Fungal aerosols: characterization and immunodetection of fungal fragments

Luftbårne soppartikler: karakterisering og

immunodetektering av soppfragmenter

Philosophiae Doctor (PhD) Thesis

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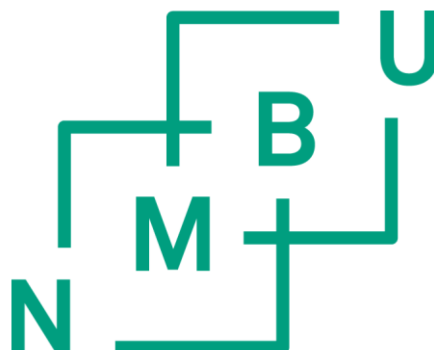
Ås (2015)



Thesis number 2015:26

ISSN 1894-6402

ISBN 978-82-575-1277-4

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Acknowledgments

The present work has been carried out at the National Institute of Occupational Health (NIOH), in the Division of Toxicology and Biology at the Department of Chemical and Biological Working Environment in the period October 2010 – march 2015 including a short research stay of three months at Allergy and Clinical Immunology Branch (Health effects Laboratory Division, Center for Disease Control and Prevention, Morgantwon, WV USA). This work was financially supported by the Research Council of Norway. I would like to thank all for providing me excellent working conditions.

My sincere gratitude goes to my supervising team: Dr Wijnand Eduard, Dr Anne Straumfors, Dr Asbjørn Skogstad at NIOH, Dr Brett James Green at NIOSH, Dr Ida Skaar at the Norwegian Veterinary Institute, Dr Linda Hjeljord and Prof. Arne Tronsmo at the Norwegian University of Life Science for guidance, for sharing their knowledge and skills throughout this project.

I would like to thank Elin Rolen at the Section of Mycology at the Norwegian Veterinary Institute for helping me with fungal isolates. Thanks also due to Else Marie Aasen at the Section of Environmental Microbiology at the Department of Chemistry, Biotechnology and Food Science – Norwegian University of Life Science for providing me different fungal and bacterial isolates.

Further, I would like to thank Dr Ajay P Nayak and Angela Lemon for their technical assistance and help during my short research stay at Allergy and Clinical Immunology Branch Lab at Center for Disease Control in Morgantown.

I do not forget all my colleagues at NIOH and especially at KBA and TOKS for creating a friendly milieu.

Special thanks also due to my friend Manjit Singh for his moral support and proof reading my manuscripts and thesis.

Also thanks to Berit Normann for the moral and material support.

But most of all, I want to thank my mother Kayi Lossa and my deceased father Albert Dossou Afanou for providing me the chance to get an education. To my brothers and sisters, thanks for the support and love.

Finally, I wish to express my love to my dear Ida K. Normann and our two kids, Aisha Akossiwa and Levi Boniface for their patient and understanding during these years.

May JAH bless all of you!

Abbreviations

ABPA: allergic bronchopulmonary aspergilliosis
AED: aerodynamic equivalent diameter
AR: aspect ratio (length:width)
A*w*: water activity
CFU: colony forming unit
DAPI: 4’, 6’-diamidino-2-phenyl-indole
DNA: deoxyribonucleic acid
ELISA: Enzyme-linked immunosorbent assay
FCM: flow cytometry
FDA: fluorescein diacetate
FE (SEM): field emission (Scanning lectron microscope)
FSSST: fungal spore source strength tester
GC/MS: gas chromatography with mass spectrometric detection
HIA: Halogen immuno assay
HPLC: high performance liquid chromatography
IgG: immunoglobulin G
IgE: immunoglobulin E
IgY: immunoglobulin Y
ITS: internal transcribed spacer
LAL: Limulus amoebocyte lysate
m^{-3}: per cubic meter
mL: milliliter
MVOC: microbial volatile organic compounds
NAGase: N-acetyl-beta-glucosaminidase
NAHA: N-acetyl hexosaminidase (NAHase)
NET: neutrophil extracellular traps
µg, ng, pg: microgram, nanogram, picogram
NIOH: National institute of occupational health, Norway
PCR: polymerase chain reaction
PLFA: phospholipid fatty acids
PM_1: particulate matter with aerodynamic diameter ≤1µm
$PM_{2.5}$: particulate matter with aerodynamic diameter ≤2.5µm
$PM_{2.5-10}$: particulate matter with aerodynamic diameter between 2.5µm and 10µm
QPCR: quantitative PCR
RNA: ribonucleic acid
SF: submicronic fragments
SPG: Stami particle generator
µm: micrometer
^oC: degree Celsius
%: percent

Summary

Indoor environments with high moisture problems often reveal colonization of indoor materials by fungi with release of airborne fungal particles. Exposure to airborne fungal particles has been associated with various negative health effects particulary in the respiratory tract. However, epidemiological and exposure studies based on spore particles could not confirm such an association. Exposure to fungal fragments including submicronic fragments, in addition to spores has been therefore suggested to play an important role in the observed adverse health effects. But, detection and enumeration of these fragments in air samples have not been possible. We aimed in the present thesis to characterize the composition, the morphology and the origin of fungal aerosols and further to develop an appropriate method for detection and enumeration of fragments in air samples using field emission scanning electron microscopy (FESEM).

