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The Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals

139. Fungal spores

Wijnand Eduard

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Preface

The main task of the Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals (NEG) is to produce criteria documents to be used by the regulatory authorities as the scientific basis for setting occupational exposure limits for chemical substances.

For each document NEG appoints one or several authors. Evaluation is made of all relevant published, peer-reviewed original literature found. The document aims at establishing dose-response/dose-effect relationships and defining a critical effect. No numerical values for occupational exposure limits are proposed.

Whereas NEG adopts the document by consensus procedures, thereby granting the quality and conclusions, the author is responsible for the factual content of the document.

The evaluation of the literature and the drafting of this document on *Fungal spores* were made by Dr Wijnand Eduard, National Institute of Occupational Health, Norway. The draft document was discussed within the group and the final version was accepted by NEG October 10, 2006. The following individuals participated in the elaboration of the document:

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All criteria document produced by the Nordic Expert Group may be downloaded from *www.nordicexpertgroup.org*.

Gunnar Johanson, Chairman of NEG

Contents

Preface

Abbreviations and acronyms

1. Introduction 1
   1.1 Delimitations 1
2. Species characterisation 2
3. Biological and physical properties 2
   3.1 Fungi 2
   3.2 The compost reaction 3
   3.3 Spores 3
   3.4 Composition of the fungal cell wall 6
   3.5 Actinomycetes 7
   3.6 Metabolites 7
   3.7 Summary 8
4. Occurrence, production and use 9
5. Measurement methods and strategies for assessment of workplace exposure 10
   5.1 Measurement methods for airborne fungal and actinomycete spores 11
      5.1.1 Culture-based methods 11
      5.1.2 Microscopic counting 12
      5.1.3 The CAMNEA method 12
      5.1.4 Other non-culture methods 13
      5.1.5 Discussion 14
   5.2 Specific IgG antibodies 14
   5.3 Measurement strategies 15
   5.4 Conclusions 15
6. Occupational exposure data 16
   6.1 Highly contaminated environments 16
   6.2 Common indoor environments 17
   6.3 Conclusions 21
7. Uptake, distribution and elimination 21
   7.1 Uptake 21
   7.2 Distribution 21
   7.3 Elimination 22
      7.3.1 *In vivo* studies 22
      7.3.2 *In vitro* studies 25
      7.3.3 Discussion 26
8. Mechanism of toxicity 27
   8.1 The innate and adaptive response to fungi 27
   8.2 Allergic responses 30
   8.3 Non-allergic responses 32
   8.4 Summary 32
9. Biological monitoring 33
   9.1 Markers of exposure 33

9.2 Markers of effect 34
10. Effects in animals 36
10.1 Dose considerations 36
10.2 Irritation 37
10.3 Sensitisation 37
10.4 Effects of single exposure 38
10.4.1 Mortality 38
10.4.2 Inflammatory markers 38
10.4.3 Blood gas parameters 41
10.4.4 Lung function 41
10.4.5 Discussion 42
10.5 Effects of short-term exposures (up to 90 days) 51
10.6 Mutagenicity and genotoxicity 53
10.7 Effects of long-term exposure and carcinogenicity 53
10.8 Reproductive and developmental studies 53
10.9 Discussion 59
11. Observations in man 60
11.1 Irritation 60
11.1.1 Highly exposed populations 60
11.1.2 Populations exposed to common indoor air 61
11.2 Sensitisation 62
11.2.1 Human challenge studies 62
11.2.2 Epidemiological studies 63
11.3 Effects of single exposure 67
11.4 Effects of short-term exposure 67
11.4.1 Provocation tests 67
11.4.2 Epidemiological studies 68
11.4.3 Discussion of effects of short-term exposure 72
11.5 Effects of long-term exposure 73
11.5.1 Epidemiological studies 73
11.5.2 Discussion of effects of long-term exposure 80
11.6 Genotoxic and carcinogenic effects 82
11.7 Reproductive and developmental effects 82
12. Dose-effect and dose-response relationships 87
12.1 Dose considerations and extrapolation from animal studies 87
12.2 Effects related to single and short-term exposure 87
12.2.1 Animal studies 87
12.2.2 Challenge studies of symptomatic subjects 89
12.2.3 Epidemiological studies 89
12.3 Effects related to long-term exposure 90
13. Previous evaluations by national and international bodies 97
14. Evaluation of human health risks 97
14.1 Assessment of health risks 97
14.1.1 Airway and lung inflammation 97
14.1.2 Respiratory function 98
14.1.3 Respiratory symptoms 99

14.1.4 Asthma 99
14.1.5 Hypersensitivity pneumonitis and organic dust toxic syndrome 100
14.1.6 Studies in common indoor environments 100
14.1.7 The role of specific organisms 101
14.2 Groups at extra risk 101
14.3 Scientific basis for an occupational exposure limit 102
14.4 Evaluations in common indoor environments 103
15. Research needs 103
16. Summary 105
17. Summary in Norwegian 106
18. References 107
19. Data bases used in the search for literature 121
Appendix 1. Terms as used in the present document 122
Appendix 2. Synonyms 125
Appendix 3. *In vivo* studies of spore distribution 126
Appendix 4. *In vivo* studies of spore elimination from the lung 127
Appendix 5. *In vitro* studies of spore elimination 129
Appendix 6. Epidemiological studies of highly exposed populations 134
Appendix 7. Epidemiological studies of populations exposed to common indoor air levels 140
Appendix 8. Human challenge studies using spores 143
Appendix 9. Maximum allowable concentrations of fungi and actinomycetes issued in the Russian Federation 145

Abbreviations and acronyms

ACGIH	American Conference of Governmental Industrial Hygienists
AED	aerodynamic diameter
bw	body weight
CAMNEA	collection of airborne microorganisms on nucleopore filters, estimation and analysis
cfu	colony forming units
CI	confidence interval
ECP	eosinophilic cationic protein
EU	endotoxin units
FEV_1	forced expiratory volume in one second
FVC	forced vital capacity
IFNγ	interferon gamma
Ig	immunoglobulin
IL	interleukin
LD_{50}	lethal dose for 50% of the animals at single exposure
LOEL	lowest observed effect level
MCP	monocyte chemoattractant protein
MEF_{25}	maximum expiratory flow at 25% of FVC
MIP	macrophage inflammatory protein
MVOC	microbial volatile organic compound
NEG	The Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals
NO	nitric oxide
NOEL	no observed effect level
ODTS	organic dust toxic syndrome
OEL	occupational exposure limit
OR	odds ratio
PaO_2	arterial oxygen tension
PMN	polymorphonuclear leukocytes (mainly neutrophils)
PPR	prevalence proportion ratio
RAST	radio-allergosorbent test
ROS	reactive oxygen species
SEM	scanning electron microscope
sp/spp	species (singular/plural)
TGFβ	transforming growth factor beta
Th cells	T helper cells
TLco	lung transfer factor for carbon monoxide
TLR	toll-like receptor
TLV®	threshold limit value
TNFα	tumour necrosis factor alpha

1. Introduction

Fungi are heterophilic organisms, i.e. dependent on dead or living organisms for their growth. They can quickly colonise all kinds of dead organic material and are together with bacteria the most important organisms that recycle organic material. Fungi are ubiquitous and have been estimated to comprise approximately 25% of the global biomass (138, 224).

Fungi replicate by formation of sexual and asexual spores. Many species produce spores, which are easily dispersed into the air and can be transported over long distances, even across the globe; for an overview see Shinn *et al* (222). We therefore inhale substantial numbers of fungal spores from outdoor and indoor air. Exposure levels are highly variable because fungi can rapidly multiply when conditions for growth are favourable, and some species release their spores on specific times of the day. *Actinomycetes* are a group of bacteria that resemble the fungi in growth and replication, and may also be present in indoor and outdoor air.

Spores of fungi and actinomycetes have been recognised as occupational hazards; reviews by Lacey and Crook, and Malmberg (116, 128). In 1950-1980 several species were identified as causes of hypersensitivity pneumonitis (also called allergic alveolitis) in a number of occupations including farmers, malt workers and wood workers. Exposure levels in these occupations regularly exceed 10^6 spores/m^3 and can be as high as 10^{10} spores/m^3. These levels are much higher than levels usually found outdoors, which seldom exceed 10^4 spores/m^3. Fungal spore exposure has also been associated with indoor air problems, especially in damp buildings. Airborne concentrations in such environments may be higher than outdoors, but levels rarely exceed 10^4 colony forming units/m^3 (cfu/m^3), which correspond to approximately 10^5 spores/m^3; review by Levetin (122).

Fungal spores are complex agents that may contain multiple hazardous components. Health hazards may differ across species because fungi may produce different allergens and mycotoxins, and some species can infect humans.

1.1 Delimitations

Delimitations of this document are needed due to the complexity of the subject. Infectious diseases will not be addressed. Also genetically altered fungi are omitted since limited information is available on occupational health risks. $\beta(1\rightarrow3)$-Glucans, which have been studied as fungal agents (review by Douwes (42)), are also excluded because they are not specific to fungi but can also be found in plants and bacteria. Mycotoxins have been subject for review elsewhere (27, 96) since they are of great concern in the public health sector from the perspective of oral intake of contaminated food. However, mycotoxins present in spores will be considered as their presence increases the toxicity of the spores. The actinomycetes are included because they have similar effects on health as fungal spores and may represent important health risks to working populations.

Terms as used in this document are presented in Appendix 1.

2. Species characterisation

The fungi comprise a large group of organisms including mushrooms, moulds, and yeasts. The fungi are placed in the kingdom Mycota, and most fungi relevant to human health belong to the division Amastigomycota. Fungi are traditionally identified by morphological characteristics of colonies and reproductive structures including spores, and conditions for growth in culture. These characteristics are compared to the characteristics of type species described in the literature and stored in type collections. The use of molecular biological methods is rapidly increasing, however. Important genera in occupational environments are shown in Table 3.

Many species have been described in the literature under different names, and have later been recognised as the same species. Appendix 2 aims at identifying species described in older publications cited in this review. The most recent names have been used in this document.

3. Biological and physical properties

General references to this topic are Gregory (70), Al-Doory and Domson (5), Burge (22) and Green (64).

3.1 Fungi

Fungi are eukaryotic organisms that lack chlorophyll and depend on other organisms for their supply of nutrients. Most fungi are saprophytic, i.e. live on dead organic material. Fungi play an important role in the ecosystem in the recycling of nutrients. However, as fungi can exploit all organic materials, they may also damage food, wood and textiles as well as building materials in buildings with humidity problems. Fungi may even invade living organisms and infect plants, animals, and humans. Plant pathogenic fungi are of major concern for farmers as they cause significant damage to crops.

Filamentous fungi (moulds and mushrooms) grow as branched multicellular filamentous structures (hyphae) that collectively form the mycelium. Fungi need organic material, oxygen, and water for growth. As oxygen and organic material are readily available in most environments, access to water is usually the limiting factor. A water content above 12-15% in materials such as grain and wood is usually sufficient to sustain fungal growth, but some fungi can even grow in materials with lower water content if the humidity of the air is high, e.g. above 85% relative humidity.

Temperature has a major influence on the growth of microorganisms. Most fungal and actinomycete species are mesophilic and show optimal growth at 15-30 °C. Psychrophilic and psychrotolerant species grow at lower temperatures, e. g. *Cladosporium herbarum* can grow at temperatures down to -5 °C. Thermophilic species have growth optima above 30 °C. Fungi may also grow outside the

optimal temperature range. However, higher temperatures may kill organisms while lower temperatures are less lethal. *Aspergillus fumigatus* is the most important opportunistic infectious agent with a growth optimum close to the human body temperature. pH conditions for growth are often broad with optima around pH 6, but few fungi grow below pH 3 or above pH 9.

3.2 The compost reaction

When organic material with sufficient water content is stored for a long time, psychrophilic or mesophilic organisms may start to grow. The metabolic activity produces heat, which may raise the temperature in the stored material and modulate fungal growth depending on the growth optima of the microorganisms present. Proliferation of thermophilic species may raise the temperature even further. The maximum temperature that stored material may reach mainly depends on the original water content (116). The material may eventually catch fire due to ignition of volatile organic compounds produced by the microorganisms (MVOCs). When nutrients are depleted, microbial growth slows down and temperature declines again. Water content, storage temperature and storage time are therefore important determinants of the level and the complexity of the microbial contamination in organic materials.

3.3 Spores

Fungi that are adapted to aerial dispersion replicate by non-motile sexual and asexual spores. Fruiting bodies may grow from the mycelium of a single organism and produce asexual spores (Figure 1). Sexual reproduction involves fusion of the mycelia of two different mating strains and subsequent development of sexual spores. Yeasts grow as single cells and replicate by budding under wet conditions and do not become easily airborne. However, some yeast species have both cellular and mycelial growth and may produce airborne spores dependent on environmental conditions.

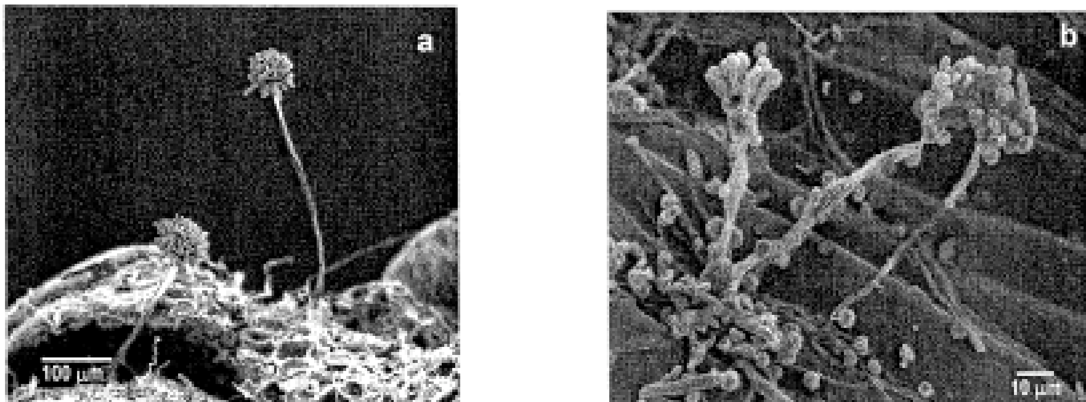

Figure 1. Fungal colonisation of grass seeds. The micrographs show mycelium, spores, and fruiting bodies from an *Aspergillus* (a) and a *Penicillium* species (b). Micrographs by Lene Madsø.

Both sexual and asexual spores are resting, metabolically inactive units surrounded by a thick wall that protects the organism from the environment. Spores can tolerate extreme physical conditions and may survive until conditions become favourable for growth. Many species liberate their spores into the air. As spores often have a small size they can stay airborne for long periods and be transported over large distances. Thus, spore formation is important for survival, and replication, as well as dispersion of the fungus.

Spore sizes range typically from 2 to 10 μm between species and vary even between spores from the same species. The physical diameter of spherical and smooth spores is probably similar to their aerodynamic diameter (AED), but spores with elongated shape and/or surface ornamentation have smaller AEDs than the diameter of a sphere with the same volume as the spore because of larger drag forces in air. It should be noted that airborne spores may have a smaller size than quoted in manuals since the size of hydrated spores observed with the light microscope is usually reported, whereas spores rapidly desiccate when dispersed in air. The specific gravity may have some influence on the AED as well since the density of spores varies from 0.4 to 1.5 g/cm^3. The AED of airborne spores is also dependent on humidity. The size of spores from five species did not change at a relative humidity ranging from 30% to 90%, but the AED increased by 11 to 27% at 100% relative humidity (194). This may have some effect on the deposition of inhaled spores in the airways (Chapter 7.1), but it is not known how rapid fungal spores absorb water vapour as many spore types are hydrophobic.

Spores may be released aggregated as chains or clumps that are expected to have larger AEDs than single spores. Figure 2 shows some examples of spores and aggregates. The increase of the AED with aggregate size was relatively small, however (191). AEDs of fungal spores measured in the laboratory are shown in Table 1.

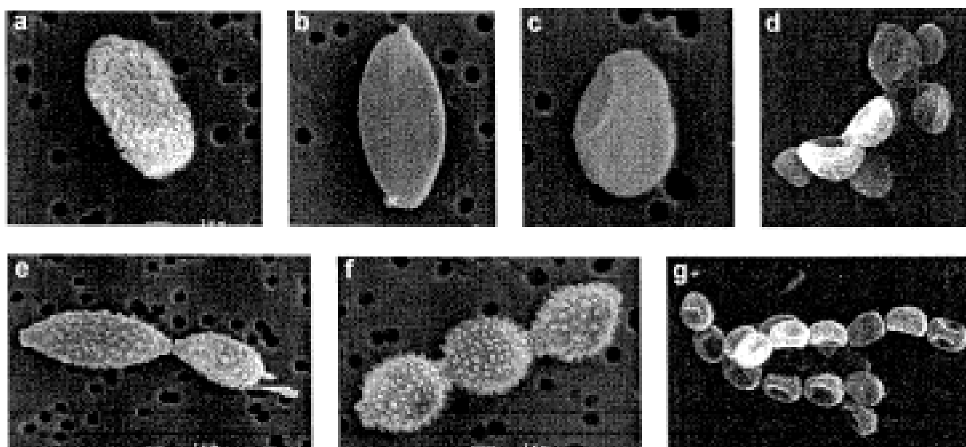

Figure 2. Fungal spores and aggregates with different morphology collected from sawmill, farm, and common indoor environments. Single spores (a, b and c), small aggregates (e and f) and large aggregates (d and g) are shown. Micrographs by Wijnand Eduard.

Table 1. Aerodynamic diameter (AED) of single and aggregated spores from fungi and actinomycetes released from culture plates.

Species	AED[a] (μm)	Reference
Fungi		
Aspergillus fumigatus	2.0-2.7	(172)
	1.9-2.2	(126)
	2.1-2.2	(194)
Cladosporium cladosporoides	2.3-2.5	(126)
	1.7-1.9	(194)
Penicillium brevicompactum	2.0-2.2	(194)
	2.1-2.4	(193)
Penicillium chrysogenum	2.6-3.0	(126)
Penicillium melinii	2.7-2.8	(194)
Stachybotrys chartarum	4.5	(225)
Actinomycetes		
Micromonospora halophytica	1.3	(195)
Streptomyces albus	0.9-1.9	(193)
	0.85	(195)
Thermoactinomyces vulgaris	0.57	(195)

[a] Geometric mean.

Spores from some genera such as *Fusarium* and *Stachybotrys* are produced in slimy aggregates, which are dispersed outdoors by raindrops (splash dispersal). The sticky spores may adhere and infect nearby plants or fall to the ground. Spores from these species can also be found airborne, however. The release of spores from the plant material probably occurs by mechanical friction during harvest and further handling of grain.

Most airborne fungal particles are single or aggregated spores whereas hyphal fragments have only occasionally been reported. However, recent experimental studies have demonstrated that spores as well as fungal fragments smaller than spores can be released from fungal cultures by an air stream. This has been shown for several species, e.g. A*spergillus versicolor, Cladosporium cladosporoides, Penicillium melinii, Streptomyces albus, Trichoderma harzianum* and *Ulocladium* sp., but not *Penicillium chrysogenum* (61, 62, 107, 108, 127). Previously Sorenson *et al* reported the liberation of hyphal fragments from cultures of *Stachybotrys chartarum* (225). The fungal origin is supported by the presence of antigens in the particle size fraction that only contained fragments (62), and by the staining of fragments with a DNA/RNA fluorochrome (127). Recent environmental and occupational studies indicate that the presence of hyphae may have been over-looked (66, 72).

3.4 Composition of the fungal cell wall

Mycelial fungal and yeast cell walls are relatively thin, typically 0.2 μm (Figure 3). The main constituents vary in composition between fungi of different taxa. The cell wall is made up of a matrix containing β(1→3)-, α(1→3)- and β(1→6)-glucans, glycoproteins and lipids reinforced by chitin (polyacetyl glucose-amine) or cellulose fibres. Pigments like melanin may also be present. The lipid constituent ergosterol is commonly present and is used as a chemical marker for fungal mass (Chapter 5.1.4). The fungal cell wall has an outer layer mainly consisting of glycoproteins that determine the antigenic properties of the cell. The cell wall has also been shown to contain high levels of fungal allergens (18). The secretion of hydrophobins (small, hydrophobic proteins) from growing hyphae has recently been described. The hydrophobins coats the outer surface of aerial hyphae and spores. The hydrophobin coating allows hyphae to attach to hydrophobic surfaces and hosts, and play an important role in fungal infections; review by Wessels (244). The hydrophobins have also been shown to be allergenic (242). Pathogenic species may have an extracellular mucous layer on the cell surface that contains adhesins, molecules that allows the fungus to adhere to the host/substrate, and enzymes that may help to invade the host or liberate nutrients. Spores have much thicker cell walls than mycelia (Figure 3). The outer layer is hydrophobic and contains lipids and proteins. The inner cell wall is similar to the mycelial wall and consists of a β(1→3)-glucan matrix reinforced with chitin fibrils. The spore wall may liberate antigens that may induce allergic responses after deposition in the respiratory system (32, 143, 161). All these components are primary metabolites as they are essential for the survival of the microorganisms.

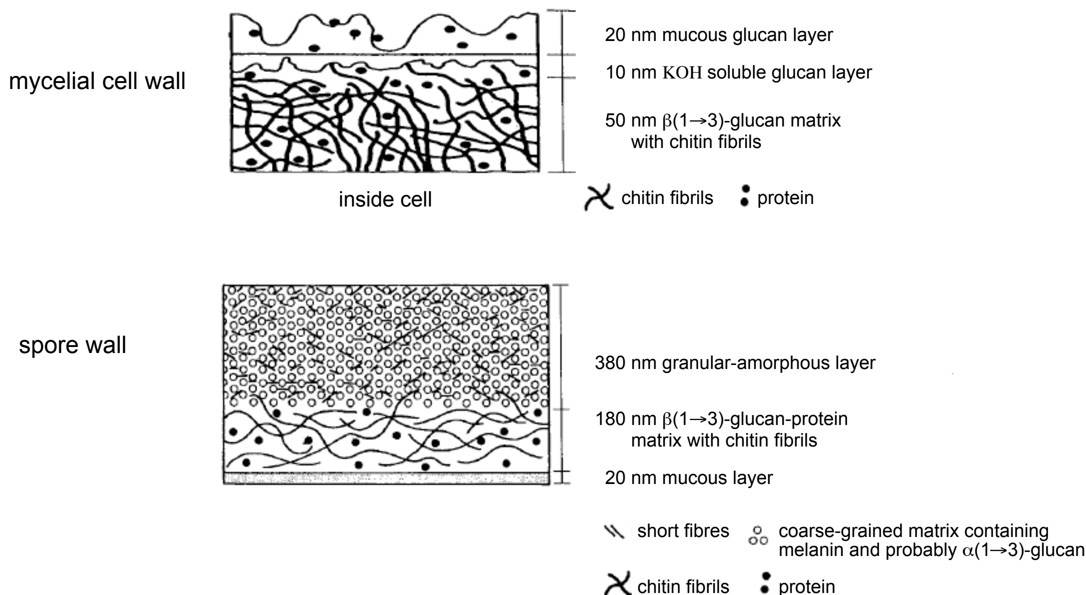

Figure 3. Structure of the mycelial cell and spore walls of the button mushroom *Agaricus brunnescens* (137, 188).

3.5 Actinomycetes

The actinomycetes are Gram-positive bacteria that mimic the fungi as they grow as branched cell chains and reproduce by production of asexual spores. Spores from actinomycetes are smaller than fungal spores, typically 0.5-1.5 μm, but otherwise have similar shape and surface characteristics. Most species are mesophilic and are abundant in soil. They usually require higher water content for growth than fungi. Thermophilic species such as *Thermoactinomyces* sp. and *Saccharopolyspora rectivirgula* grow at higher temperatures than the fungi, up to 60-65 °C, and may dominate the microbial biota in composting material at temperatures exceeding approximately 50 °C.

3.6 Metabolites

Primary metabolites are constituents of microorganisms that are essential for survival of the organism, such as the cell wall components (described in Chapter 3.4). Microorganisms may also produce secondary metabolites, which are compounds that are not vital but may be beneficial to the organism, e.g. mycotoxins, which are used in the competition with other organisms and, enzymes. MVOCs are formed during both the primary and the secondary metabolism. The different metabolites are briefly described below.

Mycotoxins are potent toxins that a number of fungi can produce and excrete in the substrates they colonise. These toxins may also be present in spores from toxigenic species (225). Mycotoxins have mainly been studied as contaminants of food and feed; review by CAST (27). Especially aflatoxins produced by *Aspergillus flavus* and *Aspergillus parasiticus* have obtained much attention because of their carcinogenic and toxic properties. Other important mycotoxins are the trichothecenes produced by *Fusarium* and *Stachybotrys* species, fumonisins and zearalenone produced by *Fusarium* species, and ochratoxin A produced by *Aspergillus ochraceus* and *Penicillium verrucosum*. Many mycotoxins are immunotoxic. However, the trichothecene mycotoxins are immunostimulating at lower doses; review by Bondy and Pestka (15).

The production of mycotoxins strongly depends on growth conditions. A toxic strain of *Stachybotrys chartarum* e.g. did not produce satratoxins when grown on pine or on other materials at a relative humidity below 84% (158).

Some mycotoxins are antibiotics, i.e. they are highly toxic to bacteria, but have low toxicity in humans. These antibiotics include penicillins produced by *Penicillium chrysogenum* and cephalosporins produced by *Cephalosporium* species. A review of penicillins was recently published by the Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals (NEG) (142).

Many fungal enzymes are glycopeptides with allergenic properties and fungi may therefore represent a risk of allergic disease (113). Enzymes are present in spores, and are released in greater amounts during germination of spores (65, 140) and mycelial growth (94). Purified fungal enzymes such as α-amylase from *Aspergillus oryzae* (90), cellulase, xylanase, phytase and α-amylase from

Trichoderma reesei (239), and phytase from *Aspergillus niger* (41), have been shown to be potent occupational allergens in the food industry.

Fungi may produce a variety of allergens. For example, an international allergen nomenclatural committee has approved 17 different allergens from *Aspergillus fumigatus*, 6 allergens from *Alternaria alternata,* and 10 allergens from *Cladosporium herbarum.* A smaller number of allergens (1 to 3) have been recognised in three *Aspergillus* and four *Penicillium* species (113).

A few fungal species are commonly recognised as aeroallergens. Species from the genera *Cladosporium*, *Alternaria,* and *Aspergillus* are most often involved in fungal allergy and have been studied in detail; reviews by Hoffman, and Kurup *et al* (86, 113). These species are also most prevalent in outdoor air (115). Several other species and genera have been related to asthma in asthmatic patients (113) and in working populations, (review by Lacey and Crook (116)), but they are not commonly included in allergy tests, probably because their occurrence in outdoor air is low (115). It is therefore possible that other species than *Cladosporium*, *Alternaria,* and *Aspergillus* spp. have been underestimated as causal agents of fungal allergy.

Spores have mainly been studied as respiratory allergens as they have been regarded as the most prevalent airborne fungal particles. This may be a simplification, as hyphae (Chapter 3.1) may not have been recognised. Furthermore, fungi may excrete allergens that may be present in other particles as well (190). The increased production of allergens in the germination phase suggests that viable spores may be more allergenic than dead spores if germination occurs in the respiratory tract (65, 140) and Sercombe *et al* recently demonstrated that germinating spores in the nasal cavity of healthy subjects were common (216). Górny *et al* showed in an experimental study that fungal fragments smaller than spores were liberated from sporulating cultures, and that these fragments had high antigenic activity (62).

Microorganisms produce a large number of MVOCs. These compounds include alcohols, aldehydes, ketones, esters, terpenes, and sulphur and nitrogen compounds. MVOCs are mainly regarded to be side-products of the primary metabolism during synthesis of DNA and amino and fatty acids. However, the division between primary and secondary metabolism is not absolute and it is likely that MVOCs are formed during both (111). The production of MVOCs strongly depends on the substrate and environmental conditions and many compounds denoted MVOCs may also originate from non-microbial sources. A separate criteria document on MVOCs has been produced by NEG (111).

3.7 Summary

Fungal spores are very different from chemical agents as spores may contain multiple components such as allergens, antigens, polysaccharides like the $\beta(1\rightarrow3)$-glucans, and mycotoxins. Many species produce spores that differ not only with respect to composition but also morphology (size, shape and aggregation), which may influence the deposition in the respiratory system. Fungi are

living organisms that multiply rapidly under favourable conditions and may colonise organic material when water is available in sufficient quantity. Many fungi produce large numbers of spores that are adapted to aerial dispersion. High exposure situations are therefore often related to handling of mouldy material. Actinomycetes are Gram-positive bacteria for which the growth and sporulation resemble that of filamentous fungi, but they have smaller spores. Recently described phenomena are the liberation of small fragments from fungal colonies and the presence of airborne hyphal fragments.

4. Occurrence, production and use

Fungi are found in practically all habitats. Fungi liberate spores into the air, and spores can be transported over long distances by air currents because of their small size. Fungal spores are therefore ubiquitous in outdoor air. Spores are also present in indoor air as they enter the indoor environment with outdoor air. Indoor fungal levels may increase further by culture of the organisms, by unintended proliferation or by handling of mouldy materials.

Outdoor levels vary between regions and seasons over a range of < 20 to $>10^5$ cfu/m^3; review by Gots *et al* (63). Outdoor levels are highest in warm regions and in the summer and autumn in temperate regions, and lowest in the winter in subarctic regions, e.g. $<10^2$ cfu/m^3 or $<10^3$ spores/m^3 in Finland; review by Pasanen (171).

Fungal spores in outdoor air enter the indoor environment by natural and mechanical ventilation but spore levels in common indoor environments are usually lower since spores are removed by filtration and/or they settle due to lower air velocities indoors. In spite of this, fungi in office buildings and houses have frequently been studied as a health risk. Fungi may grow on many types of building materials if the humidity of the air and/or in the materials is sufficient. A typical cause of fungal growth is unattended water leakage. After the energy crisis of the 1970s, buildings in temperate regions were built more tightly and with higher thermal isolation to reduce energy costs. Lower air exchange rates and inadequate construction methods repeatedly resulted in condensation of water and microbial growth in the walls, floors, and roofs. Although the presence of fungi can often be demonstrated in damp buildings, the levels of airborne fungi indoors are mostly similar or only moderately elevated compared to outdoor levels, and rarely exceed 10^4 cfu/m^3. Measurements of airborne fungi may therefore fail to detect indoor fungal growth. Specific quantitation of *Penicillium, Aspergillus, Stachybotrys chartarum* and yeasts may improve such assessments as these fungi are indicative of humidity problems while outdoor levels are often low; review by Levetin (122). In subarctic climates, assessments of fungal growth in buildings are preferably performed in the winter due to low outdoor levels (Chapter 5.3).

Although the assessment of mould problems by measurements of airborne fungi is problematic, it is interesting that remediation of humidity problems in buildings seems to reduce building-related health problems.

Table 2. Working populations potentially exposed to fungal and actinomycete spores (228-232).

Occupation	Working population in thousands				
	Denmark 2005	Norway 2005	Sweden 2004	Finland 2005	Iceland 2005
Agriculture and forestry	65	59	36	116	7
Agriculture	61	55	23		
Forestry	4	4	13		
Food industry	72	44	63	nf	nf
Wood industry	14	9	36	nf	nf
Wood processing	7	4	40	nf	nf
Furniture	19	nf	44	nf	nf
Total working population	2 400	2 800	4 200	2 500	160

nf: not found.

Fungal spore levels can be much higher at workplaces where the presence of fungi is related to production and the contribution from natural outdoor levels is usually negligible. Fungi are used in the food industry, e.g. for production of dairy products, alcoholic beverages, bread, and soy sauce, in the biotechnological industry for production of citric acid, antibiotics, and enzymes, and for composting of plant debris and the organic fraction of domestic waste. Fungi can also be produced as an end-product, e.g. baker's yeast and mushrooms. Exposure levels often exceed 10^6 spores/m^3 and can be as high as 10^{10} spores/m^3; reviews by Lacey and Crook, and Malmberg (116, 128). The highest levels occur when fungi colonise organic materials unintentionally. These conditions are usually avoided because fungal contamination may lead to economical losses. Exposure levels in general are therefore much lower. Consequently, exposure levels may show high variability in industries such as agriculture and sawmills (Table 5-6).

The numbers of people employed in work environments with potential fungal exposure in the Nordic countries are listed in Table 2. Important fungal and actinomycete genera in these work environments are shown in Table 3.

5. Measurement methods and strategies for assessment of workplace exposure

For reviews of bioaerosol measurement methods, see Eduard and Heederik (51) and Douwes *et al* (44).

Table 3. Fungal and actinomycete genera frequently found in work environments (116).

Genus	Environments
Fungi	
Alternaria	Outdoors, fruit stores
Aspergillus	Malt workers, tobacco workers, agriculture, mushroom growers, citric acid production, organic waste, compost, common indoor air
Cladosporium	Agriculture, outdoors
Penicillium	Cheese workers, malt workers, cork workers, fruit stores, penicillin production, sawmill workers, common indoor air
Rhizopus	Sawmill workers, malt workers
Stachybotrys	Common indoor air, damp buildings
Actinomycetes	
Saccharopolyspora	Agriculture, composting and compost handling
Thermoactinomyces	Agriculture, bagasse workers, composting and compost handling

5.1 Measurement methods for airborne fungal and actinomycete spores

Spores can be collected by impaction on semi-solid nutrient plates or glass slides, by impaction in liquid (impingers) or on filters. Most sampling instruments are stationary except filters, which are well adapted for personal sampling. Samples may be quantified by culture-based or non-culture methods.

5.1.1 Culture-based methods

Culture-based methods rely on growth of collected organisms into recognisable colonies on semi-solid nutrient plates under standardised conditions. The results are expressed as cfu. Different media and conditions for cultivation such as temperature, time, and use of growth inhibitors are applied to culture different groups of organisms. Culture methods have several important weaknesses. A major limitation is that dead fungi and those that cannot grow under the conditions chosen for culture remain undetected. Moreover, a colony may originate from a single spore as well as a large aggregate. When spores are dispersed in a liquid, e.g. impinger sampling and filter samples that are resuspended before analysis, substantially higher numbers of cfu are detected than when spores are collected and cultured directly on nutrient plates because aggregates break up when suspended in a liquid. Sampling time is limited to 30 minutes for impactors and most impingers. Impactors and filters expose microorganisms to sampling strain, which may affect the viability of the collected organisms. This problem is probably less pronounced for fungal spores than vegetative cells because in general spores tolerate environmental stress better. To prevent overloading, the sampling time in highly contaminated environments must be very short when impactors that collect spores directly on nutrient plates are used. Finally, not only spores but also hyphal

fragments may grow into colonies. Recent studies indicate that such fragments may represent a significant proportion of airborne fungal particles (66, 72).

The lower detection limit of a count is four colonies[1]. The detection limit of a concentration is obtained by division with the sampled volume of air. As an example, the lower detection limits range from 4 to 150 cfu/m^3 for a 400-hole single stage impactor sampling at 28 l/min for 1 to 30 minutes. Impactors also have an upper detection limit when colonies are detected beneath all holes. This limit is approximately $4 \cdot 10^4$ cfu/m^3 for 1 minute sampling with a 400-hole single stage impactor at 28 l/min. Impactor and filter samples can be cultivated by dilution plating, which increases both the lower and the upper detection limits because only a fraction of the collected fungi are cultured.

5.1.2 Microscopic counting

Microscopic counting is the traditional non-culture method for quantification of fungal spores. Most fungal spores have distinct morphology and are easily recognised with light, fluorescence, and scanning electron microscopy (SEM) (Figure 2). The SEM shows surface structure with most detail as this microscope has the highest resolution. However, the light microscope may provide other information as internal structure and colour can be observed. Least morphological detail is obtained with the fluorescence microscope, but stained microorganisms are more easily observed among other particles, and fungi may be stained specifically with antibody-fluorochrome conjugates. The latter technique is seldom applied, however. Some classification is possible when spores are counted with a microscope, but seldom at the species level.

The fluorescence microscope underestimated the number of spores by approximately 75% compared to the light and SEM in a Scandinavian interlaboratory study (52).

The detection limit of microscopic counts is 4 counted spores[1], and is further dependent on the magnification, the size of the view field, number of counted fields and the sampled volume of air. A typical detection limit is $2 \cdot 10^4$ spores/m^3 when counting 50 fields at 2 000x with a SEM and a sampling time of 8 hours at 2 l/min. Light and fluorescence microscope counts are usually performed at lower magnification (approximately 1 000x) and view fields are typically larger, which decreases the detection limit. Samples for counting by the fluorescence microscope are usually resuspended and only a subsample is counted, which increases the detection limit again.

5.1.3 The CAMNEA method

Combined analysis by microscopy and culture is possible with the so-called CAMNEA method (collection of airborne microorganisms on nucleopore filters,

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[1] The smallest concentration or amount of a component of interest that can be detected by a single measurement with a stated level of confidence. Results based on counting, such as colony and microscopic counts, are approximately Poisson distributed. The Poisson distribution with a mean of 4 contains values >0 in approximately 98% of the distribution. A value of 4 is therefore commonly used as the detection limit.

estimation and analysis). Bioaerosol samples are collected on smooth polycarbonate filters, resuspended in a liquid and subsamples of the suspension can be analysed by different methods such as culture and fluorescence microscopy (168). The detection limits are higher since the suspension is divided between the two methods (Chapters 5.1.1 and 5.1.2).

5.1.4 Other non-culture methods

Other non-culture methods quantify fungal metabolites by chemical, biochemical, immunochemical, and molecular biological methods. These metabolites can either be non-toxic or active agents such as allergens and glucans.

The former category includes ergosterol, a lipid component that is commonly found in fungi and algae but not in terrestrial plants (156). The ergosterol content of spores and mycelium differs across species with a factor of 3-6 (10, 139), however, making quantitative analysis difficult.

Similarly, β(1→3)-glucans have been measured as markers of fungi, using biochemical and immunochemical methods. Although such glucans are found in fungal mycelium and spores, some bacteria, algae and terrestrial plants may contain glucans as well. The specificity of β(1→3)-glucans as markers of fungal exposure thus depends on the presence of other glucan sources. However, glucans from different sources have been shown to have inflammatory effects in animal studies and should therefore be studied as specific agents in bioaerosols independent of their role as marker of fungal exposure (42).

Antigens can be used as more specific markers of fungi (3) although cross-reactivity of antibodies with other species than the one used for antibody production is a problem. For example, monoclonal antibodies against *Penicillium brevicompactum* showed substantial cross-reactivity with other *Penicillium* as well as *Aspergillus* species and even with species from other genera. In addition, sample collection and preparation methods had a major influence on the results (213). Similar results were reported for *Aspergillus versicolor* (214).

Molecular biological methods probably have the greatest potential for quantification of microbial exposure. However, these methods are at present most developed for qualitative assessment of fungi (247, 249, 257). Quantitative methods based on real-time polymerase chain reaction have recently been described (8, 258). The specificity of these methods may complicate the assessment of a diverse fungal biota, as many assays with different primers have to be applied. Alternatively, less specific primers may allow the detection of broader groups of organisms e.g. the genus *Cladosporium* (259). The unit of quantification is different from microscopy and culture as the number of fungal genomes is quantified, which includes DNA from spores and hyphae.

Fungal fragments with smaller particle size than spores have recently been detected in laboratory studies using particle counters and SEM (62, 107). The occurrence of such fragments in the environment has not been described, however. Larger hyphal fragments can be detected by immunomicroscopy (66) and have been detected in a residential environment (67).

5.1.5 Discussion

Most measurement methods for microorganisms estimate different entities. At present there is no general agreement on how fungal spore exposure should be assessed. The demonstration of fungal particles other than spores further complicates the assessment of fungal exposure, and their importance is not yet clear.

Filter sampling is generally preferred since personal sampling is straightforward and can be carried out according to criteria for health related size fractions (28). Filter samples can be analysed by a variety of non-culture methods. In epidemiological studies mainly culture-based and microscopic methods have been used and both methods have shown exposure-response relationships in the majority of the studies (review by Eduard (47)) with no clear difference between methods used for quantification of fungal exposure.

However, culture-based methods suffer from several well-documented errors. The cfu is at best a semi-quantitative measure. The main advantages of culture-based methods are that microorganisms can be identified and that the detection limit is lower than with microscopic methods, which is particularly important in environments with common indoor exposure levels. The best measurement strategy at present seems to be the combined use of culture-based methods for identification of species and non-culture methods for quantification of the exposure. By further development of quantitative molecular biological methods more specific measurements of fungal spores with low detection limits may be possible.

