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Good summary , includes endotoxins

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FOCUS

Bioaerosol exposure assessment in the workplace: the past, present and recent advances†‡

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Louis Pasteur described the first measurements of airborne microorganisms in 1861. A century later, the inhalation of spores from thermophilic microorganisms was shown to induce attacks of farmers' lung in patients with this disease, while endotoxins originating from Gram-negative bacteria were identified as causal agents for byssinosis in cotton workers. Further epidemiological and toxicological studies have demonstrated inflammatory, respiratory, and pathogenic effects following exposure to bioaerosols. Exposure assessment is often confounded by the diversity of bioaerosol agents in the environment. Microorganisms represent a highly diverse group that may vary in toxicity. Fungi and bacteria are mainly quantified as broad groups using a variety of viable and nonviable assessment methods. Endotoxins and β(1 → 3)-glucans are mainly measured by their activity in the *Limulus* amebocyte lysate assay, enzymes by immuno-chemical methods and mycotoxins by liquid chromatography-mass spectrometry. Few health-based occupational exposure limits (OELs) are available for risk assessment. For endotoxins, a health-based OEL of 90 endotoxin units m^{-3} has been proposed in the Netherlands. A criteria document for fungal spores recently proposed a lowest observed effect level of 100 000 spores m^{-3} for non-pathogenic and non-mycotoxin producing species based on inflammatory respiratory effects. Recent developments in bioaerosol assessment were presented at the Organic Dust Tromsł Symposium including molecular biological methods for infectious agents and organisms that are difficult to cultivate; studies of submicronic and hyphal fragments from fungi; the effect of biodiversity of microorganisms in asthma studies; and new/improved measurement methods for fungal antigens, enzymes and allergens. Although exposure assessment of bioaerosol agents is complex and limited by the availability of methods and criteria, the field is rapidly evolving.

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Environmental impact

This paper describes current methods for the measurement of exposure to bioaerosols at the workplace, and occupational exposure limits that are available for risk assessment. The assessment of health risks from bioaerosol exposure is complex due to diversity of the agents. Few regulatory occupational exposure limits have been adopted for bioaerosol agents, but proposed health-based exposure limits for endotoxins and fungal spores can be used. New developments are shortly reviewed, including molecular biological methods; studies of submicronic and hyphal fragments from fungi; the effect of biodiversity of microorganisms on asthma; and new/improved measurement methods for fungal antigens, enzymes and allergens. These methodological advancements are expected to aid in exposure and risk assessments of bioaerosol exposure in the future.

Background

Bioaerosols (syn. organic dust) are commonly defined as aerosolized particles with a biological origin. These particles originate from all types of organisms and can be dispersed into the air by a variety of abiotic and biotic mechanisms. In the occupational environment, examples of bioaerosols include fungal and bacterial spores/cells, fungal hyphae, pollen, viruses and amoebae, aggregates of these particles, and fragments of larger organisms including cotton and wood dust, flour, skin scales, animal dander, textile and paper fibres. Metabolites and excreta are also included in this context.

In 1861, the first measurements of airborne microorganisms were reported by Louis Pasteur in the journal *Annales des Sciences Naturelles*.[1] A century later, research into the role of bioaerosols in occupational diseases was mainly focused on non-infectious diseases. Pepys first demonstrated that the inhalation of spores from thermophilic actinomycetes could induce attacks of farmers' lung in patients with the disease.[2] During the 1970–80s byssinosis among cotton workers was an important research topic. Gram-negative bacteria and the endotoxins that are located in the outer cell wall of these bacteria were shown to be the most likely causal agents for this disabling disease.[3] Since then, epidemiological and toxicological studies have demonstrated exposure–response associations with different agents, including enzymes and allergens.[4]

From 1985 to 2003 four meetings were organized by R. Rylander and the ICOH Organic Dust Committee on causative agents for organic dust related disease; the so-called Skokloster meetings.[5–8] These meetings made important contributions to the diagnosis of organic dust-related diseases by specifying the difference between the potentially disabling disease, hypersensitivity pneumonitis, and the benign organic dust toxic syndrome. Both of these diseases are characterized by febrile symptoms.[5] Acute irritation of the airways and eyes is also prevalent among bioaerosol exposed workers and was termed "mucous membrane irritation".[6] A number of possible causal agents were identified:[6]

- non-pathogenic bacteria and fungi
- microbial components:
- endotoxins
- β(1 → 3)-glucans
- mycotoxins
- allergens (from plants, microorganisms, insects and animals)
- enzymes—*e.g.* amylases, proteases, proteins.