For this purpose, fungal particles were experimentally generated from *Aspergillus fumigatus, A. versicolor* and *Penicillium chrysogenum,* and analyzed in a FESEM for particle types, size, shape, origin and their enummeration. Following the characterization, a novel indirect immunolabelling method was developed using polyclonal chicken IgY against *Aspergillus versicolor* as primary antibodies and gold conjugated polyclonal goat anti-chicken as secondary antibodies. Before the immunolabelling, particles were immobilized and fixed on poly-L-lysine coated polycarbonate filters. The novel method was tested in a proof of principle experiment using indoor air samples collected during renovation of a mold contaminated building.

The composition of the *in vitro* generated fungal aerosols revealed particles of two origins: spore particles described as single spores, aggregates of 2, 3, 4 and ≥5 spores, and fragments particles that were classified by size as 0.2–1µm fragments (submicronic fragments), 1–2µm fragments, 2–3.5µm fragments and ≥3.5µm fragments. Single spores were dominant in aerosols from *A. fumigatus*, while submicronic fragnments represented the largest fraction in the aerosols collected from *A. versicolor* and *P. chrysogenum.* In addition, experimentally aerosolized submicronic fragments mainly originate from mycelial fragmentation, but a small

fraction of spore fragments were observed in experiments with the *Aspergillus* isolates. Based on these species related variations of the aerosols composition, our data support the need to include a broader range of particle types in the assessment of fungal exposure.

Further, generated fungal particles could be classified in three groups of shape based on their aspect ratios. There are near spherical particles that regroup specifically single spores; oblong particles that comprised various particle types such as fragments with length <3.5µm and some spores aggregates (2–5 spores), and the fiber-like particles that included long chained and clustered spore aggregates and large fragments (≥3.5 µm). The shape and size diversity indicated that different fungal particles will have different aerodynamic behaviour in the respiratory tract. In this regard, it is likely that deposition of inhaled fungal particles will occur in all regions in the respiratory tract where they may elicite various adverse reactions.

In the proof of concept experiment with a novel indirect immunodetection with FESEM, we identified that, 13% of the total particle counted in the aerosols were fungal particles, and of which 79% were fragments particles and 21% spore particles. Further, 39%, 40%, 19% and 2% of the total fungal particle counts were submicronic fragments, large fragments, single spores and spore aggregates, respectively. The novel method was thus usefull for detection, enumeration and characterization of fungal particles including submicronic fragments in a complex matrix although the submicronic fragments fraction was slightly underestimated. Identification of fragments from different fungal species and discrimination between fungal fragments from actinomycetes was not possible. The results in this thesis indicate that fungal aerosol composition is complex in morphology and origin, and support the recommendations to consider broader range of fungal particles in exposure assessement of fungi.

Sammendrag

Innemiljøer med fuktproblemer utvikler ofte soppvekst på byggmaterialer som kan føre til frigjøring av luftbårne soppartikler som kan bære allergener, antigener og mykotoksiner. Eksponering for soppartikler har vært assosiert med ulike helseproblemer knyttet til luftveiene, særlig allergi og astma. Epidemiologiske studier basert på eksponeringskarakterisering av sporepartikler kunne ikke bekrefte en slik assosiasjon. Eksperimentell forekomst av soppfragmenter i størrelsen <1µm, i tillegg til sporene, ble derfor foreslått å spille en viktig rolle i de observerte helseproblemene ved eksponering. Hittil, fantes ikke en metode som muliggjør deteksjon og telling av disse fragmentene i luftprøver. Vi satte derfor som mål å karakterisere sammensetningen, morfologien og opprinnelsen til de forskjellige typer soppartiklene som kunne bli luftbårne og utvikle en metode for påvisning og telling av fragmenter i luftprøver ved hjelp av skanning elektronmikroskopi.

