596) were used. Endotoxin was assayed with a quantitative kinetic chromogenic LAL method (Kinetic-QCL no.50-650 U; Bio Whittaker; lot no 4L3020) as described earlier (14). *Escherichia coli* O55:B5 endotoxin (Bio Whittaker; lot no 3L2350) was used as standard endotoxin. The endotoxin potency of this standard was 15 endotoxin units (EU)/ng. Samples were analyzed in duplicate in two

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independent test runs and did not show inhibition or enhancement of the LAL assay at sample dilutions used.

ß-(1→3)-analysis

ß-(1→3)-glucan was assayed with a specific inhibition enzyme immuno assay (EIA) which was developed and described recently.[15] The sensitivity of this assay was 40 ng/ml and the reproducibility expressed as a coefficient of variance was 20% for occupational dust samples.[15] Samples were analyzed in duplicate in two independent test runs.

Micro-organism sampling

Ambient micro-organism concentrations were assessed using the N-6 modification of the Andersen sampler[16] at a flow rate of 28.3 l/min and a sampling time of 1 minute for total bacteria and molds, and 6 minutes for gram negative bacteria. The following culture media (Oxoid, Hampshire, UK) were used: Tryptone Soy Agar (TSA) for total bacteria, TSA with crystal violet for gram negative bacteria and Dichloran Gelatin 18 (DG18) agar for molds. All samples were taken in duplicate at 1.5 m above ground level. After sampling, agar plates were incubated for 4 days at 24°C (molds) or for 2 days at 37°C (bacteria). After incubation total number of colonies were counted and adjusted for the likelihood that more than one particle passed each impactor hole.[17] If culture plates were found to be overloaded (> 400 CFU per plate), additional ambient measurements were performed using a sampling time of 15 minutes and petri dishes loaded with glycerol-gelatin medium allowing dilution (10, 100 and 1000 times) prior to culturing on TSA or DG18 plates.

Questionnaire and lung function

Workers completed a self-administered Dutch version of the questionnaire on respiratory symptoms of the British Medical Research Council.[18] Forced expiratory lung function measurements were performed at one occasion at the start of the working shift using a Vicatest-5 dry rolling spirometer (Jaeger, Breda, The Netherlands), calibrated before and after the measurement session. The measurements and data selection were performed according to the recommendations of the European Respiratory Society (ERS).[19] Parameters derived from the flow-volume curves were: Forced Vital Capacity (FVC), Forced

Expiratory Volume in one second (FEV_1), Maximal Mid-Expiratory Flow (MMEF) and Peak Expiratory Flow (PEF).

Nasal lavage

Parallel to personal exposure monitoring, repeated -Mondays and Fridays for three weeks- pre and post-shift nasal lavages were performed in 14 compost workers. A control group consisting of 10 university staff members and students, received nasal lavage only once on Monday prior and after work. For the lavage the subject tilted the head backwards and closed the nasopharynx with the tongue. Then 5 ml sterile PBS (37°C) was instilled in each nasal cavity with a polystyrene pipet, the fluid was held for 10 seconds while the subject held his breath, and it was subsequently expelled into a conical centrifuge tube via a polyamide gauze filtered funnel, essentially as described by Steerenberg *et al*.[20] Lavage fluid recovery was measured before centrifuging for 10 minutes at 250 x G. The average recovery of nasal lavage fluid was 5.7 ml (approximately 60%) for compost workers and 6.2 ml for controls and no significant differences in recovery were found between pre and post-shift NAL. Supernatants were used for the measurement of the inflammatory markers myeloperoxidase (MPO), eosinophil cationic protein (ECP), interleukin-8 (IL8), nitric oxide (NO), albumin, ureum and uric acid. Part of the pellet was fixed in an equal volume of 4% formalin for total cell counts. The remainder was used for cytospins which were stained with May-Grünwald Giemsa staining for cell differentials (neutrophils, eosinophils, mononuclear cells and epithelial cells). MPO, ECP and IL8 were measured by RIA, FIA and EIA respectively, as previously described.[20] NO was measured using a nitric oxide analyzer 280 (NOA 280; Sievers) extended with a purge-vessel. Albumin, uric acid and urea concentrations were determined on a COBAS FARA and a COBAS BIO clinical chemistry analyzer (F. Hofmann-La Roche, Basel, Switzerland), respectively.

Protocols for nasal lavage and lung function were approved by the Medical and Ethical Committee of the Agricultural University in Wageningen.

Statistical analysis

Statistical analyses were carried out with SAS statistical software (SAS 6.12; SAS institute, Cary, N.C.). Because exposure data were best described by a lognormal distribution average airborne dust, endotoxin and ß(1→3)-glucan

concentrations were expressed as geometric means (GM) with geometric standard deviations (GSD). Pearson correlations were calculated between ln-transformed exposure data. In the evaluation of NAL, dependency in the data due to repeated measurements was avoided by using the median value of the repeated measurements in each worker in the statistical analyses. This way the 160 available NAL values of each parameter measured in the NAL were reduced to 14 pre- and 14 post-shift median values as well as 14 post-pre-shift differences and 14 post/pre-shift ratios. Absolute (post minus pre-shift) and relative (post/pre-shift ratio) cross-shift differences were calculated to express acute inflammatory responses in the 14 workers. Workers and controls were compared by using group median levels based on the 10 observations of the controls and 14 observations of the workers. Differences between workers and controls were tested with a non-parametric Wilcoxon 2-sample test. Data were expressed graphically as group median values. Paired comparison between Monday and Friday NAL and pre and post-shift NAL was performed by using a Wilcoxon signed rank test. Non detectable exposure and inflammatory mediator concentrations were given a value of two thirds of the detection limit.[21]

Results
Personal and ambient geometric mean airborne dust, endotoxin and ß(1→3)-glucan levels for the various job titles are given in table 1. No large differences in exposures existed among technical personnel, supervisors and production workers, while exposures in bulldozer operators were significantly lower ($p < 0.05$). Ambient bio-aerosol concentrations were in general substantially lower compared to personal monitored exposure (table 1). Canteen, offices (in portable buildings) and workshop (in the compost hall) also showed elevated exposure levels, due to their location very close to the main process hall. Personal dust, endotoxin and ß(1→3)-glucan levels were moderately correlated ($R = 0.6-0.7$; $p < 0.01$). All culture plates used for sampling mold and total bacteria were overgrown using the direct method, indicating high ambient microorganism levels with counts of more than 95,000 Colony Forming Units (CFU) per m^3 (maximum possible count for direct method; $n = 3$). Gram-negative bacteria levels ranged from 400 - 19,400 CFU/m^3 ($n = 5$). Indirect measures using dilution media indicated total bacteria and mold levels of up to $5.5 * 10^9$ and $3.6 * 10^6$ CFU/m^3, respectively ($n = 7$).

Table 1. Personal and ambient geometric mean (GM) airborne dust, endotoxin and ß(1→3)-glucan exposures with geometric standard deviation (GSD; between brackets).

Personal	Dust (mg/m^3)			Endotoxin (EU/m^3)			ß(1→3)-glucan (μg/m^3)		
	GM[*]	n[†]	Min-Max	GM[*]	n[†]	Min-Max	GM[*]	n[†]	Min-Max
bulldozer operators	0.5 (3.1)	6/18	<0.3[‡] - 12.2	75 (3.0)	13/14	<6[‡] - 357	0.54 (3.7)	8/14	<0.15[‡] - 4.83
technical personnel	1.5 (2.2)	6/6	0.7 - 7.3	373 (3.7)	5/5	141 - 3,544	4.85 (5.0)	5/5	1.03 - 53.23
supervisors	1.8 (3.0)	11/11	0.5 - 22.8	418 (2.3)	9/9	107 - 1,678	4.28 (2.0)	9/9	1.40 - 10.38
production workers	1.3 (2.1)	38/42	<0.3[‡] - 5.3	527 (1.7)	34/34	220 - 1,712	3.62 (2.4)	31/32	<0.15[‡] - 13.18
Ambient									
canteen + offices[§]	0.4 (1.5)	11/17	<0.3[‡] - 0.8	101 (1.9)	14/14	30 - 231	0.36 (2.4)	9/16	<0.15[‡] - 1.93
workshop[11]	0.4 (2.5)	1/5	<0.3[‡] - 2.2	74 (7.9)	5/5	8 - 2,016	0.57 (6.1)	3/5	<0.15[‡] - 12.30
Process hall[¶]	0.6 (2.1)	23/33	<0.3[‡] - 3.8	133 (2.5)	29/29	10 - 366	0.65 (4.1)	17/27	<0.15[‡] - 16.21

[*] Geometric means were calculated by including non-detectable results (non-detectable results were set at two-thirds of the detection limit).