5.2 Specific IgG antibodies

Specific serum immunoglobulin (Ig)G antibodies to fungi may develop in humans and animals after continued exposure to airborne fungal spores. These antibodies have been studied as a marker of hypersensitivity pneumonitis but their presence is primarily related to repeated exposures. Specific serum IgG is most useful as an indicator of fungal exposure in the diagnosis of hypersensitivity pneumonitis where documentation of exposure is required (174, 197, 205) (Chapter 9.1).

Although development of serum IgG is a biological response (Chapter 9) IgG levels have been shown to be associated with exposure levels during the preceding months. IgG in serum can be used as a marker of exposure in populations working in highly contaminated environments. However, many species cross-react, individual differences in the antibody response are large and the precision of individual serum IgG antibody levels as an exposure estimate is relatively poor. The precision can be improved if evaluations can be based on group mean levels of similarly exposed individuals. Changes in exposure level can also be detected with better precision from intra-individual IgG changes; review by Eduard (45). In building-related illness, where exposure levels are usually low, the value of IgG is very limited; review by Trout *et al* (237).

To sum up, serum IgG antibody levels may only provide semi-quantitative information on fungal exposure and are difficult to interpret as exposure levels.

However, IgG antibodies are fairly specific and serum levels reflect exposure during the preceding months (45). IgG antibodies therefore indicate previous exposure to fungi, which can be valuable in case studies where measurement data are rarely available. IgG antibodies have been used in epidemiological studies in the past but are now mainly used to document exposure in the diagnosis of hypersensitivity pneumonitis.

5.3 Measurement strategies

Exposure assessment of fungal spores in highly contaminated work environments is similar to that of chemical agents. Personal sampling is preferred. This is straightforward for non-viable methods and even possible for some culture-based methods. There is no general understanding of the relevant sampling time, however. In environments where the exposure arises from contamination of handled materials, exposure levels may show high variability even within days (123). Collection of samples over extended periods will reduce this variability and reduce the number of samples that have to be analysed. At present the commonly used 8-hour sampling time seems appropriate in the absence of better information on relevant sampling time.

Exposure levels in common indoor environments without apparent sources of fungal exposure, e.g. in offices, are much lower than in environments where mouldy material is handled. Fungal spore levels in common indoor air are indeed expected to be similar or lower than outdoors because fresh air is often filtered before entering the ventilation system. Even in damp buildings spore levels are often lower than outdoors. Nevertheless, indoor air problems seem to be related to moisture-damage and fungal growth in buildings (16) and recognition of such damage is important for remediation of the problem. Inspections of buildings for fungal contamination and/or humidity problems by competent investigators and analysis of settled dust, floor dust and building material samples for the presence of fungi have therefore been recommended (1, 4, 75, 95). If such inspections are negative, airborne fungi can be measured to detect fungal growth in the building that is hidden from observation, e.g. within walls or ventilation systems. Indoor levels must be compared to outdoor levels to evaluate if concentrations of fungal species that may proliferate indoors are elevated. As fungal levels in outdoor air can be substantial, especially in the summer and autumn, comparisons are based on species that are rare in outdoor air. It is important that outdoor fungal levels are measured several times a day because of their high within-day variability (226). It is an advantage in subarctic regions to evaluate fungi in common indoor air in the winter when outdoor fungal levels are low. This strategy is adopted in Finland (192).

5.4 Conclusions

At present fungal spores can only be reliably quantified by microscopic counting. Viable as well as non-viable spores are counted but cannot be discriminated.

Culturable fungi can be quantified by culture-based methods but only semi-quantitatively as spore aggregates are counted as single colonies and also mycelium can be detected. However, many factors influence the results, and results obtained by different methods or even by the same method in different environments are difficult to compare. The main advantages of culture-based methods are the possibility to identify fungal species, and the higher sensitivity compared to microscopic counting.

Other methods may also be used to detect fungal agents including ergosterol, glucans, antigens, and allergens, which may indicate the presence of fungal particles. None of these methods discriminate between spores and mycelium. Results can therefore not be interpreted directly as spore levels. The detection of IgG antibodies can be useful in case studies of hypersensitivity pneumonitis where exposure data are not available.

The assessment of fungi in common indoor environments has a different focus than in highly contaminated work environments where fungi represent a risk for respiratory disease. The detection of water-damage and fungal growth is the main goal of investigations in buildings and measurement of airborne fungi is only one of several methods that can be applied for this purpose. Culture-based methods are currently preferred for detection of airborne fungi in common indoor environments.

6. Occupational exposure data

6.1 Highly contaminated environments

Occupational environments with high levels of exposure to fungi are found in agriculture, wood and food industry, and waste handling. Bioaerosol exposure at such workplaces is usually complex and fungi and actinomycetes comprise only some of the agents present. However, in some environments fungal spores are the main component of the bioaerosol and the exposure can even be limited to one or a few species. In such environments, specific organisms have been associated with hypersensitivity pneumonitis and asthma (Table 4).

When fungal colonisation occurs unintentionally, high spore concentrations may be generated when mouldy materials are handled. Work processes, preventive measures, and task performance further influence exposure levels. Exposure levels may therefore show large variability. This is clearly seen in farm work, where exposures during some tasks may differ by more than 4 orders of magnitude (Table 5).

Many exposure studies of non-agricultural occupations have been carried out. The number of epidemiological studies including quantitative exposure assessments is much smaller, however. The exposure data from these epidemiological studies are summarised in Table 6 as they are of greater importance for the present review. Exposure levels often exceed 10^4 cfu/m^3 or 10^5 spores/m^3.

Table 4. Workplaces in which fungi and actinomycetes have been associated with hypersensitivity pneumonitis and asthma; summarised from Lacey and Crook (116).

Workplace	Source	Species
Agriculture	Mouldy hay	*Saccharomonospora viridis* *Saccharopolyspora rectivirgula* *Thermoactinomyces vulgaris* *Aspergillus umbrosus*
	Grain	*Aspergillus fumigatus*
	Grain (maize)	*Aspergillus flavus*
	Straw	*Aspergillus versicolor*
	Mushroom compost	*Saccharomonospora viridis* *Saccharopolyspora rectivirgula* *Thermoactinomyces vulgaris* *Aspergillus fumigatus*
	Mushrooms	*Lentinus edodus* *Pleurotus ostreatus*
Dairy	Cheese	*Penicillium camembertii* *Penicillium rockeforti* *Penicillium casei*
Brewery	Malted barley	*Aspergillus fumigatus* *Aspergillus clavatus*
Citric acid fermentation	Culture fluid	*Aspergillus niger*
Compost	Compost	*Aspergillus fumigatus*
Cork industry	Cork	*Penicillium glabrum*
Sugar mill	Bagasse	*Thermoactinomyces vulgaris* *Thermoactinomyces sacchari*
Tobacco factory	Tobacco	*Aspergillus fumigatus*
Wood industry	Maple bark	*Cryptostroma corticale*
	Coniferous wood	*Rhizopus microsporus* *Penicillium* spp.

6.2 Common indoor environments

Exposure levels in common indoor environments without fungal problems are much lower than in the highly contaminated environments described above. A review of 10 studies including non-complaint commercial buildings showed average levels of 17-1 200 cfu/m^3 and 610-1 000 spores/m^3, which amounted to 6-120% of outdoor levels (63). Additional studies of office buildings typically show fungal levels below 10^3 cfu/m^3, rarely exceeding 10^4 cfu/m^3 (Table 7). The main source of fungi in office environments is outdoor air. As outdoor air is often filtered before it enters the ventilation system and/or fungi settle due to lower air velocities in the buildings, common indoor fungal levels are expected to be lower

Table 5. Exposure levels of culturable and countable fungi and actinomycetes during farm work (46).

Task	Agent	Exposure range [a, b]							
		10^2	10^3	10^4	10^5	10^6	10^7	10^8	10^9
Handling of									
grain	Fungi			<—	—	—	—	—>	
hay	Fungi			<—	—	—	—>		
	Fungi						<···>		
	Actinomycetes								<···>
straw	Fungi			<—	—	—	—	—>	
compost	Actinomycetes						<···	···>	
Animal tending									
dairy/cattle	Fungi		<—	—	—	—	—>		
	Fungi					<···	···>		
	Actinomycetes					<···	···>		
swine	Fungi	<—	—	—>					
poultry	Fungi	<—	—>						

[a] Whole lines show colony forming units/m³ (cfu/m³) and dotted lines spores/m³.
[b] One cfu/m³ corresponds approximately to 10 spores/m³.

than levels in outdoor air. This is also observed in the studies summarised in Table 7. In buildings with fungal growth levels indoors may be somewhat higher than outdoors but differences are usually small. Spores are easily dispersed by heating, ventilation and air-conditioning systems, and contamination of such systems is an important cause of increased indoor fungal levels (145).

An important activity that may contaminate indoor environments is the remediation of mouldy building materials. Fungal levels may then exceed 10^5 cfu/m³ (189).

In non-problem indoor environments outdoor fungi dominate with *Cladosporium* and to some extent *Alternaria* and *Penicillium* as the dominating species. In buildings with fungal problems levels of *Penicillium* and to some extent *Aspergillus* and yeasts are more often elevated compared to outdoor air. Very humid conditions favour growth of *Stachybotrys chartarum*.

Levels of culturable fungi are much lower than fungi counted by microscopical techniques. Russell *et al* found that culturable fungi amounted to 9% of counted spores in dwellings (208). This is also found in comparative studies in highly contaminated environments; review by Eduard and Heederik (51).

Table 6. Exposure to fungi and actinomycetes in epidemiological studies of highly exposed working populations.

Population	Exposure				Reference
	Method	Agent	Culturable organisms cfu/m^3, range	Countable spores spores/m^3, range	
Farmers	Personal sampling	Fungi		$0\text{-}2 \cdot 10^7$	(49)
Farmers	Personal sampling, job exposure matrix[a]	Fungi		$0\text{-}4 \cdot 10^7$	(50)
Sawmill workers (wood trimmers)	Personal sampling	Fungi	$1 \cdot 10^2\text{-}4 \cdot 10^6$		(76)
Sawmill workers (wood trimmers)	Personal sampling	Fungi	$3 \cdot 10^3$ [b]	$1 \cdot 10^5$ [b]	(38)
Sawmill workers (wood trimmers)	Personal sampling	Fungi		$4 \cdot 10^5\text{-}2 \cdot 10^7$	(54)
Saw-/chip mill workers	Personal sampling	Fungi	$3 \cdot 10^3\text{-}7 \cdot 10^4$ [c]		(6)
Joinery workers	Personal sampling	Fungi	$4 \cdot 10^3\text{-}2 \cdot 10^4$ [c]		(6)
Waste collectors	Personal sampling	Fungi Actinomycetes		$0\text{-}2 \cdot 10^6$ $0\text{-}1 \cdot 10^6$	(79, 80)

cfu: colony forming units.
[a] Job exposure matrix: exposure estimated from information on performed tasks and other determinants, which had been validated by personal exposure measurement in a subset of the workers.
[b] Median.
[c] Range of arithmetic means.

Table 7. Culturable airborne fungi in office buildings.

Environment	Fungal species (in ascending prevalence order)	Total fungal levels, cfu/m³	Reference
4 buildings *California, USA*	*Cladosporium*; non-sporulating *Aspergillus/Penicillium*	5-420 (15-35% of outdoor levels)	(212)
84 randomly selected buildings throughout *USA*	Non-sporulating *Cladosporium*; *Penicillium*; yeasts; *Aspergillus*	0-4 000 (in 5% of the buildings were indoor levels > outdoor levels)	(248)
4 buildings, 21 offices, sampled 1 year *Boston, Massachusetts, USA*	Non-sporulating *Penicillium*; *Cladosporium*; yeasts; *Aspergillus*	1-620	(29)
1 700 buildings (46% office), inspected because of complaints, water damage or fungal growth throughout *USA*	*Cladosporium*; *Penicillium*; non-sporulating *Aspergillus*	1- >10 000 82 (median indoors) 540 (median outdoors)	(221)
2 buildings without fungal problems, 6 buildings with fungal problems continental, *USA*	*Penicillium*	16-280 (11-86% of outdoor levels) 150- > 3 000 (<18-170% of outdoor levels)	(144)
15 buildings without preceding selection *Silesia, Poland*	*Penicillium*	50-1 700 (summer) 18-110 (winter)	(173)
3 non-problem offices, 2 offices with health complaints, 5 offices with fungal but no health problems *Prague, Czech Republic*	Not specified	nd-20 nd-70 190-330	(110)
1 air-conditioned building, 1 naturally ventilated building *Paris, France*	*Penicillium*; *Cladosporium*	17 (arithmetic mean, 4% of outdoor levels) 210 (arithmetic mean, 44% of outdoor levels) range 32-1 100	(169)
28 randomly selected day-care centres *Taipei, Taiwan*	*Penicillium*; *Cladosporium*; yeasts; *Aspergillus*	1 200 ± 3.0 (geometric mean ± geometric standard deviation) (120% of outdoor levels)	(124)

cfu: colony forming units, nd: not detected.

6.3 Conclusions

Exposure levels in highly contaminated environments often exceed 10^4 cfu/m^3 and 10^6 spores/m^3 and may even exceed 10^8 spores/m^3. In indoor environments without fungal contamination, levels are generally below 10^3 cfu/m^3 and lower than in outdoor air. Fungal levels in buildings with fungal growth may be somewhat higher than in buildings without such contamination. Exposure levels in highly contaminated environments are thus several orders of magnitude higher than in common indoor environments without fungal contamination.

7. Uptake, distribution and elimination

7.1 Uptake

The major exposure route of fungal spores is inhalation.

The size, shape and surface structure of the individual spores as well as aggregates are of major importance for their aerodynamic behaviour and thus the region where inhaled spores deposit in the airways. Individual spores of fungi are typically 2-10 μm in size. Spores of actinomycetes are smaller, typically 0.5-1.5 μm. Due to the variable spore size of single spores, the presence of aggregates, differences in density, and absorption of water vapour by inhaled spores, fungal spores may be expected to deposit in the whole respiratory system. A substantial fraction of the smaller spore types may reach the alveoli, while the larger spore types (>7-10 μm) do not. Aggregates, which are larger may deposit to a greater extent in the lower and upper airways rather than in the alveoli. Spores of the outdoor fungus *Alternaria alternata* are so large that they are not expected to reach the alveoli.

Gastro-intestinal exposure may occur via mucociliary clearance of spores that have deposited in the airways (and are swallowed when mucous reaches the throat).

Some pathogenic fungi may infect the skin, but such organisms are beyond the scope of this review.

7.2 Distribution

A limited number of *in vivo* studies have addressed dissemination of spores from the airways to other organs. All studies used *Aspergillus fumigatus* or *Aspergillus terreus*. In addition, a *Penicillium* species and *Rhizopus oryzae* were included in two of the studies, respectively. The studies are summarised below and described in more detail in Appendix 3.

Two studies showed that spores of *A. fumigatus* disseminated to the spleen, liver, and kidneys in rabbits (112) and mice (241). The latter study also showed that viable counts had cleared from these organs after 10 days. However, in a study by Schaffner *et al*, mice exposed to *A. fumigatus* spores showed no dissemination to other organs (210). The latter study used more realistic conditions

as airborne spores were inhaled and the dose may have been lower ($4 \cdot 10^3$ spores/g bw) than in the study by Waldorf *et al* (241) who exposed mice intranasally to a 20 times higher dose. However, Schaffner *et al* (210) estimated the dose after 2 hours by culture of lung tissue and probably underestimated the applied dose.

A study of spores of *R. oryzae* intranasally instilled in mice showed that spores migrated to the liver, spleen, and kidneys and were still present in the liver and spleen 10 days after exposure (241). Two studies of *A. terreus* spores administered intratracheally to rats and rabbits showed only minor migration of spores to the tracheobronchial lymph nodes (68, 165).

In a rabbit study, Thurston *et al* found a small fraction of spores in the digestive system 6 hours after aerosol exposure to *A. fumigatus* and a *Penicillium* sp. (235). No fungi could be cultured a week after exposure. No other organs than the lung and the digestive system were studied.

In summary, these studies show different results even for the same species *A. fumigatus*. Dissemination to other organs than the lung and the digestive system was observed only for the facultative pathogenic organisms *A. fumigatus* and *R. oryzae*. However, only one non-pathogenic fungus, a *Penicillium* species, has been studied, and all studies showing dissemination to other organs applied intratracheal or intranasal instillation, which may have caused local overload of macrophages (Chapters 10.1 and 12.1). One study found that inhaled spores of *A. fumigatus* and a *Penicillium* sp. reached the digestive system suggestive of mucociliary clearance. However, the proportion of the inhaled dose was small and the fungi were cleared within a week, indicating that effects on the digestive system are minor.

7.3 Elimination

7.3.1 In vivo studies

Elimination of spores from lung tissue and phagocytosis and destruction of spores by pulmonary alveolar macrophages have been studied for several species of fungi and laboratory animals. The studies are summarised here (Table 8) and more details are given in Appendix 4.

In three of the studies animals were exposed to aerosols of *Aspergillus fumigatus* spores and the deposited dose was estimated by culture of lung tissue. Colony counts declined in Guinea pigs, rabbits and immunised mice, although small numbers were detectable after ≥ 6 days (210, 235, 240). Counts of *Penicillium* sp. declined more slowly in rabbits inhaling spore aerosols, and colonies were still observed after 3 weeks (235). *Saccharopolyspora rectivirgula* counts declined much slower in aerosol exposed Guinea pigs (240). In a second study on *S. rectivirgula* in Guinea pigs, 10^2-10^3 times higher doses were applied by intratracheal instillation and a rapid decline in colony counts was found (256), similar to the results obtained for *A. fumigatus*. Elimination was delayed when exposure to *S. rectivirgula* spores was combined with hay dust, compared with what was observed after exposure to spores alone (256). In two other studies using intratracheal instillation of *Candida albicans* cells in Guinea pigs, and *Aspergillus*

Table 8. Elimination of spores of fungi and actinomycetes from the lung in animal studies.

Micro-organism	Animals: Species	Animals: No/group[a]	Exposure: Route	Exposure: Dose as specified in the study	Exposure: Dose estimated as spores/g bw[b]	Observation time	Elimination: From lung tissue (% of initial dose)	Elimination: Mechanistic observations	Reference
Fungi									
Aspergillus fumigatus									
	Mouse, immunised	9	ae	$3 \cdot 10^5$ cfu/g lung after 2 h	$4 \cdot 10^3$	6 d	2 d: <1% 6 d: +		(210)
	Guinea pig	100	ae	$7 \cdot 10^6$ cfu after 2 h	$4 \cdot 10^4$	12 d	1 d: 10% 4 d: 0.1% 10 d: +		(240)
	Rabbit	6	ae	$5 \cdot 10^5$-$4 \cdot 10^6$ cfu/g lung after 1 h	$1 \cdot 10^4$-$8 \cdot 10^4$	3 w	1 d: 1-10% 2 d: 0.1-2% 1.5-3 w: nd		(235)
	Mouse	10	i.n.	$1 \cdot 10^6$ spores	$4 \cdot 10^4$	10 d		18 h: 70% of spores in AM were killed, 10 d: no germination.	(241)
	Rabbit	5	i.t.	$1 \cdot 10^7$ spores	$4 \cdot 10^3$	4 h		1 h: 53% of observed spores in AM, 4 h: 22%.	(112)
Aspergillus terreus									
	Rabbit Rat	Not given	i.t. i.t.	$7 \cdot 10^6$ spores $5 \cdot 10^7$ spores	$4 \cdot 10^3$ $1 \cdot 10^5$	2 d		Rapid uptake by AM, complete after 3 h. Very few PMN observed.	(68)
	Rabbit Rat	2 2	i.t. i.t.	$7 \cdot 10^6$ spores $5 \cdot 10^7$ spores	$4 \cdot 10^3$ $1 \cdot 10^5$	24 h	3 h: 100% 24 h: 20%	0 h: 48% of observed spores in AM, 24 h: 98% in rats. Qualitatively similar results in rabbits.	(165)
Candida albicans									
	Guinea pig	20	i.t.	$1 \cdot 10^7$-$12 \cdot 10^7$ cells	$3 \cdot 10^4$-$4 \cdot 10^5$ cells	5 d	3 h: 100% 8 h: 3% 24 h: 0.1%		(240)

Table 8. *Cont*. Elimination of spores of fungi and actinomycetes from the lung in animal studies.

Micro-	Animals		Exposure			Observation	Elimination		Reference
organism	Species	No/group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]	time	From lung tissue (% of initial dose)	Mechanistic observations	
Penicillium chrysogenum									
	Mouse	Not given	i.n.	$1 \cdot 10^4$ spores	$4 \cdot 10^2$	24 h		3-24 h: Spores were phagocytised and digested by AM.	(33)
Penicillium sp.									
	Rabbit	3	ae	$1 \cdot 10^5$-$5 \cdot 10^5$ cfu/g lung after 1 h	$2 \cdot 10^3$-$1 \cdot 10^4$	3 w	1 d: 7-30%, 2 d: 4-7%, 3 w: 0.02-0.03%		(235)
Rhizopus oryzae									
	Mouse	10	i.n.	$1 \cdot 10^6$ spores	$4 \cdot 10^4$	10 d		18 h: 20% of spores in AM were killed, 10 d: no germination.	(241)
Actinomycetes									
Saccharopolyspora rectivirgula									
	Guinea pig	90	ae	$1 \cdot 10^6$-$8 \cdot 10^6$ cfu after 2 h	$3 \cdot 10^3$-$3 \cdot 10^4$	2 m	2-3 w: 10%, 2 m: +	Mycelial growth in anti-AM serum treated animals.	(240)
	Guinea pig	30	i.t.	800 μg spores	$4 \cdot 10^6$	7 d	1 d: 10%, 3 d: 0.1%, 7 d: +		(256)
	Guinea pig	30	i.t.	800 μg spores + 75 mg hay dust	$4 \cdot 10^6$	9 d	5 d: 100%, 9 d: 10%		(256)

[a] Number of animals in exposed and control group.
[b] Dose estimated as described in Chapter 10.1.
+: a few colony counts were observed, ae: aerosol, AM: alveolar macrophages, cfu: colony forming units, i.n.: intranasal, i.t.: intratracheal, nd: not detectable, PMN: polymorphonuclear leukocytes.

terreus spores in rats and rabbits most microorganisms had been cleared after one day (165, 240). A study of mice intranasally exposed to *Rhizopus oryzae* showed culturable fungi in the lungs 10 days after exposure, although no quantitative data were presented (241).

To sum up, although several studies found that the major part of culturable fungi was eliminated from the lung after a few days, elimination was not complete after a week and could last considerable longer for *R. oryzae* and *S. rectivirgula*. The applied doses in the latter studies did not exceed the overload limit of 60 μm^3/macrophage suggested by Morrow (147) (Chapter 8.1).

Spores of *A. fumigatus, A. terreus,* and *Penicillium chrysogenum* were rapidly phagocytised by alveolar macrophages in mice, rats, and rabbits (33, 68, 112, 241). Phagocytised spores of *A. fumigatus,* and *P. chrysogenum* were killed by mouse alveolar macrophages (33, 241). However, spores of *A. fumigatus* seemed to resist rabbit alveolar macrophages in one study (112). Mouse alveolar macrophages prevented germination of spores of *Rhizopus oryzae* but did not kill the spores (241). The role of alveolar macrophages in prevention of spore germination was demonstrated by mycelial growth of *S. rectivirgula* in the lungs of Guinea pigs that had been depleted of alveolar macrophages by treatment with anti-alveolar macrophage serum (240). It was also observed that alveolar macrophages migrated from peripheral alveoli to alveoli adjacent to the respiratory bronchioles (68).

In conclusion, the alveolar macrophages are the primary line of defence against fungal spores. Alveolar macrophages killed spores from most tested species, and if spores withstood macrophage attack, germination was prevented.

7.3.2 In vitro studies

In vitro studies have been carried out with viable and killed spores of various microbial species using different cell types, and a variety of responses were studied. The studies are described in more detail in Appendix 5.

Spores from *Aspergillus fumigatus, Aspergillus candidus* and *Penicillium ochrochloron* attached readily to different phagocytic cells. *P. ochrochloron* spores were more easily phagocytised than *A. fumigatus* spores, and heat-killed *A. fumigatus* spores were phagocytised more rapidly than inert particles (152, 153, 199). Ingested spores of *Aspergillus flavus* and *Aspergillus niger,* but not *A. fumigatus,* were killed by rabbit alveolar macrophages and the latter even germinated after 4 hours (112).

Killing of microorganisms by phagocytes involves reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and nitric oxide (NO). ROS production by different types of phagocytic cells differed between species of fungi and actinomycetes (84, 207, 218, 220). Differences were also observed between isolates of the same species and even the same strain grown on different substrates using a mouse macrophage cell line (85, 206). Similar differences were also observed for NO and inducible NO synthase production (81, 82, 84, 91, 92, 105, 150, 151, 176, 206).

The production of cytokines as interleukin (IL)-6, IL-1, IL-1β, and tumour necrosis factor alpha (TNFα) by phagocytic cells, and cytotoxicity in a mouse macrophage cell line varied between species, cell types and the medium used for culture of the microorganisms including building materials (91, 153, 182, 206, 219). Different results were even reported for similar tests carried out by the same laboratory (81, 82, 85, 91, 92, 148-151, 176, 204). Cytotoxic strains of *Stachybotrys* produced satratoxin but did not induce IL-6 and TNFα in a mouse macrophage cell line while non-toxic strains induced these inflammatory mediators (157), indicating that mycotoxins impair the cytokine response.

Positive interactions were found between S*tachybotrys chartarum* with the actinomycete *Streptomyces californicus* on IL-6 production, apoptosis, cell-cycle arrest, and caspase-3 enzyme activity in a mouse macrophage cell line, while no or weaker responses were found for NO and TNFα production (92, 176). Two other fungi did not show such interactions with this actinomycete.

Spore viability has shown different effects on the elicited inflammatory responses of macrophages to spores. The response to viable and dead spores was similar regarding superoxide anion induction, NO production, and phagocytosis. However, killed spores induced less ROS than viable spores while both higher and lower cytotoxicity was observed in a mouse macrophage cell line. These studies included many fungal and actinomycetes species (84, 218). Viable *A. fumigatus* induced production of a variety of cytokines whereas killed spores did not (182). However, Shahan *et al* found no difference in macrophage superoxide production between viable and killed spores in a similar study of this fungus (218). A recent study by Hohl *et al* showed a large increase in TNFα and macrophage inflammatory protein (MIP)-2 in murine alveolar macrophages after exposure to live *A. fumigatus* spores, while no response was observed after exposure to heat-killed spores (88). However, when spores were incubated, the inflammatory response of heat-killed spores increased with incubation time indicating that germination of spores alters their inflammatory potential.

7.3.3 Discussion

The *in vivo* studies demonstrated that most fungal spores were eliminated from the lung within a few days, although elimination was not complete and could last considerably longer for *Rhizopus oryzae* and *Saccharopolyspora rectivirgula*. It was also observed that alveolar macrophages phagocytised the spores, and although some spores maintained viability, they did not germinate. One study demonstrated that the alveolar macrophages were crucial in germination prevention.

The exposure levels used in *in vivo* studies were rather high. The lowest dose was $2 \cdot 10^3$ spores/g bw, which corresponds to a human dose of $>10^8$ spores. Such doses can be inhaled during an 8-hour workday at exposure levels $>10^7$ spores/m^3, i.e. at the higher end of occupational exposures and many orders of magnitude higher than in environments with common indoor exposure to fungal spores (Tables 5-7). However, the dose was estimated by culture of lung tissue 2 hours after aerosol exposure and the actual dose may have been substantially higher

(Chapter 10.1). Thus, the delayed elimination of spores of some species may not be relevant for populations working in highly contaminated environments except in extreme exposure situations.

In vitro studies further documented that alveolar macrophages bind spores rapidly to their cell walls. The alveolar macrophages phagocytise attached spores, kill ingested spores by NO and ROS and may produce cytokines. Differences in induced macrophage NO and ROS production were observed between fungal species, isolates of the same species and even isolates grown on different substrates, but results were not consistent across studies. Similarly, results on production of cytokines such as TNFα and IL-6 were not consistent although these and other cytokines were demonstrated in response to spores from many species. The use of different cell types in *in vitro* tests may account for some of the differences together with the presence of secondary metabolites in the spore preparations.

Some *in vitro* studies showed that spores of fungi and actinomycetes were able to resist and even kill alveolar macrophages although results were not consistent.

The decline of culturable microorganisms in lung tissue may also be due to migration of spores to other organs. This was shown for the opportunistic pathogens *A. fumigatus* and *R. oryzae* that seem able to resist pulmonary defence mechanisms.

Some results are indicative of mucociliary clearance of spores in the respiratory tract. Alveolar macrophages with phagocytised spores migrated from the peripheral alveoli to those closest to the terminal bronchioles. The detection of microorganisms in the digestive system indicates that they probably had been swallowed.

The effect of viability was explored in *in vitro* studies and one *in vivo* study. Although results were variable, there are indications that the elicited response from viable spores can be stronger than that from dead spores when they are able to germinate.

8. Mechanism of toxicity

Viable microorganisms such as spores of fungi and actinomycetes are different from chemical agents, as they may be able to germinate and proliferate in the host. When the host's defence systems fail, toxicity may arise, leading to various clinical outcomes such as organic dust toxic syndrome (ODTS), mucosal membrane irritation, and chronic restrictive and obstructive lung diseases such as hypersensitivity pneumonitis and asthma.

8.1 The innate and adaptive response to fungi

The alveolar macrophages are the first line of defence against microorganisms at the alveolar level. Their role in elimination of fungal spores has been described in Chapter 7.3. Alveolar macrophages phagocytise spores and migrate to the airways where they are removed by mucociliary clearance. Morrow reviewed studies on migration of rat alveolar macrophages from the alveoli into the airways (147). The

migration was impaired when the total volume of phagocytised non-soluble particles exceeded approximately 60 μm^3 per alveolar macrophage. If this clearance limit also applies to fungal spores, it corresponds to human alveolar doses of $6 \cdot 10^9$ to $9 \cdot 10^{10}$ of spores with sizes 5 and 2 μm, respectively, as the human lung contains approximately $6 \cdot 10^9$ alveolar macrophages (233). This relates to exposure levels of 10^9-$2 \cdot 10^{10}$ spores/m^3 during a work shift assuming an alveolar ventilation of 10 l/min for 8 hours. Such levels are rare in the work environment (Chapter 6.1) but have been reported in relation to fever attacks typical of ODTS (Chapters 8.3 and 11.4.2). It is therefore possible that clearance overload leading to prolonged presence of fungal spores at the alveolar level is a contributing factor to ODTS.

The defence of human and murine macrophages against hyphae is not effective, which has been shown for the pathogenic species *Aspergillus fumigatus* and *Histoplasma capsulatum* (109, 210). However, human neutrophils destroyed the mycelial form of *Aspergillus fumigatus* and *Rhizopus oryzae* by attaching to the hyphae and by extra-cellular production of ROS (40, 210).

Several receptors have been found on human and murine phagocytic cells, i.e. the alveolar macrophages, neutrophils and dendritic cells, by which these cells attach to inhaled microorganisms. The Toll-like receptors (TLR) are probably the most important receptors for microbial recognition. It has been shown that TLR2, TLR4, TLR6, and TLR9 on mouse and human phagocytes bind to the pathogenic fungi *Aspergillus fumigatus, Cryptococcus neoformans,* and *Candida albicans* (12); review by Netea *et al* (155). The binding of *A. fumigatus* to TLR2 and TLR4 promoted fungicidal activity in neutrophils through different oxidative pathways, while TLR3, TLR5, TLR6, TLR7, TLR8 and TLR9 further modulated the response (13). Other innate receptors involved in fungal recognition are the IL-1 receptor that is structurally related to the TLRs, the mannose receptor and the Dectin-1 receptor that binds to β-glucans (12, 19, 154). These receptors seem to act in combination in the recognition of pathogenic microorganisms. For example, TLR2, TLR4, the Dectin-1 receptor and the mannose receptor have been shown to bind to different molecules on the cell wall of *Candida albicans* yeast cells (154).

Fungal spores and hyphae bind differently to the innate pattern recognition receptors. Conidia of *Aspergillus fumigatus* were recognised by TLR2, TLR4 and TLR9 on human and murine neutrophils while hyphae were only recognised by TLR4 (13). Gantner *et al* found that yeast cells of *Candida albicans* bound to the Dectin-1 receptor on murine macrophages (59), which the authors ascribed to β-glucan being exposed on the outer cell wall at the scars formed by the budding yeast cells. Hyphae were not recognised probably because the β-glucan in the hyphal cell wall was covered by other compounds. The Dectin-1 receptors on murine and human macrophages were also found to bind preferentially to germinating spores of *A. fumigatus* compared to resting spores. This was similarly explained by disruption of the layer that covers the β-glucan on the spore wall during spore germination (60, 88). Thus, the composition of the cell wall surface has a major effect on the binding to phagocytic cells.

Other pattern recognition receptors are present on surfactant proteins A and D that are produced by epithelial cells in the alveoli and bronchi. These proteins play an important role in the lung by reducing the surface tension on the alveoli, but they also contain a mannose receptor that binds to a range of sugars that can be present on invading microorganisms including fungi; review by Turner (238). After binding, the surfactant proteins can activate complement directly, or they can stimulate phagocytosis by binding to collectin receptors on phagocytic cells; review by Crouch (37).

Recent publications have addressed the role that antigen presenting pulmonary dendritic cells play in the immune responses to fungi. Dendritic cells phagocytise pathogens and present their antigens to precursor T-helper (Th) cells, which may mature into Th1 and Th2 cells. The Th1 cells mediate a cellular response by stimulating macrophages to phagocytise the pathogen and T-cells to develop into cytotoxic T-cells that phagocytise and kill specific organisms. The Th2 cells stimulate B-cells to proliferate into specific antibody producing plasma cells. The Th1 and Th2 cells further modulate the type of antibodies that are produced by secretion of different cytokines. Th1 cells promote the development of IgG antibodies that bind specifically to antigens and facilitate their uptake by phagocytic cells. Th2 cells may stimulate B-cells to produce IgE antibodies and can also induce proliferation of mast cells and eosinophilic leukocytes, which are important characteristics of the allergic response. The balance between Th1 and Th2 cells is thus important for the type of immune response that is mounted against invading organisms. Recently, T regulatory cells involved in suppression of the Th2 response and thought to prevent sensitisation and allergic disease were described; review by Robinson *et al* (200). However, their role in fungal allergy is not clear.

Mouse dendritic cells have been shown to respond differently to spores and hyphae (19). Resting spores of *Aspergillus fumigatus* were more efficiently phagocytised than swollen conidia and hyphae *in vitro*. *In vivo* mouse alveolar dendritic cells with phagocytised spores translocated through the alveolar epithelial barrier migrated to the thoracic lymph nodes and the spleen and underwent functional maturation. Dendritic cells produced IL-12p70 and TNFα after ingestion of spores, which primed the maturation of precursor Th cells to interferon gamma (IFNγ) producing Th1 cells, while ingestion of hyphae induced IL-4, IL-10 and TNFα production and priming of IL-4 producing Th2 cells (Figure 4).

Similar findings were reported in an *in vitro* study of *Candida albicans*. Dendritic cells that had ingested hyphae primed Th2 cells in mice, while dendritic cells primed Th1 cells after ingestion of yeast cells (167). A difference from *Aspergillus fumigatus* was, however, that hyphae of *Candida albicans* escaped from the phagosomes, while yeast cells were destroyed. The response of dendritic cells to fungi therefore seems to depend both on the species and on the cellular form of the organism. The production of fungal allergens during germination and mycelial growth is likely to play a role (Chapter 3.6). It seems therefore possible that viable spores may induce an allergic response if they germinate in the airways.

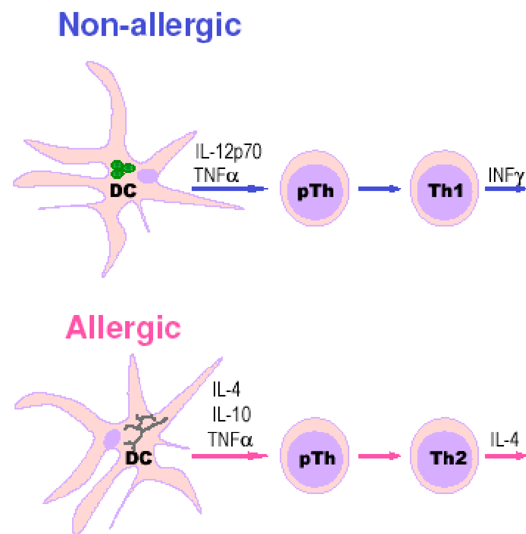

Figure 4. Differential maturation of precursor T helper cells (pTh) by dendritic cells (DCs). A non-allergic response was observed after DCs ingested *Aspergillus fumigatus* spores, while ingestion of hyphae induced an allergic response. Elaborated from Bozza *et al* (19).

Some support for this hypothesis is found in *in vivo* studies of viable and/or germinating spores (Chapters 10.3, 10.4.2 and 10.5).

Also epithelial cells seem to be involved in interactions with fungi. It has been proposed that proteases present in fungi and other allergens may facilitate passage of allergens through the epithelial barrier; review by Kauffman and van der Heide (106).

Mycotoxins may further modulate the immune response, as many mycotoxins are cytotoxic to immune cells. The mycotoxins citrinin, gliotoxin and patulin induced a stronger suppression of human Th1 cells than Th2 cells *in vitro*, which favours an allergic response to the toxin producing fungi (245). Mycotoxins can also have other health effects (Chapter 3.6).

8.2 Allergic responses

Allergy has been defined in different ways. In this document the definitions of allergy and hypersensitivity pneumonitis given by the European Academy of Allergology and Clinical Immunology are used (99) (Appendix 1).

IgE-mediated allergy is probably the most important allergic mechanism in the general population. Exposure of sensitised individuals (i.e. with elevated IgE antibodies) to the corresponding allergen causes mast cells to release histamine and other inflammatory mediators. In asthmatic patients histamine release causes constriction of the bronchi by smooth muscle contraction, and cytokines attract eosinophils. The subsequent eosinophilic inflammation further constricts the airways by swelling of the mucosa resulting in airway obstruction that can be life

threatening. In patients with allergic rhinoconjunctivitis the mast cells produce the same inflammatory agents resulting in sneezing, running or blocked nose, and eye irritation. Although not all individuals with detectable IgE are symptomatic, the presence of specific IgE antibodies is a predictor of future allergic disease if exposure to the allergen continues; review by Platts-Mills (179).

Few occupational studies of IgE-sensitisation to fungi have been published. These studies provide conflicting information on the occurrence of sensitisation and IgE-mediated diseases in working populations exposed to fungi. This may be due to the limited number of available assays for detection of IgE to fungal allergens in serum and the low sensitivity of these assays (Chapter 11.2.2).

Individuals with IgE against commonly occurring allergens also have greater risk to develop IgE against occupational allergens such as fungal amylase in bakers (89) and rodent allergens in laboratory animal workers (77). Such individuals are called atopic and their identification is important as they may represent a sensitive group. Although fungal allergy is quite common in atopic individuals of the general population, the role of atopy for fungal allergy in occupational populations is unclear.

Another type of allergic disease that seems to be more important in populations occupationally exposed to fungi is hypersensitivity pneumonitis, which is also called allergic alveolitis. This disease is characterised by alveolar and bronchiolar inflammation, and is caused by inhalation of spores from fungi and actinomycetes, and also by other allergens. Typical symptoms and clinical findings are attacks with fever, chills, dry cough, dyspnoea, basal crepitations, nodular bilateral X-ray changes, malaise and headache, and declines in forced vital capacity (FVC) and gas diffusion capacity. IgG but not IgE can usually be detected. The attacks are similar to those of ODTS (Chapter 8.3) but patients with ODTS recover within a few days without persisting symptoms or clinical findings. Acute attacks of hypersensitivity pneumonitis occur a few hours after episodes with massive exposure to spores of fungi and/or actinomycetes. Recurrent attacks may eventually progress into pulmonary fibrosis if exposure continues, and may even be fatal (174, 205). Patients typically work in environments where high exposures occur repeatedly.

Hypersensitivity pneumonitis is thought to be an allergic disease, although the mechanisms are not entirely clear. The disease is not IgE-mediated, as IgE antibodies are not generally found in patients with hypersensitivity pneumonitis. Specific IgG antibodies to fungi can often be demonstrated in serum of these patients indicating a type III allergic reaction. However, the role of IgG antibodies in the disease is unclear as these antibodies are common in healthy exposed individuals. The presence of serum IgG antibodies is therefore regarded as a marker of exposure and not of disease (24) (Chapter 9.1). Additionally, type IV hypersensitivity is suggested by the presence of granuloma in the lungs of the patients (Chapter 11.5.1.), and by granuloma formation in animal studies (Chapters 10.4 and 10.5) indicating the involvement of cytotoxic T-cells. Host factors may play a role as well because only a small proportion of the exposed individuals develop the disease (17) (Chapter 11). Neutrophilic inflammation is

generally observed in this disease and production of the pyrogenic cytokines TNFα and IL-6 by neutrophils probably explain the fever attacks that are typical for hypersensitivity pneumonitis.