For å kunne karakterisere soppartikler som kunne være luftbårne, ble soppartikler generert eksperimentelt fra *Aspergillus fumigatus*, *A. versicolor* og *Penicillium chrysogenum*, og ble analysert og kvantifisert i en "field emission scanning elektronmikroskop" (FESEM) for partikkeltype, størrelse, form og opprinnelse. Etter karakteriseringen, ble det utviklet en indirekte immunmerkingsmetode ved å bruke polyklonale kylling IgY mot *Aspergillus versicolor* som primære antistoffer i kombinasjon med gullkonjugert polyklonale antistoffer fra geit som sekundære antistoffer. Partiklene som skulle analyseres ble først immobilisert og fiksert med glutaraldehyd på poly-L-lysin-belagt polykarbonat filter. Så ble partiklene merket med primært IgY antistoff mot *A. versicolor* og med gullkonjugerte sekundært antistoff som kunne detekteres i FESEM. Denne metoden ble testet på innendørs luftprøver tatt fra en muggkontaminert bygning under renovering.

Sammensetningen av de genererte soppartiklene viste partikler av to opprinnelser: sporepartikler beskrevet som enkelsporer, aggregater av 2, 3, 4 og ≥5 sporer, og fragmentpartikler klassifisert som 0,2-1µm fragmenter, 1-2µm fragmenter, 2-3,5µm fragmenter og ≥3,5µm fragmenter. Andel av enkelsporer var dominerende i genererte aerosolen fra *A. fumigatus*, mens 0,2-1µm fragmentene representerte den største fraksjonen i aerosolene fra *A. versicolor* og *P. chrysogenum*. De genererte 0,2-1µm fragmentene kom, i

hovedsak, fra mycelfragmentering, men en liten andel kom også fra spore fragmentering med *Aspergillus* kulturer som vi tror vil være lite sannsynlig i naturen. Basert på de art relaterte variasjonene observert i partikkelsammensetningen, vil det være nødvendig å detektere flere typer sopp partikler når forekomst av sopp i luften skal vurderes. Videre ble soppartiklene klassifisert i tre ulike grupper basert på morfologien. Vi observerte nær sfæriske partikler som består spesielt av enkelsporer; avlange partikler som består av fragmenter med lengde <3,5µm og noen sporeaggregater (2-5 sporer), og fiberlignende partikler som grupperer langkjedete sporeaggregater og lange fragmenter (≥3,5µm). Denne morfologiske diversiteten indikerer at forskjellige soppartikler ville ha forskjellige aerodynamiske egenskaper i luftveiene. Det er derfor sannsynlig at de kan deponere i ulike regioner i luftveiene og forårsake ulike immune reaksjoner.

I de innendørs luftprøvene, ble det identifisert 13% av totalt antall partikler som soppartikler hvorav 79% av de soppartiklene var fragmenter og 21% sporer. Videre, var 39%, 40%, 19% og 2% av de soppartiklene forholdende 0,2 – 1µm fragmenter, >1µm fragmenter, enkelsporer og sporeaggregater. Denne nye metoden viste seg effektivt for detektering, telling og karakterisering av forkjellige soppartikler i kompleks matriks selv om 0,2 – 1µm fragmenter kunne være underestimert. Identifikasjon av forskjellige sopparter og diskriminering mellom soppfragmenter fra actinomycetes ikke var mulig. Resultatene i denne avhandlingen indikerer at soppartiklenes sammensetning er kompleks i morfologi og opprinnelse, slik at undersøkelser av forekomst av soppartikler i luften bør ta hensyn til at fragmenter kan forekomme i stor mengde i tillegg til sporer.

1. Introduction

1.1. Fungal biology

The Kingdom Fungi comprises eukaryotes that are unicellular (yeast) and/or multi-cellular (filamentous fungi) organisms (1–3). About 1.5 million fungal species are estimated to exist but, only between 80000 and 120000 species are described so far, making fungi the least investigated kingdom (3, 4). Yeasts are unicellular organisms whereas filamentous fungi grow by multi-cellular filaments (hyphae) that develop into complex networks known as mycelium. Reproduction is accomplished asexually by mean of conidia or sporangiospores (anamorphic fungi) or sexually by ascospores, basidiospores and zygospores (telomorphic fungi). Fungi are ubiquitous organisms and represent about 25% of the total organic biomass on earth (5, 6). Three distinctive modes of life are known: saprophytism, parasitism, and symbiotism. Saprophytic fungi exploit and degrade dead organisms for energy supply. They play the primary decomposer role in the biogeochemical cycling of elements in nature (7). Parasitic ones feed on living organisms, while symbiotic or mycorrhizal fungi have mutualistic relationships with other living organisms. Beyond this classification, there are some fungi that can switch from one mode of life to another in order to adapt to environmental conditions. A symbiont (eg.: *Candida spp*) can become parasitic pathogen under certain conditions. Similarly, a soil saprophytic fungus (eg.: *Aspergillus fumigatus*) can become pathogenic upon inhalation and deposition in warmer, moist environments in the lungs of an immune compromised subject. Fungi are chemo-heterotrophic organisms meaning they require organic materials as energy and carbon source. Most fungi are aerobic, but some as *Saccharomyces cerevisiae* (Baker´s yeast) are facultative anaerobic and rumen yeasts are strictly anaerobic. Due to the abundance of organic matter and the availability of oxygen in most environments, it is often the availability of water that limits fungal establishment and growth. Growth of fungi indoors is therefore conditioned by the accessibility of moisture. Moisture availability is usually expressed as water content in organic materials (%) or water activity (A*w*). A*w* is defined as the ratio of vapor pressure in the substrate material by the vapor pressure of pure water. Fungal growth can start on substrate material containing 12–15% water content per weight