[†] Number of samples with detectable concentrations / total number of samples.

[‡] Below detection limit (airborne dust concentration, 0.3 mg/m^3; airborne endotoxin concentrations, 8.5 EU/m^3; airborne ß(1→3)-glucan concentration, 0.15 μg/m^3)

[§] Canteen and offices were located in portable buildings close to the process hall.

[11] The workshop is located in the process hall directly near a compost tunnel.

[¶] Area for unloading and sieving organic waste, waste composting in concrete tunnels and transfer of compost.

Gender and smoking status differed significantly between workers and non-exposed controls, while age was approximately the same (table 2). Most workers had been employed for less than 6 months. None were previously employed in the waste industry or in jobs with high organic dust exposures. Half of the 14 workers reported symptoms of bronchitis and the majority (11) reported nasal symptoms (frequent sneezing, runny and stuffy nose and hayfever). Symptoms of asthma were reported by only one subject. The lung function variables FVC, FEV_1 and PEF were on average higher (108%, 102% and 126%, respectively) than age- and height-specific predicted values (19), while the MMEF was on average 16% lower than expected.

Table 2. population characteristics of controls and workers

	Controls (n = 10)	Compost workers (n = 14)
Male	6 (60%)	14 (100%)[**]
Mean age in years (SD)	29.2 (8.2)	28.2 (4.7)
Current smoker	1 (10%)	9 (64.3%)[***]
Mean duration of employment in months (range)	-	6 (1-23)

[**] $p < 0.05$, [***] $p < 0.01$; Fisher's exact test.

In total 188 nasal lavages were performed of which 8 were lost due to technical errors in the laboratory. Of the 180 remaining NALs 160 came from compost workers and 20 from controls. As shown in Table 3, median pre-shift levels of total cells, MPO, IL8, NO and albumin were significantly higher in workers compared to controls, suggesting a chronic or sub-chronic upper airway inflammation in the workers. Similar results were obtained when only pre-shift NALs performed on Mondays were compared with controls (not including Friday NAL of workers). Post-shift total cells and levels of most inflammatory markers were also increased in the workers. This was only partially explained by the difference in pre-shift values between workers and controls, since exposed workers also showed a cross-shift increase in most inflammatory markers, whereas in controls total cells, MPO, IL8 and ECP in NAL showed a cross-shift decrease and NO, uric acid, urea and albumin levels in controls did not change much.

Table 3. Median pre-shift and post-shift values and post- minus pre-shift values (with min-max in parentheses) for inflammatory markers (total cells; cells/ml, MPO; ng/ml, IL8; pg/ml, ECP; ng/ml, NO; pmol/ml, uric acid; nmol/ml, urea; µmol/ml, albumin; µg/ml) in NAL of compost workers and controls.

Inflammatory marker		Controls (n = 10)		Workers (n = 14)	
		median	*(range)*	*median*	*(range)*
Cells	pre-shift	0.9	(0.2 - 120.3)	4.3[**]	(0.7 - 17.0)
*10³	post-shift	0.5	(0.2 - 1.5)	7.0[***]	(1.0 - 16.2)
	post minus pre-shift	-0.3[#]	(-118.9 - 590.3)	2.1[**,#]	(-7.0 - 10.6)
MPO	pre-shift	13.5	(6.4 - 73.0)	46.7[**]	(8.6 - 131.2)
	post-shift	10.3	(5.6 - 203.8)	52.5[**]	(7.8 - 154.0)
	post minus pre-shift	-1.6	(-58.4 - 192.3)	11.0	(-68.1 - 89.4)
IL8	pre-shift	34.8	(8.0 - 416.3)	159.7[***]	(46.7 - 374.2)
	post-shift	21.4	(7.0 - 166.7)	173.3[***]	(53.0 - 409.4)
	post minus pre-shift	-11.4[#]	(-249.6 - 33.2)	8.8	(-81.6 - 117.2)
ECP	pre-shift	1.8	(1.3 - 9.5)	1.5	(1.3 - 7.2)
	post-shift	1.3	(1.3 - 6.3)	1.9	(1.3 - 6.3)
	post minus pre-shift	-0.5	(-8.2 - 0.8)	0.0	(-6.4 - 1.9)
NO	pre-shift	6.7	(6.7 - 6.7)	28.9[***]	(9.0 - 57.6)
	post-shift	6.7	(6.7 - 6.7)	61.4[***]	(6.7 - 173.1)
	post minus pre-shift	0.0	(0.0 - 0.0)	27.2[***,###]	(0.0 - 132.7)
Uric	pre-shift	6.2	(3.4 - 15.9)	8.6	(2.2 - 17.3)
Acid	post-shift	8.3	(3.8 - 18.0)	12.0	(1.9 - 30.5)
	post minus pre-shift	0.3	(-6.5 - 11.1)	3.1[##]	(-2.0 - 20.6)
Urea	pre-shift	0.3	(0.2 - 0.4)	0.4	(0.2 - 0.6)
	post-shift	0.3	(0.2 - 0.7)	0.5[**]	(0.2 - 1.0)
	post minus pre-shift	0.0	(-0.1 - 0.2)	0.1[**,##]	(-0.0 - 0.7)
Albumin	pre-shift	62.0	(59.2 - 102.3)	70.7[**]	(62.7 - 89.7)
	post-shift	62.7	(59.3 - 78.2)	69.0[**]	(62.6 - 86.5)
	post minus pre-shift	0.8	(-24.1 - 7.6)	0.1	(-7.4 - 6.4)

[**] $p < 0.05$, [***] $p < 0.01$; Workers vs controls; Wilcoxon rank sum test

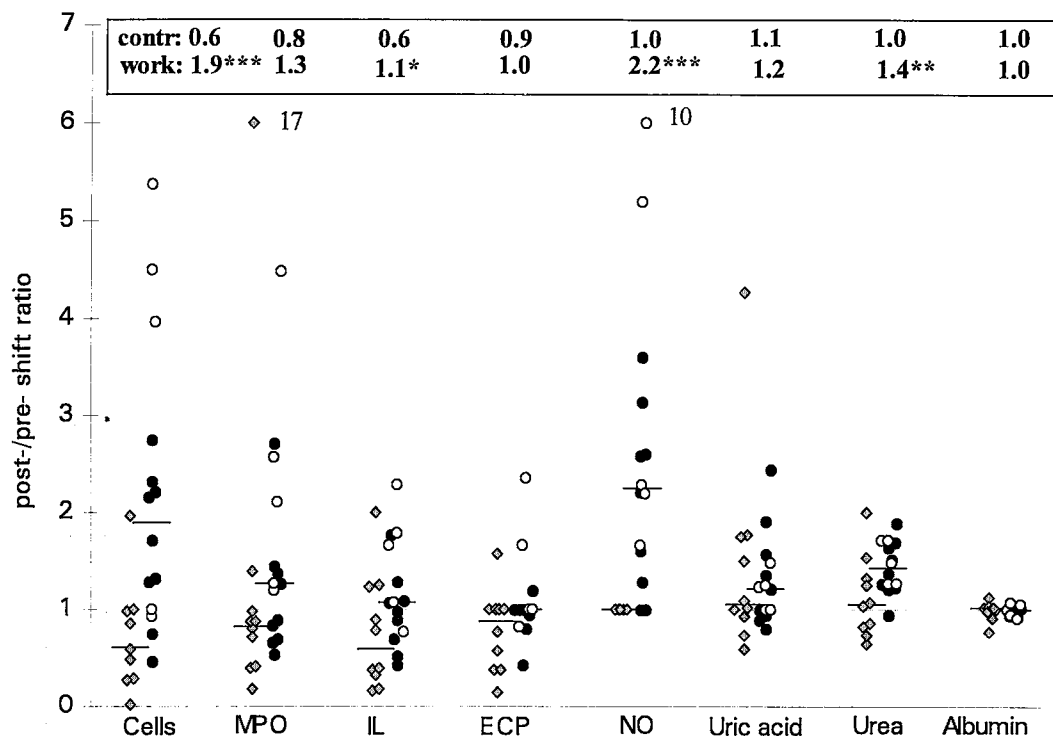[#] $p < 0.1$, [##] $p < 0.05$, [###] $p < 0.01$; post minus pre-shift values deviate from '0'; Wilcoxon signed rank test

Fig 1. Post/pre-workshift ratios for inflammatory markers in NAL of controls (shaded diamonds; n=10 for all parameters except cells, n=9 for cells) and compost workers (circles; n=14). Open circles represent the non smoking workers. Ratios > 6 were set at value 6 in the graph. NO and ECP concentrations were non detectable in NALs of all controls, and 6 controls and 7 workers, respectively. Median group levels are listed in box in upper part of the figure (* p<0.1, ** p<0.05, *** p<0.01).