Although the mechanisms of hypersensitivity pneumonitis are not entirely clear, the allergic nature of the disease is clearly demonstrated by bronchial challenge to fungal extracts and even spores in numerous case studies and a few epidemiological studies (Chapters 11.2.1 and 11.5.1.).

8.3 Non-allergic responses

Chronic bronchitis and ODTS are generally regarded as non-allergic diseases (56, 170). ODTS is also described as toxic alveolitis or inhalation fever (56, 187). ODTS patients develop febrile attacks with chills, headache, malaise, cough and dyspnoea 4-8 hours after massive exposure to some substances, e.g. metal fumes, organic particles and spores from fungi and actinomycetes. IgG antibodies against fungi are often not detectable. Patients recover within a few days without functional and X-ray changes or persisting symptoms, which distinguishes this condition from hypersensitivity pneumonitis.

Mucous membrane irritation may be due to allergic rhinoconjunctivitis, but is prevalent also in individuals without atopy. A substantial proportion of the patients with diagnosed asthma and rhinoconjunctivitis do not have atopy or eosinophilic inflammation but probably neutrophilic inflammation (43, 175, 254). Non-allergic diseases are therefore important outcomes in populations exposed to fungi.

Most experimental and animal studies report non-allergic inflammation after challenge to fungal spores. The following mechanisms are supported by these studies (reviewed in Chapter 7). Alveolar macrophages phagocytise spores and destroy spores in phagolysosomes by ROS and by non-oxygen dependent mechanisms involving lysosomal enzymes. Spores from some species, and hyphae in general, are resistant to destruction by alveolar macrophages. The alveolar macrophages can produce a variety of cytokines, among which IL-8 is most important to attract neutrophils. The neutrophils can phagocytise and destroy hyphae and swollen spores by oxidative and non-oxidative mechanisms. The fungicidal agents are present in granules within the neutrophils and are released inside the cells, but they can also be excreted and cause tissue damage. Both activated alveolar macrophages and neutrophils can produce the pyrogenic cytokines TNFα and IL-6, which provides a likely explanation for febrile symptoms during attacks of ODTS.

8.4 Summary

The inflammatory response to spores is mainly non-allergic in occupational populations although allergic diseases such as allergic asthma, allergic rhino-conjunctivitis, and hypersensitivity pneumonitis can be induced by exposure to fungi. The allergic and non-allergic responses indicate that different inflammatory

mechanisms are involved, many of which are not fully elucidated. Fungi are recognised by phagocytic cells with innate receptors. They become phagocytised and induce both innate and adaptive immune responses. Hyphae and germinating spores are able to induce allergic responses, which may be due to allergen production during germination and growth.

Allergic asthma and rhinoconjunctivitis are IgE-mediated while hypersensitivity pneumonitis is most likely due to cellular hypersensitivity (type IV allergy) and/or IgG-mediated type III allergy.

Non-allergic asthma and rhinoconjunctivitis are at least as prevalent as the allergic forms of the diseases, and are probably mediated by neutrophilic inflammation. ODTS is characterised by similar acute symptoms as hypersensitivity pneumonitis, but the chronic effects seem to be absent. The fever attacks in both outcomes are probably caused by macrophage and neutrophilic inflammation. Macrophage overload may play a role in ODTS.

9. Biological monitoring

9.1 Markers of exposure

Specific IgG antibodies to *Saccharopolyspora rectivirgula*, *Aspergillus, Penicillium,* and *Rhizopus* species in serum have been used as markers of exposure in studies of farmers and sawmill, cork, tobacco, and malt workers.

The importance of specific IgG antibodies as marker of airborne exposure depends on the absence of other exposure routes as the development of these antibodies is a general response to antigen exposure. In earlier studies, IgG antibodies have been detected by precipitation of the antibody-antigen complex in a diffusion gel, and are called precipitins. This method is semi-quantitative but is easy to perform with several antigens simultaneously. Enzyme-linked immunosorbent assays have been developed for quantitative measurements.

IgG antibodies may appear after repeated exposure to airborne fungal spores. This has been shown in several animal studies. An interesting study is the one by Thurston *et al* who exposed rabbits once a day for 1-10 days to aerosols of *Aspergillus fumigatus* spores with doses ranging from $4{\cdot}10^5$ to $2{\cdot}10^6$ cfu/g lung tissue (236). They found that precipitating antibodies only developed after exposure to the highest dose ($2{\cdot}10^4$ spores/g bw corresponding to a human dose of more than 10^9 spores) and that exposure had to be repeated at least once. This is in agreement with the mechanism for immunisation through development of IgG antibodies (201). After a single exposure few or no animals developed IgG antibodies (114, 160, 164, 165, 235, 236) except in one study where rabbits were exposed to a very high dose of *A. fumigatus* (approximately $2{\cdot}10^6$-$5{\cdot}10^6$ spores/g bw) (146). Cooley *et al* exposed mice intranasally to doses of $1{\cdot}10^4$ spores of *Penicillium chrysogenum* once a week for 6 weeks. Both killed and viable spores caused decreased specific IgG_{2a} levels, although total IgG_{2a} levels increased after exposure to killed spores (33). The presence of specific IgG_{2a} antibodies before

exposure indicates that the animals had been exposed to *P. chrysogenum* or other fungi cross-reacting with this species before the challenges, however. Thurston *et al* found a relatively high threshold for precipitin formation against *A. fumigatus* in rabbits (236), which may explain why an increase in specific IgG_{2a} levels was not found in the study by Cooley *et al* (33).

Only two epidemiological studies were found that examined the relationship between serum IgG levels and fungal exposure levels. In wood trimmers, levels of serum IgG against *Rhizopus microsporus* and *Paecilomyces variotii* were associated with exposure levels of spores from these fungi in the preceding months (53). In school children, however, levels of serum IgG against fungi from water-damaged schools were poorly correlated with airborne levels (93). This may be due to the relatively low exposure level in this environment, and to exposure to similar or even higher levels in their homes or outdoors. The authors therefore did not recommend serum IgG levels as markers of exposure in schools.

Inter-individual differences in the serum antibody response are large and absence of IgG antibodies does not prove that a worker has not been exposed. Group mean IgG levels of similarly exposed individuals indicate exposure with better precision and have been used successfully as markers of exposure in epidemiological studies of populations working in highly contaminated environments, e.g. farmers and sawmill workers. Changes in the intra-individual IgG levels indicate relative changes in exposure with better precision than a single individual measurement because the inter-individual variability is eliminated; review by Eduard (45).

To sum up, individual IgG antibody levels are difficult to interpret as exposure levels, and their use as markers of exposure has not been validated. However, the presence of IgG antibodies may indicate exposure to relatively high levels of specific fungi, which is valuable in the diagnosis of hypersensitivity pneumonitis. It should be noted that the detection of IgG antibodies is not a diagnostic tool of the disease, as healthy individuals can have high IgG antibody levels (Chapter 8.2).

9.2 Markers of effect

The demonstration of specific IgE antibodies to allergens is an important tool in the etiologic diagnosis of atopic disease. Allergens from *Cladosporium herbarum, Alternaria alternata,* and *Aspergillus fumigatus* have been best characterised and are used in commercial IgE tests (113). The skin prick test and the radioallergo-sorbent test (RAST) in serum have been used predominantly but these methods are mainly semi-quantitative. The allergenic composition of spores, mycelia, and culture fluid is different and fungal extracts also vary between strains. There is therefore a need for standardisation of fungal allergens (25, 209). Most allergen extracts have been prepared from culture fluid or mycelia. Positive tests thus indicate an allergic response to the fungus, but not necessarily to its spores since the allergen composition of the extract may deviate from that of spores. Another problem, which complicates the diagnosis of fungal allergy, is that allergic individuals can be sensitised to some but not all allergens from the same species

and no allergen is common to all allergic individuals (113). Major allergens have been recognised, however. For example, 85% of the patients allergic to *A. fumigatus* were positive when tested with the allergen Asp f 1 and 80% of the patients allergic to *Alternaria alternata* were positive to Alt a 1. However, only 50% of patients allergic to *C. herbarum* were positive to Cla h 1. Antigen extracts of this fungus used in diagnostic tests should therefore contain a combination of *Cladosporium* allergens. On the other hand, closely related (e.g. *Alternaria, Stemphylium* and *Curvularia* species) and more distantly related fungi (e.g. *C. herbarum* and *A. alternata)* may contain or produce similar allergens. Allergic individuals may therefore cross-react to fungi they have not been exposed to (86, 121). RAST analyses can be carried out by the Pharmacia CAP system, which is an automated system that has been calibrated against the World Health Organization (WHO) IgE standard and allows quantitation of total and allergen-specific IgE. Recently an automated enzyme-linked immunosorbent assay system has been developed, which may replace RAST for the detection of IgE in serum.

The RAST and the skin prick test may underestimate serum IgE against fungi. Griese *et al* found a better sensitivity (90%) and specificity (86%) for a histamine-release test with fungal extracts compared with skin prick tests and RASTs in asthmatic children using bronchial provocation as the standard (71). Similar results have been reported by Nolte *et al* but the sensitivity (67%) and specificity (80%) were lower (162). In one study the presence of IgE antibodies against *Penicillium chrysogenum* was demonstrated by a histamine-release test in individuals who did not respond in skin prick tests and a fluorometric enzyme immunoassay (Magic Lite test) (120). The histamine-release tests were performed by exposure of circulatory basophils passively immunised with serum from the subjects to spores from fungi that had been isolated from the environment. The role of IgE in these tests was validated by testing sera from unexposed individuals and sera from subjects where IgE had been removed as negative controls. The authors claim that whole spores that can be used in histamine-release tests provide a better allergen source than allergens extracted from culture fluid, mycelium, or spores. This assumption is reasonable since allergen extracts contain only soluble allergens and proteolytic enzymes may have degraded allergens during purification. Furthermore, spores from fungi that are isolated from the environment can be used. However, the concentration of spores needed to induce histamine release seems very high although the number of basophils was not given, 0.06-0.5 mg spores/ml, which is approximately equal to $1 \cdot 10^7$-$5 \cdot 10^7$ spores/ml.

Nasal lavage and sputum induction by inhalation of a hypertonic saline aerosol have been used to study inflammatory markers and mediators in small-scale epidemiological studies of populations occupationally exposed to fungi (Chapter 11). Their usefulness as markers of fungal diseases is unclear at present.

10. Effects in animals

10.1 Dose considerations

Only animal studies in which the respiratory system was exposed to whole spores have been included. Exposure is either by aerosol inhalation, intratracheal, or intranasal instillation.

All dose estimates were recalculated to estimate the number of spores/g bw as a common unit since doses had been expressed in different ways. Viable counts of spore suspensions were assumed to be half of the total number of spores unless viability had been assessed. In case of aggregation this is an underestimation. However, spore suspensions used for intratracheal or intranasal instillation were often homogenised, filtered, and inspected before use. Spore weights were computed from the mean of size ranges obtained from other references, as this information was seldom supplied in the studies. In addition, a globular shape and a specific gravity of 1 g/cm^3 were assumed. In aerosol inhalation studies, either airborne concentrations and exposure durations were given or viable counts in lung tissue obtained quickly (up to 4 hours) after the challenge. The latter method is sometimes used in instillation studies as well. Culture of lung tissue is likely to underestimate the applied dose substantially as only 0.3% of an intratracheally instilled dose could be cultured from lung tissue 4 hours after instillation (114). Inhaled doses in animal studies were computed by multiplication of the airborne concentrations with the respiration rate of the animal (26). Finally, the applied doses were normalised by the number of spores/g bw using tables of body and lung weight (133).

Intratracheal and intranasal instillation circumvent the normal deposition processes in the airways, and distribute spores deeper into the lung. In rats, four times more cerium oxide with a mass median AED of 2.2 μm reached the lung after intratracheal instillation compared to inhalation (180). Further, intratracheally instilled dust was less homogeneously distributed in the lung compared with inhaled aerosols, with little dust reaching the periphery. This may lead to local overload conditions even if the average dose does not exceed the overload limit suggested by Morrow (147) (Chapter 8.1). Pritchard *et al* recommended not to extrapolate dose-response relationships from animal studies that applied intratracheal instillation to the human condition (180).

The tracheobronchial structure and dimensions of the human lung differ from the lungs of laboratory animals, especially small rodents. Based on different deposition models in the airways and dose metrics Jarabek *et al* estimated that the exposure level applied in an animal experiment has to be multiplied by a factor of 0.03-0.2 for particle number per alveolus, and by 1-7 for particle mass per unit area for particle sizes ranging from 0.3 μm to 6 μm (98). Finally, the degree of aggregation of fungal spores is often unknown, a problem that applies to human as well as animal studies.

Macrophage overload may occur when the alveolar dose of spores 2 and 5 μm in size exceeds approximately 10^6 and 10^5 spores/g bw in humans, respectively.

These limits are similar in rats that have approximately $2.8 \cdot 10^7$ alveolar macrophages, or $9 \cdot 10^4$ per g bw assuming a body weight of 300 g, while humans have approximately $6 \cdot 10^9$ alveolar macrophages, or $1 \cdot 10^5$ per g bw assuming a body weight of 60 kg (147, 233) (Chapter 8.1). Overload may even occur at lower levels after intratracheal instillation due to uneven distribution in the lung.

Comparisons between studies can be difficult because of methodological differences. Comparisons within studies are therefore more reliable and are given most weight.

A neglected issue in studies using instillation of spore suspensions is that spores may be metabolically activated. Green *et al* showed that germinating spores had higher allergen content than resting spores (65). It is therefore possible that the responses in studies using intranasal or intratracheal instillation of spore suspensions differ from results from aerosol exposure to dry spores.

10.2 Irritation

No studies of irritation have been found.

10.3 Sensitisation

Only two experimental studies of sensitisation with whole spores were found. Hogaboam *et al* exposed non-sensitised mice, and mice sensitised by injections with *Aspergillus fumigatus* antigen intratracheally to a single dose of $5 \cdot 10^6$ spores ($2 \cdot 10^5$ spores/g bw) of *A. fumigatus* (87). Both groups developed specific serum IgE antibodies after 3 days that lasted at least to the end of the observation period of 30 days. The IgE level in the sensitised group was 5 times higher. Cooley *et al* exposed mice intranasally to $1 \cdot 10^4$ spores ($7 \cdot 10^2$ spores/g bw) of *Penicillium chrysogenum* once a week for 6 weeks (33). After exposure to killed spores specific serum IgE levels decreased while specific serum IgE and IgG_1 levels increased after exposure to a spore preparation containing 25% viable spores.

The two studies indicate that viable spores can induce serum IgE. It should be noted, however, that both studies applied spore suspensions, which may have activated the spores and induced allergen production. Exposure to non-viable spores did not induce IgE antibodies, and IgE levels even decreased indicating that the animals had developed some degree of sensitisation before the experiment. This was not mentioned in the paper. Sensitisation might have occurred from ambient exposure, as *P. chrysogenum* is prevalent in common indoor air although the animals were kept in a high efficiency particulate air (HEPA) filtered room. The induction of IgG_1 antibodies in mice after exposure to viable spores in the latter study also indicates the involvement of Th2 cells and an allergic response.

10.4 Effects of single exposure

Effects of single exposures other than irritation and sensitisation have been studied in eleven species of fungi and three species of actinomycetes in rabbits, rats, mice and Guinea pigs. The studies are summarised in Table 9.

10.4.1 Mortality

Mortality was observed after a single exposure to toxic and non-toxic *Stachybotrys chartarum* in rat pups, with lethal doses for 50% (LD_{50}) and 18% (LD_{18}) of the animals of $3{\cdot}10^5$ spores/g bw and $8{\cdot}10^5$ spores/g bw, respectively (250). For *Aspergillus fumigatus* an LD_{11} of $6{\cdot}10^5$ spores/g bw was found in adult mice (210), and mortality in rats varied from 0/3 to 3/3 after exposure to $6{\cdot}10^6$ spores/g bw of five different strains (117). The *A. fumigatus* strains from the latter study were studied for production of the mycotoxins gliotoxin but none produced the toxin. Although the presence of mycotoxins in the spores is likely to be an important factor for mortality, the toxicity of these fungi was not solely dependent on mycotoxins. In one study of *A. fumigatus* the dose was quantified by culture of lung tissue and substantially underestimated (210). At the applied doses overload of alveolar macrophages is very likely, which may contribute to toxicity.

10.4.2 Inflammatory markers

Bronchoalveolar lavage

Neutrophils and/or alveolar macrophages increased in rats, mice and Guinea pigs after exposure to *Aspergillus fumigatus, Aspergillus versicolor*, *Penicillium spinulosum,* toxic *Stachybotrys chartarum, Saccharopolyspora rectivirgula* and *Streptomyces californicus* (58, 87, 101-103, 185, 250) and in sensitised mice also after exposure to *Cladosporium herbarum* (74). Lymphocyte counts were increased after exposure to toxic *S. chartarum* and exposure of sensitised animals to *A. fumigatus* (87, 185, 250). Alveolar macrophage counts decreased after exposure of mice to the highest dose of *P. spinulosum* (102). Eosinophil counts were increased after exposure of mice to *Alternaria alternata* (74), Guinea pigs to *S. rectivirgula* (58) and rats to toxic *S. chartarum* (185). In sensitised mice, eosinophil responses were observed to *A. fumigatus* (87), *A. alternata* and *C. herbarum* and in non-sensitised mice also to *A. alternata* (74).

Many studies showed transient increases in lung cell counts after exposure to relatively high doses of $2{\cdot}10^5$ spores/g bw or more. However, one study showed that alveolar macrophage counts decreased after exposure to a very high dose of $4{\cdot}10^6$ *P. spinulosum* spores/g bw (102). Comparisons within studies showed a stronger response to toxic *S. chartarum* compared to a non-toxic strain of the fungus (184, 250), and to *A. alternata* compared to *C. herbarum* (74). Dose-related responses were observed for *A. versicolor* (103) and toxic *S. chartarum* spores (184). The responses in alveolar macrophage, neutrophil, eosinophil, and lymphocyte counts were stronger in sensitised animals than in non-sensitised animals (74, 87). Eosinophil counts were induced by *A. alternata, C. herbarum* (in sensitised animals), toxic *S. chartarum* and *S. rectivirgula* (58, 74, 87). In one

of the studies, four fungal species including *A. fumigatus* did not induce eosinophils or any other cells by doses up to $2{\cdot}10^5$ spores/g bw. Spores were applied by inhalation, which reduces the dose that reach the alveolar region compared to intratracheal or intranasal instillation applied in the other studies (58).

Increases in the number of inflammatory cells were parallelled by increases in the cytokines TNFα, IL-1β, and IL-6 and markers of tissue damage, i.e. lactate dehydrogenase, albumin and haemoglobin in rats (184, 185, 250), in mice (101, 103) and in specific pathogen free mice (57, 102, 103). Myeloperoxidase increased after exposure to toxic and non-toxic *S. chartarum* (184). The responses to *A. versicolor, Cladosporium cladosporoides,* non-toxic and toxic *S. chartarum* were dose-related (57, 103, 184). In studies of *A. alternata* and *C. herbarum* increases in IL-4, IL-5 and IL-13 in sensitised mice were also reported, indicative of an allergic response (74). The increased levels generally returned to normal within 3-28 days (57, 101, 103, 185, 250).

One study of specific pathogen free mice that were intratracheally exposed to *A. fumigatus* indicated that viable spores induced a large increase of neutrophils in bronchoalveolar lavage fluid, which was not observed when heat-killed spores were applied. However, when spores were incubated until swollen before heat-killing, a similar inflammatory response was seen as induced by viable spores (88). The applied dose ($1{\cdot}10^7$ spores/animal, approximately $4{\cdot}10^5$ spores/g bw) was high compared to the doses used in the other studies. The study indicates that agents produced during germination can be responsible for the inflammatory response.

Increased surfactant production was observed after exposure of specific pathogen free mice to toxic *S. chartarum* but only minor changes after exposure to *C. cladosporoides* (131). These results indicate that alveolar type II cells are involved, but it is not clear how these changes contribute to the inflammatory process.

Lung tissue

In many studies, increases in inflammatory cells in lung tissue, mainly alveolar macrophages, lymphocytes, and polymorphonuclear leukocytes (PMN) were observed. These studies included *Aspergillus fumigatus, Aspergillus versicolor, Penicillium spinulosum, Saccharopolyspora rectivirgula, Streptomyces californicus, Streptomyces thermohygroscopicus* and toxic and non-toxic *Stachybotrys chartarum,* and exposed Guinea pigs (255, 256), rabbits (30), rats (30, 87, 117, 250) and mice (101-103, 159), of which two studies used specific pathogen free animals (102, 103). The increase in inflammatory cells was observed after ≥3 days and was temporary in most studies within the observation times of 1-26 weeks. Exposure levels were often high, 10^5-$4{\cdot}10^7$ spores/g bw, and overload of the alveolar macrophages is likely. One study of *A. fumigatus* and two studies of toxic and non-toxic *S. chartarum* showed inflammation that did not return to normal. However, the applied doses were so high that mortality was observed in two of the

studies (117, 159, 250). In three studies, histopathological changes in lung tissue were dose-related (101, 102, 250).

No inflammation of lung tissue by formation of granuloma and fibrosis was observed within 4 hours after exposure to *A. fumigatus, Aspergillus terreus, Penicillium aurantiogriseum, Phanerochaete chrysosporium, Rhizopus stolonifera* and *S. rectivirgula* in pathogen free rabbits (165) and Guinea pigs (58). The doses were 10^2 to $2{\cdot}10^5$ spores/g bw and were applied by inhalation except for *A. terreus* that was instilled intratracheally. However, granuloma formation was observed after intratracheal instillation of *A. terreus, A. fumigatus, A. versicolor, Cladosporium cladosporoides,* and toxic and non-toxic *S. chartarum* spores in rabbits, rats, mice and specific pathogen free mice (68, 103, 114, 117, 159, 165, 183), and aerosol exposure of rabbits to *A. fumigatus* (235). Most granulomas contained spores, alveolar macrophages and PMN, but also granulomas with eosinophilic and basophilic granulocytes, germinating spores and hyphae were observed in two studies of *A. fumigatus* (117, 235). Granuloma formation was temporary in three studies (103, 114, 235) with observation times of 3-4 weeks, while the other studies were shorter (1 week or less) except one high-level exposure study in which mortality was observed (117). It seems therefore possible that granuloma formation may regress within a few weeks unless exposure is extremely high. The inhaled dose of *A. fumigatus* in the study by Thurston *et al*, $2{\cdot}10^4$ spores/g bw, was estimated by culture (235) and may have been more similar to the intratracheal dose applied by Land *et al* ($6{\cdot}10^6$ spores/g bw) (117). In all studies except the study by Green *et al* (68) doses of $\geq 1{\cdot}10^5$ spores/g bw were applied. Macrophage overload can therefore not be ruled out at least locally due to intratracheal instillation.

In sensitised mice, eosinophil counts in lung tissue increased transiently after intratracheal exposure to $2{\cdot}10^5$ *A. fumigatus* spores/g bw and fibrosis developed. The eosinophilic inflammation in lung tissue was much weaker and no fibrosis was observed in non-sensitised mice (87). However, Thurston *et al* observed eosinophils in non-sensitised rabbits after exposure to *A. fumigatus* by inhalation (235). The applied dose was probably much higher (see previous paragraph).

A special finding in studies of non-toxic and toxic *S. chartarum* is the presence of erythrocytes and haemorrhage in the lung as well as increased haemoglobin levels (159, 183-185, 250).

Two studies of specific pathogen free mice exposed to *A. fumigatus* showed increased levels of the inflammatory markers IL-3, IL-4, IL-18, IFNγ, transforming growth factor beta (TGFβ) and monocyte chemoattractant protein (MCP)-1/CCL2 in lung tissue and a decreased level of IL-10 (14, 87). Most changes returned to baseline levels within an observation time of 4 weeks.

Zaidi *et al* exposed Guinea pigs to *Saccharopolyspora rectivirgula* spores ($3{\cdot}10^6$-$4{\cdot}10^6$ spores/g bw) and 75 mg hay dust or bagasse (255, 256). More intense cellular inflammation was observed in lung tissue after exposure to hay dust combined with *S. rectivirgula* than after exposure to the agents alone. The inflammation had cleared after 30 days. Fibrosis developed after exposure to hay dust with and without *S. rectivirgula* with no differences between the two

exposure regimes, but no fibrosis occurred after exposure to *S. rectivirgula* alone (256). Bagasse dust alone induced small granulomas, *S. rectivirgula* alone induced pneumonitis, and interstitial fibrosis developed after combined exposure to these agents (255).

These results suggest that development of inflammation in lung tissue takes more than 4 hours. However, the studies with short observation time applied lower doses than those of longer duration. Intratracheal instillation was mainly applied, and overload may have contributed to the inflammatory effects. Most changes returned to normal within a few weeks except after very high doses. Three species of actinomycetes and eight fungal species showed inflammatory responses including *A. fumigatus*, and toxic and non-toxic *S. chartarum*. Signs of eosinophilic inflammation were observed after exposure to *A. fumigatus* in three studies.

Bagasse dust but not hay dust seems to interact with *S. rectivirgula*. The applied doses of hay and bagasse dust correspond to approximately 300 μm^3/alveolar macrophage assuming that Guinea pigs have the same number of alveolar macrophages as rats ($2.5 \cdot 10^8$). This dose exceeds by far the overload limit of 60 μm^3/alveolar macrophage suggested by Morrow (147) (Chapters 8.1 and 10.1). Overload conditions are therefore very likely in these studies and such exposure levels seem unlikely in the work environment. The observed interaction effects may thus be irrelevant.

10.4.3 Blood gas parameters

Arterial oxygen pressure and other blood parameters were measured in four studies using rabbits exposed to airborne *Aspergillus terreus* spores at levels from $4 \cdot 10^2$ to $2 \cdot 10^5$/g bw. Levels were measured in lung tissue except the highest level that was estimated from the applied aerosol (166). In the other studies exposure levels were underestimated because lung tissue was cultured after aerosol exposure (11, 23, 164). Decreased arterial oxygen pressure as well as reductions in platelet counts, haptoglobin and complement were observed, although platelet counts in one study were unchanged (11, 23, 164, 166). A dose-response association for arterial oxygen pressure was found in one study. The latter study also included *Aspergillus fumigatus,* which reduced arterial oxygen pressure after exposure to a ten-fold lower dose than *A. terreus* (164). The dose estimated from the applied aerosol was probably below the level where macrophage overload would occur (approximately 10^6 spores/g bw for the 2-2.5 μm spores of *A. terreus*).

10.4.4 Lung function

Only two studies included lung function parameters. Bronchial hyperreactivity developed in mice sensitised to *Aspergillus fumigatus* and in non-sensitised animals after intratracheal exposure to *A. fumigatus* spores (87). Airway resistance increased in rat pups intratracheally exposed to spores of toxic *Stachybotrys chartarum* but not after exposure to non-toxic spores (250). These changes

returned to normal within 2-4 weeks except in sensitised mice exposed to *A. fumigatus*.

In one study, respiratory symptoms were reported. Land *et al* exposed rats intratracheally to a single dose of *A. fumigatus* of five different strains (117). The animals developed dyspnoea and tachypnea after exposure to four of the strains and there were clear differences between the fungal strains. No gliotoxin was detected in the spores of these strains.

In all studies, intratracheally administered spores from pathogenic species were used and relatively high doses applied, $1 \cdot 10^5$ to $6 \cdot 10^6$ spores/g bw.

10.4.5 Discussion

Inflammatory changes in bronchoalveolar lavage and lung tissue, lung function changes, and mortality were observed after exposure to diverse fungal and actinomycete species in different types of laboratory animals. In several studies overload of the alveolar macrophages is a problem, which may have aggravated the response. However, such high exposure levels have been measured in relation to attacks of ODTS (Chapters 8.1 and 11.4.2).

In general, most effects were temporary and returned to baseline levels within weeks in spite of possible overload. *A. fumigatus* and toxic *S. chartarum* generally elicited stronger responses than other species or non-toxic *S. chartarum* spores in comparative studies. Dose-effect relationships were demonstrated in many studies for various species, mainly for changes in inflammatory cells and markers in bronchoalveolar lavage and inflammatory changes in lung tissue. The dose-effect relationships in some of the studies may have been generated artificially due to local aggregation of spores after intratracheal instillation (Chapter 10.1).

Granulomas in lung tissue contained spores, alveolar macrophages, neutrophils and lymphocytes. However, after exposure to *A. fumigatus* also signs of allergic inflammation were observed as indicated by granulomas with eosinophils, increases in eosinophils and IL-4 in lung tissue, and increases in serum IgE. Increases in eosinophils were also found in bronchoalveolar lavage after exposure to toxic *S. chartarum* spores, and to *A. alternata* and *C. cladosporoides* in sensitised animals.

One study addressed effects on lung surfactant in bronchoalveolar lavage. Toxic *S. chartarum* induced stronger increases than *C. cladosporoides*. These results indicate that alveolar type II cells are involved in the inflammatory process but it is not clear how.

Table 9. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/ group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Alternaria alternata									
	Mouse	No	5	i.n.	$2 \cdot 10^5$ spores	$1 \cdot 10^3$	24 h	*Non-sensitised animals*. BAL: Increased eosinophils. No changes in cytokines. *Sensitised animals*. BAL: Increased AM, lymphocytes, neutrophils and eosinophils. Increased IL-4, IL-5 and IL-13.	(74)
Aspergillus fumigatus									
	Guinea pig	No	Not specified	ae	$7 \cdot 10^5$-$3 \cdot 10^7$ spores/m^3, 4 h	$6 \cdot 10^1$-$3 \cdot 10^3$	4 h	BAL: No changes in AM, lymphocytes, neutrophils and eosinophils. Lung histology: No changes.	(58)
	Rabbit	No	4	ae	$4 \cdot 10^4$-$3 \cdot 10^5$ cfu/g lung	$4 \cdot 10^2$-$3 \cdot 10^3$	24 h	Blood: Decrease in P_aO_2; dose-related complement decrease.	(164)
	Rabbit	No	3	ae	$2 \cdot 10^6$ cfu/g lung	$2 \cdot 10^4$	3 w	Lung histology: Transient granuloma formation with spores, lymphocytes and heterophils; eosinophils present in granulomas with germinated spores.	(235)
	Mouse	No	5-18	ae	$2 \cdot 10^5$-$8 \cdot 10^6$ cfu/animal	$2 \cdot 10^4$-$6 \cdot 10^5$	28 d	Mortality: 2/18 animals died 21 d after the highest applied dose.	(210)
	Rabbit	No	2	i.t.	$1 \cdot 10^7$ spores/animal	$4 \cdot 10^3$	21 d	Lung histology: Transient granulomas with fungi, and central necrosis (7-14 d).	(114)
	Mouse	Yes	4-5	i.t.	$5 \cdot 10^6$ spores/animal	$2 \cdot 10^5$	30 d	*Non-sensitised animals*. Lung tissue: Transient increase in MCP-1/CCL2. No changes in MCP-3/CCL7 and MCP-5/CCL12. *Sensitised animals*. Lung tissue: Increase in MCP-1/CCL2.	(14)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/ group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Aspergillus fumigatus									
	Mouse	Yes	5	i.t.	$5 \cdot 10^6$ spores/animal	$2 \cdot 10^5$	30 d	*Non-sensitised animals*. Blood: Increased serum IgE. BAL: Increase in macrophages; transient increases in neutrophils. Lung tissue: Increased IL-18; transient increases in IL-4, IL-13, eosinophils, and lymphocytes; transient decrease in IL-10. Lung function: Transient increases in bronchial hyperreactivity. *Sensitised animals*. Stronger increase in serum IgE BAL: Similar increases in macrophages and transient increases in neutrophils. Lung tissue: Stronger increase in IL-18; stronger transient increases in IL-4, eosinophils, and lymphocytes; similar transient increase in IL-13 and transient decrease in IL-10 (all effects compared to non-sensitised animals). Lung function: Increased bronchial hyperreactivity. Sensitised animals also had increased lymphocytes in BAL, goblet cell hyperplasia, fibrosis and hydroxylproline in lung tissue, and transient increases of eosinophils in BAL, and IFNγ and TGFβ in lung tissue.	(87)
	Mouse	Yes	4	i.t.	$1 \cdot 10^7$ spores/animal	$4 \cdot 10^5$	24 h		(88)
Heat-killed								BAL: No changes.	
Heat-killed, germinated								BAL: Increased neutrophils.	
Viable								BAL: Increased neutrophils.	
	Rabbit	No	25	i.t.	100-200 mg spores/ animal	$2 \cdot 10^5$-$5 \cdot 10^5$	8 d-9 w	Precipitins against *A. fumigatus*.	(146)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Aspergillus fumigatus									
5 strains	Rat	No	3	i.t.	$1{\cdot}10^9$ spores/animal	$6{\cdot}10^6$	25 w	Mortality in different strains: 0/3–3/3. Symptoms: Dyspnoea and tachypnoea. Lung histology: Large regions with PMN inflammation, granulomas with basophils, hyphae and necrosis; granulomas with PMN and spores; granulomas with AM, epithelial and Langhans cells; increased basophils and PMN.	(117)
Aspergillus terreus									
	Rabbit	No	3/6	ae	$4{\cdot}10^4$-$3{\cdot}10^5$ cfu/g lung	$4{\cdot}10^2$-$3{\cdot}10^3$	24 h	Blood: Dose-related decrease in PaO_2 and complement.	(164)
	Rabbit	No	4	ae	$3{\cdot}10^5$ cfu/g lung	$4{\cdot}10^3$	4 h	Blood: Decreased PaO_2 and platelet counts.	(23)
	Rabbit	No	12-16	ae	$4{\cdot}10^5$ cfu/g lung	$6{\cdot}10^3$	24 h	Blood: Decreased PaO_2 and increased haptoglobin.	(11)
	Rabbit	No	8-14	ae	80 mg spores/m^3, 30 min	$2{\cdot}10^5$	24 h	Blood: Decreased PaO_2, no change in $PaCO_2$, temporarily increased white blood cells, no change in platelets, increased PMN/lymphocytes ratio. Lung histology: No change.	(166)
	Rabbit	Yes	2	i.t.	$7{\cdot}10^6$ spores/animal	$4{\cdot}10^3$	2 h	Lung histology: No inflammation.	(165)
	Rat	No	Not specified	i.t.	$7{\cdot}10^6$ spores/animal	$4{\cdot}10^3$	48 h	Lung histology: Granuloma-like lesions with AM, central necrosis and free spores.	(68)
	Rat	No	2	i.t.	$5{\cdot}10^7$ spores/animal	$1{\cdot}10^5$	1 w	Lung histology: Formation of granuloma with spores, AM, PMN and lymphocytes.	(165)
Aspergillus versicolor									
	Mouse	Yes	3-10	i.t.	$5{\cdot}10^6$ spores/animal	$2{\cdot}10^5$	28 d	BAL: Transiently increased TNFα, IL-6, neutrophils. Lung histology: Transient inflammation with neutrophils, macrophages and lymphocytes and some granulomas.	(103)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/ group [a]	Route	Dose as specified in the study	Dose estimated as spores/g bw [b]			
Aspergillus versicolor									
	Mouse	Yes	3-10	i.t.	10^5-10^8 spores/animal	$1{\cdot}10^4$-$4{\cdot}10^6$	24 h	BAL: Dose-related increase in TNFα, IL-6, neutrophils, AM, albumin, lactate dehydrogenase, and haemoglobin. Lung histology: Dose-related inflammation with neutrophils, macrophages, and lymphocytes.	(103)
Cladosporium cladosporoides									
	Mouse	Yes	5	i.t.	$3{\cdot}10^1$-$3{\cdot}10^3$ spores/g bw	$3{\cdot}10^1$-$3{\cdot}10^3$	96 h	BAL: Albumin, lactate dehydrogenase, IL-1β not significantly changed. Dose-related temporary increase in IL-6 and TNFα.	(57)
	Mouse	Yes	Not specified	i.t.	$5{\cdot}10^5$ spores	$2{\cdot}10^4$	72 h	BAL: Minor changes in surfactant production.	(131)
	Mouse	Yes	5-6	i.t.	$1{\cdot}10^6$ spores	$7{\cdot}10^4$	96 h	Lung histology: Granuloma formation with AM, PMN increasing with observation time. Transiently reduced alveolar air space.	(183)
Cladosporium herbarum									
	Mouse	No	5	i.n.	$2{\cdot}10^5$ spores	$1{\cdot}10^3$	24 h	*Non-sensitised animals*. BAL: No changes. *Sensitised animals*. BAL: Increased AM, neutrophils, eosinophils, lymphocytes, IL-4, IL-5 and IL-13.	(74)
Penicillium sp.									
	Rabbit	No	3	ae	$3{\cdot}10^5$ cfu/g lung	$4{\cdot}10^3$	3 w	Lung histology: No changes. Transient ungerminated spores in AM in lung tissue.	(235)
Penicillium spinulosum									
	Mouse	Yes	3-10	i.t.	$5{\cdot}10^6$ spores/animal	$2{\cdot}10^5$	28 d	BAL: Transient increases in neutrophils and monocytes. Lung histology: Transient inflammation with neutrophils and monocytes.	(102)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/ group [a]	Route	Dose as specified in the study	Dose estimated as spores/g bw [b]			
Penicillium spinulosum									
	Mouse	Yes	3-10	i.t.	$1·10^5$-$5·10^7$ spores/ animal	$1·10^4$-$2·10^6$	24 h	BAL: Increased TNFα, IL-6 and neutrophils, decreased macrophages. Lung histology: Dose-related neutrophilic inflammation.	(102)
Penicillium aurantiogriseum									
	Guinea pig	No	Not specified	ae	$1·10^9$ spores/m^3, 4 h	$1·10^5$	4 h	BAL: No changes in AM, lymphocytes, neutrophils and eosinophils. Lung histology: No change.	(58)
Phanerochaete chrysosporium									
	Guinea pig	No	Not specified	ae	$2·10^9$ spores/m^3, 4 h	$2·10^5$	4 h	BAL: No changes in AM, lymphocytes, neutrophils and eosinophils. Lung histology: No change.	(58)
Rhizopus stolonifera									
	Guinea pig	No	Not specified	ae	$2·10^7$spores/m^3, 4 h	$2·10^3$	4 h	BAL: No changes in AM, lymphocytes, neutrophils and eosinophils. Lung histology: No change.	(58)
Stachybotrys chartarum									
Low-toxic	Mouse	No	4	i.n.	$1·10^6$ spores/animal	$5·10^4$	3 d	Weight increase. Lung histology: Moderate inflammation with neutrophils, macrophages, haemorrhage, and granulomas.	(159)
Non-toxic	Rat	No	4	i.t.	$3·10^3$-$3·10^4$ spores/g bw	$3·10^3$-$3·10^4$	24 h	BAL: Increased MPO, no other changes (see below). No weight change.	(184)
Non-toxic	Rat pup	No	12	i.t.	$1·10^5$-$8·10^5$ spores/g bw	$1·10^5$-$8·10^5$	3-14 d	LD_{18}: $8·10^5$ spores/g bw; dose-related reduction in growth rate. Lung histology: Increased AM, spores within AM, mild interstitial pneumonia. BAL: No changes in AM, lymphocytes, neutrophils, TNFα, IL-1β and haemoglobin.	(250)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Stachybotrys chartarum									
Toxic	Mouse	No	4	i.n.	$1 \cdot 10^6$ spores/animal	$5 \cdot 10^4$	3 d	Weight loss. Lung histology: Severe inflammation with neutrophils, macrophages, haemorrhage, granuloma, and necrosis.	(159)
Toxicity not specified	Mouse	Yes	2-4	i.t.	$5 \cdot 10^5$ spores	$2 \cdot 10^4$	72 h	BAL: Increased production of surfactant and accumulation of "used" surfactant.	(131)
Toxic	Rat	No	4	i.t.	$3 \cdot 10^3$-$3 \cdot 10^4$ spores/g bw	$3 \cdot 10^3$-$3 \cdot 10^4$	24 h	BAL: Dose-related increases in MPO, lactate dehydrogenase, haemoglobin, albumin and PMN; no changes in AM, lymphocytes and eosinophils. Dose-related weight loss.	(184)
Toxic	Rat	No	4	i.t.	10^7 spores/animal	$3 \cdot 10^4$	3 d	BAL: Increases in LDH, haemoglobin, AM, lymphocytes, and eosinophils; transient increase in albumin and PMN; no changes in MPO. Weight loss.	(185)
Toxic	Rat pup	No	12	i.t.	$1 \cdot 10^5$-$8 \cdot 10^5$ spores/g bw	$1 \cdot 10^5$-$8 \cdot 10^5$	3-14 d	LD_{50}: $2.7 \cdot 10^5$ spores/g bw; dose-related reduction in growth rate. Lung histology: Dose-related haemorrhage, increased AM, spores within AM, mild interstitial pneumonia. BAL: Transient increases in AM, lymphocytes, neutrophils, TNFα, IL-1β and haemoglobin. Transient increase in airway resistance.	(250)
Toxic	Mouse	Yes	5-6	i.t.	$1 \cdot 10^6$ spores	$7 \cdot 10^4$	96 h	Lung histology: Granuloma formation with AM, PMN increasing with observation time. Transient erythrocytes and hemosiderin formation. Decreased collagen labelling in granulomas compared to controls and *Cladosporium cladosporoides*. Reduced alveolar air space.	(183)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Stachybotrys chartarum									
Toxic, tri-chothecene producing	Mouse	Yes	5	i.t.	$3 \cdot 10^1$-$3 \cdot 10^3$ spores/g bw	$3 \cdot 10^1$-$3 \cdot 10^3$	96 h	BAL: Dose-related increase in albumin, IL-1β, IL-6 (intermittent for IL-1β and for IL-6 at the highest dose). Increased TNFα (intermittent at lowest dose) and increased lactate dehydrogenase.	(57)
Toxic atranone producing	Mouse	Yes	5	i.t.	$3 \cdot 10^1$-$3 \cdot 10^3$ spores/g bw	$3 \cdot 10^1$-$3 \cdot 10^3$	96 h	BAL: Dose-related increase in albumin, IL-1β, IL-6 (intermittent for IL-1β and for IL-6 at the highest dose). Increased TNFα (intermittent at lowest dose) and increased lactate dehydrogenase.	(57)
Actinomycetes									
Saccharopolyspora rectivirgula									
	Guinea pig	No	Not specified	ae	$3 \cdot 10^8$ spores/m^3, 4 h	$3 \cdot 10^4$	4 h	BAL: No changes in AM, lymphocytes. Increased neutrophils and eosinophils. Lung histology: No change.	(58)
	Guinea pig	No	30	i.t.	800 μg spores/animal	$4 \cdot 10^6$	26 w	Lung histology: Transient changes: inflammation with AM and PMN; thickened alveolar septa.	(256)
	Guinea pig	No	30	i.t.	700 μg spores/animal	$3 \cdot 10^6$	13 w	Lung histology: Transient changes: increased PMN and AM; interstitial pneumonitis; slight fibrotic changes; lymph node infiltration by lymphocytes.	(255)

Table 9. *Cont*. Animal experiments with single exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure			Observation time	Effects	Reference
	Species	Specific pathogen free	No/ group[a]	Route	Dose as specified in the study	Dose estimated as spores/g bw[b]			
Actinomycetes									
Streptomyces californicus									
	Mouse	No	5-10	i.t.	$2 \cdot 10^7$-$3 \cdot 10^8$ spores/ animal	$1 \cdot 10^6$-$2 \cdot 10^7$	7 d	BAL: Transient increases in IL-6 and TNFα (both also in serum), AM, neutrophils, albumin, lactate dehydrogenase, haemoglobin, and total protein. Lymphocytes increased after 7 d, iNOS increased in BAL cells after 24 h only. Lung histology: Transient dose dependent neutrophilic inflammation.	(101)
Streptomyces thermohygroscopicus									
	Rabbit and rat	No	ca 10	i.t.	20 μg spores/g bw	$4 \cdot 10^7$	7 d	Lung histology: Transient inflammation with PMN, AM, phagocytosis of spores by AM.	(30)

[a] Number of animals in exposed and control groups.
[b] Dose estimated as described in Chapter 10.1.
ae: aerosol, AM: alveolar macrophages, BAL: bronchoalveolar lavage, cfu: colony forming units, IFNγ: interferon gamma, IL: interleukin, i.n.: intranasal, iNOS: inducible nitric oxide synthase, i.t.: intratracheal, $LD_{50\ (or\ 18)}$: lethal dose for 50% (or 18%) of the animals at single exposure, MCP: monocyte chemoattractant protein, MPO: myeloperoxidase, $PaCO_2$: arterial carbon dioxide tension, PaO_2: arterial oxygen tension, PMN: polymorphonuclear leukocytes, TGFβ: transforming growth factor beta, TNFα: tumour necrosis factor alpha.