and even at lower water content if the air relative humidity is above 85%. The minimum water activity requirement for fungal growth is in the range 0.64–0.9 (8, 9). Depending on the minimum A*w* required for growth, fungi can be classified as hydrophilic (A*w* >0.90) or xerophilic (A*w*: 0.64–0.90). Another important parameter is temperature. Fungi can be classified in three categories depending on the temperature ranges that support growth: the psychrophiles (0–20°C), the mesophiles (20–45°C) and the thermophiles (45–55°C). Most fungi in indoor environments are mesophiles (10). Given that fungi are ubiquitous in most environments and have great adaptation ability to grow on diverse organic materials, occurrence of condensation on internal surface or water leakage problems may induce growth of different groups of fungi (10). Growth of toxigenic fungi indoors may lead to mycotoxin production followed by release of fungal aerosols containing mycotoxins. However, health problems associated with inhalation of mycotoxins are unknown (11).

1.2. Health problems associated to fungal aerosols

Several adverse health outcomes such as irritation, allergy, infection or mycosis and mycotoxication have been linked to fungal aerosol exposure (12). All molds produce a variety of substances that are potentially antigenic or allergenic and which are carried by airborne fungal spores or fragments (13) (12). Fungal aerosols can penetrate the human body through eyes, nose and mouth. Deposition on the mucous membrane initiates irritation that may result in conjunctivitis, allergy or asthma that are IgE mediated. Exposure to mold in damp buildings increases also the risk for hypersensitivity pneumonitis, chronic rhino-sinusitis and allergic fungal sinusitis (14). Hypersensitivity pneumonitis, chronic rhino-sinusitis and allergic fungal sinusitis, in contrast to allergy and asthma, are mediated by IgG and Tcell immune response. Between 3 and 10% of the world population is estimated to have IgE mediated allergy against mold (15), and 5% are predicted to develop clinical allergy, making mold exposure an important public health issue in many countries (16).

Most fungi are saprophytes, not pathogens. However, some saprophytic fungi can cause infection in subjects with reduced immunity. As an example *Aspergillus fumigatus* and other *Aspergillus* species can invade the lungs, causing allergic bronchopulmonary aspergillosis (ABPA). Similarly sinusitis is also caused by fungal invasion of tissues (12). Molds produce secondary metabolites such as antibiotics and mycotoxins during their

growth. Most mycotoxins are cytotoxic for human cells, and interfere with RNA and DNA synthesis (11, 17). In addition, many mycotoxins have immunosuppressive effect and increase susceptibility to infectious diseases (11). Some mycotoxins are carcinogenic or may increase susceptibility to cancer. Common toxigenic fungi in moisture damaged buildings are *Penicillium*, *Aspergillus* and *Stachybotrys* species. Toxins such as citrinin (*P. citrinum, P. expansum, P. viridicatum*), ochratoxin (*P. cyclopium, P. viridicatum*) and patulin *(P. expansum, P. roquefortii*) are nephrotoxic (11). Toxins from *Aspergillus* species such as aflatoxin (*A. flavus, A. parasiticus*), sterigmatocystin (*A. versicolor*) and tremorgenic toxins in conidia from *A. fumigatus* are carcinogenic (11). Macrocyclic tricothecenes (from *Stachybotrys chartarum*) have been attributed to health problems including headaches, sore throat, hair loss, flu symptoms, diarrhea, fatigue, dermatitis, general malaise, and psychological depression (18). Genera like *Alternaria, Epicoccum, Fusarium, Paecilomyces, Trichoderma* and *Cladosporium* have also been reported to produce toxins that can be inhaled through fungal aerosols. However there is no evidence of disease caused by inhalation or respiration of mycotoxins in non- occupational settings (12).