Thus post minus pre-shift values for cells and most inflammatory markers were higher in compost workers compared to the controls, reaching statistical significance for total cells, NO and urea (table 3). Because pre-shift levels of inflammatory markers were substantially different for controls and workers, cross-shift inflammatory responses were also studied by comparing the relative differences between pre- and post shift NAL concentrations of workers and controls. In figure 1 median post/pre-shift ratio's of total cells and inflammatory mediators are shown for both groups, also indicating increased levels of total cells, MPO, IL8, NO and Urea in compost workers compared to controls, reaching statistical significance for total cells, IL-8, NO and urea. No differences ($p \geq 0.2$) in cross-shift inflammatory responses were observed between NAL's performed on Mondays and Fridays.

Differences in smoking habits between controls and workers could have contributed to the observed differences in pre-shift levels and/or cross-shift changes in NAL inflammatory markers between the two groups. There were, however, no statistical differences in pre-shift NAL levels between smoking ($n = 9$) and non smoking workers ($n = 5$). In fact, concentrations of pre-shift NAL inflammatory markers were even lower in smokers than in non-smokers (fig 2). Cross-shift changes in concentrations of cells and inflammatory markers were also smaller in NAL of smoking workers than of non-smoking workers (fig 1), being statistically significant only for MPO ($p < 0.05$).

In most NALs of both compost workers and controls the majority of cells were leucocytes (60-85%), of which almost all were neutrophils (table 4). Eosinophils were found in 3 of the controls and in none of the workers. No significant difference in proportion of cell types in pre-shift NAL existed between workers and controls, whereas in post-shift NAL of workers a significantly higher proportion of neutrophils was observed, with a corresponding lower proportion of epithelial cells (table 3). Apparently the wash out effect that presumably caused the lower cell numbers in post-shift NAL of the controls primarily affected the number of (neutrophilic) leucocytes and not the number of epithelial cells. The cross-shift increase in total cells and an equal proportion of neutrophils in pre- and post-shift NAL of the workers was thus most likely due to an active influx of neutrophils.

No clear trends with exposure were found for cross-shift inflammatory responses (data not shown). A weak dose-response trend was observed,

however, with pre-shift NAL parameters and bio-aerosol exposure. Based on the repeated endotoxin exposure measurements, individual geometric mean (GM) exposures were calculated for each worker. Subsequently, workers were divided in 'low' (n = 7) and 'high' (n = 7) exposure groups based on their individual geometric mean endotoxin exposure, using the group median as cut-off point (461 EU/m^3 [range of individual GMs: 55-773 EU/m^3]). Pre-shift inflammatory markers were higher in the high exposed group reaching statistical significance for MPO ($p < 0.1$) and urea ($p < 0.05$; figure 2). Almost identical results were obtained when 'high' and 'low' ß(1→3)-glucan and dust exposure groups were compared.

Table 4. Cell differentials (percentages with min-max) in NAL of controls and compost workers

Cell type		Controls (n = 10)[†]	workers (n = 14)
Leucocytes	pre-shift	77.0 (0.0-99.4)	84.7 (7.0-99.2)
	post-shift	60.0 (0.0-84.5)	86.1 (12.5-97.0)[**]
Neutrophils	pre-shift	77.0 (0.0-89.3)	82.9 (6.8-99.0)
	post-shift	60.0 (0.0-83.5)	84.0 (13.0-97.0)[**]
Eosinophils	pre-shift	0.0 (0.0-6.7)	0.0 (0.0-0.0)[**]
	post-shift	0.0 (0.0-5.1)	0.0 (0.0-0.0)[**]
Mononuclear	pre-shift	0.0 (0.0-4.4)	0.4 (0.0-27.1)
	post-shift	0.0 (0.0-1.4)	0.5 (0.0-4.4)
Epithelial	pre-shift	23.0 (0.6-100.0)	15.4 (0.8-93.2)
	post-shift	40.0 (15.4-93.2)	13.9 (3.4-87.5)[**]

[†] n = 9 for all cell determinations in post-shift measurements
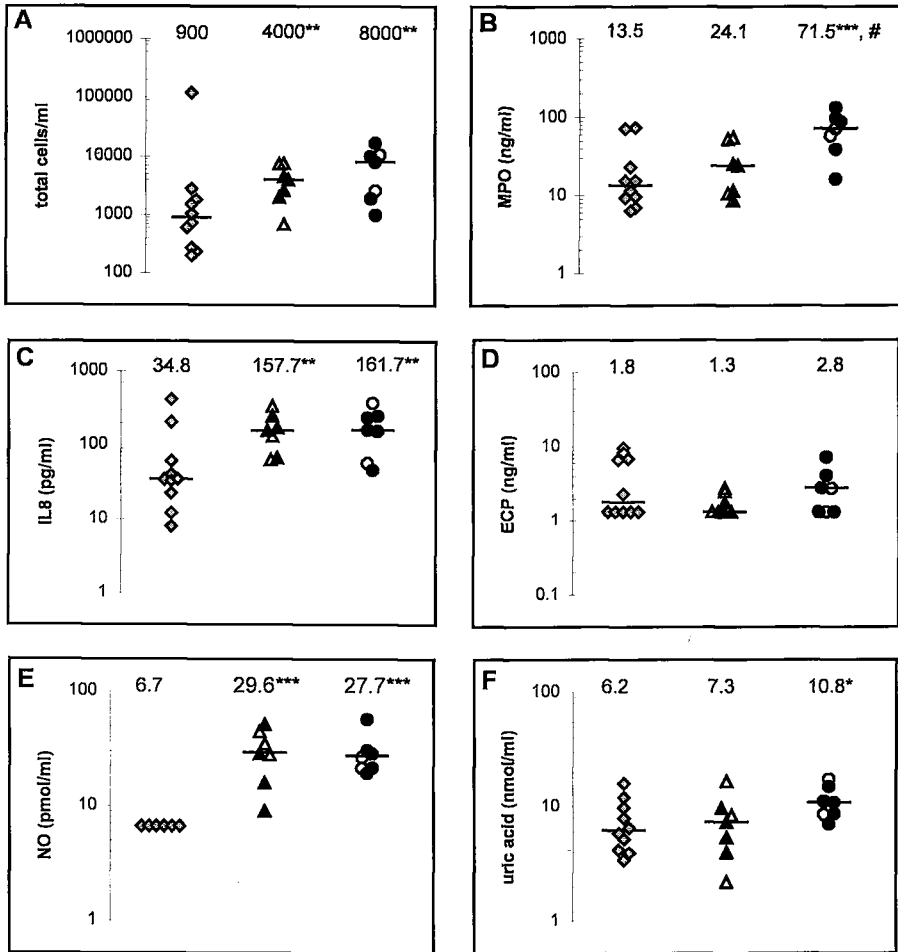[**] $p < 0.05$ vs controls; Wilcoxon rank sum test

Fig 2: Pre-shift concentrations of inflammatory markers (A; total cells; B; MPO, C; IL8, D; ECP, E; NO, F; uric acid, G; urea, H; albumin) in NAL of controls (shaded diamonds), workers with mean endotoxin exposure of less than 460 EU/m³ (triangles), and workers with mean endotoxin exposure of more than 460 EU/m³ (circles). Open symbols (triangles and circles) represent the non-smoking workers. pre-shift NO and ECP concentrations were non detectable in NALs of all controls, and 5 controls and 7 workers, respectively. Median group levels are listed in upper part of the figure (* p<0.1, ** p<0.05, *** p<0.01; comparing controls with workers, # p<0.1, ## p<0.05; comparing 'low' exposed with 'high' exposed workers).
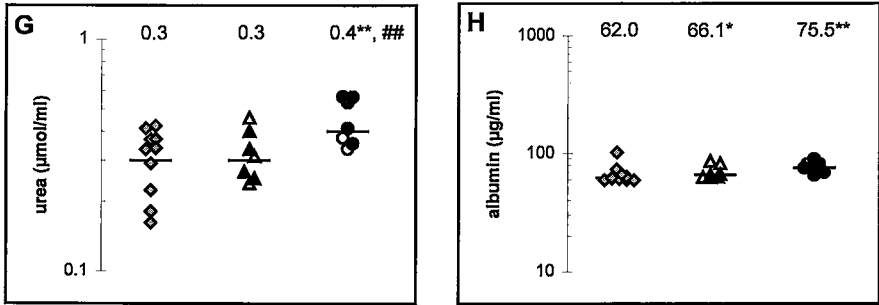
135