10.5 Effects of short-term exposures (up to 90 days)

Seven short-term exposure studies have been found that are summarised in Table 10. The one study that applied aerosol exposure is also described in the text.

Fogelmark *et al* exposed Guinea pigs (n=not given) for 4 hours per day, 5 days per week for 3 and 5 weeks to dry aerosols of *Aspergillus fumigatus* ($7 \cdot 10^5$ and $3 \cdot 10^7$ spores/m^3), *Rhizopus stolonifera* ($2 \cdot 10^7$ spores/m^3), *Phanerochaete chrysosporium* ($2 \cdot 10^9$ spores/m^3), *Penicillium aurantiogriseum* ($1 \cdot 10^9$ spores/m^3), and *Saccharopolyspora rectivirgula* ($3 \cdot 10^8$ spores/m^3). The animals were sacrificed 24 hours after the last exposure. Spore concentrations were quantified by microscopic counting of filter samples. Alveolar macrophage, neutrophil, eosinophil and lymphocyte counts increased in bronchoalveolar lavage fluid for all examined species, except alveolar macrophage and neutrophil counts after exposure to the lowest dose of *A. fumigatus*. The strongest responses were observed for *P. aurantiogriseum* followed by *S. rectivirgula*. Lung histology revealed changes ranging from slight cell infiltration of the alveoli after exposure to the lowest dose of *A. fumigatus* to severe cell infiltration in alveoli and interstitial tissue, alveolar wall thickening and granuloma formation after exposure to *P. aurantiogriseum* and the highest dose of *A. fumigatus* (58).

The Fogelmark *et al* (58) study is the most important study, as it is the only study in which animals were exposed to spore aerosols. The exposure pattern was not very different from workplace exposure, and five species were included of which *A. fumigatus* at two levels. Exposure levels may have been underestimated because spores were washed off cellulose ester filters before microscopic counting, and such filters are not suitable for resuspension of collected particles. The weakest responses were observed after exposure to $7 \cdot 10^5$ spores/m^3 of *A. fumigatus* with slight signs of lung inflammation. Responses to other species were stronger, but exposure levels were higher and varied between species. However, some generalisations can be made; 1) spores from all species induced increased counts of alveolar macrophages, lymphocytes, neutrophils and eosinophils in bronchoalveolar lavage fluid; 2) all spore types induced histopathological changes; 3) the responses did not decline within the observation period; 4) higher exposure levels of *A. fumigatus* induced stronger responses than lower levels; and 5) differences between species were observed: *A. fumigatus* was more toxic than *Rhizopus stolonifera* and *Phanerochaete chrysosporium, and Penicillium aurantiogriseum* was more toxic than *P. chrysosporium*. The lowest observed effect level (LOEL) was $7 \cdot 10^5$ *A. fumigatus* spores/m^3, which corresponds to $4 \cdot 10^5$ spores/m^3 for an 8-hour time weighted average exposure level.

Dose-related lung inflammation was observed in mice exposed intranasally to spores of *Stachybotrys chartarum* (50 and $5 \cdot 10^3$ spores/g bw applied twice weekly for 3 weeks). Spores with high levels of mycotoxins induced stronger inflammation than spores with low levels (160). No histopathological changes were observed in the thymus, spleen, or intestines, which are the target organs for trichothecene mycotoxins.

In mice intratracheally exposed to spores of the actinomycete *Streptomyces californicus* ($1\cdot10^2$-$1\cdot10^6$ spores/g bw applied weekly for 6 weeks), dose-related responses were found in bronchoalveolar lavage fluid for alveolar macrophages, neutrophils, albumin and haemoglobin, and in lung tissue for activated T-cells and non-T-lymphocytes (104).

Che *et al* studied the effect of exposure duration in rabbits and rats that were intratracheally exposed to *Streptomyces thermohygroscopicus* (approximately $4\cdot10^7$ spores/g bw weekly during 7-10 weeks. Lung histology of both species showed increased numbers of inflammatory cells as well as granulomas after 2-3 weeks. The granulomas had regressed after 7-10 weeks but were followed by fibrosis (30). The exposure level in this study was higher than in any other study in Table 10 (based on spore numbers), but the observation time was also longer. The applied dose amounts to a volumetric dose of approximately 16 μm^3/alveolar macrophage in rats. This is below the overload limit of 60 μm^3/alveolar macrophage suggested by Morrow (147) (Chapter 8.1) but intratracheal administration may still have created overload conditions locally in the lung (Chapters 10.1 and 12.1).

Cooley *et al* studied the role of spore viability in mice intranasally exposed to *Penicillium chrysogenum* ($7\cdot10^2$ spores/g bw weekly for 6 weeks) (33). Spores that had been killed by methanol treatment induced non-allergic responses in blood with increased neutrophil counts and total IgG_{2a}, although specific IgG_{2a} and specific IgE decreased. In bronchoalveolar lavage fluid IFNγ increased. Viable spores (25% viability) induced allergic responses in blood with increased total IgE, specific IgE, specific IgG_1 and eosinophils, and decreased specific IgG_{2a}. In bronchoalveolar lavage fluid, eosinophils, IL-4 and IL-5 increased, and also lung histology showed some eosinophils.

Schwab *et al* (215) extended the study by Cooley *et al* (33) to a 100 times lower dose (7 spores/g bw weekly for 11 weeks) and used non-sensitised mice and animals sensitised to *P. chrysogenum*. Inflammatory responses were only observed in sensitised mice exposed to viable spores in serum (increased IgE and IgG_1), bronchoalveolar lavage (increased eosinophils) and lung tissue (eosinophilic and neutrophilic inflammation).

Havaux *et al* tested sensitised and non-sensitised mice with spores of *Alternaria alternata* and *Cladosporium cladosporoides*. In non-sensitised animals both allergic (eosinophils in bronchoalveolar lavage (only *A. alternata)* and upregulated eotaxinreceptors in lung tissue) and non-allergic responses (neutrophilic inflammation and upregulated receptors for MIP-1α, MIP-2 and MCP-1 in lung tissue) were found. In sensitised animals, more and stronger responses were observed as well as airway hyperreactivity. The responses to *A. alternata* spores were stronger than to *C. cladosporoides* but qualitatively similar (74).

Thus, non-allergic and allergic responses to fungal spores of eight species were observed after repeated exposures in seven studies (30, 33, 58, 74, 104, 160, 215). In one of those, the applied dose was 10 - >1 000 times lower than in the other studies, and inflammatory responses, including allergic responses in bronchoalveolar lavage, lung tissue and blood were observed in sensitised animals that

had been exposed to viable spores (215). As expected, prior sensitisation increased the intensity of allergic inflammation, but also non-allergic inflammation was increased. Dose-related responses were observed in all three studies that applied more than one dose level (58, 104, 160). The importance of viability of the spores was demonstrated in two of the studies (33, 215) and it seems likely that at least a fraction of the spores were viable in the other studies although this was not mentioned. Two studies of two *Streptomyces* species showed only non-allergic inflammation (30, 104), but these results cannot be generalised to actinomycetes because *Saccharopolyspora faeni* induced eosinophils in bronchoalveolar lavage fluid (58).

In summary, repeated exposure studies demonstrated long-lasting inflammatory changes in bronchoalveolar lavage fluid, lung tissue and blood. Differences between species that partly depended on the presence of mycotoxins, and dose-related responses were generally observed.

Allergic responses to spores of various fungal species were observed in all studies. Viable spores induced allergic responses in contrast to non-viable spores. In one study with very low exposure allergic and non-allergic inflammation was only observed in sensitised mice exposed to viable spores.

A single study showed that trichothecene mycotoxins in *S. chartarum* spores did not affect the target organs for these toxins after oral exposure.

A LOEL of $7 \cdot 10^5$ *A. fumigatus* spores/m^3 was observed in Guinea pigs in the only study applying aerosol exposure (4 hours) (58).

10.6 Mutagenicity and genotoxicity

No studies have been found.

10.7 Effects of long-term exposure and carcinogenicity

Several mycotoxins are recognised carcinogens, e.g. the aflatoxins and ochratoxin A. Inhalation of spores containing the toxin as well as particles originating from the substrates with fungal growth represent potential sources of exposure to working populations (27). Experimental studies of the carcinogenicity of the pure substances have been carried out but no studies of spores from fungi applied via the respiratory route have been found.

10.8 Reproductive and developmental studies

No studies have been found.

Table 10. Animal experiments with repeated exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure				Effects	Reference
	Species	Specific pathogen free	No/ group	Route	Dose as specified in the study, spores	Dose estimated as spores/g bw	Frequency		
Fungi									
Alternaria alternata									
	Mouse	No	5	i.n.	$2 \cdot 10^5$	$1 \cdot 10^3$	1/d for 3d	*Non-sensitised animals*. BAL: Increased eosinophils. Lung histology: Neutrophilic inflammation. Lung tissue: Upregulated eotaxin, MIP1α, MIP-2 and MCP-1 receptors. *Sensitised animals*. BAL: Increased AM, lymphocytes, neutrophils and eosinophils. Lung histology: Neutrophilic and eosinophilic inflammation. Alveolar wall thickening and goblet cells. Lung tissue: Upregulated eotaxin, MIP1α, MIP-2 and MCP-1 receptors. Airway hyperreactivity.	(74)
Aspergillus fumigatus									
	Guinea pig	No	Not specified	ae	$7 \cdot 10^5/m^3$, 4 h	60	5 d/w for 5 w	BAL: No change in AM and neutrophils, increased lymphocytes and eosinophils. Lung histology: Slight cell infiltration of the alveoli. All after 3 and 5 w.	(58)
	Guinea pig	No	Not specified	ae	$3 \cdot 10^7/m^3$, 4 h	$3 \cdot 10^3$	5 d/w for 5 w	BAL: Increased AM, neutrophils, lymphocytes and eosinophils. Lung histology: Cell aggregation in alveoli, alveolar wall thickening with interstitial cells and granuloma formation. All after 3 and 5 w.	(58)

Table 10. *Cont*. Animal experiments with repeated exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure				Effects	Reference
	Species	Specific pathogen free	No/ group	Route	Dose as specified in the study, spores	Dose estimated as spores/g bw	Frequency		
Cladosporium herbarum									
	Mouse	No	5	i.n.	$2 \cdot 10^5$	$1 \cdot 10^3$	1/d for 3d	*Non-sensitised animals*. BAL: No changes. Lung histology: No changes. Lung tissue: Upregulated eotaxin, MIP1α, MIP-2 and MCP-1 receptors. *Sensitised animals*. BAL: Increased AM, neutrophils, eosinophils and lymphocytes. Lung histology: Neutrophilic and eosinophilic inflammation. Alveolar wall thickening and goblet cells. Lung tissue: Upregulated eotaxin, MIP1α, MIP-2 and MCP-1 receptors. Airway hyperreactivity.	(74)
Penicillium chrysogenum									
Non-viable	Mouse	No	6	i.n.	$1 \cdot 10^4$	$7 \cdot 10^2$	1/w for 6 w	Blood: Increased total IgG_{2a}, neutrophils; decreased specific IgE and IgG_{2a}. BAL: Increased IFNγ. Lung histology: Lymphoid aggregates.	(33)
Viable	Mouse	No	6	i.n.	$1 \cdot 10^4$	$7 \cdot 10^2$	1/w for 6 w	Blood: Increased total IgE, specific IgE and IgG_1, eosinophils; decreased specific IgG_{2a}. BAL: Increased eosinophils, IL-4, IL-5. Lung histology: Lymphoid aggregates + some eosinophils.	(33)
Non-viable	Mouse	No	5-8	i.n.	$1 \cdot 10^2$	7	1/w for 11 w	*Non-sensitised animals*. Blood: Unchanged total IgE, IgG_1 and IgG_{2a}. BAL: No production of cytokines. Lung histology: No inflammation. *Sensitised animals*. Blood: Unchanged total IgE, IgG_1 and IgG_{2a}. BAL: No production of cytokines. Lung histology: No inflammation.	(215)

Table 10. *Cont*. Animal experiments with repeated exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure				Effects	Reference
	Species	Specific pathogen free	No/group	Route	Dose as specified in the study, spores	Dose estimated as spores/g bw	Frequency		
Penicillium chrysogenum									
Viable	Mouse	No	5-8	i.n.	$1 \cdot 10^2$	7	1/w for 11 w	*Non-sensitised animals*. Blood: Unchanged total IgE, IgG_1 and IgG_{2a}. BAL: No production of cytokines. Lung histology: No inflammation. *Sensitised animals*. Blood: Increased total IgE and IgG_1. BAL: Increased eosinophils, no production of cytokines. Lung histology: Eosinophilic and neutrophilic inflammation.	(215)
Penicillium aurantiogriseum									
	Guinea pig	No	Not specified	ae	$1 \cdot 10^9/m^3$, 4 h	$1 \cdot 10^5$	5 d/w for 5 w	BAL: Increased AM, neutrophils, lymphocytes and eosinophils. Lung histology: Defined granulomas. All after 5 w.	(58)
Phanerochaete chrysosporium									
	Guinea pig	No	Not specified	ae	$2 \cdot 10^9/m^3$, 4 h	$2 \cdot 10^5$	5 d/w for 5 w	BAL: Increased AM, neutrophils, lymphocytes and eosinophils. Lung histology: Severe cell infiltration in alveoli and interstitial tissue, alveolar wall thickening and granuloma formation. All after 5 w.	(58)
Rhizopus stolonifera									
	Guinea pig	No	Not specified	ae	$2 \cdot 10^7/m^3$, 4 h	$2 \cdot 10^3$	5 d/w for 5 w	BAL: Increased AM, neutrophils, lymphocytes and eosinophils. Lung histology: Cell infiltration in alveoli and interstitial tissue, alveolar wall thickening. All after 3 and 5 w.	(58)
Stachybotrys chartarum									
Non-toxic	Mouse	No	7	i.n.	$1 \cdot 10^3$	50	2/w for 3 w	Blood: Increased eosinophils, red blood cells, haemoglobin, haematocrit, and lymphocytes. Lung histology: No changes. No HPC in other organs.	(160)

Table 10. *Cont*. Animal experiments with repeated exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure				Effects	Reference
	Species	Specific pathogen free	No/ group	Route	Dose as specified in the study, spores	Dose estimated as spores/g bw	Frequency		
Stachybotrys chartarum									
Non-toxic	Mouse	No	7	i.n.	$1{\cdot}10^5$	$5{\cdot}10^3$	2/w for 3 w	Blood: Increased eosinophils, red blood cells, haemoglobin, and haematocrit. Lung histology: Mild to moderate inflammation. No HPC in other organs.	(160)
Toxic	Mouse	No	3	i.n.	$1{\cdot}10^3$	50	2/w for 3 w	Blood: Increased platelets and haematocrit. Lung histology: Mild to moderate inflammation. No HPC in other organs.	(160)
Toxic	Mouse	No	8	i.n.	$1{\cdot}10^5$	$5{\cdot}10^3$	2/w for 3 w	Blood: Increased eosinophils, red blood cells, haemoglobin, and haematocrit. Lung histology: Severe inflammation with AM, neutrophils and lymphocytes associated with spores. No HPC in other organs.	(160)
Actinomycetes									
Saccharopolyspora rectivirgula									
	Guinea pig	No	Not specified	ae	$3{\cdot}10^8/m^3$, 4 h	$3{\cdot}10^4$	5 d/w for 5 w	BAL: Increased AM, neutrophils, lymphocytes and eosinophils. Lung histology: Cell infiltration in alveoli and interstitial tissue, alveolar wall thickening. All after 3 and 5w.	(58)
Streptomyces californicus									
	Mouse	No	16	i.t.	$2{\cdot}10^3$-$2{\cdot}10^7$/ animal	$1{\cdot}10^6$-$2{\cdot}10^7$	1/w for 6 w	BAL: Dose-related increase in AM, neutrophils, albumin and lactate dehydrogenase. No change in TNFα, IL-6. Lung tissue: Dose-related increase in activated T-cells and non-T-lymphocytes.	(104)

Table 10. *Cont*. Animal experiments with repeated exposures to spores from fungi and actinomycetes.

Micro-organism	Animals			Exposure				Effects	Reference
	Species	Specific pathogen free	No/ group	Route	Dose as specified in the study, spores	Dose estimated as spores/g bw	Frequency		
Streptomyces thermohygroscopicus									
	Rabbit	No	ca 5	i.t.	20 μg/g bw	$4 \cdot 10^7$	1/w for 2-3 w	Lung histology: Increased AM, granuloma with AM, epithelial giant cells and lymphocytes.	(30)
	Rat	No	ca 5	i.t.	20 μg/g bw	$4 \cdot 10^7$	1/w for 2-3 w	Lung histology: AM, granuloma with AM, epithelial giant cells and lymphocytes.	
	Rabbit	No	ca 10	i.t.	20 μg/g bw	$4 \cdot 10^7$	1/w for 7-10 w	Lung histology: AM, lymphocytes, fibrosis, less granulomas than after exposure for 2-3 w that regressed over time.	(30)
	Rat	No	ca 10	i.t.	20 μg/g bw	$4 \cdot 10^7$	1/w for 7-10 w	Lung histology: AM, lymphocytes, fibrosis, less granulomas than after exposure for 2-3 w that regressed over time.	

ae: aerosol, AM: alveolar macrophages, BAL: bronchoalveolar lavage, HPC: histopathological changes, Ig: immunoglobulin, IFNγ: interferon gamma, IL: interleukin, i.n.: intranasal, i.t.: intratracheal, MIP: macrophage inflammatory protein, MCP: monocyte chemoattractant protein, TNFα: tumour necrosis factor alpha.

10.9 Discussion

Single and repeated exposure studies showed proinflammatory and inflammatory responses in the lung. In bronchoalveolar lavage fluid, increases in alveolar macrophages, PMN as neutrophils and eosinophils, pro-inflammatory cytokines such as TNFα and IL-6, and markers of tissue damage as lactate dehydrogenase, albumin and haemoglobin were observed. In lung tissue, alveolar macrophages, PMN, lymphocytes, pro-inflammatory cytokines such as IL-3, IL-4, IL-18, TNFα, TGFβ and MCP-1/CCL2 increased, IL-10 decreased and granulomas were formed. Only one study observed fibrosis (90 days after repeated exposures to high doses of an actinomycete). A major difference between single and repeated exposure studies is that most changes were temporary after exposure to a single dose, whereas repeated exposures generally induced responses that persisted throughout the observation period.

Several single and three repeated exposure studies demonstrated dose-effect associations. However, in most studies the maximum applied doses were so high that overload seems likely. Only in the studies by Olenchock *et al*, Fogelmark *et al* and Nikulin *et al* were dose levels below 10^4 spores/g bw applied (58, 160, 164). These studies showed dose-effect associations between arterial oxygen tension and a single exposure to *Aspergillus terreus*, between inflammatory cells in bronchoalveolar lavage and inflammatory changes in lung tissue and prolonged exposure to aerosols of *A. fumigatus* spores, and between severity of lung inflammation and repeated intranasal exposures to toxic and non-toxic *Stachybotrys chartarum,* respectively. All tested species induced inflammatory effects but differences between species were observed in several studies. Species differences partly depended on mycotoxin production by the microorganism, as has been shown in several studies of *S. chartarum*. However, species not known as toxin producers, e.g. a strain of *A. fumigatus* not producing gliotoxin, *S. rectivirgula,* and *Penicillium aurantiogriseum,* also elicited strong responses. It cannot be ruled out, however, that microorganisms may produce toxins that have not yet been identified.

Many studies reported non-allergic inflammatory responses involving TNFα, IL-6, alveolar macrophages and neutrophils. However, as the majority of the studies applied single exposures, development of an adaptive immune response was prevented. Interestingly, in all repeated exposure studies including various fungi allergic inflammation were observed together with non-allergic inflammation. Viable spores and prior sensitisation were important determinants of the allergic response (33).

11. Observations in man

Studies with quantitative information on spore exposure, mainly obtained by culture-based methods and microscopic methods, are reviewed. Spore levels measured by culture methods were multiplied by a factor of 10 to estimate airborne spore levels, although the ratio spore/cfu varied from 1.5 to 100 in comparative studies; for a review see Eduard and Heederik (51). Studies using specific serum IgG antibodies as measure of exposure are excluded, as immunoglobulin levels are difficult to extrapolate to airborne spore levels. Human challenge studies using fungal extracts are included as these studies provide information on the specificity of the response.

Case reports (human challenge studies) are summarised in Tables 11-12 and Appendix 8, epidemiological studies of highly exposed populations in Table 13 (symptoms), Table 14 (objective outcomes), and Appendix 6, and epidemiological studies of populations with common indoor exposure in Table 15 and Appendix 7. Tables 13-15 include only studies where associations were adjusted for confounders. If no specific levels were reported for an increased response, the median of the reported exposure levels was used to indicate the exposure level where the outcome was increased.

Diseases are described shortly in Appendix 1 and in more detail in Chapter 8.

11.1 Irritation

This chapter includes eye, nose, and throat irritation (Tables 13, 15). Symptoms and effects of the lower airways are reviewed in Chapters 11.4 and 11.5.

11.1.1 Highly exposed populations

Irritation symptoms in the eyes and nose were recorded by a questionnaire simultaneously with exposure measurements in a study of 89 Norwegian farmers (49). Exposure to many agents was measured including fungal and actinomycete spores, bacteria, endotoxins, glucans, *Aspergillus/Penicillium* antigens and silica. The prevalence of eye irritation was significantly elevated after exposure to $2 \cdot 10^4$-$5 \cdot 10^5$ fungal spores/m^3 (odds ratio (OR) 8.3, 95% confidence interval (CI) 1.0-70 compared to exposure below $2 \cdot 10^4$ fungal spores/m^3) and was not associated with any of the other agents. The prevalence of nasal symptoms was elevated after exposure to $2 \cdot 10^4$-$5 \cdot 10^5$ fungal spores/m^3 (OR 4.1, 95% CI 0.88-19) and significantly after exposure to $5 \cdot 10^5$-$2 \cdot 10^7$ fungal spores/m^3 (OR 6.0, 95% CI 1.3-28). Nasal symptoms were also associated with silica exposure and both agents seemed to induce these symptoms, as the correlation between the two exposures was not very strong.

Work-related cough, nasal irritation and sore throat have been studied by questionnaires in cross-sectional studies of wood workers in Norway and Australia, and were associated with fungal exposure (6, 54). In wood trimmers, exposure to $2 \cdot 10^6$ spores/m^3 was associated with a 50% relative increase in self-reported cough, nasal irritation and sore throat (54). However, this study may suffer

from reporting bias as work-related symptoms had been recorded conditional on handling of mouldy timber. In joinery, sawmill and chip mill workers an increased prevalence of throat irritation was observed at an exposure level of $3{\cdot}10^4$ cfu/m^3 or $3{\cdot}10^5$ spores/m^3 (6). The dominant fungi in these studies were *Rhizopus microsporus (*42%), and *Penicillium/Aspergillus* species (71%), respectively.

In both studies of wood workers associations were adjusted for age, smoking and gender but not for atopy. Other relevant exposures such as wood dust (6, 54) and endotoxins, glucans and Gram-negative bacteria (6) were either adjusted for (54) or studied in separate models (6). In the latter study, endotoxins were also associated with sore throat, but more weakly than fungal spores. Confounding of the association with fungal exposure is not likely because endotoxin exposure was only moderately correlated with fungal exposure. The farmer study (49) is the most reliable as all relevant short-term exposure was measured. This study indicates LOELs for nose irritation of approximately $3{\cdot}10^6$ spores/m^3 (geometric mean of the range $5{\cdot}10^5$ to $2{\cdot}10^7$ spores/m^3) and for eye irritation of approximately $1{\cdot}10^5$ spores/m^3 (geometric mean of the range $2{\cdot}10^4$ to $5{\cdot}10^5$ spores/m^3) calculated to equal 8-hour exposures. The Australian sawmill study (6) indicates a LOEL for sore throat of $3{\cdot}10^5$ spores/m^3 based on average exposure levels. No single species was more potent in the data analysis.

11.1.2 Populations exposed to common indoor air

Li *et al* studied 264 day-care workers from 28 randomly selected day-care centres in Taiwan. Nasal congestion and discharge were associated with total fungal levels, and nasal congestion also with *Aspergillus* levels (geometric means 1 200 cfu/m^3 and 32 cfu/m^3, respectively, n not given). *Cladosporium* and *Penicillium* were the dominating species. Associations were adjusted for age, gender, and education, but not for smoking. House dust mite allergens were measured in settled dust but were not associated with any outcome. The study suggests that *Aspergillus* may be more irritating than other species as *Aspergillus* comprised only a small fraction of all culturable fungi (124). Alternatively, this association might be due to a correlation with the total fungal count, as such correlations were not assessed.

Purokivi *et al* compared eye symptoms in 37 employees from a school building with moisture problems and fungal growth with 23 employees from a school without such problems in Finland at the end of spring term (181). Symptom prevalences were not significantly different. Fungal levels in the problem and non-problem buildings in the winter were 29 and 6 cfu/m^3 (geometric mean), respectively. No confounder adjustments were carried out, however.

Roponen *et al* studied nasal, laryngeal, and eye symptoms in 41 randomly selected Finnish teachers (203). Exposure to fungi was measured by personal sampling during 24 hours at home and 8 hours at work and the teachers were dichotomised into groups with low and high exposure. Exposure levels in these groups were 12 cfu/m^3 (median, range 0-31 cfu/m^3) and 67 cfu/m^3 (median, range 31-270 cfu/m^3), respectively. The prevalences of all symptoms were similar in the

two groups. Exposure to bacteria was also measured. The influence of smoking and asthma status was evaluated by stratified analysis.

Thus, only the study by Li *et al* found associations between nasal irritation and fungi (124). This may be due to much higher exposure levels in this study compared to the other studies. Li *et al* used random selection and controlled for confounders, which strengthen the reliability of their results, although the exposure assessment is poorly described. The geometric mean provides a LOEL estimate of approximately $1 \cdot 10^4$ spores/m^3. The study by Roponen *et al* (203) has a strong design as both home and work exposure were assessed. The exposure level of the highest exposed group in this study, median 67 cfu/m^3 or approximately 700 spores/m^3, thus represent a no observed effect level (NOEL) for irritation.

11.2 Sensitisation

Sensitisation is often understood as the presence of serum IgE antibodies to a specific allergen. However, also hypersensitivity pneumonitis patients are more sensitive to exposure to allergens, which indicates that the sensitisation is not mediated by IgE. It is more likely that cytotoxic T-cells are involved (99, 174, 205) (Chapter 8.2). The hypersensitivity can be shown by bronchial challenge and such studies are therefore also included here.

11.2.1 Human challenge studies

Fungi and actinomycetes have been identified as causes of hypersensitivity pneumonitis and asthma by bronchial challenge of patients with the suspected antigens. Provocations have mainly been carried out with extracts of the organisms to avoid late responses that may be provoked by spores. Positive tests are strong evidence of sensitisation when performed with specific microorganisms from the work environment. Challenge studies therefore provide important information about the aetiology of diseases associated with fungi.

Provocation tests were carried out with close surveillance of patients (177), but are no longer recommended because of the risk of severe late reactions and lack of standardised antigens (197, 205). Exposure challenge to the workplace atmosphere is regarded sufficient to demonstrate an association with the disease, but identification of the causal agent(s) is difficult when the patient is exposed to many different agents.

The focus of this chapter is on species that may cause sensitisation, while the applied doses are discussed in Chapter 11.4.1.

Table 11 summarises studies on hypersensitivity pneumonitis including more than a single case. The provocations were carried out with spores or extracts of the implicated organism, or by exposure to work environments with one dominating species. A relevant non-occupational study (7) is also included in the table.

Studies reporting a single case identified *Aspergillus clavatus, Aspergillus glaucus, Lentinus edodes, Aspergillus fumigatus, Saccharomonospora viridis, Scopulariopsis brevicaulis* and *Ustilago esculenta* as cause of hypersensitivity pneumonitis in a malt worker (198), two mushroom workers (132, 251), a green-

house worker (253), a logging worker who also raised pigs (69), a tobacco worker (118) and a traditional handicraft worker (252), respectively.

The case studies show that hypersensitivity pneumonitis can be induced by several species of *Aspergillus* and *Penicillium* as well as mushrooms, smuts, wood-rot fungi, and actinomycetes. Most patients tested positive for a single species. Ando *et al* (7) even showed that the response was specific to a serotype. Thus, the sensitisation seems to be specific although widely different species are involved.

Challenge studies of asthmatic cases are summarised in Table 12. Reports describing a single case identified *Neurospora* sp. and *Serpula lacrymans* as causal agents of asthma in a plywood factory worker (34) and in a patient from a home with extensive dry rot (163), respectively. The latter patient was also diagnosed with hypersensitivity pneumonitis.

Only four occupational studies of asthmatic patients have been found. In two of these studies specific IgE or skin prick tests were negative (39, 217) indicating that the asthma was non-allergic. In three studies of hypersensitivity pneumonitis patients, obstructive responses were also observed (55, 69, 178) and one patient in the study by Edwards *et al* developed an immediate asthmatic response (55). These studies indicate that fungi and actinomycetes may also induce asthma attacks.

Two studies of nasal hypersensitivity were found. Thirteen furniture workers with positive skin prick tests to fungi and three with elevated total IgE from a population of 268 workers from five factories were challenged by nasal provocation with fungal extracts. Five workers had positive tests but fungal species were not reported (246). Seuri *et al* tested all 14 employees from a small hospital with repeated water damage (217). Total fungal levels exceeded 500 cfu/m^3 at several locations, and approximately half of the counts were of *Sporobolomyces salmonicolor*. Nasal provocations with *S. salmonicolor* extract were positive in 11 workers, but skin prick tests with *S. salmonicolor* were negative in all 12 employees tested. Three of the employees were atopic as evaluated by skin prick tests.

No studies of ODTS patients challenged by bronchial provocation tests have been found, probably because of the benign course of the disease (Chapter 8.3).

The asthma and nasal hypersensitivity studies show that the sensitisation to fungi was IgE-mediated only in a fraction of the cases and in hypersensitivity pneumonitis patients IgE is usually absent. It seems therefore likely that other mechanisms are involved. The specificity of the response is most clearly demonstrated in hypersensitivity pneumonitis studies, where extracts of several species were often applied.

Most challenge studies used extracts. Aerosols of extracts differ from aerosols of spores by metabolite composition, particle size, and solubility. In spite of this, typical attacks of hypersensitivity pneumonitis and asthma were observed after provocation with extracts.

11.2.2 Epidemiological studies

Few studies have been found on IgE-mediated sensitisation to fungi.

Thomas *et al* found no elevation of IgE against fungi in 19 workers with work-related symptoms (cough, wheeze or dyspnoea) from a population of 197 coffee workers exposed to $3 \cdot 10^3$-$2 \cdot 10^4$ fungal cfu/m^3 using species isolated from the work environment (234). IgE levels to green coffee beans and castor beans were elevated, 14% and 15%, respectively.

However, Zhang *et al* reported high prevalences of IgE and positive skin prick tests to *Aspergillus fumigatus*, 27% and 19%, respectively, and to *Rhizopus nigricans*, 52% and 24%, respectively, in 130 Chinese tobacco workers (260). Fungi were measured by sedimentation on culture plates showing that these fungi were also abundant in the air.

Studies by Larsen *et al* (120) and Meyer *et al* (136) on staff members from a school contaminated by fungi failed to demonstrate IgE against species found on building construction materials by skin prick tests or Magic Lite tests in staff members. However, serum IgE against fungi isolated from the school building was demonstrated by histamine-release tests using blood basophils isolated from the participants (120) or by passive immunisation with the participants serum (119, 136). No fungal levels were reported.

In 39 greenhouse workers, a high rate of sensitisation to fungi (18%) and flowers (21%) by skin prick testing was found (141). The workers were exposed to $5 \cdot 10^3$ fungal cfu/m^3.

As no studies have been published with information on exposure levels of the specific organisms, unpublished results of a Norwegian population of wood trimmers are interesting. Results communicated by B Rydjord (National Institute of Public Health, Oslo, submitted for publication) show that levels of IgE against *Rhizopus microsporus* were elevated in 1 of 343 sawmill workers. The most intensively exposed workers in this population were wood trimmers exposed to a median level of $3 \cdot 10^6$ spores/m^3, about half of which were from *R. microsporus* (54). Levels of IgE against *R. microsporus* in healthy blood donor controls were elevated in 2 of 100.

The information on sensitisation to fungal spores in working populations with relatively high exposure levels is contradictory. It is surprising that sensitisation was practically absent among sawmill workers who were exposed to high levels of *Rhizopus microsporus* spores. The reason for this is not understood as tobacco workers showed a high prevalence of sensitisation to the related fungus *Rhizopus nigricans*.

The higher prevalence of sensitisation found by the histamine-release test in the indoor air studies (120, 136) may be due to the use of whole spores of fungi isolated from the workplace, which seems more relevant than the use of fungal extracts in RASTs and skin prick tests. It is also possible that the histamine-release test is more sensitive than skin prick tests and RASTs, although good agreement has been reported for fungal allergens in children (71, 162). Both indoor air studies were performed by the same research group, and confirmation of their findings in other indoor air studies and in highly exposed populations is needed (see also Chapter 9.2).

Table 11. Case-reports: Hypersensitivity pneumonitis confirmed by provocation testing.

Occupation / environment	Species	Patient data	Challenge, BPT	Response	Reference
7 farmer's lung patients working with mouldy hay	*Saccharopolyspora rectivirgula*	In addition to hypersensitivity pneumonitis, asthma (n=4) and bronchitis (n=1)	Whole culture extract	6 responded with fever, aches, malaise, and 5 showed FVC and FEV_1 decline.	(178)
16 farmers working with mouldy hay	*Saccharopolyspora rectivirgula*	X-ray (n=14), obstructive (n=8) and restrictive (n=4) lung function changes	Fungal extract	3 responded with fever, lung function changes (not specified).	(55)
19 cork workers	*Penicillium glabrum*	No info on patient selection	Culture plate extract	Fever, cough, dyspnoea, malaise, crackles, TLco decline.	(9)
4 mushroom workers	*Pleurotus ostreatus*	Fever, chills, dyspnoea, dominated, cough in 2/4	Spore suspension (dose ca 10^7 spores)	After 6-8 h fever, chills, muscle pain, dyspnoea, leukocytosis, decreased FVC.	(35)
5 mushroom workers	*Lentinus edodes*	Fever, chills, cough, dyspnoea, pain in muscles	Spore suspension (dose ca $3 \cdot 10^7$ spores)	After 6 h fever, chills, muscle pain, dyspnoea, leukocytosis and decreased FVC. TLco and PaO_2 decreased in 1/5.	(36)
17 patients with summer type hypersensitivity pneumonitis	*Trichosporon cutaneum*	Fever, cough, dyspnoea, IgG [a] T-lymphocytosis (BAL), granulomatous/hypersensitivity pneumonitis	Culture filtrate	16 responded with fever, cough, dyspnoea, rales, increase in white blood cells, often serotype specific.	(7)
6 patients with hyper-sensitivity pneumonitis to wood-rot fungi	*Serpula lacrymans* (n=3) *Geotrichum candidum* (n=2) *Aspergillus fumigatus* (n=2)	Breathlessness, X-ray changes and positive lung biopsy	Culture filtrate	Late reaction with ≥20% fall in TLco and/or FEV_1.	(21)

[a] Specific for the fungus used in the challenge.
BAL: bronchoalveolar lavage, BPT: bronchial provocation test, FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, Ig: Immunoglobulin, PaO_2: arterial oxygen tension, TLco: lung transfer factor for carbon monoxide (measure of gas diffusion).

Table 12. Case-reports: Asthma confirmed by provocation testing.