1.3. Fungi in indoor and outdoor air

Fungi are commonly present in outdoor environments which usually represent an important reservoir for introduction into indoor environments (19). Airborne fungal particles are transient outdoors and likely transported into indoor environments. Small variations have been found in the profile of fungal species identified in indoor as compared to outdoor air (20). Indeed, fungal species belonging to *Drechslera* and *Alternaria* genera have been predominantly recovered in outdoor air samples while *Aspergillus* and *Penicillium* were dominant in samples from indoor environments. Moreover species belonging to *Cladosporium, Epicoccum* and *Fusarium* were found in both environments (21). In contrast, Chew et al studied 496 houses without mold problems in the Boston area (USA) and did not find any significant difference between the species profiles identified in indoor and outdoor samples. Isolates belonging to *Cladosporium, Penicillium, Alternaria, Botrytis, Aureobasidium, Eurotium, Aspergillus, Wallemia, Pithomyces, Paecilomyces,* and yeasts were common in air samples from both environments (22). This was also confirmed in another study from Georgia (USA) by Horner and coauthors who demonstrated that species

belonging to *Cladosporium, Penicillium, Aspergillus, Epicoccum,* yeast, *Alternaria* and *Curvilaria* were found in indoor samples from houses without mold contamination as well as in outdoor air samples (23). From Norwegian buildings with humidity problems, the profile of fungal genera identified by a microscopic method comprise *Cladosporium* (22%), *Penicillium* (15%), *Aspergillus* (15%), *Chaetomium* (9%), *Stachybotrys* (7%), *Acremonium* (7%) and *Ulocladium* (6%) (24). Taxonomically, most species growing in indoor environments are saprophytes and belong to the Zygomycotina, Ascomycotina and Basidiomycotina divisions (25).

1.4. Characteristics of fungal aerosols

During colonization of indoor environments, fungal cultures release microbial volatile organic compounds (MVOCs), spores and hyphal fragments that are known as fungal aerosols. Fungal aerosols are acknowledged to impact negatively on health of exposed people (14, 26). In this thesis, the term "fungal aerosols" will be used to designate airborne fungal spores and fragments.

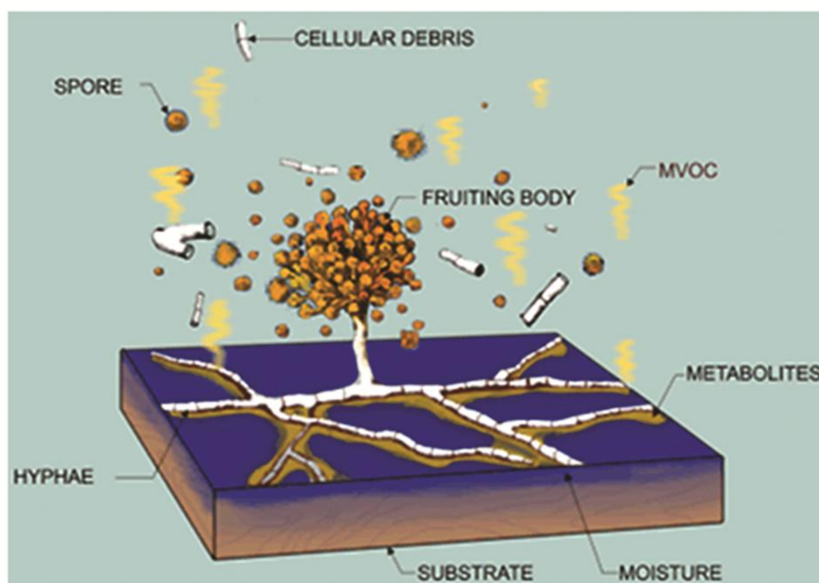

Figure 1: Schematic overview of substrate colonized by filamentous fungi.
(From http://www.wbdg.org/resources/env_iaq.php; 4th March 2015)

Fungal aerosols include a diverse array of particles/propagules, whole or fragmented, dead or viable that passively or actively become airborne from fungal cultures (27, 28) (**Figure 1**). Characterization of fungal aerosols until recently has mainly been focused on spores as

major airborne fungal particles, although some early studies demonstrated the presence of hyphal fragments (29, 30). Focus on fungal particles other than spores increased with the demonstration of large amounts of smaller fragments aerosolized during *in vitro* aerosolization experiments (31). Many tested indoor species in *in vitro* experiments released fragments smaller than spores in addition to spores (31–37). Furthermore, the use of *in vitro* aerosolization approaches on pure cultures of a few selected indoor fungi helped to improve our knowledge on the effect of different parameters such as fungal species, growth substrate, age, air currents, aerosolization regime, air humidity and vibration on the release of fungal spores and fragments into the air. Also the complex composition of the fungal aerosols has been revealed during *in vitro* aerosolization experiments. Single spores, aggregated spores, fragmented spores and fragmented hyphae have been reported in different studies (31–34, 38–41). However there are knowledge gaps on the size and shape characteristics of each particle type in the fungal aerosols.

1.4.1. Fungal spores

Spores are specialized microscopic cells that are actively or passively dispersed from fungal colonies in order to colonize new and suitable environments. These spores can be formed asexually or sexually. Asexual (vegetative) spores are those most commonly encountered in indoor environments and are those referred to in this thesis.