Fig. 2G and 2H; see page 135

Discussion

Results indicated work related acute and possibly (sub-)chronic upper airway inflammation in this relatively small group of compost workers. Compared to controls a cross-shift increase in levels of neutrophils, MPO, IL8, NO and urea was shown and for most of these markers elevated pre-shift levels were observed as well. Levels of eosinophils and ECP did not show a cross-shift or pre-shift increase. Exposure to microbial agents in this work environment apparently induces upper airway inflammation in which neutrophil infiltration and activation predominates, whereas eosinophils play a minor role. Specific serologic IgE determination against a mix of various molds (*Penicillium notatum, Cladosporium herbarum, Aspergillus fumigatus, Alternaria tenuis*) showed only one positive reaction out of 12 tested workers (data not shown), also suggesting that a work related IgE-mediated type I inflammation, like in occupational asthma, does not seem plausible. We presume that epithelial cells and activated neutrophils in the nose released IL-8 following exposure, contributing to chemoattraction and activation of more neutrophils, resulting in increased levels of cells, IL-8 and MPO. NO was most likely produced by nasal epithelium and possibly macrophages.[22,23] NO measured in the NAL fluid may, however, not have accurately reflected endogenously produced NO, because of high exogenous sources (1.0 - 1.7 ppm in compost hall compared to 20-40 ppb in a control room). The relation between exogenous NO and NO levels in NAL fluid is, however, as yet not clear. Elevated urea levels suggested increased vascular endothelial permeability.

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Differences between controls and workers with respect to smoking habits and gender may have biased our results. No differences in inflammatory response were noted, however, between men and women within the group of controls (data not shown), while smoking was associated with slightly decreased pre-shift NAL concentrations and lower cross-shift inflammatory responses. Thus if smoking biased our results, this most probably would have led to an underestimation of the actual difference in acute inflammatory response between controls and workers in our study, since there were more smokers in the group of workers (64%) than in the controls (10%). A similar difference between smokers and non-smokers has been demonstrated in workers exposed to fuel oil ash.[12] Another study showed no difference between smokers and non-smokers in ozone induced lower airway inflammation.[24]

Total cells (mainly neutrophils), MPO, IL8, NO and albumin levels in pre-shift NAL of workers were clearly higher than in the controls (table 3) and they were weakly associated with bio-aerosol exposure. No differences in pre-shift levels were found between Mondays and Fridays. It is not clear whether the increased levels of cells and cytokines indicate chronic inflammation or a delayed inflammatory response from previous exposure, 16 - 64 h before NAL. Other studies showed that intense acute lower airway inflammation can persist for at least 24 h following bio-aerosol exposure.[8,9] Whether bio-aerosol exposure, as encountered by the compost workers, can induce an acute inflammatory response that is still measurable after two days without exposure remains, however, unclear.

In controls a cross-shift decrease was observed in the number of cells and levels of cytokines, most likely due to a washout effect. Washout of cells primarily affected the number of neutrophils and not epithelial cells. Washout of cells at 18 h and 48 h after nasal lavage has been described earlier.[6,25] The differences between post and pre-shift cell and inflammatory marker levels in NAL of workers are thus in fact underestimated due to these washout effects, which - after 8 h - amounted to approximately 40 % as observed in the controls.

Two recent studies, using both NAL and BAL, showed acute intense upper and lower airway inflammation in healthy naive subjects exposed to bio-aerosols in swine confinement buildings.[8,9] Another study showed moderate upper respiratory airway inflammation in grain workers.[13] In these studies inflammation was characterized by an influx of neutrophils[13] with increased levels of IL-1,

TNF-a, IL-6 and IL-8,[8,9] and absence of eosinophils like in our study. Endotoxin exposure in one of these studies was weakly correlated with post exposure IL-6 and albumin levels in BAL but not in NAL,[9] while in the other studies no relation was found with endotoxin exposure. The cross-shift effects in our study were also not clearly associated with endotoxin and ß(1→3)-glucan exposure. Nevertheless we presume that these agents played a role in the observed upper airway inflammation, since mean endotoxin exposure levels in our study, ranging from 75 - 530 EU/m^3 (5 - 35 ng/m^3), exceeded the level of 10 ng/m^3, recently suggested to be a 'no effect level' for airway inflammation.[26] ß(1→3)-glucans, recently suggested to be associated with respiratory symptoms,[30,31] also have pro-inflammatory capacities.[28,29] At present, however, little is known at what levels ß(1→3)-glucans can initiate airway inflammation. Little variation in exposure, the small number of subjects, and a large intra-individual variation in inflammatory markers may explain why a relation with acute inflammation was not found. Besides endotoxins and ß(1→3)-glucans, other microbial but also non-microbial components may have accounted for the observed inflammatory effects.

The magnitude of acute airway inflammation in the compost workers was less than observed in healthy volunteers exposed to swine dust,[8,9] in which cells and cytokines increased 10 - 40 fold after a single 3 h exposure compared to a 2 - 3 fold increase measured in our study. Dust as well as endotoxin exposure were, however, 5-20 times higher in the swine dust studies. Another explanation might be that we studied chronically exposed workers (mean duration of employment was 6 months) who may have developed a tolerance for the exposure resulting in only a moderate airway inflammation, a phenomenon that has been described previously for various other endotoxin-exposed occupational populations. This is also in concordance with results from two other studies[13,32] which showed only moderate airway inflammation - in the same order of magnitude as described in this paper - in workers (pig farmers and grain workers) with similarly high organic dust and endotoxin exposure as in the pig dust exposure study with naive subjects.

Lung function was normal in the studied compost workers but prevalence of nasal symptoms and symptoms of bronchitis were high. As yet, it is not clear whether the observed upper airway inflammation is an early indicator of respiratory injury causing the high prevalence of nasal and bronchitis symptoms.

Large scale longitudinal studies are needed to investigate this.

In conclusion, work related upper airway inflammation was indicated in compost workers, most likely representing non-immune or possibly type III, but not type I allergic inflammation. These inflammatory responses were most probably due to bio-aerosol exposure. Specific causal agents could, however, thus far not be identified.

Acknowledgements

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Chapter 9

General discussion

9.1 Introduction

It is well recognized that exposure to airborne non-infectious micro-organisms poses a respiratory health risk for individuals living in damp and moldy homes and for workers in industries where microbial exposure is abundant. Although much effort has been put into the measurement of exposure to micro-organisms in many studies, dose-response relationships with respiratory health effects have been difficult to establish. This is mainly due to the lack of good quantitative methods for exposure assessment. The studies described in this thesis validated an existing method to measure bacterial endotoxin and explored new methods for measuring mold components in the environment, particularly ß(1→3)-glucans and extracellular polysaccharides (EPS). Endotoxin in the work environment is a well-known cause of occupationally induced airway obstruction and, according to a few studies, endotoxin in the residential indoor environment may also be causally related to adverse respiratory health effects. ß(1→3)-glucans, cell wall components of molds and also of some bacteria and most plants, are known to elicit a wide range of inflammatory reactions in vitro, but at present there is only limited evidence for a causal role in the development or aggravation of respiratory symptoms. Mold EPS do not have known pathogenic properties themselves but may be used as genus-specific markers of mold exposure. The various assays were used to study levels of these microbial components in the home and occupational environment, and to investigate their relationships with respiratory health in children or workers, respectively.

9.2 Validity of methods for assessment of microbial concentrations

The methods studied in this thesis have obvious advantages for use in epidemiological studies because measurements can be performed on samples that are stored frozen, and costs per sample are relatively low. In addition, measurement of endotoxin and possibly also ß(1→3)-glucan has direct relevance to the health effects that are studied. An important question that remains, however, is whether these methods provide valid assessment of microbial concentrations.

For comparison no 'golden standards' are available and we thus instead compared our methods with a frequently applied culture-based method. ß(1→3)-glucan and EPS-*Asp/Pen* in house dust were weakly correlated with total culturable fungi in house dust, and an even weaker correlation was found between EPS-*Asp/Pen* and counts of culturable *Penicillium* and *Aspergillus* spp. Lack of stronger associations with culturable fungi can have several reasons: 1) ß(1→3)-glucan is, as previously mentioned, not a specific marker of fungal mass because it also originates from other sources[1]; 2) only culturable fungi were counted, while fungal ß(1→3)-glucan and EPS-Asp/Pen most likely represent total fungal biomass, including non-culturable spores and mycelia; and 3) CFU measurements were performed on different samples collected from the same location, whilst it is known from a previous study that CFU counts in house dust samples taken from the same location (within a 6-week interval) show poor reproducibility.[2] Variability in isolated genera and species between duplicate samples was even more substantial in that study. The weak correlations with CFU counts may thus be largely due to the limitations of culture-based methods. Our study showed that EPS-*Asp/Pen* levels were much stronger associated than CFU counts with reported damp and mold spots in the home, which may indicate that the EPS-assay provides a better method to assess indoor fungal concentrations. Several other studies have also found only weak associations between culturable mold counts in house dust or in the air, and signs of mold and dampness in the home.[3-8] In another recent study we showed that ß(1→3)-glucan, EPS-*Asp/Pen* as well as endotoxin levels in house dust were positively associated with the presence of a container for compostable house hold waste (as a suspected source of microbial growth in the home) and negatively associated with the frequency with which the container was emptied an/or cleaned (Wouters *et al*, in preparation). This suggests that these measures indeed provide valuable information about microbial sources indoors.