The 3rd meeting further addressed these agents as well as experimental models.[7] The last meeting focused on susceptibility to organic dust and exposure assessment.[8]

Risk assessment and exposure limits

Risk assessment of bioaerosol-exposed workers is complicated by the diversity of agents in occupational environments and by few occupational exposure limits (OELs) set by regulatory organizations. Regulatory OELs have been adopted for cotton, grain, wood, flour, organic dust, and subtilisins, Table 1. These limits are based on dust levels in relevant industries and do not consider specific components present in the dust. Even the OEL for "particulates not otherwise regulated"[9] is used in lack of more specific OELs. The only exception is for subtilisin; however, this specific regulatory OEL does not seem to protect against IgE sensitization.[11] In Russia, regulatory OELs for bioaerosols have been adopted for a number of fungal and actinomycetes species and range from 10^3 to 10^4 cells m^{-3};[12] however, the scientific documentation for these exposure limits is difficult to find.[13] The European Union has published Directive 2000/54/EC on the protection of workers from health risk related to biological agents.[14] This directive deals mainly with the risk of infectious agents and gives guidance on health surveillance and containment levels. However, exposure limits of neither infectious nor non-infectious biological agents are given.

In cotton factories, exposure–response associations for byssinosis were strongest in the departments where raw cotton was first handled,[15] indicating that the active agent(s) were partly removed during carding. Later studies showed that endotoxins from bacterial contamination of raw cotton were the most likely causal agent of byssinosis.[3]

Specific OELs are required to protect workers health. However, bioaerosol research has thus far only resulted in proposed exposure limits for endotoxins and fungal spores. In the Netherlands, 90 endotoxin units m^{-3} has been proposed as the OEL for endotoxins on the basis of acute respiratory effects.[16] Recently, a lowest observed effect level (LOEL) of 100 000 spores m^{-3} for non-pathogenic and non-mycotoxin producing fungal species has been proposed in a criteria document based on inflammatory respiratory effects.[13] Guidelines for fungi in indoor environments have also been proposed by several organisations; however, these criteria have been developed for the assessment of indoor mould problems and are not health-based.[17,18] In industries that utilize or manufacture enzymes, in-house occupational exposure guidelines derived minimal effect levels (60 ng m^{-3}).[19] For other agents, risk assessments may be based on exposure–response associations found in relevant epidemiological studies, *e.g.* β(1 → 3)-glucans and allergens, but lack of standardization of measurement methods represents a great challenge.[20,21] Since exposure–response relations have been described especially for sensitizing agents, standard setting seems most promising for these agents.[22] Few

Table 1 Occupational exposure limits in USA and Norway

Agent	ACGIH[a], USA[9]	Norway[10]
Raw cotton dust[b]	0.2 mg m^{-3} (<15 μm AED)	0.2 mg m^{-3} (<15 μm)
Grain dust (oat, wheat, barley)	4 mg m^{-3} (total dust)	None
Flour dust	0.5 mg m^{-3} (inhalable dust)	3 mg m^{-3} (inhalable dust)
Wood dust[c]	0.5–1 mg m^{-3} (inhalable dust)	1–2 mg m^{-3} (total dust)
Organic dust	None	5 mg m^{-3} (total dust)
Particulates not otherwise regulated	10 mg m^{-3} (inhalable dust)	10 mg m^{-3} (total dust)
Subtilisin (protease from *Bacillus subtilis*)	60 ng m^{-3} (total dust, STEL[d])	60 ng m^{-3} (total dust)

[a] American Conference of Governmental Industrial Hygienists. [b] Measured stationary with a vertical elutriator. [c] Dependent on species. [d] Short term exposure limit.

exposure–response relations have been described for fungal constituents like glucans and extracellular polysaccharides (EPS) and this complicates a standard setting process.