Occupation / environment	Species	Patient data	Challenge, BPT	Response	Reference
4 asthmatic patients, occupation unknown	*Alternaria alternata*	Mild asthmatic, skin prick test [a]	Dry spores and extracts (positive response after $9 \cdot 10^4$ spores)	Immediate and late response (spores only), 35% decreased specific airway conductance.	(125)
3 asthmatic patients, occupation unknown	*Penicillium* sp.	Mild asthmatic, skin prick test [a]	Dry spores and extracts (positive response after $6 \cdot 10^4$ spores)	2 responded with immediate and late (spores only) 35% decreased specific airway conductance.	(125)
7 farmer's lung patients working with mouldy hay	*Saccharopolyspora rectivirgula*	Additional asthma (n=4) and bronchitis (n=1)	Whole culture extract	6 responded with fever, aches, malaise, and 5 showed FVC and FEV_1 decline.	(178)
16 farmers working with mouldy hay	*Saccharopolyspora rectivirgula*	X-ray (n=14), obstructive (n=8) and restrictive (n=4) lung function changes	Fungal extract	Immediate asthmatic response in 1 subject.	(55)
3 tomato growers	*Verticillium albo-atrum*	Cough, wheeze, breathlessness, variable peak flow, (1/3 skin prick test positive [a], 0/3 IgE positive [a])	Mycelia extract	Immediate (n=3) and late (n=2) FEV_1 decline.	(39)
6 employees from hospital with water damage (total population 14)	*Sporobolomyces salmonicolor*	Bronchial hyperreactivity (3/5), respiratory symptoms (4/6), skin prick test [a] (0/6), atopy (1/6)	Fungal extract	Doubling of airway resistance within 10 min (n=3) and >15% reduction in peak expiratory flow after 6 h (n=1), reversible by asthma medication, cough, dyspnoea and headache in ≥1.	(217)

[a]Specific for the fungus used in the challenge.
BPT: bronchial provocation test, FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, Ig: immunoglobulin.

11.3 Effects of single exposure

Inhalation tests of naive subjects have not been performed because of concerns of inducing irreversible sensitisation and allergy.

Brinton *et al* described an incident among novice college students (20). The students participated in a "hay party" in a poorly ventilated room where the floor had been covered with straw that emitted dense clouds of dust that did not settle rapidly. Fifty-five of 67 students developed typical ODTS symptoms with cough, muscle aches, and fever. Although no microbiological investigations were performed it seems likely that the straw emitted large numbers of spores from fungi and/or actinomycetes. As the incident occurred in students, previous exposure to such levels is unlikely except for those raised on a farm. These findings indicate that prior sensitisation is not needed for development of attacks of ODTS.

11.4 Effects of short-term exposure

This chapter includes provocation tests of patients with respiratory disease inhaling spores of organisms suspected of causing the disease, and epidemiological studies of acute responses recorded concurrent with exposure measurements. Both study designs may demonstrate effects of short-term exposure in repeatedly exposed individuals, and reflect exacerbations of pre-existing health effects.

11.4.1 Provocation tests

Bronchial and nasal provocation tests have been carried out to study the role of fungi and actinomycetes in hypersensitivity pneumonitis, allergic asthma, and allergic rhinitis (Chapter 11.2.1). In these tests, subjects are exposed to either a single dose or increasing doses of an agent until a reaction of sufficient intensity occurs or the maximum predefined dose has been reached.

Studies that have applied challenge with spores are summarised in Tables 11-12, and are described in more detail in Appendix 8. Bronchial provocations with *Aspergillus clavatus, Lentinus edodes,* and *Pleurotus ostreatus* were positive in malt - and mushroom workers with hypersensitivity pneumonitis after inhaled doses of 10^9, $3 \cdot 10^7$ and 10^7 spores, respectively. The dose of *Aspergillus clavatus* was specified as 20 mg of dry spores without further details, while the latter two doses were based on occupational exposure levels causing attacks of hypersensitivity pneumonitis (35, 36, 198). Only a single dose was applied in these studies.

One study was performed in patients with mild non-occupational asthma using increasing doses of spores of *Alternaria alternata* and a *Penicillium* sp. Seven patients with strongly positive skin prick tests to these fungi were challenged with graded doses (10-fold increments) of the organism they were allergic to. The spores were mixed with lactose and inhaled with Spinhalers, probably during one or a few inhalations (number not specified). The minimum doses for a positive

bronchial provocation test were $9 \cdot 10^4$ *A. alternata* spores and $6 \cdot 10^4$ *Penicillium* sp. spores (125).

Eight employees from moisture-damaged schools with building-related symptoms but without asthma were challenged with aerosols of *Penicillium chrysogenum* and *Trichoderma harzianum* at single concentrations during 6 minutes of $6 \cdot 10^5$ and $3.5 \cdot 10^5$ spores/m^3, respectively. No changes in mucosal or systemic symptoms, lung function or leukocytes in blood were observed compared to placebo exposure. Participants had positive *P. chrysogenum* histamine-release tests for detection of specific serum IgE but *T. harzianum* tests were negative. Skin prick tests to these fungi were also negative (135).

These findings suggest that asthma attacks may be induced at lower dose levels than hypersensitivity pneumonitis in IgE-sensitised individuals. However, the number of studies is small, the species vary and it is not known if lower doses could have induced a positive response in the hypersensitivity pneumonitis studies as single dose levels were applied. The study on asthma patients (125) indicates LOELs in a sensitive group of $2 \cdot 10^4$ *A. alternata* spores/m^3 and $1 \cdot 10^4$ *Penicillium* sp. spores/m^3 calculated to equal 8-hour exposures by dividing the applied doses with 5 m^3 assuming a minute ventilation of 10 l/min during 8 hours. The findings in the employees from moisture-damaged schools with building-related symptoms indicate NOELs of $8 \cdot 10^3$ *P. chrysogenum* spores/m^3 and $4 \cdot 10^3$ *T. harzianum* spores/m^3 calculated to equal 8-hour exposures in individuals with IgE to *P. chrysogenum* but not to *T. harzianum*. However, the significance of the detection of IgE by the histamine-release test used in the latter study (135) is uncertain (Chapter 9.2).

In one study, symptom-free garbage recycling workers and retired garbage workers with occupational asthma were tested by nasal provocation with a suspension of *A. fumigatus* spores containing approximately $6 \cdot 10^7$ spores/ml. No differences in inflammatory markers or nasal volume were observed between the two groups or when compared to sham challenge (223).

11.4.2 Epidemiological studies

Further information on short-term exposure was obtained from epidemiological studies of work-related symptoms and short-term functional changes, e.g. cross-shift change in lung function. Work-related irritation symptoms are reviewed in Chapter 11.1. Other outcomes are summarised in Appendix 6-7 and studies with confounder adjustment have been summarised in Tables 13-15.

Highly exposed populations: Fever attacks of ODTS and hypersensitivity pneumonitis

Patients who develop febrile attacks often recover within a few days without functional and X-ray changes or persisting symptoms. This condition is different from hypersensitivity pneumonitis (Chapter 11.5.1) and is called organic dust toxic syndrome (ODTS), toxic alveolitis or inhalation fever (56, 187) (Chapter 8.3). The yearly incidence has been estimated to 10-190 per 10 000 farmers (56). Febrile attacks may also occur in farmers with hypersensitivity pneumonitis. The

role of fungal spores in ODTS is often clear as attacks rapidly develop after the farmer handled mouldy materials emitting large numbers of spores.

Malmberg *et al* found that 6 farmers with diagnosed ODTS experienced fever attacks after exposure to $14 \cdot 10^9$ spores/m^3, and 4 farmers with hypersensitivity pneumonitis reported fever attacks after exposure to $3 \cdot 10^9$ spores/m^3 (arithmetic means) (130). Another difference was that farmers with ODTS had carried out extraordinary tasks such as removal of spontaneously heated grain that lasted typically 1 hour, while farmers with hypersensitivity pneumonitis carried out work that they did once or twice daily with a duration of 15-30 minutes. Maximum exposure levels in 17 farmers without febrile symptoms were much lower, $1 \cdot 10^8$ spores/m^3 (arithmetic mean). Exposure had been measured when the work causing a fever attack was repeated. The exposure level calculated to equal an 8-hour exposure was $2 \cdot 10^9$ spores/m^3 in farmers with ODTS, $1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3 in farmers with hypersensitivity pneumonitis, and $3 \cdot 10^6$-$1 \cdot 10^7$ spores/m^3 in non-symptomatic farmers. No species dominated and the proportion of actinomycetes ranged from <1 to 96%.

Malmberg *et al* reported in another paper that the fever reactions were not attributable to endotoxins (129), as exposure levels were similar in farmers experiencing fever attacks and non-symptomatics although levels were high, 300 000 EU[2]/m^3.

These results indicate a LOEL for fever attacks of $2 \cdot 10^9$ spores/m^3 in farmers with ODTS and a LOEL for individuals with hypersensitivity pneumonitis of $1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3.

Highly exposed populations: Work-related cough

Work-related cough was significantly elevated (OR 3.9, 95% CI 1.0-15) in 89 farmers after exposure to $5 \cdot 10^5$-$2 \cdot 10^7$ fungal spores/m^3 calculated to equal an 8-hour exposure and adjusted for confounders except atopy. Exposure to many other agents was measured but none were associated with cough (49).

In 22 municipal waste handlers, cough was associated with exposure to fungal spores but not with endotoxins and bacteria. The median exposure level of the symptomatic workers was $3 \cdot 10^5$ spores/m^3. Confounding by smoking and age was evaluated qualitatively (78).

In both studies, cough was associated with short-term exposure to fungal spores. Exposure was measured on the same day as symptoms were recorded, a design eliminating the between day variability provided that the symptoms are a response to short-term exposure. In the farmer study by Eduard *et al* (49) a higher exposure level was associated with an increased prevalence of cough, approximately $3 \cdot 10^6$ spores/m^3 (geometric mean of the range $5 \cdot 10^5$-$2 \cdot 10^7$ spores/m^3, calculated to equal an 8-hour exposure) than in the waste handler study (78). This may either be a real difference, which reveals differences in the fungal flora in the

[2] EU = endotoxin units, relative measure obtained by comparison to a standard endotoxin prepared from *E. coli.* A Dutch occupational exposure limit of 200 EU/m^3 has been adopted.

two environments, or be due to a shorter exposure time in the farmer study. However, the small sizes of the populations and the lack of exposure data at the species level prevent exploration of such hypotheses. Other bioaerosol agents such as bacteria, endotoxins, and glucans were not associated with cough. Thus, LOELs of $3 \cdot 10^5$ spores/m^3 are indicated in waste handlers and $3 \cdot 10^6$ spores /m^3 in farmers.

Highly exposed populations: Lower airway inflammation

Heldal *et al* studied 25 organic waste collectors and measured inflammatory cells and markers in induced sputum on Monday morning and Thursday morning before work (80). Inflammatory changes were observed during this period but no correlations were found with fungal spore exposure (median $2 \cdot 10^5$ spores/m^3). The IL-8 increase was correlated with exposure to glucans, but glucan exposure was only weakly associated with fungal spores. This association is therefore more likely due to glucans from non-fungal origin.

Highly exposed populations: Lung function

Lung function and fungal exposure was studied during four workdays in 29 wood trimmers from two sawmills with median exposures of $1 \cdot 10^4$ cfu/m^3 and $3 \cdot 10^5$ cfu/m^3, respectively. The FVC decline was significant in the sawmill with highest exposure. FVC decline as well as forced expiratory volume in one second (FEV_1) and FVC as % of predicted at the end of the 4-day period were correlated with exposure to fungi. Lung function had also been measured three months earlier but no exposure measurements were performed at that time. Declines in FVC and FEV_1 were observed after 3 months in workers from the sawmill with the highest exposure, while the lung function in workers from the other sawmill did not change (76). No information on production changes was provided. It seems likely, however, that the exposure levels were similar during the follow-up as it takes time to implement preventive measures and to sort all unsorted dry timber in stock.

FEV_1 and maximum expiratory flow at 25% of FVC (MEF_{25}) declined during the workweek in 28 wood trimmers and correlated with exposure to fungi (median $1 \cdot 10^5$ spores/m^3 dominated by *Rhizopus* and *Paecilomyces*) (38). Exposure to dust, terpenes, bacteria, and endotoxins were also measured and were low, except to bacteria (medians 0.3 mg/m^3, 10 ppm, $1 \cdot 10^5$ cells/m^3 and 20 EU/m^3, respectively).

No adjustments were made for confounders in neither study, but studying intra-individual changes in lung function reduces confounding by gender and age since subjects are compared with themselves. The effect of smoking was evaluated by separate analysis of smokers and non-smokers, but had no major influence on the associations. In the study by Dahlquist *et al* (38), exposure to total dust, endotoxins and terpenes was low and not likely to cause lung function changes. Microscopical counts of bacteria were similar to fungal counts but were not evaluated.

Both studies showed associations between lung function changes and exposure to fungi, which was most consistent for FEV_1. The study by Dahlquist *et al* (38)

indicates that associations with lung function are observable at median exposure levels of $1 \cdot 10^5$ spores/m^3. A more than 10 times higher level is indicated by the study by Hedenstierna *et al,* however (76).

Highly exposed populations: Upper airway inflammation

Nasal lavage fluid was analysed for inflammatory markers in two studies.

Roponen *et al* studied 11 sawmill workers and found no differences between NO, TNFα, IL-4, IL-5 and IL-6 levels in nasal lavage after work compared to samples collected during vacation. The exposure level of fungal spores was $6 \cdot 10^5$ spores/m^3 (median, mainly *Rhizopus* and *Penicillium*) and exposure to endotoxins and terpenes was also measured (202).

Heldal *et al* performed nasal lavage in 31 municipal waste handlers on Monday morning and Thursday morning. Exposure to fungal spores (median $2 \cdot 10^5$ spores/m^3), bacteria, actinomycetes spores, endotoxins, and glucans was measured between the lavages. The increase in proportion of neutrophils from Monday to Thursday and the level on Thursday was associated with exposure to fungal spores, whereas other inflammatory cells, myeloperoxidase, IL-8 and eosinophilic cationic protein (ECP) were not. Possible confounding by smoking and age was evaluated qualitatively (79).

Both studies agree in not finding associations between fungal spore exposure and inflammatory cytokines. Heldal *et al* (79) found associations with neutrophilic lymphocytes, which indicate an inflammatory response. Roponen *et al* (202) did not study inflammatory cells. Thus, there is some evidence for a cellular response to fungal spores at a level of $2 \cdot 10^5$ spores/m^3. No changes in the level of inflammatory mediators were found, however. Associations with IL-8 were expected because neutrophils are attracted by IL-8. Possible explanations are high intra- and interindividual variability and different temporal developments of the IL-8 and neutrophil responses. A LOEL of $2 \cdot 10^5$ spores/m^3 for neutrophilic infiltration is thus indicated.

Highly exposed populations: Nasal congestion

Heldal *et al* studied nasal congestion in 31 municipal waste handlers by acoustic rhinometry (79). A correlation was found between change in nasal congestion and fungal spore exposure (median $2 \cdot 10^5$ spores/m^3). The association was not due to confounding or exposure to other agents (bacteria, actinomycetes spores, endotoxins and glucans). A LOEL of $2 \cdot 10^5$ spores/m^3 is thus indicated by this study.

Populations exposed to common indoor air

Roponen *et al* studied 41 randomly selected teachers (203). Exposure to fungi and bacteria were measured at work and at home. Irritation, non-specific symptoms and the levels of NO, IL-4, IL-6 and TNFα in nasal lavage were similar in groups with high and low exposure to fungi (medians 12 cfu/m^3 and 67 cfu/m^3). IL-1β was elevated in the high exposure group but not significantly. Particles were resuspended from the filters and tested for cytokine production with a mouse RAW264.7 macrophage cell line. In this assay samples of the high exposure

group showed stronger IL-1β and IL-6 responses than samples of the low exposed group, while no differences in TNFα and cell viability were observed.

These results are not likely to be due to random variation because the intra-individual variability of the cytokines in the nasal lavages and the mouse macrophage tests was relatively low, subjects were randomly selected, and exposure at home was included in the exposure assessment. Separate analyses in smoking and asthma status subgroups showed that the findings were not due to confounding. This study thus indicates that the two groups were exposed to particles with different inflammatory potential, but that this did not result in observable changes in nasal lavage. This may be due to differences between the individual responses as the correlation between IL-1β, IL-6 and TNFα levels in the nasal lavages and the macrophage assays were low. However, inflammatory reactions of cells in the human nose may be different from isolated cells of a mouse macrophage cell line. Furthermore, the exposure levels may have been too low. The median exposure of the highest exposed group, 67 cfu/m^3, is regarded as a NOEL for irritation and non-specific symptoms, and inflammatory markers in the upper airways. This estimate represents a 24-hour time weighted average.

11.4.3 Discussion of effects of short-term exposure

Inhaled doses inducing a 35% increase in airway conductance in asthmatic patients after bronchial challenge were $6 \cdot 10^4$ *Penicillium* sp. spores and $9 \cdot 10^4$ *Alternaria alternata* spores (125). The patients were sensitised to the fungus used in the challenge and thus represent a susceptible group. The dose was inhaled over a short time period, but when the minimum dose for a positive challenge response was calculated to equal an 8-hour exposure by dividing the applied dose with 5 m^3 assuming a minute ventilation of 10 l/min during 8 hours, concentrations of $1 \cdot 10^4$ and $2 \cdot 10^4$ spores/m^3 were found for the two fungal species, respectively.

In employees from moisture-damaged schools with building-related symptoms and sensitised to *P. chrysogenum* but without asthma, NOELs of $6 \cdot 10^5$ *P. chrysogenum* spores/m^3 and $3.5 \cdot 10^5$ *T. harzianum* spores/m^3 were found by bronchial challenge to a single concentration for 6 minutes (135). Also this group may be susceptible although less than the former. The applied doses are equal to $4 \cdot 10^3$-$8 \cdot 10^3$ spores/m^3 for 8 hours.

In highly exposed working populations (farmers, sawmill workers and waste handlers), LOELs of $1 \cdot 10^5$-$2 \cdot 10^5$ spores/m^3 were found for nasal congestion, eye irritation, sore throat, and lung function changes (6, 38, 49, 76, 79). A higher LOEL of $3 \cdot 10^6$ spores/m^3 was found for nose irritation in farmers, but the prevalence was already increased (OR 4.1, 95% CI 0.88-19) at an exposure level of $1 \cdot 10^5$ spores/m^3, although not significantly (49). No changes in inflammatory markers were found in the upper or lower airways in four studies of sawmill workers, waste handlers and teachers (79, 80, 202, 203). However, in waste handlers neutrophils in nasal lavage but not in induced sputum were associated with fungal spore exposure at a median level of $2 \cdot 10^5$ spores/m^3 (79).

Cough seems to be related to higher exposure levels with LOELs of $3 \cdot 10^5$ spores/m^3 in a waste handler study (78) and $3 \cdot 10^6$ spores/m^3 in a study of farmers (49).

Even higher levels induced fever. Bronchial challenge of patients with hypersensitivity pneumonitis using the main species found in the environment *(Aspergillus clavatus, Pleurotus ostreatus* and *Lentinus edodes)* induced typical attacks including fever in mushroom workers (35, 36) and a malt worker (198). The applied doses are equal to $2 \cdot 10^6$-$2 \cdot 10^7$ spores/m^3 (calculated to equal an 8-hour exposure). An epidemiological study in farmers (130) indicated even higher exposure levels, $1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3 in hypersensitivity pneumonitis patients, $2 \cdot 10^9$ spores/m^3 in ODTS patients, and $3 \cdot 10^6$-$1 \cdot 10^7$ spores/m^3 in non-symptomatic farmers (equal to an 8-hour exposure). The farmers were exposed to spores from various fungal and actinomycete species.

The response in bronchial challenge studies of patients with hypersensitivity pneumonitis or asthma shows species specificity (Chapter 11.2.1), whereas attacks of ODTS seem not to require previous sensitisation (Chapter 8.3). The other studies provided little information on the importance of specific fungi.

11.5 Effects of long-term exposure

11.5.1 Epidemiological studies

Epidemiological studies of hypersensitivity pneumonitis, asthma, chronic bronchitis, and systemic symptoms are reviewed as well as studies of chronic lung function loss and X-ray changes.

Highly exposed populations: Hypersensitivity pneumonitis

The role of fungal spores in occupational respiratory disease was probably first recognised in hypersensitivity pneumonitis. The disease is characterised by alveolar and bronchiolar inflammation, and is caused by inhalation of spores from fungi and actinomycetes, and also by other allergens. Acute attacks are similar to ODTS. Recurrent attacks may eventually progress into pulmonary fibrosis. IgG but not IgE can usually be detected (Chapter 8.2). The incidence of hypersensitivity pneumonitis is lower than that of ODTS, 2-30 and 10-190 per 10 000 farmers/year, respectively (56).

Ávila and Lacey diagnosed 26 patients with hypersensitivity pneumonitis among 648 cork workers in a cross-sectional study (prevalence 4%) (9). *Penicillium glabrum* (previous name *frequentans*) was the dominating fungus with exposure levels ranging from $1 \cdot 10^6$ to $7 \cdot 10^7$ spores/m^3 in different departments. Current exposure was not associated with hypersensitivity pneumonitis. However, as the disease develops after prolonged exposure and workers changed jobs frequently, current exposure was not a good measure of relevant exposure. The role of *P. glabrum* was demonstrated by bronchial provocation of 19 cork workers (9) (Table 11). Although information on exposure levels in the past is lacking, these may not be very different from current exposure levels as no mention was made of preventive measures in spite of the large number of workers with hypersensitivity

pneumonitis. It seems therefore possible that hypersensitivity pneumonitis can develop after prolonged exposure to the mean levels found in this study, approximately 10^7 spores/m^3.

Wenzel and Emanuel found 5 cases of hypersensitivity pneumonitis with interstitial pneumonitis and granuloma among 37 papermill workers (prevalence 14%) exposed to spores of *Cryptostroma corticale* (243). Cases developed during periods when exposure was highest (mean level probably $5 \cdot 10^6$ *C. corticale* spores/m^3). There is uncertainty about this level, however, because stationary sampling was applied and the presented data did not describe the measurement unit.

Both studies suggest that prolonged exposure to approximately 10^7 spores/m^3 of *P. glabrum* and *C. corticale* can induce hypersensitivity pneumonitis, but these are crude estimates since exposures were poorly documented and results not adjusted for confounders.

Highly exposed populations: Asthma

Current physician diagnosed asthma was studied in a cross-sectional study of 1 614 farmers. Annual exposure levels to fungal spores (one year equalled geometric mean $2 \cdot 10^6$ spores/m^3), bacteria, endotoxins, and ammonia were estimated (50). Exposure to fungi was positively correlated with asthma in the highest exposed ($4 \cdot 10^6$-$4 \cdot 10^7$ spores/m^3) non-atopic farmers with OR 1.7 (95% CI 1.0-2.7). In atopic farmers, exposure to fungi was negatively correlated with asthma with OR 0.28 (95% CI 0.10-0.78). The associations with fungal spores were stronger than with endotoxins and of similar strength as with ammonia. Associations were adjusted for age, smoking, gender, and asthma in the family.

Year-average exposure levels were estimated because it was not clear when asthma was initiated and occupational asthma may take years of exposure to develop. Atopic status was important as the association was reversed in atopic farmers. Endotoxins and amonia may also explain these associations as exposure levels were correlated, but ammonia as a cause of asthma seems not biologically plausible, as the annual exposure level was 4 ppm.

In three studies, indicators of asthma were investigated (6, 31, 141). Variability of peak expiratory flow was studied in 72 waste collectors and exposure was estimated by a job exposure matrix including total fungi (level approximately $2 \cdot 10^5$ cfu/m^3), bacteria and endotoxins (31). Peak flow variability was significantly increased in the group with the highest exposure to *A. fumigatus* (level approximately 10^4 cfu/m^3), but was not associated with the other agents.

Wheezing was studied in 82 joinery and 108 sawmill and chip mill workers (6). Exposure to fungal spores (arithmetic mean $3 \cdot 10^4$ cfu/m^3), dust, Gram-negative bacteria, endotoxins, and glucans was measured. Wheezing was associated with fungal exposure (OR 1.4, 95% CI 1.0-2.0) but more strongly with respirable glucans. The association between wheezing and fungal exposure may be due to glucan exposure because these exposures were strongly correlated (r=0.76-0.86).

Monsó *et al* studied wheeze and asthma attacks in 39 greenhouse workers and measured exposure to endotoxins, bacteria, and fungi (median $5 \cdot 10^3$ cfu/m^3) (141).

No associations were found but the number of cases was small, especially of asthma attacks (n=3).

Current exposure is probably a valid exposure estimate for peak flow variability and wheezing, because these outcomes are likely to be a response to recent exposure. In the first two studies, the role of other bioaerosol components such as bacteria and endotoxins was also evaluated. The waste collector study (31) indicated that peak flow variability was a specific response to *A. fumigatus* but associations were not adjusted for confounders. The greenhouse worker study (141) was small and included only three subjects with asthma attacks. The results of the wood worker study (6) are therefore the most reliable. In that study, wheezing was more strongly associated with glucans than fungi. This may, however, indicate a response to a fungal component as glucans and fungi were correlated.

In summary, wheezing was associated with fungal exposure in wood workers exposed to approximately $3 \cdot 10^5$ spores/m^3 (arithmetic mean) (6). This level is lower than indicated by the increased prevalence of asthma in non-atopic farmers, approximately $1 \cdot 10^7$ spores/m^3 (geometric mean of the range) (50). This might indicate that non-atopic asthma is induced by higher exposure levels than wheezing. However, atopy was not evaluated in the wood worker study and the association with wheezing might be due to the atopic individuals in the population. In the farmer study, atopic individuals had been excluded in the analysis of non-atopic asthma and this association was therefore not confounded by atopy. Furthermore, the exposure in the asthma study among farmers was estimated with considerable random error, which may have attenuated exposure-response associations. Wheezing is not specific to asthma as the prevalence of wheezing is much higher than that of asthma. The negative association with atopic asthma in farmers is special (50). This may be due to a protective effect, but may also be explained by selection, either by retirement or by changing production. There is uncertainty about the role of fungi because levels of other bioaerosol agents that were associated with these outcomes were also correlated with fungal levels.

Highly exposed populations: Chronic bronchitis

Chronic bronchitis and productive cough were studied in 72 waste collectors. Symptoms were monitored during two weeks, and exposure to bacteria, endotoxins and culturable fungi was estimated by a quantitative job exposure matrix. Both outcomes were significantly increased in the group with highest exposure to *A. fumigatus* (exposure level approximately 10^4 cfu/m^3) but these outcomes were not associated with total fungi (exposure level approximately $2 \cdot 10^5$ cfu/m^3). The results were not adjusted for confounders, however (31).

Chronic bronchitis was also studied by a general questionnaire in 951 waste collectors and 423 park workers (73). Exposure of waste collectors to culturable and total fungi, inorganic dust, organic dust, irritant gases and fumes was estimated by a quantitative job exposure matrix. The waste collectors were categorised in two groups; those exposed to low ($<2 \cdot 10^5$ cfu/m^3) and high ($\geq 2 \cdot 10^5$ cfu/m^3) levels, respectively. The groups were of very different sizes (at the 96%-tile)

without justification, and no other exposure data were presented. The exposure of the park workers was not measured and assumed to be low. Chronic bronchitis was significantly elevated in the low exposure group (prevalence proportion ratio (PPR) 1.9, 95% CI 1.0-3.6) for both culturable fungi and fungal spore count. The PPR increased further in the high exposure group to 2.5 (95% CI 0.6-11) for countable fungi and 2.7 (95% CI 0.7-11) for culturable fungi, but not significantly.

Alwis *et al* studied bronchitis, phlegm, and breathlessness in 82 joinery workers, and 108 sawmill and chip mill workers (6). Breathlessness (OR 4.8, 95% CI 1.4-17) was most strongly associated with fungal exposure in sawmill and chip mill workers (arithmetic mean $4{\cdot}10^4$ cfu/m^3). Phlegm (OR 2.0, 95% CI 1.0-3.9) was also associated with fungal exposure in joinery workers (arithmetic mean $1{\cdot}10^4$ cfu/m^3). Endotoxin exposure was also associated with phlegm, but the association was slightly weaker. Confounding of the association with fungal exposure is not likely because endotoxin exposure was only moderately correlated with fungal exposure. Exposure to fungi was also associated with bronchitis in joinery workers (OR 3.0, 95% CI 1.3-6.6, arithmetic mean $1{\cdot}10^4$ cfu/m^3) and sawmill/chip mill workers (OR 2.9, 95% CI 1.1-7.2, arithmetic mean $4{\cdot}10^4$ cfu/m^3). The associations with glucans were stronger (OR 20, 95% CI 10-41), however, and the association between bronchitis and fungal exposure may be due to glucan exposure because these exposures were strongly correlated (r=0.76-0.86). The associations with glucan exposure may also reflect fungal exposure because glucans are a structural component of fungi.

Morning cough was associated with exposure to fungal spores in 107 wood trimmers (54). Exposure to different spore types (42% were from *Rhizopus microsporus)*, pine dust, and spruce dust was measured. Work-related chest tightness was associated with *Rhizopus microsporus* spore and wood dust exposure but this association may suffer from reporting bias because the symptom had been recorded conditional on handling of mouldy timber. Exposure levels of $2{\cdot}10^6$ spores/m^3 predicted an OR of 1.5 for both associations. The study did not include an external reference group. Therefore, the lowest exposed wood trimmers ($4{\cdot}10^5$ spores/m^3) were used as an internal reference group.

To sum up, symptoms suggestive of chronic bronchitis were associated with fungal exposure in five populations with three different occupations. Two studies of waste collectors specifically reported chronic bronchitis. In one of those, no association was found with total fungi (exposure level approximately 10^4 cfu/m^3) but associations were not adjusted for confounders (31). An association was reported in the other study but the expsure level is unclear (73). A LOEL of $2{\cdot}10^6$ spores/m^3 was indicated for morning cough in the wood trimmer study (54), but this is a high estimate because the lowest exposed group was exposed to $4{\cdot}10^5$ spores/m^3 and lower exposure levels could thus not be explored.

The associations with breathlessness and phlegm reported by Alwis *et al* (6) and morning cough by Eduard *et al* (54) in wood workers seem reliable because the exposure of wood workers is mainly to wood dust and fungal spores. It is therefore likely that the association between chronic bronchitis symptoms and exposure to fungal spores is real. LOELs of $1{\cdot}10^5$ and $4{\cdot}10^5$ spores/m^3 were

indicated for phlegm in joinery workers and for breathlessness in sawmill and chip mill workers, respectively. In wood trimmers, a LOEL of $2 \cdot 10^6$ spores/m^3 was found for morning cough, but this is probably a high estimate because the internal reference group in this population was relatively highly exposed.

In two studies, stronger associations between bronchitis symptoms and specific fungi were found than with total fungi; *A. fumigatus* in waste collectors and *R. microsporus* spores in wood trimmers (31, 54). However, both studies suffer from methodological weaknesses.

Highly exposed populations: Lung function

Reduced restrictive lung function (described as a "restrictive effect" but not further documented) was observed in 648 cork workers (9). *Penicillium glabrum* was the dominating fungus and total spore levels ranged from $1 \cdot 10^6$ to $7 \cdot 10^7$ spores/m^3 in different departments. Restrictive lung function was not associated with spore exposure but increased from 8% in workers employed for up to 5 years to 29% after more than 30 years of employment. Impaired lung function is expected to be associated with long-term exposure as it may take years before the decline is sufficiently large to be detectable. Duration of employment is probably a reasonable estimate of cumulative exposure because workers changed jobs frequently. It is possible that current exposure levels were not very different from the past (see page 73). These results may therefore indicate that long-term exposure to approximately 10^7 spores/m^3 induced the restrictive lung function impairment.

Johard *et al* compared 19 wood trimmers exposed to fungi (median $1 \cdot 10^5$ spores/m^3) with 19 not occupationally exposed subjects, all non-smokers (100). Lung function, (vital capacity, FEV_1, diffusion capacity for CO) was not significantly different compared to the controls. These results suggest a NOEL of $1 \cdot 10^5$ spores/m^3 for long-term decline in FVC and FEV_1 decline. However in another publication of this study an association was found between fungal spore exposure and short-term decline in FEV_1 and MEF_{25} (38).

Highly exposed populations: Systemic symptoms

Ivens *et al* studied diarrhoea in 950 waste collectors with 387 outdoor municipal workers as reference (97). Inhaled doses of culturable fungi, total fungi and endotoxins were estimated by a quantitative job exposure matrix for the waste collectors but not the reference group. The waste handlers were categorised in three exposure groups of very different sizes. Diarrhoea was significantly elevated in the lowest exposed waste collectors (PPR 3.0, 95% CI 1.9-4.9) for fungal spores, and increased in a dose-response manner in the more highly exposed groups. Adjustments for confounders were applied but not for other agents although similar associations were observed for endotoxins.

No other studies of systemic symptoms have been found.

Highly exposed populations: X-ray changes

Wenzel and Emanuel observed diffuse reticulo-nodular infiltrates by chest X-ray in 40% of 37 papermill workers exposed to spores of *Cryptostroma corticale*

(243). These changes were observed during summer and winter, and the mean exposure was approximately $5 \cdot 10^6$ *C. corticale* spores/m^3. There is, however, uncertainty about these levels (see *Highly exposed populations: Hypersensitivity pneumonitis* page 73 and Appendix 6).

Reticulo-nodular changes were observed by chest X-ray in 57% of 648 cork workers (9). *Penicillium glabrum* was the dominating fungus and total spore levels ranged from $1 \cdot 10^6$ to $7 \cdot 10^7$ spores/m^3 in different departments. The X-ray changes correlated with current exposure to fungal spores in non-smokers but not with duration of employment.

Both studies indicate that X-ray changes occur in populations exposed to fungal spores and wood or cork dust. Although confounding was not adjusted for in the papermill study, and only smoking was considered in the cork worker study, it seems likely that fungal spores may induce these X-ray changes since the prevalence of this outcome was high. Wood or cork dust is not known to induce X-ray changes, while this is a common finding in hypersensitivity pneumonitis. The cork worker study (9) suggests that exposure levels of 10^7 *Penicillium glabrum* spores/m^3 can induce the X-ray changes, and as cork workers changed jobs regularly, the correlation with current exposure levels indicates that the X-ray changes do not take very long time to develop. A similar value is indicated in the papermill study (243), but there is uncertainty about the unit of exposure in this study.

Highly exposed populations: Lower airway inflammation

Johard *et al* studied 19 wood trimmers exposed to approximately $1 \cdot 10^5$ spores/m^3 and to 10^5 bacteria/m^3, and 25 subjects not occupationally exposed to fungi, by bronchoalveolar lavage (100). Cell counts were not different, but albumin, fibronectin, and hyaluronan were significantly higher in the sawmill workers than in the controls indicating low intensity alveolar inflammation. Results were not adjusted for confounders but all participants were non-smokers. Exposures to total dust, endotoxins and terpenes were reported to be low in another publication of this study with medians of 0.3 mg/m^3, 20 EU/m^3 and 10 ppm, respectively (38).

Populations exposed to common indoor air: Respiratory symptoms

Li *et al* studied cough and phlegm in 264 day-care workers. Fungi were measured in indoor and outdoor air (geometric means 1 200 cfu/m^3 and 1 000 cfu/m^3, respectively), and house dust mite allergens in settled dust. *Cladosporium*, *Penicillium, Aspergillus,* and yeasts were prevalent species. Cough and phlegm were associated with *Aspergillus* levels (geometric mean 32 cfu/m^3) but not with other species or total fungi (124).

Menzies *et al* compared 107 office workers with work-related respiratory symptoms and 107 office workers without symptoms. Airborne fungi (mean *Alternaria* levels 1 and 0.3 cfu/m^3, respectively) were measured as well as fungi and house dust mite allergens in floor dust. Symptoms were associated with *Alternaria* exposure (OR 4.2, 95% CI 1.1-16) (134).

Purokivi *et al* compared 37 employees from a school with fungal problems with 23 employees from a school without such problems. Culturable fungi were

measured during the winter (geometric mean 29 cfu/m^3 and 6 cfu/m^3, respectively). Respiratory symptom prevalences were not significantly different at the end of spring, but no confounder adjustments were carried out (181).

Roponen *et al* recorded symptoms of the lower airways in randomly selected teachers, and exposure to fungi and bacteria was measured at home and at school. The population was divided in a low and a high exposure group (median 12 cfu/m^3 and 67 cfu/m^3, respectively). Lower airways symptoms were similar in the two groups (203).

No study found associations between respiratory symptoms and total fungi. The study by Li *et al* (124) is interesting because they used random selection and controlled for confounders, which strengthen the reliability of their results. However, it is not clear how fungal exposure was measured. Fungal levels in this study were considerable higher than in the other studies, 1 200 cfu/m^3, which is 20-500 times higher than in the studies by Purokivi *et al* (181) and Roponen *et al* (203). Menzies *et al* (134) only quoted *Alternaria* levels, which were very low. The study by Li *et al* (124) suggests a NOEL of approximately 10^4 spores/m^3, and the study by Roponen *et al* (203) a NOEL of approximately $7 \cdot 10^2$ spores/m^3 for a 24-hour exposure.

Findings in the studies by Li *et al* (124) and Menzies *et al* (134) suggest that *Aspergillus* and *Alternaria* are more potent than other species as they were associated with symptoms but were not the dominating species.

Populations exposed to common indoor air: Systemic symptoms

Two studies described in the previous chapter also studied systemic symptoms.

Li *et al* studied lethargy and fatigue in day-care workers and found associations with *Aspergillus* levels (geometric mean 32 cfu/m^3) but not with total culturable fungal levels (geometric mean 1 200 cfu/m^3) (124).

Roponen *et al* found that non-specific symptoms in randomly selected teachers were not different in individuals with low and high exposure (median 12 cfu/m^3 and 67 cfu/m^3, respectively including exposure both at work and home (203).

The day-care workers were exposed to the highest levels but associations were only found for *Aspergillus*, which constituted only a small fraction of the fungal exposure. This might indicate that *Aspergillus* species are more potent than other fungi. However, non-specific symptoms are commonly associated with dampness in buildings, but a specific role of fungi has not yet been demonstrated; review by Bornehag *et al* (16). It can therefore not be ruled out that *Aspergillus* sp. is just a marker of humidity in buildings.

Populations exposed to common indoor air: Airway inflammation

Hirvonen *et al* studied 32 school staff members of a school contaminated with fungi and 8 or 25 healthy controls from a research institution without known fungal contamination (the numbers of controls were inconsistent) (83). Increased levels of NO, TNFα and IL-6 were observed in nasal lavage after the spring and/or autumn term compared to after vacation. These levels were also higher than in external controls. Fungal levels in the school in the autumn were 7-100

cfu/m^3. However, no exposure data after vacation or of the control subjects were reported.

Purokivi *et al* compared nasal lavage and induced sputum samples of school employees from schools with and without fungal problems (181). Culturable fungi were only measured during the winter (geometric mean 29 and 6 cfu/m^3, respectively). Higher levels of IL-1 and IL-4 were found in nasal lavage and of IL-6 in induced sputum at the end of the spring term in employees from the school with fungal problems. Differential cell counts were similar. No confounder adjustments were carried out, however.

Thus, both studies found increased cytokine levels in nasal lavage and/or induced sputum after school terms. These findings cannot be related to exposure, however, due to lack of exposure data.

Populations exposed to common indoor air: Asthma and rhinitis

Seuri *et al* described a cluster of 4 asthma (6 tested) and 11 rhinitis cases in 14 employees from a hospital with water damage (217). Fungal levels exceeded 500 cfu/m^3 at three locations with a maximum of 1 400 cfu/m^3 (approximately 50% *Sporobolomyces salmonicolor).* The asthma and rhinitis cases had positive challenges with *S. salmonicolor* extract but skin prick tests with the extract were negative indicating that the asthma and rhinitis were non-allergic (see also Chapter 11.2.1). Serum IgG antibodies to typical water-damage fungi and *S. salmonicolor* were found in most employees, which further demonstrated exposure to fungi. Exposure levels had probably been higher as a water-damaged floor had been repaired a year earlier, but no microbial measurements were then carried out.

This study indicates that a high proportion of workers in a water-damaged building developed non-allergic asthma and rhinitis, and the response was specific for the fungus *S. salmonicolor* that was also the dominating species in the building. Symptoms were maintained at exposure levels of approximately 10^4 spores/m^3 but induction of the diseases probably occurred at higher levels.

11.5.2 Discussion of effects of long-term exposure

Symptoms in employees with non-allergic asthma and rhinitis in the hospital study (217) indicated a LOEL of $1 \cdot 10^4$ spores/m^3. The exposure levels inducing the diseases were probably higher. In farmers, a much higher LOEL of approximately 10^7 spores/m^3 was indicated for non-atopic asthma (50). This association may, however, have been attenuated by considerable non-differential misclassification of the exposure and have resulted in a high estimate of the effect level. Also exposure to other biological agents may explain the association in the farmer study.