9.2.1 Measurement of environmental endotoxin

A field study described by Zock *et al*,[9] performed in the potato processing industry, confirmed that 0.05 % Tween 20 increased the extraction efficiency considerably. Questions remained, however, to the generalizability of these findings for samples from other environments. Currently two large international round robin studies in which our laboratory was involved, confirmed the effect

of 0.05% Tween-20 for other types of dust (compost dust, Douwes *et al*, in preparation; swine, corn and poultry dust, Reynolds *et al*, in preparation). Increased extraction efficiency associated with the use of 0.05% Tween 20 has also been shown for extraction of rat and mouse allergens from filters collected in laboratory animal facilities.[10,11] Two studies,[12,13] using bio-aerosol samples from various other sources (swine and poultry dust, cotton dust, and endotoxin containing machining oil aerosol) also confirmed our findings that endotoxin extraction efficiency is better for glass fibre filters than for other types of filters e.g. polycarbonate, cellulose acetate and PVC.

An occupational exposure limit for endotoxins has recently been proposed in The Netherlands[14] and is considered in other countries. It is therefore important to agree on rigorous standardization with respect to analytical methods, sampling media, extraction media, and storage conditions. Based on recent experience it should be recommended to sample environmental endotoxin on glass fibre filters, elute them with 0.05% Tween 20, and store the extracts at -20°C. Repeated freezing and thawing should be avoided. This should provide the most efficient recovery of environmental endotoxin. The European committee for standardization is currently preparing a standard for the determination of airborne endotoxin, in which these recommendations will be considered.[15]

9.2.2 Measurement of environmental ß(1→3)-glucans

Previous studies on the effects of airborne exposure to ß(1→3)-glucans employed a glucan reactive preparation of *Limulus* amebocyte lysate (LAL).[16,17] It is at present not known how comparable results from the ß(1→3)-glucan EIA and the LAL assay are. There are some obvious differences that may lead to different outcomes of measurements including: 1) the LAL assay is much more sensitive i.e. 1pg/ml[17] vs 40 ng/ml; 2) the LAL assay is not entirely specific since it also reacts with other glucans and polysaccharides,[18] whereas the EIA was proven to be highly specific for ß(1→3)-glucans; and 3) activation of LAL depends on chain length and conformation of the glucans,[19,20] while that may not be the case for the EIA. A comparison between both methods using ß(1→3)-glucan-containing environmental dust samples is currently in progress.

The ß(1→3)-glucan EIA was sufficiently sensitive for measurement of samples from studies described in this thesis i.e. settled dust in homes and dust samples from occupational environments with high exposure levels. However, for studies

in environments with (much) lower levels a more sensitive method like a sandwich EIA is required.

The fact that ß(1→3)-glucans from various plants also reacted in the EIA demonstrated that ß(1→3)-glucan as measured with our method can not be regarded as an entirely specific marker for fungal biomass in the environment. This has also been demonstrated for the LAL assay.[20] It is currently not clear whether plant ß(1→3)(1→4)-glucans possess similar biological properties as fungal ß(1→3)(1→6)-glucans. Our study suggests that ß(1→3)-glucans in house dust as measured with our method, most likely including plant ß(1→3)-glucans, are a cause of increased PEF-variability or at least associated with the 'true' causal agents.

9.2.3 Measurement of environmental fungal extracellular polysaccharides

In our study we confirmed previous investigations[21,22] that showed that the EPS-*Asp/Pen* sandwich immunoassay was highly specific. House dust extracts that were stored frozen for 4 years still yielded positive results, suggesting that EPS in environmental samples are very stable, which is in accordance with a previous study that demonstrated that EPS are stable in food products even after heat treatment.[21] The EPS-*Asp/Pen* assay was sufficiently sensitive to measure EPS in house dust, but it is at present not clear whether the assay is also sensitive enough to measure airborne concentrations. Airborne personal dust samples collected in compost workers, pig farmers and waste collectors were shown to contain detectable concentrations of EPS-*Asp/Pen* in only part of the samples (Wouters *et al*, in preparation). Studies are in progress to further investigate its usefulness in the assessment of occupational airborne exposures of fungi.

In our study it was shown that EPS-*Asp/Pen* in house dust was associated with observed mold and damp spots in the home and that in addition, EPS-*Asp/Pen* was significantly but weakly associated with total culturable fungi in house dust. This would suggest that, at least in our study, EPS-*Asp/Pen* was a good marker for total fungal biomass. Whether this is generalizable to other indoor studies is unclear and probably depends on the variation of the genera present in the indoor environment that is studied. Other assays for the measurement of EPS of other genera (*Alternaria, Mucor, Cladosporium*) are currently available and should also be tested in future studies. Application of these assays should,

at least partially, determine whether certain fungal genera are more strongly associated with respiratory health effects than others, and if so, which genera are most important. Main advantage of the EPS-assay compared to other quantitative methods that measure more general fungal markers (e.g ergosterol and ß(1→3)-glucan), may thus be that important qualitative information on fungal burden in the environment can be obtained.

9.2.4 Measures of exposure

Microbial exposure of workers in the occupational environment for epidemiological studies is probably best assessed by performing personal sampling of airborne microorganisms or their components during a work day. In the home environment personal sampling is usually not an option because analytical sensitivity is not sufficient for most microbial agents. Culture-based methods may be used for airborne measurements in the home, but given the high variability of microbial concentrations over time and the relative short sampling time allowed for this method (<15 minutes), they give at best a 'snapshot' of the concentration present. Many studies therefore analyze samples of settled dust for microorganisms and their components, which may reflect exposure integrated over time. Some authors have argued that measurements in settled dust may not represent concentrations in the air,[23] because microorganisms may grow and multiply at high humidity in house dust.[24] Our study demonstrated that EPS-*Asp/Pen* and ß(1→3)-glucans measured in settled dust were associated with respiratory health, which may indicate that settled house dust measurements at least provide a good proxy for exposure. For assessment of dust mite allergen exposure this method has already been employed for many years showing an association with respiratory health in many studies. For endotoxin, ß(1→3)-glucan, and EPS-*Asp/Pen* reproducibility of settled dust measurements is at present largely unknown. One study reported a maximum 10-fold difference between mean endotoxin concentrations measured every month over a one year period in house dust from 20 Brazilian homes.[25]

When using settled dust sampling it is not known to what extent the concentration expressed per gram dust or per square meter sampled reflect the actual exposure of the occupants. In our study EPS-*Asp/Pen* levels were associated with respiratory health when expressed per g dust. ß(1→3)-glucan and endotoxin levels were associated with PEF-variability only when expressed

per m² sampled area, but not when expressed per g dust. House dust mite allergen levels were associated with PEF-variability independently of how levels were expressed. Dust mite levels per g dust and per m² were, however, very strongly correlated, whereas glucan and endotoxin levels expressed relative to dust weight were poorly correlated with absolute levels present on the floors. Strong correlation between both measures for *Der p* I concentrations has been shown previously by van Strien *et al*,[26] and they suggested, given this high correlation that both measures could be used for exposure assessment. Coloff *et al*[27] suggested that concentrations of dust mite allergens expressed per m² would give a better estimate of the potential exposure. Given these findings, both measures per m² and per g dust should be explored for microbial agents in future epidemiologic investigations.

9.3 Associations with respiratory health

We found associations between mold components measured in house dust and adverse respiratory health effects, in agreement with previous studies that consistently demonstrated an association between reported mold growth and/or home dampness, and respiratory symptoms.[3,7,28-34] An inverse association was found with bedroom EPS-*Asp/Pen* levels, which was probably due to allergen avoidance measures. Omitting subjects whom had taken obvious allergen avoidance measures in the bedroom resulted in somewhat higher odds ratios, suggesting that this assumption may indeed be valid. ß(1→3)-glucan levels measured in dust from bedroom floors and mattresses were also not positively associated with respiratory health, most likely due to the same reason. Whether ß(1→3)-glucans levels in house dust are causally related to respiratory health in asthmatic children or that the relation with respiratory health effects is due to correlation with other currently unknown pathogenic substances in house dust, is at present not clear. Animal and *in vitro* studies have shown that ß(1→3)-glucans can initiate a variety of biological responses in vertebrates (chapter 2). In addition, several recent studies suggested a role for ß(1→3)-glucans in bio-aerosol induced inflammatory responses and resulting respiratory symptoms (chapter 2). However, evidence for a causal role of ß(1→3)-glucan in environmentally induced respiratory symptoms was not very strong in these studies.