Measurement methods

Cotton, grain, wood, flour and organic dust are measured by filter sampling and gravimetry of the collected dust. Specific methods for these agents do not exist, and the composition of the dust is evaluated by expert judgement. Only subtilisins can be measured by standardized methods.[11] For most dust types, the inhalable fraction is collected which includes large particles that may cause irritation symptoms in the upper airways and eyes. Cotton dust is measured with stationary vertical elutriators that were designed to collect particles <15 μm aerodynamic diameter (AED) as this OEL aims to protect against byssinosis. However, the 50% cut-off AED of these instruments was found to be 20 μm.[23]

Sampling of bioaerosol agents in the work environment should be based on the same principles as dust sampling in general. As bioaerosols may be represented by particles of varying sizes, inhalable samplers are preferred for measurements in the workplace. The particle size selection criteria for the inhalable fraction are defined in a CEN document where the thoracic and respirable fractions are also described. The latter are relevant for outcomes in the lower airways and alveoli, respectively.[24] Compared to filter sampling, few bioaerosol samplers collect the inhalable fraction or can be used for breathing zone sampling, which is a further requirement in occupational exposure assessment.[25]

Bioaerosol samplers range from impaction devices (impactors and impingers) to cyclones and inhalable dust cassettes, the latter being most common. More elaborate measurements make use of impactor devices that can fractionate bioaerosols according to size, but few of these samplers are suitable for personal sampling.

The chemical structure and toxicity of endotoxins differs across species of Gram-negative bacteria. Therefore, the combined activity of endotoxins is assessed with the *Limulus* amebocyte lysate (LAL) assay using an enzyme system derived from the horseshoe crab. Endotoxin from *E. coli* is typically used as a reference. Monoclonal antibody-based methods have also been developed but are less sensitive than the LAL assay. Samples are collected on glass fibre filters and endotoxin quantified using a chromogenic version of the LAL assay.[26] Endotoxin exposure assessed with this method has been shown to be associated with adverse respiratory health effects in numerous epidemiological studies performed in different populations.[16] However, limitations of the LAL method include substantial interlaboratory variations (5–12 fold), and water insoluble endotoxins cannot be detected.[27,28] Endotoxins can also be estimated by gas chromatography-mass spectrometry using 3-hydroxy fatty acids as chemical markers. In sewage treatment plants, this method showed comparable results with the LAL assay.[29]

Airborne fungi and bacteria can be quantified by cultivation and non-culture based methods.[30] Cultivation methods have the advantage that species can be identified but results depend on a range of factors: the culturability of the collected microorganisms, sampling strain, the growth medium, the applied cultivation conditions, and even the presence of other species. Visible colonies are identified, counted and results are given as colony forming units (CFU). However, non-viable microorganisms may also induce health effects similar to viable microorganisms.[13] For example, non-viable fungal spores have been shown to release allergens.[31] For the purpose of estimating the total microbial exposure culture counts are only semi-quantitative because non-culturable microorganisms are not detected, only one colony may grow from an aggregate of culturable organisms, and fungi with specific nutrient requirements may not grow on the nutrient medium. In addition, cultivation techniques often require short sampling intervals to avoid overloading culture plates and/or culturability loss due to desiccation. In contrast, microscopic methods such as light microscopy, fluorescence microscopy and scanning electron microscopy have been used to quantify airborne microorganisms independent of culture methods. Samples are typically collected on filters allowing full shift personal sampling, however, these methods have limited potential for species classification.

Microorganisms represent a highly diverse group and different species may not be expected to have similar toxicity. To date, identifying etiological bioaerosol agents of adverse health effects in epidemiological studies has been a challenging task. In a recent review of the toxicological and epidemiological studies of fungi, no major differences between effect levels of spores from many species were found, except for those species that are pathogenic and/or produce mycotoxins.[13] For the assessment of dampness problems in indoor environments, the dominance of species like *Aspergillus versicolor*, *Chaetomium globosum*, *Stachybotrys chartarum*, and *Ulocladium chartarum* is used as an indicator of such problems, but these criteria are not health based and indoor exposure levels are generally well below the LOEL recently proposed for fungal spores.[13,17,18]

Molecular biological methods have the potential to quantify exposure to microorganisms independent of culturability and with high specificity. These methods allow for the specific characterization of the microbial biota. The most promising methods to be developed for standard protocols are quantitative PCR (qPCR) for total bacteria, Archae and fungi. Universal primers and probes for bacteria and Archae have been applied to agricultural and industrial environments.[32–35] Genus-or species-specific qPCR primers have also been designed for the detection of bacterial and fungal bioaerosols.[36,37] The most commonly studied genes for bacterial detection are the 16S ribosomal RNA fragments. These fragments come from highly conservative regions in the bacterial genome and can be used to identify species. The techniques and procedures used differ strongly between studies and range from DGGE (Denaturing Gradient Gel Electrophoresis) and SSCP (Single-Strand Conformational Polymorphism), which yield a limited number of species because of the limited sensitivity and high detection limit, to shotgun or deep sequencing which can yield thousands of sequences which have to be compared with libraries. Cloning the 16S PCR product and construction of a 16S gene library may be used. This approach is easily applicable to bioaerosol samples and leads to biodiversity