The negative association with asthma in atopic farmers is special (50). It may be due to a protective effect, ascribed to microbial exposure (the so-called hygiene hypothesis), but may also be due to selection. The role of other biological agents cannot be ruled out as they were correlated with exposure to fungal spores. It is

interesting, however, that none of the asthma and rhinitis cases in the hospital employee study were atopic (217).

Bronchitis symptoms were associated with fungal exposure in five populations with three different occupations. Most populations were exposed to median levels of $1 \cdot 10^4$-$4 \cdot 10^4$ fungal cfu/m^3, which indicate LOELs of $1 \cdot 10^5$-$4 \cdot 10^5$ spores/m^3. Studies performed on sawmill workers, and especially wood trimmers, are of special interest since exposure to agents other than fungal spores is usually low. For respiratory symptoms such as phlegm and breathlessness in wood workers LOELs of $1 \cdot 10^5$ spores/m^3 and $4 \cdot 10^5$ spores/m^3 were reported (6). In wood trimmers a LOEL of $2 \cdot 10^6$ spores/m^3 was found for morning cough. This is a high estimate because the population did not include individuals with low exposure (54).

No associations were found between respiratory symptoms and exposure to "total fungi" in four common indoor air studies (124, 134, 181, 203). The exposure level in the study with the highest exposure was approximately 10^4 spores/m^3, which was measured by stationary sampling. Exposure would probably have been higher if measured by personal sampling. The documentation of the measurements was poor (124). This NOEL is therefore consistent with the LOELs found for respiratory symptoms in more highly exposed populations.

The wood worker study indicates a NOEL of $1 \cdot 10^5$ spores/m^3 for lung function changes (100). A reduced FVC observed cross-sectionally in cork workers exposed to approximately 10^7 spores/m^3 may be consistent with this NOEL (9). Results were poorly documented in both studies, however.

Exposure levels of 10^7 spores/m^3 of *P. glabrum* and *C. corticale* were related to hypersensitivity pneumonitis and X-ray changes (9, 243). These exposure levels are crude estimates because of poorly documented exposure, and results not adjusted for confounders. However, the exposure was mainly to fungal spores, and wood or cork dust and only fungal spores are known to induce these outcomes. Furthermore, the prevalence of the X-ray changes was very high and cases of hypersensitivity pneumonitis were confirmed by bronchial provocation tests. It seems therefore unlikely that confounder adjustment would have changed these associations substantially.

Inflammatory markers in bronchoalveolar lavage were increased in wood trimmers exposed to 10^5 spores/m^3 whereas inflammatory cells were not (100). In two common indoor air studies, inflammatory markers in nasal lavage and induced sputum in school personnel working in a moisture-damaged building where higher than in controls and school employees working in a non-problem building, respectively, but the effects cannot be related to the measured exposure (83, 181).

An association was reported between fungi and diarrhoea in a study of waste handlers (97), but no other studies reported this outcome. Two common indoor studies did not find associations between fungal exposure and the non-specific symptoms lethargy and fatigue (124, 203).

Few studies have analysed results for specific fungi. A study of a waste handler reported associations between *A. fumigatus* and variable peak flow and bronchitis

symptoms, but no confounder adjustments were applied (31). The indicated LOEL, $1 \cdot 10^5$ spores/m^3, was somewhat lower than that for lung function changes and respiratory symptoms. In a wood trimmer study, work-related chest tightness was associated to *Rhizopus microspores* with a LOEL of $2 \cdot 10^6$ spores/m^3 but this estimate may be biased and is a high estimate since the population did not include individuals with low exposure (54). X-ray changes and hypersensitivity pneumonitis were observed in wood workers and cork workers exposed to spores mainly from *Cryptostroma corticale* and *Penicillium glabrum*, respectively (9, 243). Common indoor air studies showed associations between respiratory symptoms and *Aspergillus* sp. and *Alternaria* sp. (124, 134), and between asthma and rhinitis and *Sporobolomyces salmonicolor* (217). A role for *S. salmonicolor* is probable, as this association was further confirmed by provocation tests with the organism. The exposure levels of the specific organisms in the other two studies were very low, however. Three studies support that specific organisms may be more toxic (31, 124, 134), but these studies have important shortcomings in the exposure assessment.

11.6 Genotoxic and carcinogenic effects

No studies including fungal spore exposure have been found.

11.7 Reproductive and developmental effects

No studies including fungal spore exposure have been found.

Table 13. Epidemiological studies of highly exposed populations: Symptoms associated with fungal spore exposure. Associations have been adjusted for smoking, age, and gender. Studies are cross-sectional unless otherwise stated.

Study group	Sampling method	Exposure level, spores/m^3	Association			Comments	Reference
			Symptom	Exposure level, [a] spores/m^3	OR (95% CI)		
89 farmers	Personal	$2 \cdot 10^3$-$2 \cdot 10^7$ (8 h-TWA)	Nasal irritation	$2 \cdot 10^3$-$2 \cdot 10^4$	REF	Current work-related symptoms recorded concomitantly with exposure measurements. Also adjusted for exposure to bacteria, endotoxins, and other agents.	(49)
				$2 \cdot 10^4$-$5 \cdot 10^5$	4.1 (0.88-19)		
				$5 \cdot 10^5$-$2 \cdot 10^7$	6.0 (1.3-28) [b]		
			Eye irritation	$2 \cdot 10^3$-$2 \cdot 10^4$	REF		
				$2 \cdot 10^4$-$5 \cdot 10^5$	8.3 (1.0-70) [b]		
				$5 \cdot 10^5$-$2 \cdot 10^7$	7.0 (0.83-59)		
			Cough	$2 \cdot 10^3$-$2 \cdot 10^4$	REF		
				$2 \cdot 10^4$-$5 \cdot 10^5$	1.3 (0.28-5.7)		
				$5 \cdot 10^5$-$2 \cdot 10^7$	3.9 (1.0-15) [b]		
1 614 farmers	Personal, Job exposure matrix[c]	$2 \cdot 10^5$-$4 \cdot 10^7$ (1 yr-TWA)	Atopic asthma	$2 \cdot 10^5$-$2 \cdot 10^6$	REF	Also adjusted for exposure to bacteria and endotoxins.	(50)
				$2 \cdot 10^6$-$4 \cdot 10^6$	0.55 (0.23-1.3)		
				$4 \cdot 10^6$-$4 \cdot 10^7$	0.28 (0.10-0.78) [b]		
			Non-atopic asthma	$2 \cdot 10^5$-$2 \cdot 10^6$	REF		
				$2 \cdot 10^6$-$4 \cdot 10^6$	1.6 (0.96-2.6)		
				$4 \cdot 10^6$-$4 \cdot 10^7$	1.7 (1.0-2.7) [b]		
107 wood trimmers	Personal, group-based	$4 \cdot 10^5$-$2 \cdot 10^7$	Cough, morning	$2 \cdot 10^6$	1.5 [b]	Exposure levels that predict an OR of 1.5 in significant logistic regression models. Also adjusted for hayfever, and wood dust. Separate models with fungal species were also tested. Possible recording bias of work-related symptoms.	(54)
			Cough, WR	$2 \cdot 10^6$	1.5 [b]		
			Nasal obstruction, WR	$2 \cdot 10^6$	1.5 [b]		
			Sore throat, WR	$2 \cdot 10^6$	1.5 [b]		
		0-$7 \cdot 10^6$ *Rhizopus microsporus*	Shortness of breath, WR	$2 \cdot 10^6$	1.5 [b]		

Table 13. *Cont.*

Study group	Sampling method	Exposure level, cfu/m^3	Association			Comments	Reference
			Symptom	Exposure level, [a] cfu/m^3	OR (95% CI)		
951 waste collectors and 423 park workers (REF)	Personal, Job exposure matrix [c, d]	Not given	Chronic bronchitis	0 $< 2 \cdot 10^5$ $\geq 2 \cdot 10^5$	REF 1.9 (1.0-3.6) [b] 2.7 (0.7-11) [b, c]	Two groups of waste handlers were compared to a reference group. The exposure of the reference group was not measured and assumed to be 0. Only the cutpoint that divided two exposure categories of the waste handlers was reported.	(73)
950 waste collectors and 387 park workers (REF)	Personal, Job exposure matrix [d]	$1 \cdot 10^5$-$2 \cdot 10^7$ Weekly inhaled spore dose	Diarrhoea	0 $1 \cdot 10^5$-$1 \cdot 10^6$ $>1 \cdot 10^6$-$1 \cdot 10^7$ $>1 \cdot 10^7$-$2 \cdot 10^7$	REF 3.0 (1.9-4.9) [b] 3.5 (2.2-5.3) [b] 5.6 (2.4-13) [b]	Similar association with endotoxins. Three groups of waste handlers were compared to a reference group. The exposure of the reference group was not measured and assumed to be 0.	(97)
82 joinery workers and 108 saw-/chip mill workers	Personal	$3 \cdot 10^3$-$7 \cdot 10^4$ (arithmetic group means)	Throat irritation, WR Phlegm (joinery) Breathlessness, WR (saw-/chip mill)	$3 \cdot 10^4$ $1 \cdot 10^4$ $4 \cdot 10^4$ (arithmetic means)	OR 1.6 (1.2-2.1) [b] 2.0 (1.0-3.9) [b] 4.8 (1.4-17) [b]	Exposures to allergenic wood dust, glucans, Gram-negative bacteria and endotoxins were evaluated in separate models. Models where fungi showed the strongest or independent relationships are shown.	(6)

[a] Exposure level for which the association was calculated.
[b] Significantly different from the lowest exposed group (REF).
[c] See comment in Appendix 6.
[d] Job exposure matrix: exposure estimated from information on performed tasks and other determinants, which had been validated by personal exposure measurements in a subset of the workers.
cfu: colony forming units, CI: confidence interval, OR: odds ratio, PPR: prevalence proportion ratio, REF: reference group (OR or PPR =1), TWA: time weighted average, WR: work-related.

Table 14. Epidemiological studies of highly exposed populations: Objective outcomes associated with fungal spore exposure. Studies are cross-sectional unless otherwise stated.

Study group	Sampling method	Exposure level, spores/m^3		Association		Comments	Reference
		Range	Median [a]	Endpoint	Outcome		
28 waste handlers	Personal	$0\text{-}2 \cdot 10^6$ (8-h TWA)	$2 \cdot 10^5$ $5 \cdot 10^4$	% neutrophils in nasal lavage Δnasal volume (n=12) after 3 days of exposure	Positive correlation Negative correlation	The influence of con-founders was evaluated.	(79)
25 waste collectors	Personal	$0\text{-}2 \cdot 10^6$ (8-h TWA)	$2 \cdot 10^5$	Change in inflammatory cells and mediators in induced sputum after 3 days of exposure	Not associated to exposure levels	The influence of con-founders was evaluated.	(80)
11 sawmill workers during work vs vacation	Not specified	$2 \cdot 10^5\text{-}1.5 \cdot 10^6$ *Rhizopus* and *Penicillium* sp.	$6 \cdot 10^5$	Inflammatory markers in nasal lavage	Not associated to exposure levels	The influence of con-founders was not evaluated.	(202)
29 sawmill workers (follow-up)	Personal	$1 \cdot 10^2\text{-}4 \cdot 10^6$ cfu/m^3	$2 \cdot 10^4$ cfu/m^3	ΔFVC after 4 d of exposure	Positive correlation	Stratified analysis in smoking categories. Effect change design controls for age and gender.	(76)

[a] Central measure of the exposure distribution that showed an association with the outcome.
Δ: intra-individual change, cfu: colony forming units, FVC: force vital capacity, TWA: time-weighted average.

Table 15. Epidemiological studies of working populations exposed to common indoor levels: Symptoms associated with fungal spore exposure. Studies are cross-sectional unless otherwise stated.

Study group	Sampling method	Exposure level	Association		Comments	Reference
			Work-related symptoms (effect)	Outcome, OR (95% CI)		
264 day-care workers from 28 randomly selected day-care centers	Area	1 200 cfu/m^3 (GM) (*Penicillium* and *Cladosporium* dominated, levels indoors slightly higher than outdoors)	Nasal congestion Nasal discharge	2.0 (1.2-3.3) [a] 1.7 (1.0-2.8) [a]	Exposure measurements poorly documented. Exposure variables in models not specified. Adjusted for age, gender, and education. Separate models with other fungal species, total culturable bacteria, and house dust allergens on settled dust did not show associations.	(124)
		32 *Aspergillus* cfu/m^3 (GM)	Nasal congestion Cough Phlegm Lethargy Fatigue	1.7 (1.0-2.8) [a] 1.7 (1.1-2.8) [a] 1.8 (1.1-2.8) [a] 1.8 (1.0-3.1) [a] 2.0 (1.2-3.4) [a]		
107 office workers with and 107 without work-related respiratory symptoms	Area in offices	0-7 *Alternaria* cfu/m^3	Respiratory symptoms	4.2 (1.1-16) [a] (1-7 cfu/m^3)	Levels of other species and total fungi not given. Subjects matched on age, gender, and atopy. Adjusted for smoking, mite allergen in house dust, and difference between absolute indoor and outdoor air humidity.	(134)
41 randomly selected teachers	Personal, home+work combined	0-270 cfu/m^3	Irritation and non-specific symptoms; nasal lavage: NO, IL-4, IL-6, TNFα and IL-1β	Similar in groups with low (0-31 cfu/m^3) and high (31-270 cfu/m^3) exposure, except IL-1β that was non-significantly increased in the high exposure group	Exposure to bacteria was also studied. Confounder assessment by separate analyses in subgroups based on asthma and smoking.	(203)

[a] Significant association between exposure and symptom.
cfu: colony forming units, CI: confidence interval, GM: geometric mean, IL: interleukin, OR: odds ratio, NO: nitric oxide, TNFα: tumour necrosis factor alpha.

12. Dose-effect and dose-response relationships

12.1 Dose considerations and extrapolation from animal studies

Intratracheal and intranasal instillation circumvent the normal deposition processses in the airways, and distribute spores deeper into the lung. The dose that reaches the lung is increased compared with inhalation. Further, intratracheally instilled dust was less homogeneously distributed in the lung with little dust reaching the periphery (180). This may lead to local overload conditions and Pritchard *et al* recommended not to extrapolate dose-response relationships from animal studies that applied intratracheal instillation to the human condition (180). Such studies are therefore not considered further.

In some animal inhalation studies the applied dose was estimated by viable counts in lung tissue obtained shortly (up to 4 hours) after challenge. This method probably underestimates the applied dose to a large extent and such studies were also omitted in the dose-effect and dose-response considerations.

Due to differences between the lungs of humans and laboratory animals exposure levels applied in animal experiments should be extrapolated. These factors vary substantially from 0.03 to 7 and depend on dose metric and particle size (98). As the relevant dose metric is not clear no attempts were made to specify an extrapolation factor.

Results from human challenge studies were extrapolated from the dose that was inhaled during a few minutes to the average concentration over an 8-hour work shift. This may be an underestimation as "the concentration is often more important than duration of exposure" (98).

See also Chapter 10.1.

12.2 Effects related to single and short-term exposure

12.2.1 Animal studies

Only one out of 32 animal studies is relevant for evaluation of dose-effect and dose-response associations. In that study, Guinea pigs were exposed to airborne fungal spores for 4 hours/day, for 1 day or for 5 days/week during 3 and 5 weeks, a time frame not very different from workplace exposure (58). Four fungal and one actinomycete species were tested, but only exposure to $3 \cdot 10^8$ spores/m^3 of *Saccharopolyspora rectivirgula* showed increased neutrophil and eosinophil counts in bronchoalveolar lavage 24 hours after a single exposure for 4 hours. None of the fungal species induced a response after a single exposure. These results indicate that a single exposure to fairly high concentrations of four species, including *Aspergillus fumigatus* (approximately $3 \cdot 10^7$ spores/m^3 and similar or higher for the other fungi) for 4 hours do not induce inflammation in Guinea pigs. Table 16 summarises effects after exposures for 3 and 5 weeks. Only one dose level was applied except for *Aspergillus fumigatus* that was tested at two concentrations. Exposure levels were probably underestimated because spores

Table 16. LOELs of 5 fungal species in Guinea pigs exposed to spore aerosols for 4 hours/day, 5 days/week for 3 and 5 weeks (58).

Species	LOEL, spores/m^3	Effects
Aspergillus fumigatus	$7 \cdot 10^5$	After 3 as well as 5 w: BAL: Increased lymphocyte and eosinophil counts. Lung tissue: Slight cell infiltration of the alveoli.
Aspergillus fumigatus	$3 \cdot 10^7$	After 3 as well as 5 w: BAL: Increased AM, lymphocyte, neutrophil and eosinophil counts. Lung tissue: Cell aggression in the alveoli, alveolar wall thickening with interstitial cells and granuloma formation.
Rhizopus stolonifera	$2 \cdot 10^7$	After 3 as well as 5 w: BAL: Increased AM, neutrophil, lymphocyte and eosinophil counts. Lung tissue: Cell infiltration in alveoli and interstitial tissue, alveolar wall thickening.
Saccharopolyspora rectivirgula	$3 \cdot 10^8$	After 3 as well as 5 w: BAL: Increased AM, neutrophils, lymphocytes and eosinophils counts. Lung tissue: Cell infiltration in alveoli and interstitial tissue, alveolar wall thickening.
Penicillium aurantiogriseum	$1 \cdot 10^9$	After 5 w: BAL: Increased AM, neutrophils, lymphocyte and eosinophil counts. Lung tissue: Defined granulomas.
Phanerochaete chrysosporium	$2 \cdot 10^9$	After 5 w: BAL: Increased AM, neutrophil, lymphocyte and eosinophil counts. Lung tissue: Severe cell infiltration in alveoli and interstitial tissue, alveolar wall thickening and granuloma formation.

AM: alveolar macrophages, BAL: bronchoalveolar lavage, LOEL: lowest observed effect level.

were counted by microscopy after resuspension from membrane filters with a pore size of 0.8 μm. A fraction of the smaller spore types, e.g. from *Aspergillus* and *Penicillium* will be trapped in the filter matrix and lost in the analysis but this is not recognised in the paper (Chapter 10.5).

All species induced inflammatory changes in the lung after repeated exposure. The lowest LOEL was $7 \cdot 10^5$ spores/m^3 for *A. fumigatus* that induced slight lung inflammation. Extrapolating from 4 to 8 hours of exposure, this level corresponds to $4 \cdot 10^5$ spores/m^3.

It should be noted, however, that probably a smaller proportion of the inhaled spores reaches the alveolar region in rodents than in humans.

The following differences in inflammatory potency between the fungal and the actinomycete species can be deduced from these results: *A. fumigatus* was more

toxic than *Rhizopus stolonifera* and *Phanerochaete chrysosporium, and Penicillium aurantiogriseum* was more toxic than *P. chrysosporium*. Exposure to $3 \cdot 10^8$ *S. rectivirgula* spores/m^3 induced inflammation at a similar level as exposure to $2 \cdot 10^7$ *R. stolonifera* spores/m^3. Further differences between fungal species have been reported in other *in vivo* studies. Especially mycotoxin containing spores and spores from pathogenic fungi, e.g. *A. fumigatus*, showed the highest inflammatory potential (Chapters 10.4.5 and 10.9).

12.2.2 Challenge studies of symptomatic subjects

Human challenge studies with spores are summarised in Table 17. These studies indicate a LOEL above $1 \cdot 10^4$-$2 \cdot 10^4$ spores/m^3 for *Penicillium* species and *Alternaria alternata* in healthy subjects, as this level was the lowest that induced significant airway obstruction in asthmatic patients allergic to these fungi (125). The NOELs of $8 \cdot 10^3$ and $4 \cdot 10^3$ spores/m^3 observed for *Penicillium chrysogenum* and *Trichoderma harzianum*, respectively, in school employees with sick building syndrome (135) are consistent with the former study. Higher LOELs were found in mushroom workers with hypersensitivity pneumonitis, but these workers were only challenged with a single dose (35, 36).

12.2.3 Epidemiological studies

Epidemiological studies of effects such as short-term changes in lung function, changes in inflammatory markers in the airways, and studies where symptoms were recorded on the same day as exposure measurements were performed are considered here. Studies in highly contaminated environments are summarised in Table 18 and those in common indoor environments in Table 19. Only epidemiological studies fulfilling the inclusion criteria were included, i.e. exposure assessment based on personal sampling, objective outcomes, or self-reported symptoms if adjusted for confounders. The criteria were applied less stringent for studies of populations exposed to common indoor levels, as few studies were found. Cfu counts were multiplied by 10 in order to estimate spore counts (Chapters 5 and 11).

A study of wood trimmers showed a NOEL of $1 \cdot 10^5$ spores/m^3 and a LOEL of $3 \cdot 10^6$ spores for a 4-day change in FVC (76) (Table 18). Exposure to fungi was measured by culture. In the group with highest exposure, a decline in FVC was observed during the preceding 3 months but no exposure measurements were performed during that period. It seems unlikely, however, that exposure levels were very different from those that had been measured during the 4-day period (Chapter 11.4.2). In another study of Swedish wood trimmers, a LOEL of $1 \cdot 10^5$ spores/m^3 for a cross-week change in FEV_1 and MEF_{25} was found (38).

LOELs for nasal congestion and inflammatory markers in the nose of waste handlers were found at $2 \cdot 10^5$ spores/m^3 (79) (Table 18). NOELs of $2 \cdot 10^5$ and $6 \cdot 10^5$ spores/m^3 were found for inflammatory markers in induced sputum of the same population of waste handlers (80), and in the nose in a study of sawmill workers (n=11) (202), respectively.

LOELs for cough in waste handlers (78) and in farmers (49) were $3 \cdot 10^5$ and $3 \cdot 10^6$ spores/m^3, respectively (Table 18). In farmers, LOELs of $1 \cdot 10^5$ spores/m^3 for eye irritation and $3 \cdot 10^6$ spores/m^3 for nasal irritation were found (49). For fever LOELs of $1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3 were observed in cases of hypersensitivity pneumonitis, and $2 \cdot 10^9$ spores/m^3 in cases of ODTS (130) (Table 18).

Thus, at exposure levels of $1 \cdot 10^5$ spores of various fungi/m^3 both objective findings as well as symptoms emerge in populations working in highly contaminated environments. The findings in wood workers seem most reliable, as fungal spores are often the dominating agent besides wood dust. Studies in wood trimmers are particularly interesting as little wood dust is generated in the sorting and trimming department. Dust, endotoxin and terpene levels in trimming departments of Swedish sawmills have been shown to be low (38). Bacterial levels were similar to fungal levels. However, the counting of bacteria by fluorescence microscopy has been shown to have poor accuracy (48).

Only one study of effects related to short-term exposure in a population exposed to common indoor levels was found (203) (Table 19). This well-designed study examined inflammatory markers in nasal lavage and recorded irritation and systemic symptoms in schoolteachers, which indicated a NOEL of $7 \cdot 10^2$ spores/m^3 including exposure in the work and home environment.

12.3 Effects related to long-term exposure

Cross-sectional studies of effects such as lung function and respiratory symptoms are considered here where the effect registration is not linked individually to the day(s) that exposure was measured. The measured exposure is therefore expected to represent an average level over a certain time period. Studies in common indoor environments are summarised in Table 19 and those in highly contaminated environments in Table 20. The inclusion criteria used in Chapter 12.2.3 apply also here.

Increased albumin, fibronectin, and hyaluronan in bronchoalveolar lavage in 19 wood trimmers indicated low intensity inflammation compared to a control group of 25 healthy subjects without occupational exposure to fungi (all non-smokers) (100). The exposure level indicated a LOEL of $1 \cdot 10^5$ spores/m^3. The design was cross-sectional but no information on the exposure period was provided. It is therefore not clear whether these differences are effects of short or long-term exposure.

In a cross-sectional study of sawmill, joinery, and chip mill workers LOELs for respiratory symptoms such as phlegm, throat irritation, and breathlessness all ranged from $1 \cdot 10^5$ to $4 \cdot 10^5$ spores/m^3 (6). In another cross-sectional study of wood trimmers morning cough indicated a LOEL of $2 \cdot 10^6$ spores/m^3 (54). This is probably a high estimate because the population did not include individuals exposed below $4 \cdot 10^5$ spores/m^3.

Exposure levels that correlated with X-ray abnormalities in cork workers were approximately 10^7 spores/m^3 (9). As hypersensitivity pneumonitis was relatively prevalent (4%) a similar LOEL is indicated for this outcome as well. Restrictive

lung function was correlated with duration of employment of the cork workers indicating an association with cumulative exposure at the level of 10^7 spores/m^3. There was, however, limited information and uncertainty about previous exposure levels in this study (9).

In farmers, a LOEL of 10^7 spores/m^3 was found for asthma in non-atopic farmers (50) but this level may have been attenuated by substantial random errors in the estimated exposure.

Thus, both objective findings as well as respiratory symptoms begin to appear at exposure levels of $\geq 1 \cdot 10^5$ spores of various fungi/m^3 in populations exposed to highly contaminated environments. In cork workers, hypersensitivity pneumonitis, X-ray changes and restrictive lung function were observed at an exposure level of $1 \cdot 10^7$ spores/m^3, mainly from *Penicillium glabrum*.

In an epidemiological study of a fairly large population (n=264) of randomly selected day-care centres exposed to common indoor levels a LOEL of 10^4 spores/m^3 of various fungi for nasal symptoms was found (124).

A small cluster of asthma and rhinitis cases in a small population of hospital workers had positive bronchial challenge tests to *Sporobolomyces salmonicolor*. Exposure levels were approximately 10^4 spores/m^3 (50% *S. salmonicolor*) in the employees and could indicate a LOEL in sensitive individuals. However, exposure was poorly described (217). In a case-control study, work-related respiratory symptoms were associated with approximately 10 *Alternaria* spores/m^3. No exposure levels to total fungi or other species were reported (134).

These studies in common indoor environments indicate lower effect levels than the studies of highly exposed populations. However, the exposure assessment in the former studies was insufficient due to stationary sampling and few or an unknown number of measurements. Furthermore, these LOELs are similar to the LOELs found by bronchial provocation in a susceptible group of asthmatic patients allergic to the fungi used in the challenge, which is not very likely since LOELs are expected to be higher in healthy subjects. There is therefore insufficient support for the assumption that health effects are induced at lower fungal levels in common indoor environments than in highly contaminated environments.

Table 17. Effects observed in symptomatic subjects in challenge studies with whole spores.

NOEL	LOEL	Species	Study group	Effects	Comments	Reference
spores/m^3						
$8 \cdot 10^3$ [a] $4 \cdot 10^3$ [a]		*Penicillium chrysogenum* *Trichoderma harzianum*	8 school employees with sick building syndrome	No changes in mucosal symptoms, systemic symptoms, lung function and blood leukocytes.	Provocation tests compared to sham exposure of the same subjects.	(135)
	$1 \cdot 10^4$ [b]	*Penicillium* sp.	3 asthma patients	Immediate and late decrease in airway conductance.	Provocation tests with increasing dose with steps of 10x and interpolation between doses to find a 35% decrease in specific airway conductance.	(125)
	$2 \cdot 10^4$ [b]	*Alternaria alternata*	4 asthma patients (occupation unknown in both groups)	Immediate and late decrease in airway conductance.		
	$2 \cdot 10^6$ [b]	*Pleurotus ostreatus*	4 mushroom workers with hypersensitivity pneumonitis	After 6-8 h fever, chills, muscle pain, dyspnoea, leukocytosis, decreased FVC.	Provocations tests with a single dose.	(35)
	$6 \cdot 10^6$ [b]	*Lentinus edodes*	5 mushroom workers with hypersensitivity pneumonitis	After 6 h fever, chills, muscle pain, dyspnoea, leukocytosis and decreased FVC. TLco and PaO_2 decreased in 1/5.	Provocation tests with a single dose.	(36)

[a] Calculated to equal an 8-hour exposure by the weight factor 8 hours/exposure time.
[b] Calculated to equal an 8-hour exposure by dividing the applied dose with 5 m^3 assuming a minute ventilation of 10 l/min during 8 hours.
FVC: forced vital capacity, LOEL: lowest observed effect level, NOEL: no observed effect level, PaO_2: arterial oxygen tension,
TLco: lung transfer factor for carbon monoxide (measure of gas diffusion).

Table 18. Effects of short-term exposure in epidemiological studies of workers exposed to highly contaminated environments.

NOEL	LOEL	Species	Study group	Effects	Comments	Reference
spores/m^3						
$1 \cdot 10^5$ [a]	$3 \cdot 10^6$ [a]	Mainly *Rhizopus*	29 wood trimmers	Decline in FEV_1 and FVC during 4 days and 3 months	Follow-up study, adjusted for individual factors by studying lung function change, only IgG antibodies to *Rhizopus* were detected, exposure measured by culture methods during all days.	(76)
	$1 \cdot 10^5$ [a]	Mainly *Rhizopus* and *Paecilomyces*	28 wood trimmers	Decline in FEV_1 and MEF_{25} during 1 week	Adjusted for individual factors by studying lung function change, species were detected by culture, exposure measured on one day, bacteria were measured but not evaluated. Dust, endotoxin and terpene levels were low.	(38)
	$1 \cdot 10^5$ [b]	Various fungi	89 farmers	Eye irritation	Cross-sectional study, adjusted for confounders and other bioaerosol agents, acute symptoms and exposure recorded simultaneously.	(49)
$2 \cdot 10^5$		Various fungi	25 municipal waste handlers	Change in inflammatory cells and mediators in induced sputum after 3 days of exposure	Cross-sectional study, qualitative evaluation of smoking and age, other bioaerosol agents were studied in separate models, exposure recorded during days between sputum inductions.	(80)
	$2 \cdot 10^5$	Various fungi	31 municipal waste handlers	Increased neutrophils in nasal lavage and nasal congestion after 3 days of exposure	Cross-sectional study, qualitative evaluation of smoking and age, other bioaerosol agents were studied in separate models, exposure recorded during days between nasal lavages.	(79)
	$3 \cdot 10^5$	Various fungi	22 municipal waste handlers	Cough	Cross-sectional study, qualitative evaluation of smoking and age, other bioaerosol agents were studied in separate models and were more weakly associated with cough, acute symptoms and exposure were recorded simultaneously.	(78)
$6 \cdot 10^5$		Mainly *Rhizopus* and *Penicillium*	11 sawmill workers	Inflammatory markers in nasal lavage	Compared workers at work and on holiday, no adjustments for confounding, other bioaerosol agents were measured.	(202)

Table 18. *Cont.* Effects of short-term exposure in epidemiological studies of workers exposed to highly contaminated environments.

NOEL (spores/m^3)	LOEL (spores/m^3)	Species	Study group	Effects	Comments	Reference
	$3 \cdot 10^{6}$ [b]	Various fungi	89 farmers	Cough, nasal irritation	Cross-sectional study, adjusted for confounders and other bioaerosol agents, acute symptoms and exposure were recorded simultaneously.	(49)
	$1 \cdot 10^{8}$-$4 \cdot 10^{8}$	Various fungi and actinomycetes	4 farmers with hyper-sensitivity pneumonis	Fever attacks	Cross-sectional study, no confounder adjustment, confirmed diagnosis, exposure was measured when the work causing a fever attack was repeated.	(130)
	$2 \cdot 10^{9}$	Various fungi and actinomycetes	6 farmers with ODTS	Fever attacks		

[a] Estimated from culture counts by multiplying with a factor of 10.
[b] Calculated to equal an 8-hour exposure if exposure time was less than 8 hours by multiplying with the weight factor 8 hours/exposure time.
FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, Ig: Immunoglobulin, LOEL: lowest observed effect level,
MEF_{25} maximum expiratory flow at 25% of FVC, NOEL: no observed effect level, ODTS: organic dust toxic syndrome.

Table 19. NOELs and LOELs observed in epidemiological studies of populations exposed to common indoor environments.

NOEL (spores/m^3)	LOEL (spores/m^3)	Species	Study group	Effects	Comments	Reference
	$1 \cdot 10^{1}$ [a]	*Alternaria*	107 symptomatic and 107 non-symptomatic office workers	Work-related respiratory symptoms.	Matching on age, gender, and atopy. Adjusted for smoking, house dust mite allergen in floor dust and indoor/outdoor humidity. Not clear if personal sampling was applied. No data on other fungi, total fungi and the number of collected samples were given.	(134)
$7 \cdot 10^{2}$ [a,b]		Various fungi	41 teachers	No changes in nasal lavage: NO, IL-4, IL-5, IL-6, and TNFα. No nasal, eye, throat irritation, lower airways symptoms, and non-specific symptoms.	Population randomly selected. Personal exposure measurements at work and at home. Exposure to bacteria was also studied. Symptoms of the last week were recorded and nasal lavages were done at the end of the sampling period. Confounder assessment by separate analyses in asthmatic and smoking sub-groups.	(203)
	$1 \cdot 10^{4}$ [a]	Various fungi	264 day-care workers from 28 centres	Nasal discharge and congestion.	Day-care centres randomly selected. Stationary measurements and number of measurements not given. Exposure variables not specified in models. Adjusted for age, gender, and education. Separate models with other fungal species, total culturable bacteria, and house dust allergens on settled dust did not show associations.	(124)
	$3 \cdot 10^{2}$ [a]	*Aspergillus fumigatus*		Nasal congestion, cough and phlegm, lethargy, fatigue.		
	$1 \cdot 10^{4}$ [a]	Various fungi (50% *Sporobolomyces salmonicolor*)	14 hospital workers with asthma (n=4) and rhinitis (n=11)	Asthma and rhinitis symptoms.	IgG positive but skin prick test negative to *S. salmonicolor*. Positive provocation tests with extract of *S. salmonicolor*. Stationary measurements at 10 locations. Number of measurements not given.	(217)

[a] Estimated from culture counts by multiplying with a factor of 10.
[b] Including exposure in the work and the home environment.
IL: interleukin, Ig: immunoglobulin, LOEL: lowest observed effect level, NO: nitric oxide, NOEL: no observed effect level, TNFα: tumour necrosis factor alpha.

Table 20. Effects of long-term exposure in epidemiological studies of workers exposed to highly contaminated environments.

NOEL (spores/m^3)	LOEL (spores/m^3)	Species	Study group	Effects	Comments	Reference
	$1 \cdot 10^5$	Various fungi	19 wood trimmers and 25 controls	Inflammatory markers in bronchoalveolar lavage	All non-smokers. No adjustments for confounding or bacteria (that was measured).	(100)
$1 \cdot 10^5$		Various fungi	19 wood trimmers and 25 controls	FEV_1 and FVC	As above.	(100)
	$1 \cdot 10^5$ [a]	Various fungi	82 joinery workers	Phlegm	Cross-sectional study, adjustments for confounders, other bioaerosol agents studied in separate models were more weakly associated with the effect and were not strongly correlated with fungal exposure. Exposure was measured by culture methods.	(6)
	$3 \cdot 10^5$ [a]	Various fungi	82 joinery workers and 108 saw-/chip mill workers	Throat irritation	As above.	(6)
	$4 \cdot 10^5$ [a]	Various fungi	108 sawmill and chip mill workers	Breathlessness	As above.	(6)
	$2 \cdot 10^6$	Various fungi (42% *Rhizopus microsporus*)	107 wood trimmers	Morning cough	Cross-sectional study, adjusted for confounders and wood dust. Lowest exposure $3 \cdot 10^5$ spores/m^3.	(54)
	$1 \cdot 10^7$	*Penicillium glabrum*	648 cork workers	Hypersensitivity pneumonitis, X-ray changes; restrictive lung function related to years employed	Large cross-sectional study, adjustments for smoking (only X-ray changes). Prevalences of the outcomes were 4, 57 and 30%, respectively. No information on previous exposure.	(9)
	$1 \cdot 10^7$	Various fungi	1 614 farmers	Non-atopic asthma	Large cross-sectional study, adjustments for confounders and endotoxins (correlated with fungi). Exposure was estimated with considerable random error.	(50)

[a] Estimated from culture counts by multiplying with a factor of 10.
FEV_1: forced expiratory volume in one second, FVC: forced vital capacity, IL: interleukin, LOEL: lowest observed effect level, NOEL: no observed effect level.

13. Previous evaluations by national and international bodies

NEG evaluated microorganisms in 1991 but did not find a scientific basis for criteria or guidelines for fungal spores (128).

The Russian Federation is the only nation that has adopted official occupational exposure limits (OELs) for microorganisms. The Russian maximum allowable concentrations are given for specific fungi and actinomycetes (227). These limits appear to be based on allergenicity in animal models. The list is shown in Appendix 9. The limits range from 10^3 to 10^4 cfu/m^3, which may correspond to 10^4 to 10^5 spores/m^3. However, many of the listed species are used for production of food and antibiotics and probably have high viability when freshly cultivated. A factor of 10 for conversion of cfu to spores may therefore be too high. The species are classified according to hazard and allergenicity but no information is provided on the classifications.

The current opinion of the American Conference of Governmental Industrial Hygienists (ACGIH) is that the establishment of threshold limit values (TLVs) for total culturable or countable bioaerosols is not possible (2). Their most important arguments are: 1) that bioaerosols are generally complex mixtures of many agents, 2) the diversity of responses to microorganisms, and 3) insufficient information on exposure-response relationships.

Rao *et al* reviewed quantitative standards and guidelines for airborne fungi and found that these recommendations were primarily based on the low exposure levels in "normal" environments given either as absolute or relative levels by comparing to outdoor levels, and not on health effects data (186). A number of guidance documents have been published, e.g. by the ACGIH (1), AIHA (4), Health Canada (75), and Institute of Medicine in the United States (95). Many of these guidelines contain recommendations on prevention because dampness-related symptoms can be remediated by reducing humidity without knowledge of the causative agent.

14. Evaluation of human health risks

14.1 Assessment of health risks

14.1.1 *Airway and lung inflammation*

Only one animal study provides relevant information on effects of single exposure (58). This study showed no lung inflammation after exposure for 4 hours to $3 \cdot 10^7$ spores/m^3 of *Aspergillus fumigatus* and three other fungal species. However, neutrophil and eosinophil counts increased in bronchoalveolar lavage after a single exposure to $3 \cdot 10^8$ spores/m^3 of the actinomycete *Saccharopolyspora rectivirgula*. An exposure level of $3 \cdot 10^8$ spores/m^3 seems very high, but the high incidence of ODTS attacks among college students who participated in a party in a room where the floor was covered with mouldy straw (20) (Chapter 11.3) suggests that such exposure situations may occur.

Lung inflammation was induced by all tested fungal and actinomycete species in the animal study described above when exposures were repeated daily for 5 weeks. The lowest LOEL was $4{\cdot}10^5$ spores/m^3 for *Aspergillus fumigatus* (calculated to equal an 8-hour exposure) (58).

Four epidemiological studies in waste handlers (79, 80), sawmill workers (202) and wood trimmers (100) indicate NOELs at $2{\cdot}10^5$-$6{\cdot}10^5$ spores/m^3 and LOELs at somewhat lower levels $1{\cdot}10^5$-$2{\cdot}10^5$ spores/m^3. The study of waste handlers has the strongest design, as exposure was measured on three days between nasal lavages and sputum inductions. However, inflammatory markers and cells in induced sputum showed no association with fungal spores indicating a NOEL of $2{\cdot}10^5$ spores/m^3, while nasal lavage showed a LOEL of $2{\cdot}10^5$ spores/m^3 for increased neutrophil counts. The effects of exposure to endotoxins, glucans, and bacteria, and the potential confounders smoking, age and atopy were evaluated qualitatively and could not explain the associations with fungal exposure. All studies were relatively small, however, and there is a considerable risk of false-negative results. Furthermore, as methods for sputum induction and nasal lavage were recently developed, little is known about the development of the inflammation over time, which complicates interpretation of the results.

14.1.2 Respiratory function

Two human challenge studies present information on effect levels regarding respiratory function. In a single dose study of school employees with sick building syndrome no changes in mucosal and systemic symptoms, lung function, and blood leukocytes were found after challenge with $8{\cdot}10^3$ *Penicillium chrysogenum* spores/m^3 or $4{\cdot}10^3$ *Trichoderma harzianum* spores/m^3 (135). In the other study, asthmatic patients were exposed to successively increasing doses, and a reduced airway conductance was found after challenge with $1{\cdot}10^4$ *Penicillium* sp. spores/m^3 and $2{\cdot}10^4$ *Alternaria alternata* spores/m^3 (125). The patients had specific IgE to the fungi used in the test, while the sensitisation of the school employees was not clear (positive basophil histamine-release tests but negative skin prick tests and RASTs).