Crude unadjusted regression analyses showed an association between endotoxin levels and PEF variability which did not remain in a multiple regression

analysis after adjusting for other potential confounders including presence of pets in the home. Previous studies have shown an association between endotoxin levels in house dust and clinical severity of asthma in asthmatic adults and children.[25,35,36] In these studies, however, relationships were not adjusted for other known risk factors. In our study the presence of pets in the home was associated strongly with PEF-variability in both the crude and the adjusted regression analyses. Interestingly, presence of pets was also associated with higher endotoxin levels measured in living room floor samples (3074 vs 1569 EU/m^2; p < 0.05). Part of the effect on PEF variability associated with pets may thus be related to endotoxin exposure. Pets are also well known sources of allergens, and may thus have contributed to PEF-variability through allergic mechanisms. This seems plausible considering the high prevalence of dog or cat positive skin prick tests among the symptomatic children (up to 50% in the asthmatic group). The role of endotoxins in the home environment thus remains unclear from our data.

Some studies have suggested a type I allergic reaction as a plausible mechanism for the observed association between fungal indoor exposure and respiratory health symptoms.[33,34] Evidence for fungal induced type I allergic reactions as the primary pathophysiological mechanism was, however, weak. It is generally assumed that respiratory symptoms result from preceding airway inflammation which can either be induced by allergic IgE or IgG mediated mechanisms or by non-immune mediated mechanisms. Fungi, as well as bacteria, contain both IgE and IgG inducing allergens and pro-inflammatory and toxic agents, and are thus able to induce both inflammatory mechanisms in the airways. In our study the association between ß(1→3)-glucan and PEF-variability was observed primarily in asthmatics and/or atopics. This relationship is, however, unlikely to be based on type I allergic reactions, since ß(1→3)-glucans are considered to be non-immunogenic.[1] More pronounced lung function responses in atopics and asthmatics have previously also been shown for endotoxin, another non-allergenic component, in occupationally exposed pig farmers and experimentally challenged subjects, respectively.[37,38] Presumably, ß(1→3)-glucan and possibly endotoxin exposure may increase PEF-variability in atopic asthmatics by enhancing, non-specifically, pre-existing allergic or non-allergic inflammation. Alternatively, they may activate an independent pathway of airway inflammation that is most strongly expressed in hyper-responsive atopic asthmatics. Possible biological

mechanisms through which ß(1→3)-glucan may induce respiratory health effects thus remain unknown. The EPS study did not provide evidence for specific pathophysiological mechanisms, involved in the development or aggravation of fungal induced respiratory symptoms. The study among waste composting workers that were shown to be heavily exposed to microorganisms and their components, including endotoxin and ß(1→3)-glucan, showed that resulting upper airway inflammation was primarily a non-immune or possibly a type III allergic inflammation. Considering the endotoxin exposures, which were well above the suggested occupational exposure limit recently proposed by the Dutch health council, non-immune specific inflammation seems plausible. No evidence was observed for a type I allergic inflammation, since no ECP and eosinophils were involved in the inflammatory response, and no increased serum IgE levels against specific fungal antigens were observed. It may thus be that non-immune specific inflammation is more important than type I allergic inflammation as the mechanism underlying microbial induced respiratory health effects, but that these inflammatory processes may be stronger and/or may occur more frequently in atopics and or asthmatics.

The number of subjects involved in the epidemiological studies were small. Therefore larger and preferably prospective studies should be conducted in the future, to confirm the relationships with respiratory health and microbial exposure as measured with the methods described in this thesis.

9.4 Conclusions

- The studies reported in this thesis contribute to new methods for indoor microbial exposure assessment, that offer obvious advantages for use in epidemiological studies compared to traditionally employed culture-based methods.

- Endotoxin exposure measurements may vary considerably between laboratories using different sampling, extraction and storage protocols.

- The newly developed ß(1→3)-glucan EIA and the newly introduced EPS-*Asp/Pen* EIA offer specific and sensitive methods for assessment of indoor exposure to ß(1→3)-glucans and fungal biomass, respectively.

- House dust levels of ß(1→3)-glucan and the fungal marker EPS-*Pen/Asp* are associated with adverse respiratory health effects in children.

- ß(1→3)-glucan and endotoxin levels in the home environment are associated with certain home characteristics. Further investigations of these relations may contribute to the development of control measures.

- There is little evidence that type I allergy plays an important role in the airway inflammation induced by inhaled microbial agents. Instead, non-immune mediated direct activation of inflammatory cells seems to be the predominant mechanism underlying microbial agent induced respiratory symptoms.

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Summary

Chapter 1 summarizes the background of the study. Indoor exposure to airborne non-infectious microorganisms and their components, both in the residential and occupational environment, is well recognized as a plausible cause of acute and chronic non-infectious respiratory diseases. Dose-response relationships have, however, been difficult to establish. This is mainly due to the lack of good quantitative methods for exposure assessment of microorganisms and their components. Main objectives of the studies described in this thesis were:

1. To validate and improve methods to assess indoor exposure to non-infectious microorganisms and their components.
2. To study the role of microbial exposure in the development of respiratory symptoms in residentially and occupationally exposed populations.

Chapter 2 presents a review of the literature on exposure assessment methods of non-infectious microorganisms and health effects of endotoxins and ß(1→3)-glucans. Airborne exposure to microorganisms in the environment can be studied by counting culturable propagules in air samples or in settled dust samples. This has, however, proven to be of limited use in epidemiological studies. Non-culture-based methods are believed to give better and more reproducible estimates of microbial concentrations. Non-culture-based techniques can be divided in (1) microscopy and flow cytometry for counting individual spores or cells, and (2) chemical/biochemical methods for the measurement of specific microbial components. Particularly the last category includes methods that may be very useful in epidemiological studies. This thesis focussed on the measurement of three microbial components: bacterial endotoxin, mold ß(1→3)-glucans and mold extracellular polysaccharides. Endotoxins, pro-inflammatory cell wall components from gram negative bacteria, are well-known agents in the occupational environment that can cause non-immune mediated airway diseases. A similar role for endotoxin in the residential indoor environment has recently been suggested. ß(1→3)-glucans, cell wall components of molds and also of some bacteria and most plants, are known to elicit a wide range of inflammatory reactions in vitro, but at present there is only limited evidence for a causal role in the development or aggravation of respirato-

ry symptoms. Mold EPS do not have known pathogenic properties themselves but may be used as genus-specific markers for mold exposure.

Chapter 3 describes the influence of filter types and extraction conditions on the quantitation of airborne endotoxin with the *Limulus* amebocyte lysate test by using dusts sampled in a potato processing plant. The endotoxin extraction efficiency of 0.05% Tween-20 in pyrogen-free water was seven times higher than that of pyrogen-free water only. Two-times-greater amounts of endotoxin were extracted from glass fibre, teflon and polycarbonate filters than from cellulose ester filters. Repeated freeze (-20°C)-and-thaw cycles with commercial LPS solutions showed a 25% loss in endotoxin activity per freeze-thaw cycle. House dust extracts showed a decrease of about 20% in the endotoxin level after they had been frozen and thawed for a second time.

Chapter 4 describes a newly developed inhibition enzyme immunoassay (EIA) for the measurement of ß(1→3)-glucans in the environment. The laminarin based calibration curve in the inhibition EIA ranged from ≈ 40 - 3,000 ng/ml (15 - 85% inhibition). The assay was shown to be very specific and to detect heat extractable ß(1→3)-glucan in dust samples collected in a variety of occupational and environmental settings. Based on duplicate analyses of dust samples a coefficient of variation was calculated of approximately 25%.

Chapter 5 describes endotoxin and ß(1→3)-glucan levels in house dust from 25 German houses, and the relations with home characteristics. Endotoxin and ß(1→3)-glucan were readily detectable in all samples and were highest on living room floors and lowest in mattresses. Dust, endotoxin and ß(1→3)-glucan levels were 2-3 times higher on living room floors of centrally heated houses built after 1970 compared to older individually heated houses. This was not found for mattresses and bedroom floors. No associations between biocontaminant levels and other home characteristics were found. ß(1→3)-glucan levels were significantly but not very strongly correlated with culturable fungi.