assessment. This approach was used to evaluate bacterial[33] and archaeal biodiversity in swine barns.[34] The use of different techniques complicates comparisons across studies. Most molecular studies conducted so far are exploratory. A typical meta-genomic study includes a very limited number of samples (due to expense of sequencing costs), thus variability between environments or over time is yet poorly understood. However, the results generated from these studies are exciting and provide greater insight into personal and occupational exposures. Surveys of outdoor environments (soil and water samples) reveal the presence of thousands, sometimes even millions, of different species.[38] A significant fraction of the clone sequences appear to be novel, although quality control issues resulting from amplification of DNA may complicate interpretation.[39] Täubel *et al.* studied bacterial diversity on skin samples and mattress and floor dust samples.[40] Analysis of samples from four houses showed that mattress dust samples are dominated by Gram-positive bacteria. The mattress samples had a microbial spectre which came closest to the human skin, suggesting that shedding of microorganisms by the occupants of the houses determined to a large extent the microbial flora of the mattress. Bacterial diversity appeared strongly dependent on location, and exposure in close proximity of the farmer differs from further away from sources.[41] Few studies have associated microbial diversity with health risks. The association between microbial exposures and the protective effect for asthma and allergy has been put in a completely new perspective by recently published results from two independent population surveys.[42] A direct association was found between environmental microbial diversity and protective effects for asthma and atopy.

For fungal aerosols recent developments in molecular technologies have enabled the differentiation of DNA sequence variation to characterize fungal diversity. Several genomic loci have been used for sequence comparison; however, the internal transcribed spacer region of fungal nuclear rRNA is the most widely utilized. Previous studies using this molecular screening approach have provided new insight into the diversity of fungal bioaerosols within the indoor built environment.[43] However, like most exposure assessment methodologies, limitations associated with extraction are important considerations that require further optimization.[44] Recently, universal qPCR for the detection of fungi in indoor environments has been employed to identify potentially contaminated environments. Several options are available for group- or genus-specific primers.[45] Viruses can also be quantified from aerosols but specific protocols have to be designed for each virus since no "universal" markers are available. The quantification of viruses in bioaerosols has been described in industrial,[46] agricultural[47] and in laboratory-generated aerosols[48] where filtration and extraction methods have been compared. Methods for sampling airborne viruses have also been reviewed.[49]

Molecular techniques have been most often used for measurement of single species in the air using qPCR techniques. Examples exist where zoonotic microorganisms like *Coxiella burnetii*, responsible for Q-fever clusters after transmission from goats or sheep through the air, have been measured in the air.[50] The most extreme example is *Archaea*, formerly classified as *Archaea*-bacteria, nowadays considered a separate domain. Some *Archaea* species live in the gut of ruminants and lead to human exposure in animal husbandry and farming. They are very difficult to culture because they are extreme anaerobes and sensitive to oxygen. Levels in stable air appeared extremely high up to 10^8 per m^3 on the basis of qPCR measurements of species specific 16S rRNA.[34] However, there are currently no exposure criteria for evaluation of occupational measurements performed with molecular biological methods.

β(1 → 3)-Glucans can be quantified by a version of the LAL assay. Immunoassays for β(1 → 3)-glucans have also been described but these methods had much lower sensitivity than the LAL-based method. A recent study described improvements of these immunoassays that allowed the measurement of β(1 → 3)-glucans in air samples.[20,51] β(1 → 3)-Glucans are often regarded as markers of fungi, but these agents can also be found in some plants and bacteria, and may also induce airway inflammation.[20] Other markers, such as ergosterol for fungi, can be measured,[30] but few epidemiological studies have included these agents, and their health relevance is at present unclear.

Occupational exposure to high (>5 kDa) and low molecular weight (haptens) antigens may result in allergic sensitization and exacerbate respiratory diseases such as occupational asthma (OA). More than 250 high-molecular-weight allergens have been characterized in OA and these include a variety of proteins derived from organic dusts, including fungi.[52,53] Typically, these allergens can be quantified by immunochemical methods such as enzyme-linked immunosorbent assays (ELISA) if antigen-specific monoclonal or polyclonal antibodies are available.[54]

Mycotoxins can be analysed by liquid chromatography-mass spectrometry and in some cases ELISAs (*e.g.* aflatoxins and trichothecenes). However, these methods are currently not sensitive enough to detect mycotoxin levels in personal samples. Indirect assessment by analysing settled grain dust or detecting mycotoxin-producing species in personal samples of grain farmers by real-time PCR has been reported.[55,56] In spite of the high toxicity of mycotoxins, exposure levels and health risks from airborne mycotoxin exposure are mainly unknown.