Many species in the Ascomycota and Basidiomycota phyla can actively eject their spores by the mechanisms known as osmotic pressure and surface tension discharge (42). Spores have various morphologies (spherical, oval, ellipsoidal, oblong, star-like etc…), sizes and surface ornamentations that are used in microscopic identification. The aerodynamic equivalent diameter (AED) of spores varies between 1.5 and 50 µm (27), but most frequently in the range of 1.7 - 10 µm for indoor fungi (43). Spores are released into the air as single, clustered or chained aggregates (**Figure 2**)(44).

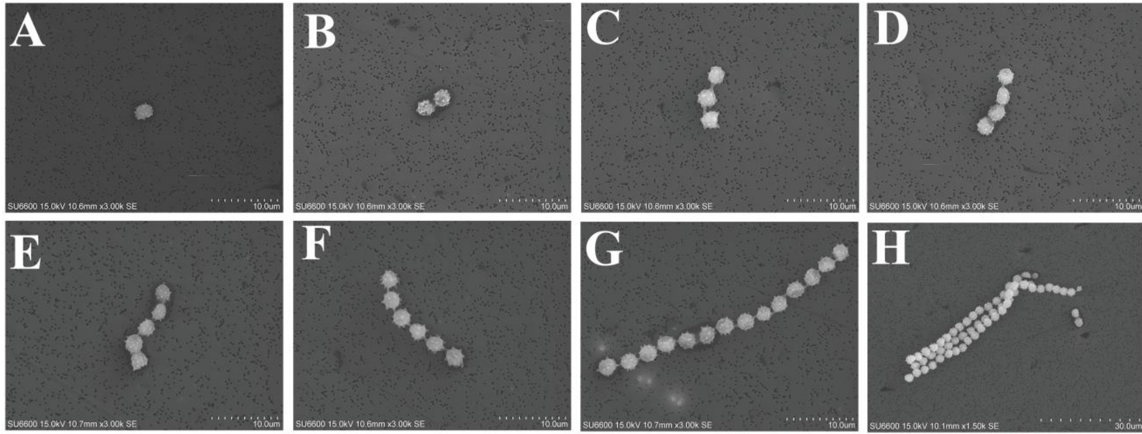

Figure 2: Micrographs of various fungal spore particles from *A. versicolor*. Single spore (A), Spore aggregates (B-H) (*Micrographs by Anani K. Afanou*)

Spore aggregates from the same colony may vary considerably by number of spores, sizes and shapes, and these characteristics remain poorly described. Genera like *Aspergillus* and *Penicillium* are recognized to produce and release unicellular spores, often in clustered or chained aggregates, while *Alternaria*, *Epicoccum* and many other species mostly produce multiple-celled spores (**Figure 3**)(25).

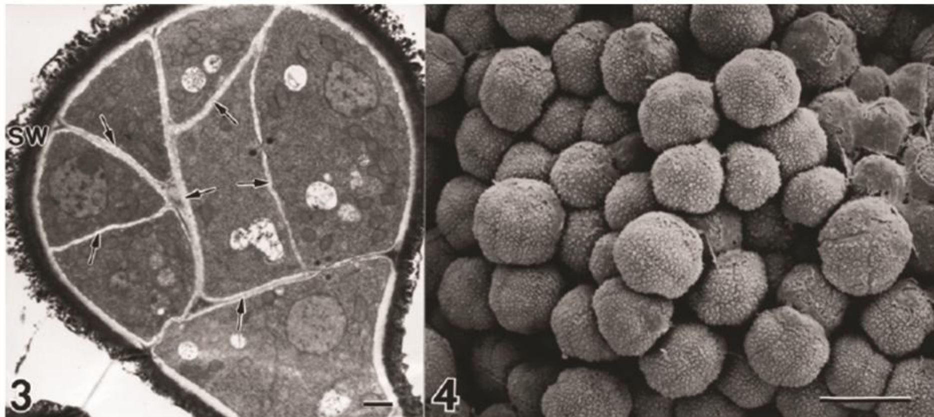

Figure 3: Micrographs of multiple cells spores from *Epicoccum sp*.
(Adapted from (45))