Chapter 6 describes a sandwich EIA for the measurement of extracellular polysaccharides of *Aspergillus* and *Penicillium* species (EPS-*Asp/Pen*) in house dust, as a marker for fungal biomass. Extracts of house dust samples from

homes of 31 children with, and 29 children without chronic respiratory symptoms were analysed. EPS-*Asp/Pen* were detectable in all house dust extracts, with highest concentrations in living room floor dust (compared to bedroom and mattress dust). EPS-*Asp/Pen* levels were 2-3 times higher on carpeted floors than on smooth floors. EPS-*Asp/Pen* were significantly but not very strongly correlated with total culturable fungi. EPS-*Asp/Pen* levels in living room floor dust were positively associated with occupant-reported home dampness and with respiratory symptoms. This was not observed for EPS-*Asp/Pen* in bedroom floor and mattress dust.

Chapter 7 describes the relationship between endotoxin and ß(1→3)-glucan levels in house dust and peak flow (PEF) variability in 159 children. Children were selected based on respiratory symptoms, such that 50% had chronic respiratory symptoms, and 50% had no symptoms. Children self-monitored twice daily their PEF for a period of 16 weeks. Unadjusted regression analyses showed that living room exposure to endotoxin and ß(1→3)-glucan were significantly associated with PEF-variability in symptomatic children, and particularly in atopic children with asthma symptoms. Regression analyses adjusted for dust mite allergen levels, presence of pets in the home and type of floor cover showed the same association for ß(1→3)-glucan while for endotoxin no significant association remained. No relations were found between PEF-variability and levels of microbial agents in bedroom floor or mattress dust.

Chapter 8 describes upper airway inflammation in 14 compost workers. Airway inflammation was assessed by performing repeated pre and post-shift nasal lavages (NAL) during 3 weeks on Mondays and Fridays. Results were compared with those of pre- and post-shift NALs in ten controls on Monday. In NAL total cells, cell differentials, MPO, IL8, ECP, NO, uric acid, urea and albumin were determined. Job title-based mean endotoxin and ß(1→3)-glucan exposure levels ranged from 75-527 EU/m^3 and 0.5-4.9 μg/m^3, respectively. A cross-shift 1.4-3.2 fold increase in total cells, MPO, IL8, NO and urea was observed in NAL of compost workers compared to controls. Most cells in NAL were neutrophils, whereas eosinophils were only incidentally observed. No association between exposure level and cross-shift inflammatory responses was found. Total cell, MPO, IL8, NO and albumin levels in pre-shift NAL of workers were elevated

(1.1-4.8 fold) compared to controls and weakly associated with levels of bio-aerosol exposure. Typical type I inflammatory responses (e.g increased eosino-phils and ECP levels) were not observed.

The general discussion (**Chapter 9**) is focussed mainly on validity of methods for assessment of microbial concentrations as used in our studies, and the asso-ciations found between the measures obtained with these methods and respi-ratory health. It was concluded that:

- The studies reported in this thesis contribute to new methods for indoor microbial exposure assessment, that offer advantages for use in epidemio-logical studies compared to traditionally employed culture-based methods.
- Endotoxin exposure measurements may vary considerably between labo-ratories using different sampling, extraction and storage protocols.
- The newly developed ß(1→3)-glucan EIA and the newly introduced EPS-*Asp/Pen* EIA offer specific and sensitive methods for assessment of indoor exposure to ß(1→3)-glucans and fungal biomass, respectively.
- House dust levels of ß(1→3)-glucan and the fungal marker EPS-*Pen/Asp* are associated with adverse respiratory health effects in children.
- ß(1→3)-glucan and endotoxin levels in the home environment are associated with certain home characteristics. Further investigations of these relations may contribute to the development of control measures.
- There is little evidence that type I allergy plays an important role in the airway inflammation induced by inhaled microbial agents. Instead, non-immune mediated direct activation of inflammatory cells seems to be the predominant mechanism underlying microbial agent induced respiratory symptoms.

Samenvatting

Hoofdstuk 1 beschrijft de achtergrond van de studie. Inhalatoire blootstelling aan niet-infectieuze microorganismen en microbiële componenten, zowel in de woon- als werkomgeving, wordt algemeen beschouwd als een mogelijke oorzaak van acute en chronische luchtwegaandoeningen. Blootstelling-respons relaties zijn echter moeilijk vast te stellen. Het ontbreken van goede meetmethoden om de blootstelling aan micro-organismen en microbiële componenten te schatten in epidemiologische studies is hiervoor de belangrijkste reden. De belangrijkste doelstellingen van de studies beschreven in dit proefschrift waren:

1. Het valideren en verbeteren van meetmethoden om de blootstelling aan niet-infectieuze microorganismen en hun componenten te bepalen.
2. Het bestuderen van de rol van microbiële blootstelling in de ontwikkeling van luchtwegsymptomen in populaties die beroepsmatig of in hun woonomgeving worden blootgesteld.

Hoofdstuk 2 geeft een overzicht van de literatuur over beschikbare methoden om blootstelling aan non-infectieuze microorganismen te bepalen. Tevens worden de gezondheidseffecten van endotoxinen en ß(1→3)-glucanen besproken. Inhalatoire microbiële blootstelling kan bestudeerd worden door het tellen van levensvatbare microorganismen (kolonievormende eenheden) in luchtmonsters of in gedeponeerd stof. Deze methode heeft echter voor epidemiologische toepassingen een zeer beperkte waarde. Van de zogenaamde 'non-culture-based' methoden, methoden die niet afhankelijk zijn van de levensvatbaarheid van microorganismen, wordt verwacht dat ze betere en reproduceerbaardere schattingen geven van de microbiële blootstelling. Deze technieken kunnen worden ingedeeld in 1) microscopie en flow-cytometrie voor het tellen van individuele sporen en cellen, en 2) chemisch/biologische methoden voor het meten van specifieke microbiële componenten. Met name methoden uit deze laatste categorie lijken voor epidemiologische toepassingen zeer geschikt. In dit proefschrift worden meetmethoden voor bacteriële endotoxinen, schimmel ß(1→3)-glucanen en extracellulaire polysacchariden (EPS) besproken. Inhalatoire blootstelling aan endotoxinen, celwandbestanddelen van gramnegatieve bacteriën, kan in het arbeidsmilieu resulteren in acute en chronische non-immuun-

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specifieke obstructieve luchtwegeffecten. Enkele recente studies hebben gesuggereerd dat endotoxinenblootstelling in de woonomgeving tot verergering van luchtwegsymptomen kan leiden. ß(1→3)-glucanen, celwandcomponenten van schimmels, sommige bacteriën en de meeste planten, zijn net als endotoxinen in staat ontstekingsreacties *in vitro* te induceren, en zijn recent in verband gebracht met luchtwegsymptomen bij de mens. Bewijsvoering voor een causale rol van ß(1→3)-glucanen in de ontwikkeling van luchtwegsymptomen was echter zwak. EPS zijn zelf niet pathogeen maar kunnen mogelijk gebruikt worden als genusspecifieke markers voor schimmelblootstelling.

Hoofdstuk 3 beschrijft de invloed van filtermateriaal en extractiecondities op de detecteerbare hoeveelheid endotoxinen, geanalyseerd met behulp van een *Limulus* amebocyte lysaat test, in luchtmonsters genomen in een aardappelverwerkingsbedrijf. De endotoxinen-extractie met 0.05% Tween-20 in pyrogeenvrij water gaf 7 maal hogere endotoxinenopbrengsten dan met pyrogeenvrij water alleen. Gebruik van glasvezel-, teflon- en polycarbonaatfilters resulteerde in twee maal hogere endotoxinenopbrengsten dan wanneer cellulose-esterfilters werden gebruikt. Herhaald invriezen en ontdooien van commercieel verkrijgbaar endotoxine resulteerde in een 25% afname in endotoxinenactiviteit per vries-dooi cyclus. Endotoxinenniveaus in huisstofextracten namen 20 % af nadat ze voor een tweede keer waren ingevroren en ontdooid.

Hoofdstuk 4 beschrijft een nieuw ontwikkelde inhibitie enzym immunoassay (EIA) om ß(1→3)-glucanen te meten. Concentraties tussen de 40 en 3000 ng/ml (15 - 85% inhibitie), gebaseerd op een ijklijn van laminarin [ß(1→3)-glucan], konden met deze assay worden gemeten. De assay was zeer specifiek, en ß(1→3)-glucanen konden worden aangetoond in extracten van monsters uit uiteenlopende arbeidssituaties alsmede uit de woonomgeving. ß(1→3)-glucanen werden geëxtraheerd door stofmonsters in water te verhitten tot 120°C. Reproduceerbaarheid van de assay was goed met een CV-waarde van ongeveer 25%.