Challenges for bioaerosol exposure assessment research

Recent developments in bioaerosol exposure assessment were presented at the Organic Dust Tromsø Symposium that was organized in Norway in April 2011, including:

Multiple resistant *Staphylococcus aureus* strains were measured by molecular biological methods and were shown to be transmitted from swine to humans in several studies as well as their presence in outdoor air. Airborne *Coxiela burnetti* could also be detected in and outside goat stables.

Fungal fragments smaller than spores have been shown to be released from fungal colonies in air chamber studies. Recently termed non-gonomorphic particles, these particles are defined to have become mechanically severed from the parent mycelium but were not programmatically differentiated as separable.[57] Non-gonomorphic particles include hyphal fragments (<100 μm), chlamydospores, partial multicellular conidia, and subcellular fragments of

hyphae and conidia. Particle fragmentation can be facilitated by several biotic (fungal autolysis, hyphal vacuolation, shizolytic/rhexolytic separation, as well as prokaryote, protozoan, and micro-arthropod comminution) or abiotic processes (wind, vibration, anthropogenic, and mechanical disturbances). In some environments, larger non-gonomorphic particles (>2.5 μm) may represent a significant proportion of the fungal bioaerosol load (~56%) and are derived from species within the orders Capnodiales, Eurotiales, and Pleosporales.[57] Immunodiagnostic methods such as the Halogen immunoassay have demonstrated non-gonomorphic particles to contain antigens as well as allergens. These preliminary studies have initiated collaborative studies into the occurrence and possible health effects associated with personal exposure to non-gonomorphic particles. The contribution of respirable sized fragments to personal exposure especially in contaminated indoor and occupational environments remains less clear and is the focus of future research.

Recent studies using molecular biological methods have shown the presence of previous unnoticed micro-organisms such as the Archaebacteria in high concentrations in animal houses. Biodiversity of microorganisms assessed in genomic studies has shown promising results in asthma research.[34] The effect of Archae on lung inflammation has been recently published and shows that unsuspected agents may have great influences and impacts on human respiratory health.[58]

New/improved methods for quantifying fungal antigens, proteases, other enzymes and allergens were also presented.

Overview papers from this symposium are planned to be published elsewhere.

There is an increasing need for OELs for bioaerosols that are known to exacerbate adverse health effects: endotoxins, fungal spores, β(1 → 3)-glucans, mycotoxins, allergens and enzymes. Setting OELs requires more exposure–response data derived from a greater number of animal models and, in particular, epidemiological studies of human exposure. Standardized and reproducible measurement methods are also required to compare between studies in different environments. The lack of available monoclonal or polyclonal antibody-based immunoassays remains a great caveat in the exposure assessment field. Until more immunoassays are developed it will be challenging to establish exposure–response relationships in epidemiological studies, particularly for high-molecular-weight antigens. However, exposure levels to endotoxins and fungal spores, especially in the agricultural sector, can be extremely high and exceed the proposed limits by more than 10 fold. No doubt, this remains an area of great concern for occupational health researchers and the reduction of exposure levels in these environments is of utmost importance.

Bioaerosol exposure is usually to a heterogeneous mixture of agents that need to be considered in epidemiological studies as well as in risk assessments. In addition, exposure levels of microbial agents often show high variability. The median geometric standard deviation of endotoxin exposure was 3.4 compared to 2.5 for inhalable dust in a large database of bioaerosol exposure in agricultural and waste handling industries. As a consequence more measurements need to be conducted in order to achieve exposure estimates with similar accuracy as for chemical agents.[59]

Conclusions

Bioaerosol exposure assessment is a rapidly evolving field. As yet, OELs for organic dust seem insufficient for risk assessment in the workplace. Health-based exposure limits have been proposed for endotoxins and fungal spores that are recommended for improved risk assessments. However, more specific OELs are required as the complex composition of bioaerosols represents a major challenge for assessing risks. It may be expected that the recent methodological advancements will aid in the identification of new biomarkers of exposure. This may have wider implications for our understanding of bioaerosol mediated disease in the occupational environment and improve the assessment of bioaerosol exposure in future studies.

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