In exposure assessments, the total number of spores is commonly estimated by counting spores present in single spores and in spore aggregates or by colony forming units (CFU). Quantification of single spores and spore aggregates in environmental air samples using microscopy revealed that single spores were more prevalent than spore aggregates in samples from sawmills (>85% of total spores particles) (46) and from composting

environments (>60%) (47). Also *in vitro* generated fungal aerosols from dried cultures of *Aspergillus versicolor* and *Scopulariopsis brevicaulis* showed dominance of single spores (73 – 88 %) (48). Spore concentrations in common buildings without mold problems are often below 10^3 spores m^{-3} air (43, 49). From Cincinnati area (USA), sampling from indoor (six homes) and outdoor air revealed geometric means of 211 and 605 spores m^{-3} air, respectively (50). Another study from Brisbane (Australia) showed mean concentration of 810±389 CFU m^{-3} based on samples from 14 houses with no mold problems while outdoor air concentrations were 1133±759 CFU m^{-3} (51). From Finland, Airaksinen et al. reported 339 – 13000 CFU m^{-3} in crawl spaces (indoor) with mold contamination compared to 140 – 1126 CFU m^{-3} in outdoor air during summer time (52). Although there is no established concentration limit for non-pathogenic fungi, indoor environments with total spore levels above 300 CFU m^{-3} (53) or 10^3 m^{-3} may be suspected of having an indoor source of fungal growth, especially in the winter periods (54, 55).

Moreover, exposure assessments based on spore quantification alone could not confirm any of the associated adverse health outcomes reported from mold-contaminated indoor environments. Indoor airborne spore concentrations have been often below the lowest observed effect level (LOEL) and could not be as such associated with health effects other than allergy (43). The effects of MVOCs seem also too low to play a major role in indoor air associated health problems (56). Thus, the consideration of fungal fragments in exposure assessment is interesting as possible link to fungal adverse health effects (57).

1.4.2. Large fungal fragments

Large fragments are desiccated pieces of hyphae or conidiophores that become airborne (Figure 4). Airborne hyphal fragments have been identified as un-branched conidiophores with sizes between 5 and 20 µm (58). In indoor environments, Green et al reported hyphal fragments with size between 5 and 10 µm (59). The occurrence of hyphal fragments in outdoor air have been known for decades (29, 30) and has also been showed during *in vitro* experiments with *Stachybotrys atra* (now *S. chartarum*) (60). Their presence in air samples from mold contaminated houses and in occupational settings has also been documented (59, 61). Hyphal fragments have been estimated to comprise 6 – 56% of total fungal particles counted in outdoor air by microscopic method (59, 62, 63). However assessment of large fungal fragments in exposure studies is still not common.

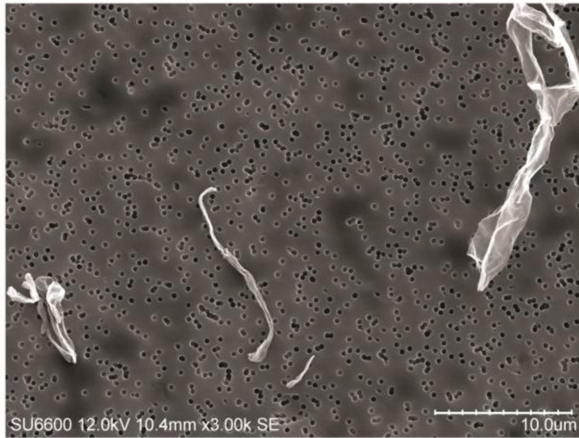

Figure 4: Fungal fragment particles from *P. chrysogenum*.
(*Micrographs by Anani K. Afanou*)

1.4.3. Small fungal fragments

Small fungal fragments have been variously defined in the literature as fungal micro-particles (0.3 – 1.3 µm in aerodynamic diameter) (34), fungal fragments (0.3 – 1.6 µm in optical diameter) (32), fungal fragments of submicrometer-size (< 1 µm in aerodynamic diameter) (36, 37, 64), and submicron fungal fragments (< 1 µm in aerodynamic diameter) (65). In this thesis, these particles will be referred to as submicronic fragments (Figure 5).

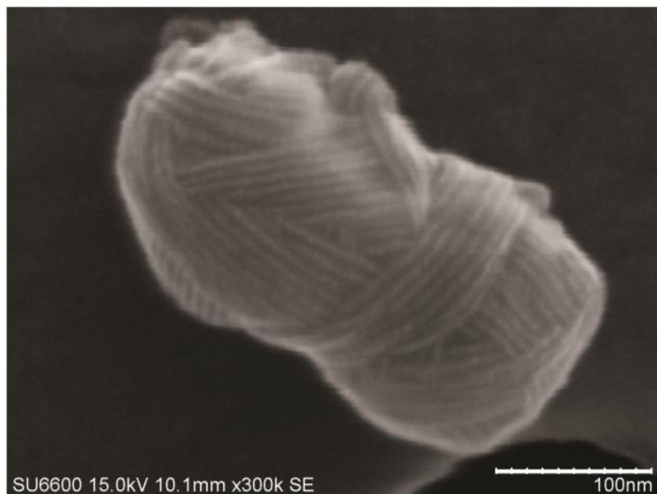

Figure 5: Fungal submicronic fragments from *A. fumigatus*. Rodlet structures visualized at high resolution. (*Micrographs by Anani K Afanou*)