In a Swedish study, wood trimmers exposed to $1{\cdot}10^5$ spores/m^3 showed no changes in FVC and FEV_1 after 4 days and 3 months, respectively (76). In the same study, wood trimmers from another sawmill exposed to $3{\cdot}10^6$ spores/m^3 showed FVC and FEV_1 declines both after 4 days with exposure measurements, and during the 3 preceding months. It seems unlikely that exposure levels were very different from those that had been measured previously (Chapter 11.4.2). An exposure-response association for changes in FVC and MEF_{25} after 3-5 days exposure to a median of $1{\cdot}10^5$ spores/m^3 was found in another study of Swedish wood trimmers (38). A LOEL of $2{\cdot}10^5$ spores/m^3 was found for increased nasal congestion after 3 days of exposure among waste handlers (79). In cork workers exposed to a mean level of approximately 10^7 spores/m^3, exposure duration was associated with restrictive lung function (9). However, limited information on current and previous exposure levels, and outcomes complicates interpretation of the latter study.

The two Swedish studies of wood trimmers reported different effect levels. A NOEL of $1{\cdot}10^5$ spores/m^3 for short-term FVC and FEV_1 decline in wood trimmers may be based on the study by Hedenstierna *et al* (76), whereas Dahlquist *et al* (38) report an exposure-response association at the same level for FEV_1 and MEF_{25} decline during a week. This may indicate that effects start to appear at exposure to $1{\cdot}10^5$ spores/m^3. The estimate in the latter study is more reliable as fungal spores were counted by SEM. This LOEL is consistent with NOELs of $4{\cdot}10^3$-$8{\cdot}10^3$ spores/m^3 observed in a single dose human challenge study (135), and with the LOELs of $1{\cdot}10^4$-$2{\cdot}10^4$ spores/m^3 found in the human challenge study of asthmatic patients (125) since LOELs are expected to be higher in healthy subjects. The LOEL for short-term increase in nasal congestion in waste handlers of $2{\cdot}10^5$ spores/m^3 (79) is also in the same region.

14.1.3 Respiratory symptoms

No respiratory and general symptoms were observed in the human challenge study of school employees after challenge with $8{\cdot}10^3$ *Penicillium chrysogenum* spores/m^3 or $4{\cdot}10^3$ *Trichoderma harzianum* spores/m^3 calculated to equal an 8-hour exposure (nasal and throat irritation, nasal congestion and headache were examined) (135). LOELs of $1{\cdot}10^5$-$4{\cdot}10^5$ spores/m^3 for short-term and long-term respiratory symptoms such as cough, eye irritation, throat irritation, phlegm and bronchitis were observed in three epidemiological studies of farmers, waste handlers, and joinery, sawmill and chip mill workers (6, 49, 78). Cough in wood trimmers and farmers, and nasal irritation in farmers indicated higher LOELs of $2{\cdot}10^6$-$3{\cdot}10^6$ spores/m^3 (49, 54). However, the wood trimmer population did not contain workers exposed to less than $4{\cdot}10^5$ spores/m^3, which precluded the exploration of symptoms at lower exposure levels. The LOEL of work-related cough in the farmers study seems genuine as exposure to other bioaerosol components was measured but these agents were not associated with cough and the internal reference group was exposed to $2{\cdot}10^3$-$2{\cdot}10^4$ spores/m^3 (49). In this study the risk of nasal irritation was already high at an exposure level of $1{\cdot}10^5$ spores/m^3 although not significantly (OR 4.1, 95% CI 0.88-19).

Thus, most associations with respiratory symptoms indicate LOELs of $1{\cdot}10^5$-$4{\cdot}10^5$ spores/m^3, which is similar to the LOELs indicated for short-term respiratory function decline. However, associations with cough and possibly nasal irritation in farmers indicate higher LOELs of $2{\cdot}10^6$-$3{\cdot}10^6$ spores/m^3.

14.1.4 Asthma

Only one epidemiological study related the prevalence of current physician-diagnosed asthma in non-atopic farmers to exposure to fungal spores and other agents (50). Annual exposure levels were estimated from task-based measurements, which is likely to have introduced substantial random error. The observed LOEL of 10^7 spores/m^3 is therefore most likely a high estimate. In addition, endotoxins and ammonia were also associated with asthma and could not be adjusted for due to high correlation between exposure measures.

In atopic farmers, an inverse exposure-response association was found (50). Studies from different countries have shown that the occurrence of atopic diseases in farming populations is lower than in the general population, especially in children. This seemingly protective effect is associated with contact with farm animals, and it was postulated in the so-called hygiene hypothesis that exposure to microbial agents, especially endotoxins from Gram-negative bacteria, mediated this protective effect; review by Schaub *et al* (211). The results of the farmer study indicate that such a protective effect may also result from exposure to the farm environment as an adult and that also exposure to fungal spores can be involved (50). It is also possible, however, that farmers with allergic asthma change their production, methods and/or tasks to lower exposure, or even quit farming, which would select asthmatic farmers away from highly exposed farm work (healthy worker effect).

14.1.5 Hypersensitivity pneumonitis and organic dust toxic syndrome

Single dose human challenge studies of patients with hypersensitivity pneumonitis found LOELs for typical attacks of the disease at $2 \cdot 10^6$ *Pleurotus ostreatus* spores/m^3 and $6 \cdot 10^6$ *Lentinus edodes* spores/m^3, calculated to equal an 8-hour exposure, respectively (35, 36). These LOELs are lower than the levels that induced fever attacks in farmers with a clinically confirmed diagnosis of the disease, $1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3 (130). The farmer study also indicates that fever attacks in farmers with ODTS develop at approximately 10 times higher exposure levels ($2 \cdot 10^9$ spores/m^3) than in farmers with hypersensitivity pneumonitis (130). Further information is obtained from a study of cork workers exposed to $1 \cdot 10^7$ spores/m^3 (mainly *Penicillium glabrum*) where 4% of the population had hypersensitivity pneumonitis and 57% X-ray changes, a typical observation in hypersensitivity pneumonitis patients, which correlated with current exposure levels (9).

These data indicate that attacks of hypersensitivity pneumonitis may develop after exposure to $2 \cdot 10^6$-$6 \cdot 10^6$ spores/m^3 of a single species, and that the disease can develop after long-term exposure to $1 \cdot 10^7$ spores/m^3. Higher LOELs were reported in the farmer study ($1 \cdot 10^8$-$4 \cdot 10^8$ spores/m^3). However, farmers were exposed to spores from various species. As hypersensitivity pneumonitis seems to be species specific (Chapter 11.2.1) the exposure level of the species that the farmers were sensitised to is likely to be lower. It cannot be ruled out, however, that the potential to induce hypersensitivity pneumonitis differs between species.

14.1.6 Studies in common indoor environments

The evidence from epidemiological studies of populations exposed to common indoor air is insufficient for conclusions about effect levels to be drawn, except for one well-designed study (203) indicating a NOEL for inflammation in the nose and respiratory symptoms of $7 \cdot 10^2$ spores/m^3 including exposure at home and at work (Chapters 12.2 and 12.3).

14.1.7 The role of specific organisms
The animal study by Fogelmark *et al* (58) shows that all tested species are capable of inducing inflammation in the airways, but differ in inflammatory potential. This is further documented in many other animal studies (Chapter 10.9). The limited number of human challenge studies with intact spores provide little information on species differences. A few epidemiological studies of long-term effects have analysed the role of specific fungi (Chapter 11.5.2). Stronger associations have been found for *Aspergillus fumigatus* in a waste handler study (31) and for *Aspergillus* and *Alternaria* sp. in common indoor air studies (124, 134). However, these studies suffer from weaknesses in the exposure assessment. The extremely low LOEL of 10 *Alternaria* spores/m^3 in the study by Menzies *et al* (134) is highly unlikely considering the challenge study by Licorish *et al* (125) where a LOEL of $2{\cdot}10^4$ spores/m^3 was found in asthmatic patients allergic to the fungus. The implicated species in these epidemiological studies represented only a fraction of the total number of fungi. In other studies, a single species dominated, e.g. *Penicillium glabrum* in a study of cork workers (9), *Rhizopus microsporus* in a study of wood trimmers (54), and *Sporobolomyces salmonicolor* in a common indoor air study (217). These studies do not indicate lower LOELs to the dominating species than observed in studies where species characterisation was not performed.

Little information is available about actinomycetes. The animal study by Fogelmark *et al* (58) indicated that a 10 times higher exposure level of spores of the actinomycete *Saccharopolyspora rectivirgula* induced inflammation in lung tissue of similar intensity as spores of the fungus *Rhizopus stolonifer* after prolonged exposure (Table 10). However, after a single exposure only *S. rectivirgula* and none of the fungal species induced inflammation. Human challenge studies have further documented that *Saccharopolyspora rectivirgula* extracts can induce attacks of hypersensitivity pneumonitis and asthma in farmers' lung patients (Tables 11-12) (55, 178). *In vitro* and *in vivo* studies further document that spores from different actinomycetes species can induce airway and lung inflammation similarly to fungal spores. It is therefore reasonable to assume that the health effects following exposure to actinomycete spores do not differ qualitatively from fungal spores, but data on effect levels are lacking.

14.2 Groups at extra risk

Patients with hypersensitivity pneumonitis respond to fungal exposure with febrile attacks at a lower level than individuals without the disease. However, the exposure levels that induce typical attacks of the disease are relatively high, $\geq 2{\cdot}10^6$ spores/m^3.

Patients with allergic asthma to fungi may have an increased risk. LOELs of $1{\cdot}10^4$-$2{\cdot}10^4$ spores/m^3 found for airway obstruction in allergic asthmatics are one order of magnitude lower than LOELs found in epidemiological studies of lung function in healthy subjects. Children should also be regarded as a vulnerable

group because they have a higher prevalence of allergic asthma to fungi; review by Bush and Portnoy (25).

The role of atopy is less clear. In a farmer study, asthma decreased with exposure to fungal spores and other agents in atopic subjects (50). This may be due to selection, but also the hygiene hypothesis offers an alternative explanation.

No information was identified on sex differences and immunodeficient people. However, fungal infections may be life threatening in immunocompromised cancer patients undergoing treatment; review by Richardson (196).

14.3 Scientific basis for an occupational exposure limit

In vitro and *in vivo* studies have shown that a large number of fungal and actinomycete species are capable of inducing inflammatory effects in phagocytic cells and lung tissue. There is also evidence from those studies that the inflammatory potency depends on species, with the mycotoxin producing *Stachybotrys chartarum* and the facultative pathogenic *Aspergillus fumigatus* showing the highest potencies. However, epidemiological studies of various respiratory outcomes indicate fairly similar potencies when comparing LOELs expressed as spores/m^3. Thus, the inflammatory potency of most species occurring in the work environment seems to be fairly similar. Furthermore, fungi with high potency have to constitute a substantial fraction of the fungal biota before a significant change in inflammatory potential can be expected.

The response of subjects with hypersensitivity pneumonitis as well as asthma is species specific as shown by human challenge studies using fungal extracts and whole spores. It cannot be derived from these findings, however, that the allergenic potential differs between species because the exposure levels leading to allergy is not known. For example, *Cladosporium*, *Alternaria,* and *Aspergillus* species are most often involved in fungal allergy but dominate also in outdoor air (115).

A series of studies including various fungal species suggests that respiratory symptoms, airway inflammation, and lung function impairment begin to appear at exposure levels of approximately 10^5 spores/m^3.

The lung function studies in wood trimmers are most interesting because the effect is objectively measured and wood trimmers are primarily exposed to fungal spores besides low levels of wood dust, bacteria, endotoxins, and terpenes. The effect of agents other than fungal spores is therefore limited. The effect of confounders was evaluated separately (smoking) or was eliminated by the follow-up design (age, height and body weight). The study by Dahlquist *et al* (38) is preferred, as fungal spores were measured by microscopic counting. A relationship was found between exposure to fungal spores and FEV_1 decline in a population exposed to a median of $1 \cdot 10^5$ spores/m^3. This level is also compatible with NOELs of $4 \cdot 10^3$-$8 \cdot 10^3$ spores/m^3 (calculated to equal an 8-hour exposure) observed in the human challenge study in which subjects inhaled a single concentration during 6 minutes (135), and with NOELs of $1 \cdot 10^4$-$2 \cdot 10^4$ spores/m^3

(calculated to equal an 8-hour exposure) found in the human challenge study where asthmatic patients inhaled graded doses by single inhalations (125).

An OEL based on the study by Dahlquist *et al* (38) is likely to represent a range of fungal species as studies of other populations suggest LOELs of similar magnitude. However, such a limit is not applicable if spores from mycotoxin producing and/or opportunistic pathogenic species are prevalent.

As the fungal spore is the unit of interest in this document, exposure assessments should be based on non-culture methods. Microscopical methods seem most suitable at present. Characterisation of the fungal biota can be achieved by cultivation and by molecular biological methods. However, the presence of other airborne fungal particles as hyphae and fungal fragments have been demonstrated (Chapters 3.3 and 3.6), but their role in respiratory disease is not clear as no animal or epidemiological studies addressing these particles have been published. *In vitro* studies suggest that the response to hyphae is different from the response to spores, but the studies have only been performed with pathogenic fungi (Chapter 8.1).

14.4 Evaluations in common indoor environments

Very few studies of acceptable quality have been found of populations exposed to common indoor environments. However, the airborne fungal biota found in such environments do not oppose the application of effect levels based on studies in highly contaminated environments on common indoor populations as well. Furthermore, in a challenge study of individuals with sick building syndrome NOELs of $4 \cdot 10^3$-$8 \cdot 10^3$ spores/m^3 were found (135). This NOEL is consistent with effect levels in highly contaminated environments since only a single dose level was applied in the human challenge study and higher NOELs are not precluded.

It is now generally accepted that dampness in buildings is related to respiratory effects, and the measurement of airborne fungal spores is just one of several ways to detect dampness (16). Several strategies are available for the evaluation of "mould problems" and many criteria have been proposed to evaluate common indoor environments (186) (Chapter 13). These criteria specify much lower levels of airborne fungi than the LOELs found in this review, but the criteria are only indirectly related to health effects as they have been proposed to identify "sick buildings".

15. Research needs

More information is needed on NOELs of spores from specific fungi at different dose levels, including mycotoxin producing and opportunistic pathogenic species. This requires animal studies as human challenge with whole spores is generally regarded unethical (Chapter 11.2.1). Studies should be carried out with aerosol exposure over extended periods.

Molecular biological methods seem well suited for non-culture based assessment of fungi. Development of quantitative methods for the measurement of specific organisms and genera is therefore needed to improve the exposure assessment in future studies. Even chemical markers of fungi can be considered, especially agents shown to have inflammatory properties such as $\beta(1\rightarrow3)$-glucans (42).

The few occupational studies that have examined allergic outcomes show contradictory results. In some highly exposed populations sensitisation to prevalent fungal species is almost absent, whereas fungal allergy is not uncommon in the general population and in working populations exposed to fungal enzymes (Chapters 3.5 and 11.2.2). There is some information in the experimental studies that deserves more attention, however. In animal studies, viable spores induced allergic responses in contrast to non-viable spores, and repeated exposure studies also demonstrated eosinophilic inflammation in addition to neutrophils and macrophages (Chapter 10.9). Mechanistic studies have shown that dendritic cells prime a non-allergic response to spores and an allergic response to hyphae (Chapter 8.1). The allergic response to viable spores and to hyphae can be due to the production of allergens by germinating spores and growing hyphae (Chapter 3.5). Furthermore, Sercombe *et al* recently demonstrated that germinating spores in the nasal cavity of healthy subjects were common (216). It is therefore important to develop methods for quantification of viable spores and hyphae and apply these in epidemiological studies.

16. Summary

Eduard W. *The Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals. 139. Fungal spores.* Arbete och Hälsa 2006;21:1-145.

Fungal spores are ubiquitous in the environment. Exposure levels in workplaces where mouldy materials are handled are much higher than in common indoor and outdoor environments. Febrile attacks of organic dust toxic syndrome (ODTS) and hypersensitivity pneumonitis may occur after episodes with excessive exposure. In common indoor environments irritation symptoms have been ascribed to fungi at much lower exposure levels, especially in damp buildings.

The specificity of the response to fungi in hypersensitivity pneumonitis and asthma patients has been clearly demonstrated by bronchial provocation with the suspected organisms. *In vitro* and animal studies have further shown differences in the inflammatory potential of fungal species. In animal studies, non-allergic responses dominated after a single spore dose but allergic responses, especially to viable spores, were also observed. Repeated exposures induced both non-allergic and allergic pulmonary inflammation.

Two human challenge studies with whole spores provide information on effect levels. A single dose study of subjects with sick building syndrome indicated no observed effect levels (NOELs) of $4{\cdot}10^3$ spores/m^3 for *Trichoderma harzianum* and $8{\cdot}10^3$ spores/m^3 for *Penicillium chrysogenum*. The investigated effects were lung function impairment, mucosal and systemic symptoms and inflammatory cells in the blood. A study of asthmatic patients allergic to *Penicillium* sp. or *Alternaria alternata* showed lowest observed effect levels (LOELs) for reduced airway conductance of $1{\cdot}10^4$ and $2{\cdot}10^4$ spores/m^3, respectively.

In epidemiological studies of highly exposed populations lung function decline, respiratory symptoms and airway inflammation began to appear at exposure levels of 10^5 spores/m^3. LOELs of $1{\cdot}10^5$-$4{\cdot}10^5$ spores/m^3 have been reported in wood workers predominantly exposed to fungal spores and wood dust. The only well-designed study on populations exposed to common indoor air indicates a NOEL of $7{\cdot}10^2$ spores/m^3. The investigated effects were inflammatory markers in nasal lavage, and eye, throat, lower airways and non-specific symptoms. Fungal species were seldom identified in the epidemiological studies.

In conclusion, the combined evidence from human challenge and epidemiological studies support fairly consistent LOELs of approximately 10^5 spores/m^3 for diverse fungal species in non-sensitised populations. However, identification of the fungal biota is required in order to document the occurrence of the more toxic mycotoxin containing species and pathogenic species before measurements are evaluated.

Further epidemiological studies of allergic responses to fungal spores and effects of pathogenic and mycotoxin containing species are recommended.

Keywords: actinomycetes, fungal spores, hypersensitivity pneumonitis, irritation, occupational exposure limit, organic dust toxic syndrome, review, toxicity

17. Summary in Norwegian

Eduard W. *The Nordic Expert Group for Criteria Documentation of Health Risks from Chemicals. 139. Fungal spores.* Arbete och Hälsa 2006;21:1-145.

Soppsporer finnes overalt i miljøet. I arbeidsmiljø hvor materialer med soppvekst håndteres kan eksponeringen for soppsporer bli mye høyere enn det som er vanlig innendørs og utendørs. Typiske feberanfall av allergisk alveolitt og "organic dust toxic syndrome" kan utløses etter massiv eksponering for soppsporer. Men også i arbeidsmiljø med mye lavere eksponering er sopp blitt assosiert med plager, for eksempel i fuktige bygninger.

Bronkial provokasjon av pasienter med allergisk alveolitt eller astma med mistenkte organismer har vist tydelig at responsen er artsspesifikk. *In vitro* og dyreforsøk har videre vist forskjeller i inflammatorisk potensial hos ulike sopparter. Hos dyr eksponert én gang dominerte ikke-allergiske responser. Allergiske responser ble også observert, spesielt på viable sporer, sporer som inneholder mykotoksiner, og etter gjentatte eksponeringer.

Provokasjon av personer med "sick building syndrome" som ble eksponert én gang viste 'no observed effect levels' (NOELs) på $4 \cdot 10^3$ sporer/m^3 for *Trichoderma harzianum* og $8 \cdot 10^3$ sporer/m^3 for *Penicillium chrysogenum.* Lungefunksjon, slimhinne og systemiske symptomer og inflammatoriske celler i blod ble undersøkt. Provokasjon av pasienter med astma som var allergiske for *Penicillium* sp. eller *Alternaria alternata* med økende doser viste 'lowest observed effect levels' (LOELs) for redusert luftveismotstand ved henholdsvis $1 \cdot 10^4$ sporer/m^3 og $2 \cdot 10^4$ sporer/m^3.

I epidemiologiske undersøkelser av høyt eksponerte yrkesgrupper ble nedsatt lungefunksjon, luftveissymptomer og luftveisinflammasjon funnet når eksponeringsnivået overskred 10^5 sporer/m^3. Undersøkelser av trearbeidere som er hovedsakelig eksponert for soppsporer og trestøv viser LOELs på $1 \cdot 10^5$-$4 \cdot 10^5$ spores/m^3. Få epidemiologiske undersøkelser av yrkesgrupper som jobber i vanlig innemiljø ble funnet og bare en hadde god design. Denne undersøkelsen viste en NOEL ved $7 \cdot 10^2$ sporer/m^3 for inflammationsmarkører i neseskyllevæske, symptomer fra øye, hals og nedre luftveier samt ikke-spesifikke symptomer. Sopp ble sjeldent identifisert i epidemiologiske undersøkelser.

Konklusjon: Resultatene fra humane provokasjonsstudier og epidemiologiske undersøkelser underbygger ganske konsistente LOELs på ca. 10^5 sporer/m^3 ved eksponering for diverse sopparter i ikke-sensibiliserte yrkesgrupper. Identifikasjon av soppfloraen er nødvendig for å dokumentere forekomst av de mer toksiske mykotoksin-produserende og patogene arter før evaluering av måleresultater.

Videre epidemiologiske undersøkelser av allergiske sykdommer og effekter av eksponering for patogene og mykotoksin-produserende arter anbefales.

Stikkord: administrative norm, aktinomycetes, allergisk alveolitt, irritasjon, review, organic dust toxic syndrome, soppsporer, toksistitet.

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19. Data bases used in the search for literature

A literature search was conducted in the Medline, Toxline, Arbline, OSH-ROM and ISI bases as well as the author's own database. Further references were obtained from reviewed papers. The last search was performed in November 2006. The following terms were used in the literature search:

Agent	***Effects***	***Occupational environments***
Fungal spores	Toxicity	Agriculture
Fungi	*In vitro*	Farmers
Moulds/Molds	*In vivo*	Mushroom cultivation
Hyphae	Animal	Sawmills
Actinomycetes	Cytotoxicity	Wood industry
	Mutagenicity	Breweries
Fungal components	Carcinogenicity	Cheese production
Mycotoxins	Teratogenicity	Indoor air, built environment, schools
Fungal antigens	Reproductive effects	Municipal waste
Fungal allergens	Human challenge studies	Sugar mills
Glucans	Provocation tests	
	Exposure-response	
Antibodies	Exposure-effect	
Fungal antibodies	Dose-response	
Fungal IgG antibodies	Dose-effect	
Fungal IgG	Respiratory effects	
Fungal IgE antibodies	Inflammation	
Fungal IgE	Lung function	
	Airway disease	
	Allergic alveolitis	
	Hypersensitivity pneumonitis	
	Asthma	
	Allergy	
	Chronic bronchitis	
	Fever	
	Epidemiology	

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Appendix 1. Terms as used in the present document

Term	Explanation
Actinomycetes	Bacteria that grow and replicate like filamentous fungi. Actinomycetes may produce large numbers of vegetative spores that are easily dispersed into the air.
Aerodynamic diameter	Diameter of a spherical particle with specific density equal to 1 g/cm^3 and the same sedimentation velocity as the actual particle.
Allergen	Antigen that can induce hypersensitivity reactions often by inducing the production of IgE antibodies.
Allergic alveolitis	See Hypersensitivity pneumonitis.
Allergy	A hypersensitivity reaction initiated by immunological mechanisms.
Antigen	Molecule that may induce the production of immunoglobulin antibodies.
Asthma	Obstructive lung disease characterised by reversible attacks of airway obstruction following exposure to allergens and non-specific irritants. In allergic asthma the response is mainly limited to specific allergens, and IgE antibodies to the allergen can be demonstrated. Non-allergic asthma does not seem to be IgE-mediated.
Atopy	The ability of an individual to produce IgE antibodies after exposure to an allergen. Atopy can be tested by the presence of IgE antibodies to common allergens in serum or by skin prick tests with these allergens.
Colony forming unit	A single microorganism or an aggregate of microorganisms that has grown into a countable colony by culture on a semi-solid nutrient medium under controlled conditions.
Conidia	Asexual spores produced by fungi.
Culturable microorganisms	Microorganisms quantified by culture-based methods.
Eukaryotic organism	Organism with cell(s) that have well defined nuclei containing the genetic material.
Fluorochrome	Fluorescent dye used to visualise specific particles in the fluorescence microscope.
Fungi	Organisms belonging to the kingdom Mycota. Fungi can be divided into filamentous fungi that form hyphae and replicate by spores, and by budding of cells (yeast).
Genus	The next lowest taxonomic category; also called family.
Gram-positive	Bacteria are divided in two main groups based on a staining technique developed by Gram. The Gram-positive bacteria differ from Gram-negative bacteria by the structure and composition of the cell wall.

Term	Explanation
Heterophilic microorganisms	Microorganisms dependent on organic material from dead or living organisms for growth.
Hyphae	Branched multi-cellular filaments formed by filamentous fungi.
Hypersensitivity	Causes objectively reproducible symptoms, or signs, initiated by exposure to a defined stimulus at a dose tolerated by normal subjects.
Hypersensitivity pneumonitis	Alveolar and bronchiolar inflammation caused by inhalation of spores from fungi and actinomycetes, and other allergens. Acute attacks are similar to ODTS (see Organic dust toxic syndrome). Recurrent attacks may eventually progress into pulmonary fibrosis. IgG but not IgE can usually be detected. Also called allergic alveolitis.
Immunoglobulin E	Antibodies that are a part of the adaptive immune response. Specific and total immunoglobulin E can be detected in serum as a measure of allergic sensitisation.
Immunoglobulin G	Antibodies that are a part of the adaptive immune response. Specific immunoglobulin G antibodies to fungal species in serum can be used as biomarkers of exposure but the precision is poor.
Impactor	Sampling device that collects particles according to inertial properties when the air stream is diverted after aspiration of the aerosol.
Impinger	Impactor that collects particles in a liquid.
Induced sputum	Collection of sputum stimulated by inhalation of a saline aerosol.
Inhalation fever	See Organic dust toxic syndrome.
Job exposure matrix	A tool to estimate qualitative or quantitative exposure levels using job characteristics.
Magic Lite test	A fluorometric enzyme immunoassay for IgE.
Mesophilic microorganisms	Microorganisms with optimal growth at temperatures between 15 and 30 °C.
Metabolites	Intermediate compounds and products of metabolism.
Mycelium	All aggregated hyphae from a single organism.
Mycotoxins	Toxic compounds produced by a number of fungal species. Mycotoxins can be excreted in substrates and may also be found in spores from such species.
Organic dust toxic syndrome	Attacks of fever/chills with respiratory symptoms and malaise that develop 4-8 hours after massive exposure to some substances, e.g. metal fumes and organic particles. The symptoms resolve within a few days. IgG antibodies against fungi are often not detectable.
Phadiatop	Combined detection of IgE against 10 common respiratory allergens by a radio-allergosorbent test. A positive test indicates the presence of atopy.

Term	Explanation
Precipitins	IgG antibodies detected in serum by the double-diffusion technique of Ouchterlony through formation of an antigen-antibody precipitate.
Primary metabolites	Metabolites essential for normal growth, development, and reproduction.
Proliferation	Rapid reproduction of microorganisms.
Psychrophilic microorganisms	Microorganisms that preferentially grow at temperatures below 15 °C.
Secondary metabolites	Metabolites that are not essential for normal growth, development and reproduction, but usually have important ecological functions. Examples are toxins such as antibiotics used in competition with other organisms, and pigments.
Sensitisation	Sensitisation is often understood as the presence of serum IgE antibodies to a specific allergen. However, hypersensitivity pneumonitis patients are also more sensitive to exposure to allergens, which is mediated by other mechanisms.
Spores	Sexual or asexual reproductive cells of fungi and actinomycetes (only asexual). Spores are metabolically inactive and tolerate environmental stress much better than vegetative cells. They are therefore important means for dispersion of organisms to other habitats.
Thermophilic microorganisms	Microorganisms that preferentially grow at temperatures above 30 °C.
Toxic alveolitis	See Organic dust toxic syndrome.
Toxigenic microorganisms	Microorganisms that have the capability to produce toxins.
Viable organisms	Living organisms capable of germination, growing, and replication. Viable organisms include organisms that can grow on nutrient plates as well as living organisms not able to grow in culture, e.g. obligate parasitic organisms.

Appendix 2. Synonyms

Recent and previous scientific names of fungi and actinomycetes used in this document.

Previous name	Current name
Fungi	
Merulius lacrymans	*Serpula lacrymans*
Penicillium cyclopium	*Penicillium aurantiogriseum*
Penicillium frequentans	*Penicillium glabrum*
Penicillium notatum	*Penicillium chrysogenum*
Rhizopus rhizopodiformis	*Rhizopus microsporus*
Stachybotrys atra	*Stachybotrys chartarum*
Actinomycetes	
Thermopolyspora polyspora	*Saccharopolyspora rectivirgula*
Micropolyspora faeni	*Saccharopolyspora rectivirgula*
Faeni rectivirgula	*Saccharopolyspora rectivirgula*

Appendix 3. *In vivo* studies of spore distribution

Green *et al* exposed rats and rabbits (n not given) intratracheally to $5{\cdot}10^7$ and $7{\cdot}10^6$ *Aspergillus terreus* spores or $1{\cdot}10^5$ and $4{\cdot}10^3$ spores/g bw, respectively. Spores were collected by washing culture plates with 0.01% Tween in saline, and were washed twice in saline. *A. terreus* spores migrated only to a minor extent to other organs. A few spore-containing alveolar macrophages were observed in the alveolar interstitium after 3 and 24 hours and in the tracheobronchial lymph nodes after 24 and 48 hours (68).

Kurup and Sheth exposed rabbits (n=2/group) intratracheally to $1{\cdot}10^7$ *A. fumigatus* spores in saline or $4{\cdot}10^3$ spores/g bw. Spores were washed from culture plates by 0.05% Tween 80 in saline and washed several times in saline. Dissimination to the kidneys and spleen was observed after 3 days (114).

Kurup exposed rabbits (n=5) intratracheally to $1{\cdot}10^7$ *A. fumigatus* spores ($4{\cdot}10^3$ spores/g bw, two strains, 90-95% viable, mainly single spores). Spores were washed from culture plates by saline and filtered. Viable spores were detected in lung tissue, liver, spleen and kidneys after 4 hours (112).

Olenchock *et al* exposed pathogen free rats (n=2/group) intratracheally to $5{\cdot}10^7$ *A. terreus* spores ($1{\cdot}10^5$ spores/g bw). Spores were collected by washing culture plates with 0.01% Tween in saline, and were washed twice in saline. The spores did not penetrate the alveolar and bronchial epithelium. Peripheral alveolar macrophages migrated from the alveoli adjacent to the bronchioles and after 3 hours some spores were also detected in the tracheobronchial lymph nodes (165).

Schaffner *et al* immunised mice by intravenous injection of viable *A. fumigatus* spores and exposed the animals to aerosols of spores blown from a culture of the same organism ($3{\cdot}10^5$ cfu/g lung tissue after 2 hours, n=9, $4{\cdot}10^3$ spores/g bw). No dissemination of the spores to the brain, spleen, liver, or kidneys was observed at 24-hour intervals up to 4 days after exposure (210).

Thurston *et al* exposed rabbits to aerosols of viable *Aspergillus fumigatus* and *Penicillium* sp. $5{\cdot}10^5$-$4{\cdot}10^6$ and $1{\cdot}10^5$-$5{\cdot}10^5$ cfu/g lung tissue (or $1{\cdot}10^4$-$8{\cdot}10^4$ and $2{\cdot}10^3$-$1{\cdot}10^4$ spores/g bw, respectively, estimated 1 hour after exposure; n=3-4 /group). Spores were collected by 1) rolling glass beads over a mature culture plate, and 2) aspiration of the spores from the beads into a filter holder. Viable spores were found in the lung and in digestive system and intestinal lymphoid tissue 6 hours after exposure. However, spore counts in gastro-intestinal tissues were much lower than in the lungs, ≤0.1% of the initial levels in lung tissue. Spore counts had cleared after 1 week. No other organs were studied (235).

Waldorf and Levitz exposed mice (n=10/group) intranasally to $1{\cdot}10^6$ spores of *A. fumigatus* and *Rhizopus oryzae* ($4{\cdot}10^4$ spores/g bw). Spores were harvested from culture plates in saline, vortex mixed, filtered and washed three times in saline (viability >95%, hyphae free). After 1 day, spores from both species were abundant in the lungs, blood, liver, spleen and kidney, but after 10 days, *A fumigatus* spores were only found in the lungs whereas *R. oryzae* spores were also found in the spleen and liver (241).

Appendix 4. *In vivo* studies of spore elimination from the lung

Cooley *et al* exposed mice intranasally to $1{\cdot}10^4$ spores of *Penicillium chrysogenum* ($4{\cdot}10^2$ spores/g bw). Spores were washed from culture plates by saline, washed with phosphate-buffered saline, sonicated, and filtered (single spores, 25% viable). The authors showed by transmission electron microscopy that spores were phagocytised and digested by alveolar macrophages from 3 to 24 hours after exposure (33).

Green *et al* exposed rabbits and rats (n not given) intratracheally to $7{\cdot}10^6$ and $5{\cdot}10^7$ *Aspergillus terreus* spores or $4{\cdot}10^3$ and $1{\cdot}10^5$ spores/g bw, respectively. Spores were collected by washing culture plates with 0.01% Tween in saline. The collected spores were washed twice in saline. Lung tissue was studied by SEM until 48 hours. The authors observed rapid uptake of the spores by alveolar macrophages that started immediately and was almost complete after 3 hours. Peripheral alveolar macrophages moved from the alveoli adjacent to the bronchioles. A few PMN were observed (68).

Kurup and Sheth exposed rabbits (n=2/group) intratracheally to $1{\cdot}10^7$ *A. fumigatus* spores in saline ($4{\cdot}10^3$ spores/g bw). Spores were washed from culture plates by 0.05% Tween 80 in saline and washed several times in saline. After 4 hours, 0.3% of the inocculum was recovered by culture of the lungs (114).

Kurup exposed rabbits (n=5) intratracheally to $1{\cdot}10^7$ *A. fumigatus* spores (two strains, 90-95% viable, mainly single spores, $4{\cdot}10^3$ spores/g bw). Spores were washed from culture plates by saline and filtered. Spores and macrophages in bronchoalveolar lavage fluid were quantified by light microscopy until 4 hours. Spores were rapidly ingested by pulmonary alveolar macrophages, 53% of the spores were ingested after 1 hour. After 4 hours, the proportion of internalised spores in alveolar macrophages had declined to 22% (112).

Olenchock *et al* exposed pathogen free rats and rabbits (n=2/group) intratracheally to $7{\cdot}10^6$ and $5{\cdot}10^7$ *A. terreus* spores ($4{\cdot}10^3$ and $1{\cdot}10^5$ spores/g bw), respectively. Spores were collected by washing culture plates with 0.01% Tween in saline, and were washed twice in saline. Spores and macrophages in lung tissue were quantified by SEM until 24 hours. Spores were rapidly phagocytised by alveolar macrophages in rats (42% after 0 hours and 98% after 24 hours) and in rabbits (only qualitative observations). After 24 hours only 20% of the spores found at 0-3 hours were observed (165).

Schaffner *et al* immunised mice by intravenous injection of viable *A. fumigatus* spores and exposed the animals to aerosols of spores blown from a culture of the same organism ($3{\cdot}10^5$ spores/g lung tissue after 2 hours, n=9, $4{\cdot}10^3$ spores/g bw). Lung tissue was cultured until 6 days. Cfu counts in lung tissue decreased by >99% from 2 to 48 hours after exposure, but were still found in one animal after 6 days (210).

Thurston *et al* exposed rabbits by inhalation to *A. fumigatus* spores, $5{\cdot}10^5$-$4{\cdot}10^6$ cfu/g lung tissue after 1 hour (n=6, $1{\cdot}10^4$-$8{\cdot}10^4$ spores/g bw) and *Penicillium* sp. spores, $1{\cdot}10^5$-$5{\cdot}10^5$ cfu/g lung tissue after 1 hour (n=3, $2{\cdot}10^3$-$1{\cdot}10^4$ spores/g bw).

Spores were collected by 1) rolling glass beads over a mature culture plate, and 2) aspiration of the spores from the beads into a filter holder. Culture counts in lung tissue were performed during 3 weeks. Cfu counts of *A. fumigatus* in lung tissue reduced to 1-10% after 24 hours, 0.1-2% after 48 hours and were not found after 1.5-3 weeks. Cfu counts of *Penicillium* sp. reduced more slowly, 7-30% after 24 hours, 4-7% after 48 hours and 0.02-0.03% after 3 weeks (235).

Voisin *et al* exposed Guinea pigs by intratracheal instillation of $1{\cdot}10^7$-$12{\cdot}10^7$ cells of *Candida albicans* (n=20/group, $3{\cdot}10^4$-$4{\cdot}10^5$ cells/g bw), and by inhalation to *A. fumigatus* for 5 minutes ($7{\cdot}10^6$ cfu after 2 hours, n=100, $2{\cdot}10^4$ spores/g bw) and *S. rectivirgula* spores for 10 minutes ($1{\cdot}10^6$-$8{\cdot}10^6$ cfu after 2 hours, n=90, $3{\cdot}10^3$-$3{\cdot}10^4$ spores/g bw). Culture counts in lung tissue were performed until day 5, 10 and 59, respectively. The preparation of spores was not described. Inhaled *A. fumigatus* spores and intratracheally instilled *Candida albicans* cells were rapidly destroyed by alveolar macrophages and spore counts in lung tissue decreased to 1% within 1-2 days. Cfu counts of *S. rectivirgula* reduced to 10% after 2-3 weeks and could still be cultured 2 months after exposure. Mycelial growth was observed in animals that had been treated with anti-alveolar macrophage serum (n=30) before exposure to *S. rectivirgula* spores (240).

Waldorf and Levitz exposed mice (n=10/group) intranasally to $1{\cdot}10^6$ spores of *A. fumigatus* and *Rhizopus oryzae* ($4{\cdot}10^4$ spores/g bw). Spores were harvested from culture plates in saline, vortex mixed, filtered and washed three times in saline (viability >95%, hyphae free). Bronchoalveolar lavage was performed 18 hours after exposure and 70% of the *A. fumigatus* spores in bronchoalveolar lavage fluid had been killed by alveolar macrophages but only 20% of the *R. oryzae* spores. However, germination of spores was not observed by histological examinations over a 10-day period for both species (241).

Zaidi *et al* exposed Guinea pigs (n=30/group) to 800 µg *S. rectivirgula* spores (approximately $4{\cdot}10^6$ spores/g bw) with and without hay dust (75 mg) by intratracheal instillation. Culture counts in lung tissue were performed until 7 days. Spores were scraped of culture plates and suspended in saline. When exposed to *S. rectivirgula* only, cfu counts in lung tissue rapidly decreased to 10% after 1 day and 0.1% after 3 days. However, when combined with hay dust spore counts remained high for 7 days (256).

Appendix 5. *In vitro* studies of spore elimination

Hirvonen *et al* exposed the mouse macrophage cell line RAW264.7 to spores from 14 actinomycete species (*Actinomyces* and *Streptomyces* species isolated from mouldy environments, 0.01-10 spores/macrophage). Spores were harvested by adding phosphate-buffered saline containing 0.0001% Triton X-100 to culture plates, and eventually killed by gamma irradiation. Cytotoxicity to the cell line and production of nitrite, iNOS and ROS by the macrophages differed across species. Killed spores induced similar amounts of nitrite, less iNOS (judged visually) and ROS. Nine species were cytotoxic and cytotoxicity of killed spores was even higher than that of viable spores for 7 of these species (84).

Hirvonen *et al* exposed the mouse macrophage cell line RAW264.7 to spores of *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Candida albicans, Stachybotrys* sp. and two *Streptomyces* spp. (0.01-10 spores/macrophage). Spore suspensions in phosphate-buffered saline were prepared but no details were given. *Streptomyces* species induced high levels of nitrite, iNOS, IL-6, and TNFα. *Cladosporium* sp., *Candida albicans,* and *Stachybotrys* sp. produced moderate amounts of nitrite and *Cladosporium* sp. also TNFα. Only *Stachybotrys* sp. was cytotoxic to the cell line (81).

Hirvonen *et al* exposed the mouse macrophage cell line RAW264.7 to viable spores of the *Streptomyces* species (used in the study above), now identified as *S. californicus* and *S. anulatus* (0.01-10 spores/macrophage). Spores were harvested by adding phosphate-buffered saline containing 0.0001% Triton X-100 to culture plates. Nitrite, IL-6 and TNFα were produced in a dose-response manner and iNOS induction was detected. No cytotoxicity was observed (82).