Hoofdstuk 5 beschrijft endotoxinen- en ß(1→3)-glucanenniveaus in huisstof van 25 Duitse woningen, en de relatie met woningkenmerken. Endotoxinen en ß(1→3)-glucanen waren detecteerbaar in alle monsters, en concentraties waren het hoogst op woonkamervloeren en het laagst in matrassen. Stof, endotoxinen

en ß(1→3)-glucanenniveaus waren 2-3 keer hoger op woonkamervloeren van centraal verwarmde huizen gebouwd na 1970 dan op woonkamervloeren in oudere woningen zonder centrale verwarming. Deze relatie werd niet gevonden voor endotoxinen en glucanen in stof van matrassen en slaapkamervloeren. Geen relaties werden gevonden met andere woningkenmerken. ß(1→3)-glucanen waren significant maar niet erg sterk gecorreleerd met concentraties levensvatbare schimmels in huisstof.

Hoofdstuk 6 beschrijft een sandwich EIA om EPS te meten van *Aspergillus* en *Penicillium* soorten (EPS-*Asp/Pen*) in huisstof, als een marker voor schimmelblootstelling. Met behulp van deze assay werden huisstofextracten geanalyseerd van woningen van 31 kinderen met, en 29 kinderen zonder chronische luchtwegsymptomen. EPS-*Asp/Pen* concentraties waren detecteerbaar in alle monsters. De hoogste concentraties werden gevonden in stof van de woonkamervloer (in vergelijking met slaapkamervloer en matras). EPS-*Asp/Pen* concentraties (uitgedrukt per gram stof) waren 2-3 keer hoger in stof van textiele dan in stof van gladde vloerbedekkingen. EPS-*Asp/Pen* niveaus waren significant maar niet erg sterk gecorreleerd met tellingen van levensvatbare schimmels in huisstof. Concentraties EPS-*Asp/Pen* in woonkamerstof waren geassocieerd met gerapporteerde vocht- en schimmelplekken in de woning en met luchtwegsymptomen van de kinderen. Deze associaties werden niet gevonden met EPS-*Asp/Pen* concentraties in stof van slaapkamervloeren en matrassen.

Hoofdstuk 7 beschrijft de relatie tussen endotoxinen- en ß(1→3)-glucanen-concentraties in huisstof en piekflow-(PEF)-variabiliteit in 159 kinderen. Kinderen werden geselecteerd op basis van luchtwegsymptomen, zodanig dat 50% chronische luchtwegsymptomen rapporteerde, en 50% geen symptomen had. Kinderen registreerden 2-maal daags hun PEF voor een periode van 16 weken. Ongecorrigeerde regressie-modellen lieten zien dat endotoxinen- en ß(1→3)-glucanenconcentraties op woonkamervloeren significant waren geassocieerd met PEF-variabiliteit in symptomatische kinderen, en met name in atopische kinderen met astma symptomen. Na correctie voor mijtallergeenconcentraties, aanwezigheid van huisdieren en type vloerbedekking bleef de associatie met ß(1→3)-glucanen ongewijzigd, terwijl geen significante associatie met endotoxinen meer gevonden werd. Er werden geen associaties gevonden tussen PEF-

variabiliteit en endotoxinen en ß(1→3)-glucanenniveaus gemeten op slaap-
kamervloeren en matrassen.

Hoofdstuk 8 beschrijft werkgerelateerde acute en (sub-)chronische luchtweg-
ontstekingen bepaald met behulp van neuslavages (NALs) in 14 GFT compos-
teerders. Herhaalde NALs zijn uitgevoerd gedurende drie weken op maandagen
en vrijdagen vóór en na het werk. Tevens zijn op één maandag NALs uitgevoerd
bij 10 controles. In NALs werden concentraties cellen, MPO, IL8, ECP, NO,
urinezuur, ureum en albumine bepaald. De op functie gebaseerde gemiddelde en-
dotoxinen en ß(1→3)-glucanenconcentraties varieerden respectievelijk van 75-
527 EU/m^3 and 0.5-4.9 μg/m^3. Een toename van een factor 1,4-3,2 in con-
centraties cellen, MPO, IL8, NO en ureum over de werkdag werd geobserveerd
bij de werknemers ten opzichte van de controles. Cellen bestonden voornamelijk
uit neutrofielen. Deze acute ontstekingen (binnen 8 uur) in de bovenste lucht-
wegen waren niet geassocieerd met de gemeten blootstelling aan endotoxinen
en ß(1→3)-glucanen. Concentraties cellen, MPO, IL8, NO en albumine waren
aanzienlijk hoger in de NAL van composteerders gemeten op maandagmorgen
(na een blootstellingsvrij weekend), dan in de NAL van de controles, en waren
wel geassocieerd met de gemeten blootstelling. Typische type I ontstekinsreac-
ties gekarakteriseerd door een toenamen in eosinofielen en ECP werden niet
geobserveerd.

Hoofdstuk 9 bediscussieert de belangrijkste bevindingen uit dit proefschrift. Met
name de validiteit van de gebruikte methoden om microbiële blootstelling te
schatten worden bediscussieerd, alsmede de aangetoonde associaties tussen
deze blootstellingsschattingen en luchtwegeffecten. De samenvattende con-
clusies van het onderzoek zijn de volgende:

- De in dit proefschrift beschreven nieuwe methoden om microbiële bloot-
  stelling te schatten in de arbeids- en woonomgeving, kunnen een belangrijke
  bijdrage leveren in toekomstige populatiestudies naar de effecten van
  microbiële blootstelling op de luchtwegen.
- Verschillen in procedures voor monstername en extractie van endotoxinen,
  en verschillende bewaarmethoden voor endotoxinen-extracten kunnen leiden
  tot grote verschillen in meetresultaten tussen laboratoria.

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- De nieuw ontwikkelde ß(1→3)-glucanen EIA en de nieuw geïntroduceerde EPS-*Asp/Pen* EIA zijn specifieke en sensitieve methoden om de blootstelling aan respectievelijk ß(1→3)-glucanen en schimmels te schatten in epidemiologisch onderzoek.

- ß(1→3)-glucanen en de schimmel marker EPS-*Asp/Pen* in huisstof zijn geassocieerd met negatieve gezondheidseffecten op de luchtwegen.

- ß(1→3)-glucanen- en endotoxinen-niveaus in woningen zijn geassocieerd met bepaalde woningkenmerken. Dergelijke kennis kan van belang zijn voor de ontwikkeling van beheersmaatregelen.

- Non-immuun gemedieerde ontstekingsreacties in de luchtwegen lijken belangrijk in de ontwikkeling van microbieel geïnduceerde luchtwegsymptomen. Daarentegen speelt Type I allergie waarschijnlijk geen belangrijke rol.

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Curriculum Vitae

Jeroen Douwes werd geboren op 12 januari 1967 in Breda. Na het behalen van zijn VWO diploma aan het Newman College in Breda in 1985, begon hij met de studie Milieuhygiëne aan de Landbouwuniversiteit in Wageningen (LUW). Deze studie werd in 1991 afgerond met als hoofdvakken gezondheidsleer, lucht-verontreiniging en -hygiëne en toxicologie. Na zijn afstuderen werd hij aan-gesteld als toegevoegd onderzoeker bij de vakgroep Humane Epidemiologie en Gezondheidsleer (van de LUW) waar hij onder andere werkte aan een arbeids-hygiënisch onderzoek onder waterzuiveringswerkers. In juli 1993 trad hij in dienst als assistent in opleiding bij de huidige vakgroep Gezondheidsleer (van de LUW), waar hij begon met het in dit proefschrift beschreven onderzoek. Vanaf juli 1997 was hij aangesteld als postdoctoraal onderzoeker bij dezelfde vak-groep. In de afgelopen jaren is hij naast zijn promotieonderzoek betrokken geweest bij een aantal grote nationale en internationale projecten op het gebied van microbiële blootstelling in de woon- en werkomgeving en gerelateerde luchtwegeffecten. Vanaf 1997 is hij actief lid van een Europese werkgroep op het gebied van bio-aërosolen (Workgroup 5), welke deel uitmaakt van de Europese Standaardisatie Commissie (CEN). In mei 1998 is hem een talent-stipendium toegekend door NWO ten behoeve van een onderzoeksverblijf van 12 maanden aan het 'Department of Medicine of the Wellington School of medicine' in Nieuw Zeeland, alwaar hij vanaf november 1998 zijn werkzaam-heden zal voortzetten.