Hirvonen *et al* exposed the mouse macrophage cell line RAW264.7 to spores of *Streptomyces anulatus* from cultures grown on 15 different substrates (0.01-10 spores/cell). Spores were harvested by adding distilled water to culture plates. Nitrite, TNFα and IL-6 were produced and cytotoxicity increased in a dose dependent manner. Only spores grown on one of the substrates induced ROS production. The substrate used for culture influenced the level of all responses except TNFα (85).

Huttunen *et al* compared the inflammatory and cytoxic responses of mouse RAW264.7 macrophage, human 28SC macrophage and human A549 lung epithelial cell lines to spores from *Streptomyces californicus, Penicillium spinolosum, Aspergillus versicolor* and *Stachybotrys chartarum* (0.2-20 spore/cell). Spores were collected with a sterile loop and suspended in Hank's balanced salt solution containing 0.0001% Triton X-100. TNFα increased in the mouse macrophage cell line for all species, but not in the human cell lines. Nitrite production increased in human lung epithelial cells exposed to *S. chartarum* and *S. californicus* and in mouse macrophages exposed to *S. californicus.* Both mouse and human macrophages exposed to *S. californicus* showed an IL-6 response, and in mouse macrophages also IL-1β was induced. Such responses were not observed in the other cell lines. All species were cytotoxic to the macrophage cell lines with

toxicity of *S. chartarum* > *A. versicolor* > *P. spinolosum*/*S. californicus*. *S. chartarum* and *A. versicolor* were also cytotoxic to the human lung epithelial cell line (91).

Huttunen *et al* exposed the mouse RAW264.7 macrophage cell line to spores of *Aspergillus versicolor, Penicillium spinulosum* and non-trichothecene producing *Stachybotrys chartarum*, either alone (0.02-2 spores/cell) or together with *Streptomyces californicus* spores (0.2 spores/cell). The preparation of the spores was not described. NO, TNFα and IL-6 was not induced by any fungal species alone, but all species were cytotoxic. *S. californicus* alone induced NO, TNFα and IL-6, and was cytotoxic. *S. chartarum* together with *S. californicus* showed a strong synergistic interaction on IL-6 production (92).

Jussila *et al* exposed a human alveolar epithelial cell line to spores from *Streptomyces anulatus* (0.001-24 spores/cell). Spores were suspended in phosphate-buffered saline but no further details were given. Nitrite and IL-6 was induced at the highest exposure levels (12-24 spores/cell) that also caused a substantial cell death (105).

Kurup exposed normal rabbit pulmonary alveolar macrophages to viable spores of *A. fumigatus* (1-3 spores/macrophage). Spores were washed from culture plates by saline and filtered. Only a small fraction of viable spores of *A. fumigatus* (19%) was killed by the macrophages after 4 hours. Activation of macrophages in the rabbits by repeated intracutaneous injections with complete adjuvant, and/or opsonisation with *anti-A. fumigatus* serum increased the fungicidal activity of the alveolar macrophages only moderately. Remaining ingested spores started germination 4 hours after exposure. Most viable spores of *Aspergillus niger* and *Aspergillus flavus* were killed by normal pulmonary alveolar macrophages within 4 hours (80-90%) (112).

Murtoniemi *et al* exposed the mouse macrophage cell line RAW264.7 to spores from *Streptomyces californicus, Penicillium spinolosum, Aspergillus versicolor* and *Stachybotrys chartarum* grown on different plasterboards under identical conditions (0.01-1 spore/cell). Different responses of the inflammatory mediators nitrite, IL-1β, TNFα and IL-6 were observed both between species and strains grown on different plasterboards. *S. californicus* showed the strongest responses. All species were cytotoxic at approximately similar levels (151).

In a similar study by Murtoniemi *et al*, different inflammatory mediator responses were also found between fungi grown on the liner and core materials when tested separately (150). Spore isolation is not clearly described in these studies (150, 151).

The same author (Murtoniemi *et al*) describes the use of a sterile swab in a similar study. In the latter study it was found that cytotoxicity did not correlate with inflammatory mediators or growth conditions for *A. versicolor* or *S. chartarum*, whereas a positive association was found between cytotoxicity and inflammatory mediators for *S. californicus* and *P. spinolosum* (148).

In another study (Murtoniemi *et al*) of the effect of plasterboard composition on biological activity of *S. chartarum* and *S. californicus* spores, a sterile plastic rod was used. The composition of the plasterboards, especially the presence of starch

in the lining material and biocides in both the lining and core material influenced nitrite, IL-6 and TNFα production as well as cytotoxicity in the mouse RAW264.7 macrophage cell line (149).

Nessa *et al* exposed human alveolar macrophages obtained by bronchoalveolar lavage of healthy volunteers to heat-killed *A. fumigatus* spores and silica particles of similar size (10 spores/macrophage). Spores were washed from culture plates with 0.02% Tween 80 solution, heat-killed at 80 °C for 20 minutes, filtered, centrifuged and washed twice with buffer. Heat-killed *A. fumigatus* spores that had attached to alveolar macrophages were ingested more rapidly than silica particles of similar size, 64 and 39%, respectively, within 30 minutes. The spores induced production of superoxide anions (153).

Nessa *et al* exposed rat alveolar macrophages to heat-killed spores of *A. fumigatus* and *Aspergillus candidus*, and silica particles of similar size (10 spores/macrophage). Spores were washed from culture plates with 0.02% Tween 80 solution, heat-killed at 80 °C for 20 minutes, filtered, centrifuged and washed twice with buffer. Heat-killed *A. fumigatus* and *A. candidus* spores that had attached to alveolar macrophages were ingested more rapidly than silica particles of similar size, 75, 74 and 33%, respectively, within 30 minutes. The spores induced production of superoxide anions after 1 hour, which level declined to ca 50% after 24 hours (152).

Nielsen *et al* (157) examined the metabolic profiles of 20 *Stachybotrys* sp. strains studied by Ruotsolainen *et al* (206). All 11 strains producing the trichothecenes satratoxin G and H were cytotoxic to the mouse macrophage RAW264.7 cell line, but only 1 of the 9 strains that did not produce these toxins. Seven of the strains that did not contain satratoxins induced IL-6 and TNFα production but none of the satratoxin containing strains (157).

Penttinen *et al* exposed the mouse RAW264.7 macrophage cell line to spores of *Stachybotrys chartarum* (mycotoxin production not specified; 0.002-0.5 spores/cell) and *Streptomyces californicus* spores (0.008-3 spores/cell) either alone, together in a ratio of 1:5 (0.01-3 spores/cell) or spores from a co-culture (same ratio and spore dose/cell). Spores were collected with a sterile loop and suspended in Hank's balanced salt solution containing 0.0001% Triton X-100. *S. californicus* alone and in combination with *S. chartarum* were cytotoxic, induced IL-6, MIP-2, nitrite and cell cycle arrest in the macrophages. *S. chartarum* alone and in combination with *S. californicus* induced apoptosis in the macrophages. Only co-cultured spores increased caspase-3 enzyme activity significantly. The effect of co-cultured spores compared to mixed spores was stronger on apoptosis, cell-cycle arrest, and caspase-3 enzyme activity but weaker on nitrite production (176).

Pylkkänen *et al* exposed the mouse macrophage cell line RAW264.7 to viable and heat-killed spores of *Aspergillus fumigatus* (0.001-1 spores/cell). Spores were harvested with a sterile loop and suspended in Hank's balanced salt solution containing 0.0001% Triton X-100. Spores were used directly or were killed by autoclaving at 120 °C for 20 minutes. Exposure to viable spores showed upregulation of TNFα mRNA and MCP-1 mRNA but TNFα and MCP-1 did not

increase. MIP-1α mRNA and MIP-1β mRNA increased but no response was observed for IL-6 mRNA, IL-1β mRNA or IL-6. The heat-killed spores did not produce any of these responses. After both 2 and 6 hours, 50% of the macrophages had phagocitised spores, and viable and heat-killed spores had been phagocitised to the same extent (182).

Robertson *et al* exposed human circulatory monocytes and PMN to opsonised spores of *A. fumigatus* and *Penicillium ochrochloron* (0.5 spores/cell). Spores were suspended in Hanks' balanced salt solution, homogenised, filtered, opsonised with autologous serum, centrifuged and washed. Most spores were associated with phagocytes after 1 hour (88% and 78% respectively). A large proportion of the *P. ochrochloron* spores were phagocytised but spores of *A. fumigatus* were to a large extend attached to the cell surface of the phagocytic cells (qualitative SEM data) (199).

Roponen *et al* exposed the mouse macrophage cell line RAW264.7 to spores of *Streptomyces anulatus* from cultures grown on sterilised and unsterilised concrete, plaster board and mineral wool (1 spore/cell). Spores were collected with sterile swabs and suspended in phosphate-buffered saline. The nitrite, TNFα and IL-6 responses as well as cytotoxicity depended on the substrates that the fungi were grown on, and sterilised plasterboard showed the strongest responses (204).

Ruotsalainen *et al* exposed human blood PMN to graded doses (1-100/cell) of viable spores of fungi isolated from common indoor air; *Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp., *Paecilomyces* sp., *Stachybotrys* sp. and two *Streptomyces* spp., as well as *Candida* sp. cells. Spores were suspended in phosphate-buffered saline containing 0.0001% Triton X-100, opsonised with human AB serum, centrifuged, and washed twice in the same solution. They found positive dose response associations between ROS production and spores of *Penicillium* sp., *Candida* sp. and *Streptomyces* spp., ∩-shaped associations with *Aspergillus* sp. and *Cladosporium* sp., no response in *Paecilomyces* sp. and a negative association in *Stachybotrys* sp. Opsonisation of the spores and cells was essential for the response (207).

Ruotsalainen *et al* exposed mouse macrophage cell line RAW264.7 to graded doses of spores of 21 strains of *Stachybotrys* sp. (0.01-1 spore/cell) isolated from mouldy buildings. Spores were harvested by adding phosphate-buffered saline containing 0.0001% Triton X-100 to culture plates. They found no induction of iNOS or production of NO. Only 3 strains induced ROS in a dose-response manner, while 7 other strains induced TNFα and IL-6. The latter strains were less cytotoxic than the remaining isolates (206).

Shahan *et al* exposed bronchoalveolar lavage cells from Guinea pigs to spores from *A. candidus A. fumigatus, A. niger, A. terreus, Eurotium amstelodami, Cladoporium cladoporoides* and *Penicillium spinulosum* ($2 \cdot 10^{-6}$-200 spores/ macrophage). Spores were suspended in Hank's balanced salt solution containing 0.01% Tween 80, vortexed, filtered and washed five times in Hank's balanced salt solution using centrifugation. Spores were killed by autoclaving in phosphate-buffered saline at 121 °C for 30 minutes. Minor differences were detected

between superoxide anion production by viable and killed spores. The responses were similar to all species except *E. amstelodami*, which induced a 2-3 times lower response than the other species (218).

Shahan *et al* exposed rat alveolar macrophages to spores of *A. fumigatus, A. niger, A. terreus, A. candidus, E. amstelodami, P. spinulosum*, and *C. cladosporoides* (100 spores/macrophage). Spores were killed by autoclaving in phosphate-buffered saline at 121 °C for 30 minutes and washed five times with phosphate-buffered saline. No further details on spore collection and treatment were given. *A. fumigatus* produced a strong TNFα response and MIP-1 mRNA was upregulated, which was not observed for the other species. Expression of mRNA to the cytokines MIP-2 and KC was upregulated by *A. fumigatus, A. candidus, A. niger* and *E. amstelodami* of which *A. fumigatus,* and *A. candidus* induced the strongest response, while no response was detected after exposure to *A. terreus* and *P. spinulosum* (219).

Shahan *et al* exposed rat alveolar macrophages to spores of *A. niger, A. candidus*, and *E. amstelodami* (10 spores/macrophage). Spores were collected with a sterile loop, suspended in phosphate-buffered saline containing 0.1% Tween 80, homogenised, filtered, centrifuged and washed, killed by autoclaving in phosphate-buffered saline at 121 °C for 30 minutes and washed five times with phosphate-buffered saline. Production of ROS was highest after exposure to *A. candidus*, intermediate after exposure to *A. niger*, and *E. amstelodami* induced ROS at similar levels as latex particles. The ROS response correlated with tyrosine kinase activation in the rat alveolar macrophage. It was also shown that the tyrosine kinase response was primarily dependent on endogenous ROS in rat alveolar macrophages (220).

Appendix 6. Epidemiological studies of highly exposed populations

Alwis *et al* performed a cross-sectional study of joinery (n=82) and sawmill/chip mill workers (n=108) from 12 work sites. Exposure to mould spores, dust, Gram-negative bacteria, endotoxins and glucans was measured by personal sampling and culture of the microorganisms (n=5-15/site, total 114 measurements). Mean exposure levels to fungi ranged from $3 \cdot 10^3$-$7 \cdot 10^4$ cfu/m^3 with an arithmetic mean of $3 \cdot 10^4$ cfu/m^3 for the combined populations, $4 \cdot 10^4$ cfu/m^3 in the saw/chip mills and $1 \cdot 10^4$ cfu/m^3 in the joineries. *Penicillium* sp. (mean frequency 54%) and *Aspergillus* sp. (mean frequency 17%, mainly *A. fumigatus*) dominated. Associations between respiratory symptoms and exposures were adjusted for age and smoking but not for other exposures. Only fungal exposure was associated with breathlessness (OR 4.8, 95% CI 1.4-17) in sawmill/chip mill workers. Throat irritation in all wood workers (OR 1.6, 95% CI 1.2-2.1) and phlegm in joinery workers (OR 2.0, 95% CI 1.0-3.9) were associated with fungal exposure. These symptoms were also associated with endotoxins (OR 1.5, 95% CI 1.1-2.1 and OR 1.8, 95% CI 1.1-2.9, respectively). The associations with fungi were not likely due to confounding as the endotoxin exposure was only moderately correlated with fungal exposure (r=0.46-0.50). Exposure to fungi was also significantly associated with wheezing (OR 1.4, 95% CI 1.0-2.0 in all workers), bronchitis (OR 3.0, 95% CI 1.3-6.6 in joinery workers and OR 2.9, 95% CI 1.1-7.2 in saw/chip mill workers), and ear inflammation (OR 13, 95% CI 5.8-190 in saw mill workers and OR 2.7, 95% CI 1.5-4.9 in all wood workers). The associations with glucan exposure were stronger, however, and the associations with fungi may be due to the strong correlation (r=0.76-0.86) between glucan and fungal exposure. However, the associations with glucan may also reflect fungal exposure because glucans are a structural component of fungi (6).

Ávila and Lacey performed a cross-sectional study of 648 workers in two cork factories exposed to mould spores, mainly from *Penicillium glabrum* (72-97% of the spores), and cork dust. Mean exposure levels of workers in different departments ranged from $1 \cdot 10^6$-$7 \cdot 10^7$ spores/m^3 (number of samples unknown) measured by light microscopy and personal sampling. It was not uncommon that workers changed jobs during their employment in the plant. X-ray showed reticulo-nodular changes in 57% of the workers, which were correlated with the current exposure level in non-smokers but not with the number of exposed years. Spirometry concluded with an "obstructive effect" in 12% and a "restrictive effect" in 29% (no measurement data). Pulmonary restriction was correlated with duration of exposure (0 - >30 years) but not with the current exposure level. A total of 26 patients with hypersensitivity pneumonitis were diagnosed (9).

Coenen *et al* studied 72 waste collectors in a cross-sectional study exposed to bacteria, endotoxins, and culturable fungi (n not given, <$4 \cdot 10^2$-$3 \cdot 10^5$ cfu/m^3 of which 10^2-$5 \cdot 10^4$ cfu/m^3 of *Aspergillus fumigatus*). Exposure was estimated by a job exposure matrix based on measurements carried out during health monitoring

and was dichotomised at $1{\cdot}10^5$ cfu/m^3 of total fungi and $2{\cdot}10^3$ cfu/m^3 of *A. fumigatus*. Peak expiratory flow variability was measured during two weeks and was significantly increased in workers with the highest exposure to *A. fumigatus*. The prevalences of chronic bronchitis and productive cough (no further information on questions) were also associated with exposure to *A. fumigatus*. No confounder adjustments were applied, however (31).

Dahlquist *et al* studied 28 wood trimmers in one sawmill during a work week. Exposure was measured by personal sampling (n=28) and analysis of dust (median 0.3 mg/m^3), terpenes (median 10 ppm), bacteria (median $4{\cdot}10^2$ cfu/m^3 and $1{\cdot}10^5$ cells/m^3), endotoxins (median 20 EU/m^3) and fungi by culture (median $3{\cdot}10^3$ cfu/m^3) and by SEM (median $1{\cdot}10^5$ spores/m^3). No significant changes in lung function were observed, but decreases in FEV_1 and MEF_{25} over the work week correlated with exposure levels. No adjustments were made for confounders (38).

Eduard *et al* studied 107 wood trimmers in 10 sawmills in a cross-sectional study. Exposure to mould spores (arithmetic mean $4{\cdot}10^6$ spores/m^3 of which 42% *Rhizopus microsporus*), and wood dust were measured during 2-4 weeks in each sawmill (n_{total}=148). Respiratory symptoms were recorded by a general questionnaire. Associations between symptoms and exposure to all agents were studied by logistic regression using mean exposure levels within each sawmill and adjustments for age, smoking, hay fever and part-time farm work. Morning cough as well as work-related cough, sore throat and nasal obstruction were most strongly associated with mould spore exposure, and work-related chest tightness with *R. microsporus* spore and wood dust exposure. Exposure levels predicting an OR of 1.5 were approximately $2{\cdot}10^6$ spores/m^3 for all associations. The associations between work-related symptoms and mould spore exposure may be positively biased due to wordings in the questionnaire, however (54).

Eduard *et al* studied 89 farmers and spouses in a cross-sectional study. Exposure was measured by personal sampling when the farmers carried out exposed farm work, and current work-related respiratory and eye symptoms were recorded by a questionnaire after the measurement was completed. Exposure to dust, bacteria, fungi (mean $1{\cdot}10^6$ spores/m^3, calculated to equal an 8-hour exposure) endotoxins, glucans, *Aspergillus*/*Penicllium* antigens, organic dust, inorganic dust, silica, ammonia, hydrogen sulphide, and nitrogen dioxide were measured. Associations were adjusted for age, smoking, and gender, but not for atopy. Cough (OR 3.9, 95% CI 1.0-15) and eye irritation (OR 8.3, 95% CI 1.0-70) were significantly elevated after exposure to $5{\cdot}10^5$-$2{\cdot}10^7$ fungal spores/m^3 and $2{\cdot}10^4$-$5{\cdot}10^5$ fungal spores/m^3, respectively. These symptoms were not associated with exposure to other agents. Nasal symptoms were significantly associated with endotoxin, dust, silica, and fungal spores (exposure level $5{\cdot}10^5$-$2{\cdot}10^7$ fungal spores/m^3, OR 6.0, 95% CI 1.3-28), of which the latter two agents were equally strong predictors. The correlation between fungal spores and silica exposure was not very strong, $r_{Pearson}$<0.6, indicating that both agents may induce nasal symptoms (49).

Eduard *et al* studied 1 614 farmers and spouses in a cross-sectional study. Annual time weighted average exposure levels were computed from task participation, mean exposure levels, and mean duration of exposed tasks. Exposure of these tasks was measured by personal sampling and analysis of dust, bacteria, mould spores, endotoxins, and ammonia. Current physician diagnosed asthma was recorded by questionnaire and a positive Phadiatope test in serum (RAST to 10 respiratory allergens) was used as criterion for atopy. Associations between all exposures and asthma were studied in atopic and non-atopic individuals separately by logistic regression using exposure levels categorised in tertiles and adjustments for age, smoking, gender, and asthma in the family. Exposure to endotoxins, fungi ($4{\cdot}10^6$-$4{\cdot}10^7$ spores/m^3), and ammonia were positively correlated with asthma in non-atopic farmers (OR 1.7, 95% CI 1.0-2.7) and negatively with asthma in atopic farmers (OR 0.28, 95% CI 0.10-0.78). The associations with fungal spores were stronger than with the other agents except ammonia, which was of approximately equal strength in the association in non-atopic farmers. Ammonia exposure at the observed level (geometric mean 4 ppm) as a cause of asthma is not biologically plausible, however (50).

Hansen *et al* studied 951 waste collectors and 423 park workers (reference group) in a cross-sectional study. Respiratory symptoms were recorded by a general questionnaire. The waste collectors' exposure to culturable and countable fungal spores was measured by culture-based and microscopical methods and further estimated by a quantitative job exposure matrix. The waste collectors were categorised in low and high exposed groups divided by a cutpoint at $1.5{\cdot}10^5$ cfu/m^3 ($3{\cdot}10^5$ spores/m^3). The groups were of very different size, n= 915 and 36, and n=917 and 34, respectively. The exposure of the reference group was assumed to be low, but no exposure information was available. Only chronic bronchitis was significantly elevated among waste collectors compared to the reference group. Associations with fungal exposure were studied with a generalised linear model adjusting for age, smoking, residential area, and exposure to inorganic dust, organic dust, irritant gases, and fumes. The prevalence of chronic bronchitis was significantly elevated in the low exposed waste collector group, PPR 1.9 (95% CI 1.0-3.6) for both culturable and countable fungal spores. The PPR increased further in the highest exposed waste collectors to 2.7 (95% CI 3.6-11) and 2.5 (95% CI 0.6-11) for culturable and countable fungal spores, respectively. The first confidence interval must be wrong because the lower confidence limit is higher than the central measure. Assuming the same ratio between the central measure and the lower confidence limit as between the higher confidence limit and the central measure, the 95% CI becomes 0.7-11. The PPR is thus not significantly different for the highest exposed group, which was probably due to the relative small size of this group (73).

Hedenstierna *et al* studied 29 wood trimmers in 2 sawmills during a working week. Lung function was measured before work on Monday and after work on Thursday. Exposure to fungi was measured by personal sampling during all shifts (median levels $1{\cdot}10^4$ cfu/m^3 and $3{\cdot}10^5$ cfu/m^3, respectively, n=105). In non-smoking workers the FVC decline during the 4 days was correlated with

the exposure levels. Similar associations were found for FVC and FEV_1 in % predicted measured on Thursday. No other confounder adjustments than stratification on smoking habits and comparison to a reference population (probably age and height) were done. The lung function had been measured 3 months earlier after the summer holiday and reductions in FVC and FEV_1 were observed only in workers from the sawmill with the highest exposure. No further changes were observed in neither of the group of sawmill workers 27 months after the first lung function measurements (76).

Heldal *et al* studied 31 municipal waste handlers in a cross-sectional study of changes of inflammatory markers in nasal lavage and nasal dimensions by acoustic rhinometry from Monday morning to Thursday morning before work. Exposure was measured on the 3 days in between by personal sampling and analysis of fungal spores (n=93; median $2 \cdot 10^5$ spores/m^3), bacteria, actinomycetes spores, endotoxins, and glucans. Thursday levels of neutrophils % and change in neutrophils % were correlated with exposure to fungal spores but not with other inflammatory cells, MPO, IL-8 and ECP. Nasal congestion was correlated both with fungal spores and glucans, which are probably independent effects as these exposures were weakly correlated. Possible confounding by smoking and age was evaluated qualitatively (79).

Heldal *et al* studied 25 organic waste collectors in a cross-sectional study of changes of inflammatory markers in induced sputum and spirometry from Monday morning to Thursday morning before work. Work-related symptoms were recorded daily. Exposure was measured on the 3 days in between by personal sampling and analysis of fungal spores (n=75; median $2 \cdot 10^5$ spores/m^3), bacteria, actinomycetes spores, endotoxins, and glucans. No associations were observed between fungal spore exposure and neutrophils %, other inflammatory cells, IL-8 and ECP, and the work-related symptoms chest tightness, cough and phlegm (80).

Heldal *et al* studied 22 household waste collectors from three different climatic regions in a cross-sectional study of acute work-related symptoms. Exposure was assessed by personal sampling and analysis of fungal spores (n=22; median $1 \cdot 10^5$ spores/m^3), bacteria, endotoxins and dust. Workers reporting cough had significantly higher exposure to fungal spores (median $3 \cdot 10^5$ spores/m^3) than non-symptomatic workers (median $6 \cdot 10^4$ spores/m^3). Cough was not significantly associated with other agents. Associations were also found between fungal spore exposure and headache and unusual tiredness, but this could be due to confounding by smoking and age, which was evaluated qualitatively (78).

Ivens *et al* studied 950 waste collectors and 387 outdoor municipal workers (reference) in a cross-sectional study. Symptoms were recorded by a general questionnaire. The doses of culturable fungi, countable fungal spores, and endotoxins inhaled by the waste collectors were estimated using a quantitative job exposure matrix, information on task duration, and lung ventilation estimates. The population was categorised in low, medium, and high exposed waste handlers by cut points $1 \cdot 10^6$ and $1 \cdot 10^7$ cfu for culturable fungi, and $2 \cdot 10^6$ and $2 \cdot 10^7$ spores for countable fungal spores, respectively. The groups were very different in size,

n= 229, 711 and 10, and 69, 813 and 68, respectively. No exposure information was available for the reference group. Diarrhoea was significantly elevated among waste collectors compared to the reference group. The association with fungal exposure was studied with a generalised linear model adjusting for smoking, alcohol consumption and psychological demand and support. The symptom prevalence was significantly elevated in the low exposed waste collectors, PPR 3.0, 95% CI 1.9-4.9 for culturable and PPR 3.5, 95% CI 1.9-6.4 for total fungi, and increased further in a dose-response manner for culturable fungi in the medium and high exposed waste collectors. Similar associations were observed for endotoxins, but no adjustments for exposure to other agents were studied (97).

Johard *et al* studied 19 non-smoking wood trimmers in one sawmill and a non-smoking control group (n=25) not occupationally exposed to fungi by broncho-alveolar lavage and lung function measurements. Exposure to fungi (median 10^5 spores/m^3) and bacteria was assessed by personal sampling (n=16). No significant changes in lung function were observed compared to 19 non-smoking controls. Cell counts in bronchoalveolar lavage were not different, but albumin, fibronectin, and hyaluronan were significantly higher in the sawmill workers indicating low intensity alveolar inflammation (100).

Malmberg *et al* studied 32 dairy farmers with febrile episodes, which had been selected from a population of approximately 6 800 Swedish farmers. Medical examinations confirmed a diagnosis of hypersensitivity pneumonitis (n=11) or ODTS (n=17). After episodes that induced fever attacks exposure was measured by repeating the task using the same materials. Personal samples were collected and spores of actinomycetes and moulds were counted by fluorescence micro-scopy and identified by culture. Farmers with diagnosed hypersensitivity pneumonitis had been exposed to $3 \cdot 10^9$ spores/m^3 (n=4, mean, range ($4 \cdot 10^8$-$4 \cdot 10^9$ spores/m^3) and had performed work that lasted 15-30 minutes, 1-2 times/day and the work had been done for several weeks. Farmers with diagnosed ODTS had been exposed to $14 \cdot 10^9$ spores/m^3 (n=6, mean, range $3 \cdot 10^9$-$20 \cdot 10^{10}$ spores/m^3) and had performed work that lasted on average 60 minutes, during 1-2 days and the work was unusual, typically removal of spontaneously heated grain. The species composition was variable and included species of *Aspergillus, Penicillium, Rhizomucor, Wallemia* and *Streptomyces* but none of the species dominated. The proportion of actinomycetes varied from <1 to 96%. Exposure of tasks with highest expected exposure was also measured in a reference group of non-symptomatic farmers. These levels were $1 \cdot 10^8$ spores/m^3 (n=17, mean, range $5 \cdot 10^6$-$6 \cdot 10^8$ spores/m^3). Endotoxin exposure of symptomatic and non-symptomatic farmers was similar (129, 130).

Monsó *et al* studied 39 greenhouse workers in a cross-sectional study of wheeze (31%) and asthma attacks (8%) during the last year. Sensitisation to moulds by skin prick testing was found in 7 workers (18%). Exposure to endotoxins, bac-teria, and moulds (n=39; median $5 \cdot 10^3$ cfu/m^3) was measured. No associations were found with these outcomes (141).

Roponen *et al* studied 11 sawmill workers with nasal lavage after a work shift and during vacation (time after the last workday not given). Exposure to endo-

toxins, terpenes, and moulds (n=10; median $6 \cdot 10^5$ spores/m^3, mainly *Rhizopus* and *Penicillium*) was measured. Nasal lavage samples were analysed for NO, TNFα, IL-4, IL-5, and IL-6. No differences were observed between nasal lavage after work and during vacation, and no associations were found with fungal spore exposure (202).

Wenzel and Emanuel examined 37 workers involved in debarking and chipping of timber in a papermill. Exposure to fungal spores was measured by stationary sampling and analysis by microscopy. Most particles were spores of *Cryptostroma corticale*, which were recognised by ovoid shape, size 4-5 μm and red-brown colour. Five workers developed severe clinical disease that was confirmed by lung biopsies showing interstitial pneumonitis and granuloma formation. All workers were examined 5 times during a 2.5-year period and 15 workers had X-ray changes on at least one occasion. Disease was initiated in the winter. During this period exposures were also the highest with a mean level of $1.3 \cdot 10^5$ spores. The mean exposure during the whole 1.5-year period was $4 \cdot 10^4$ spores (n=35). Concentration units were not given in the paper (and personal communication with the corresponding author did not provide the information), but levels are more likely to be spore counts per ft^3 as the study was performed in 1962 and published in 1967. If so, the mean exposure level is calculated to be $1 \cdot 10^6$ spores/m^3 during the whole study, and $5 \cdot 10^6$ spores/m^3 during the winter (243).

Zhang *et al* studied 130 Chinese tobacco workers and 112 controls in a cross-sectional study. Dust exposure was high (mean 14 to 30 mg/m^3 in different departments). Fungi were measured by sedimentation on culture plates. Specific IgE and positive skin prick tests to *Aspergillus fumigatus* were increased, 27% and 19%, respectively, and to *Rhizopus nigricans*, 52% and 24%. These fungi were also frequently detected on the sedimentation plates (260).

Appendix 7. Epidemiological studies of populations exposed to common indoor air levels

Hirvonen *et al* studied 32 staff members working in a mould contaminated school and 8 or 25 healthy controls (specified numbers inconsistent) from a research institute. Nasal lavage was performed after spring term, vacation, and autumn term in school staff. Culturable fungi (n not given, range 7 to 100 cfu/m^3) were measured in the school at the end of the autumn term. NO, IL-6, respiratory and systemic symptoms were higher in school staff after spring term and autimn term compared to after vacation and control subjects. TNFα was elevated only after the spring term. The findings cannot be associated to exposure levels because no measurements were performed after vacation or in the environment of the control subjects (83).

Li *et al* studied 264 day-care workers from 28 randomly selected day-care centres in Taiwan. Culturable fungi were measured indoors and outdoors, and house dust mite allergens in settled dust. Indoor and outdoor levels of culturable fungi were similar (n not given, geometric means 1 200 cfu/m^3 and 1 000 cfu/m^3, respectively). *Cladosporium* and *Penicillium* dominated with *Penicillium* levels higher indoors and *Cladosporium* higher outdoors. Other prevalent fungi were *Aspergillus* and yeasts. Associations were found between *Aspergillus* levels (geometric mean 32 cfu/m^3) and nasal congestion, cough, phlegm, lethargy, and fatigue. Significant associations were also found between total culturable fungi and nasal congestion and nasal discharge, and these associations were stronger than for *Aspergillus*. Associations were adjusted for age, gender, and education (124).

Menzies *et al* studied 107 office workers with work-related respiratory symptoms and 107 office workers without symptoms from 6 mechanically ventilated office buildings in Quebec. The non-symptomatic workers were matched on age, gender, and presence of atopic disease. Airborne culturable fungi were measured with a Burkhard personal impactor in the offices in heating, ventilating, and air conditioning systems (HVAC) and office air. However, this sampler is not suitable for the culture-based measurements that were reported. Fungi and house dust mite allergens were measured in floor dust. The mean *Alternaria* levels were 0.3 and 1 cfu/m^3 in offices of non-symptomatic and symptomatic workers, respectively, but no data on other species or total fungi were given or on the number of collected samples. Work-related respiratory symptoms were associated with *Alternaria* exposure with OR 4.2 (95% CI 1.1-16) 1-7 cfu/m^3 compared to the reference group where *Alternaria* was not detected, after adjustments for smoking, house dust mite allergen and difference between absolute indoor and outdoor water content (134).

Purokivi *et al* studied 37 employees working in a school building with moisture problems and mould growth and 23 employees from a school without such problems in Finland. Culturable moulds were measured during the winter, n=17 and 18, geometric mean 29 and 6 cfu/m^3 in the moisture-damaged and control schools,

respectively. Respiratory and eye symptoms were recorded and nasal lavage and induced sputum samples were analysed for inflammatory cytokines and cells. Higher levels of IL-1 and IL-4 were found in nasal lavage and of IL-6 in induced sputum at the end of the spring term in employees from the school with moisture problems compared to employees from the control school. This was incorrectly claimed for IL-6 and TNFα in nasal lavage in the summary. Differential cell counts were similar except for elevated eosinophil counts in atopic employees at the water-damaged school after the summer holiday. Symptom prevalences were not significantly different. Influence of confounders was assessed by separate analyses in employees with and without atopy. There were several inconsistencies in the presentation and summaries of the results, however. Differences were observed compared to biological samples collected after vacations, but these results are difficult to interpret in terms of exposure levels (181).

Roponen *et al* studied 41 randomly selected teachers in Finland. Culturable fungi and bacteria were measured by personal sampling during 24 hours at home and 8 hours at work. Nasal, eye, throat, lower airways and non-specific symptoms during the last week were recorded, nasal lavage samples were analysed for inflammatory cytokines and the combined particle mass was extracted and tested with the mouse RAW264.7 macrophage cell line for cytokine production and cell viability. The subjects were divided in groups with low (median 12 cfu/m^3, range 0-31 cfu/m^3) and high exposure to fungi (median 67 cfu/m^3, range 31-270 cfu/m^3). The study was repeated two weeks later to study intra-subject variability. Symptom prevalences and the levels of NO, IL-4, IL-6 and TNFα in nasal lavage were similar in both exposure groups. IL-1β was elevated in the high exposure group but not significantly. Influence of confounders was assessed by separate analyses in smoking and asthma status subgroups. IL-1β and IL-6 were significantly elevated in the mouse macrophage assay, but not TNFα and cell viability. Intra-individual variability of the cytokines in the nasal lavages and mouse macrophage tests was relatively low. However, the correlations between determinations of IL-1β, IL-6 and TNFα by these tests were also low (203).

Seuri *et al* described a cluster of asthma and rhinitis in a small population of 14 employees from a hospital with a history of water damage. The population was tested with bronchial provocation with histamine and a *Sporobolomyces salmonicolor* antigen extract, nasal provocation and skin prick tests with the *S. salmonicolor* antigen, skin prick tests with common respiratory allergens, and serum-IgG to fungi typically found in water-damaged buildings. Exposure to fungi was measured once by stationary sampling on 10 locations, and exceeded 500 cfu/m^3 at three locations with a maximum of 1 400 cfu/m^3. About half of the colonies were *S. salmonicolor*. Exposure levels had probably been higher as a floor had been repaired because of water damage a year earlier. Asthma was diagnosed by a doubling of the airway resistance after inhalation of aerosolised *S. salmonicolor* extract as an immediate response, or a 15% fall in peak flow during follow-up as a late response. A positive nasal provocation was diagnosed by distinct watery discharge from the nostril where the extract had been applied. Four cases of

asthma (6 employees were tested) and eleven cases of rhinitis (14 employees tested) were diagnosed. The response was probably not IgE-mediated as skin prick tests with the same antigen were negative. Serum IgG antibodies to this (8 of 13 employees) and several other fungi typical in water-damaged buildings (11 of 13) were found (217).

Appendix 8. Human challenge studies using spores

Cox *et al* challenged 4 mushroom workers with hypersensitivity pneumonitis to *Pleurotus ostreatus* to an aerosol of a spore suspension for 10 minutes. The workers inhaled a dose of 0.65 mg spores, which is approximately 10^7 spores, and developed fever, chills, muscle pain, dyspnoea, leukocytosis and FVC decreases 6-8 hours after the challenge. X-ray changes were negative (35).

Cox *et al* challenged 5 mushroom workers with hypersensitivity pneumonitis to *Lentinus edodes* to an aerosol of a spore suspension for 10 minutes. The workers inhaled a dose of 0.5 mg spores, which is approximately $3 \cdot 10^7$ spores and developed fever, chills, muscle pain, dyspnoea, leukocytosis and FVC decreases 6 hours after the challenge. One patient developed decreased TLco and decreased PaO_2. X-ray changes were negative (36).

Licorish *et al* challenged 7 mild asthmatic patients with whole spores of *Alternaria alternata* and a *Penicillium* isolate that were inhaled mixed with lactose (≤1:200 weight ratio spores:lactose) by means of a Spinhaler. Doses of $1 \cdot 10^5$-$4 \cdot 10^5$ *A. alternata* spores were sufficient to induce a 35% drop in specific airway conductance in 4 sensitised patients. The doses that induced the same effect in 2 patients sensitised to *Penicillium* were more variable, $4 \cdot 10^4$ and $5 \cdot 10^7$ *Penicillium* spores, and one patient did not respond at all. The latter patient had a weak positive skin prick test to the used antigen, however. After bronchial challenge with spore extracts only immediate reactions were observed whereas both immediate and late asthma attacks were provoked after bronchial challenge with whole spores (125).

Meyer *et al* exposed 8 employees with building-related symptoms from mould-damaged schools for 6 minutes to aerosols of *Penicillium chrysogenum* and *Trichoderma harzianum* liberated from cultures grown on gypsum boards, and placebo on different days. The selected participants had positive *P. chrysogenum* and negative *T. harzianum* histamine-release tests, but skin prick tests to the same moulds were negative. Spore concentrations were approximately $6 \cdot 10^5$ spores/m^3 and $3 \cdot 10^5$ spores/m^3 of *P. chrysogenum* and *T. harzianum*, respectively. Symptoms (nasal and throat irritation, nasal congestion and headache), lung function, and leukocytes in blood were recorded. The challenges were performed double blind. Small but significant changes were observed after challenge compared to before challenge, but the responses were similar to placebo exposure (135).

Riddle *et al* challenged a malt worker with hypersensitivity pneumonitis to a spore extract and dry spores of *Aspergillus clavatus* and to dry spores of *Penicillium brefeldianum*. The workers inhaled a dose of 20 mg of dry spores of both species which equals approximately 10^9 spores of *A. clavatus* (spore size of *P. brefeldianum* not found) and developed fever, shortness of breath, nausea, sweating, headache and aching trunk and limbs and FVC decreases 3-6 hours after the challenge to *A. clavatus*. Arterial oxygen tension (PaO_2) and lung transfer factor for carbon monoxide (TLco, measure of gas diffusion) were also decreased, neutrophils increased and eosinophils were unchanged. The symptoms were

similar to those experienced at work and the decreased FVC lasted one week. Challenge with *A. clavatus* extract or *P. brefeldianum* spores resulted only in minor changes (198).

Sigsgaard *et al* exposed 5 garbage-recycling workers without symptoms and 5 retired garbage workers with occupational asthma intranasally to *Aspergillus fumigatus* spores, 10 mg suspended in 10 ml of water for 15 minutes. No changes in the inflammatory cytokines (TNFα, IL-1β, IL-6 and IL-8) and cells or nasal volume were observed until 11 hours after the challenge (223).

Appendix 9. Maximum allowable concentrations of fungi and actinomycetes issued in the Russian Federation

Translated from Russian by Dr Natalia Romanova (Northwest Public Health Research Centre, St. Petersburg, Russia).

Microorganism	Maximum allowable concentration, cfu/m^3	Hazard class[a]	Allergenicity
Fungi			
Acremonium chrysogenum	$5 \cdot 10^3$	III	+
Ampelomyces quisqualis	10^4	III	
Blakeslea trispora	10^4	III	+
Candida scotti	10^3	II	
Candida tropicalis	10^3	II	
Candida utilis	10^3	II	
Candida valida	10^3	II	
Cryptococcus laurentii var. *magnus*	0.5 mg/m^3	II	+
Fusidium coccineum	$5 \cdot 10^3$	III	
Penicillium canescens	$2 \cdot 10^3$	III	
Saccharomyces cerevisae	0.5 mg/m^3	II	+
Actinomycetes			
Actinomyces roseolus	10^3	II	
Streptomyces aureofaciens	$5 \cdot 10^3$	III	+
Streptomyces erythreus	$3 \cdot 10^3$	III	+
Streptomyces lactis	10^4	III	
Streptomyces kanemyceticus	$5 \cdot 10^3$	III	+
Streptomyces rimosus	$3 \cdot 10^3$	III	+

[a] No information provided on the hazard classification.