Submicronic fungal fragments have been suggested to play a significant role in the observed adverse health effects related to mold exposure (57). Aerosolization experiments on pure fungal cultures in specially designed chambers led to the discovery of fungal particles smaller than spores. Actually, Kildesø et al. reported the occurrence of fragments smaller than spores from building materials colonized with *Aspergillus versicolor* and *Trichoderma harzianum* using air jets in a generator named PFLEC (Particle Field and laboratory Emission Cell) (31). Quantification of experimentally generated submicronic fragments in this and many other studies was based on automatic particle counters or sizers for enumeration (32, 35–37, 40, 66–72). Over 400 times more submicronic fragments than spores have been reported (35). Scanning electron microscopy (32, 72) and DNA or RNA staining (34) have been used to confirm the nature of submicronic fragments.

To date, there is no report on the numbers of submicronic fungal fragments whether in indoor or outdoor air. Their shape characteristics as well as toxicological properties and importance in indoor environments remain unknown. However, the occurrence of submicronic fungal fragments in indoor air samples have been reported in many studies through quantification of fungal biomarkers such as ergosterol (63, 73, 74), phospholipid fatty acids (75), antigens (32, 76), β-glucans (37, 41, 64, 65, 77–80), mycotoxins (81) and N-acetylhexosaminidase (NAHA) (82) or N-acetyl-beta-glucosaminidase (NAGase) (34, 78). In these studies, the collected aerosols were size fractionated with two stage-cyclones, triplex cyclones, multi-stage impactor or filters. However, there are limitations associated with these methods as demonstrated by the presence of spores in the submicronic fraction (67, 78, 83). Hence, it is not clear whether the reported mass of biomarkers represents the actual fraction of submicronic fragments. The presence of antigens, allergens and mycotoxins in these submicronic fragments (32, 81) furthers a paradigm shift on the mold exposure assessment that has previously focused only on spore quantification.

1.5. Fate of inhaled fungal aerosols in the respiratory tract

The respiratory tract comprises three main regions: the nasopharyngeal region (sinuses and pharynx), the trachea-bronchial region (trachea and bronchial tubes) and the alveolar regions (**Figure 6**). Deposition of inhaled particles in the respiratory tract is governed by three major mechanisms: inertial impaction, sedimentation and diffusion. Deposition by sedimentation and inertial impaction is dependent on the air velocity, shape, size and

density of the particles. The radius of the airways is also of great importance for particle impaction. Sedimentation of particles in the respiratory tract is not only a function of particle size, shape and density, but is also dependent on gravitational and centrifugal forces. Diffusion is dependent on the particle size and the absolute temperature in the respiratory system. Furthermore the mechanism of interception associated with fiber particles and the electrostatic precipitation associated with particles with electrostatic charges are also probable (84, 85).

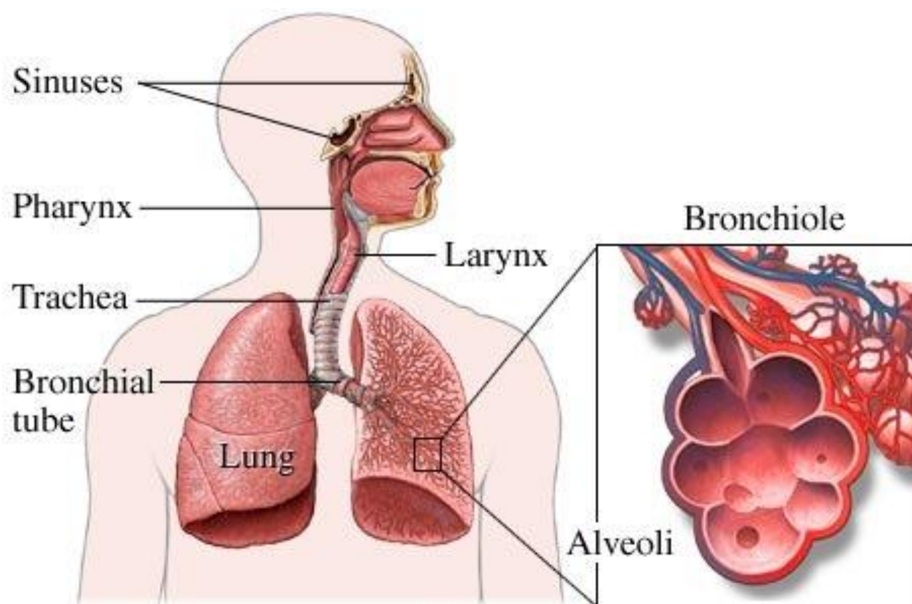

Figure 6: Schematic illustration of human respiratory tract (From http://www.webmd.com/lung/respiratory-system; 4th mars 2015)

Considering that there is great variation in size and shape of fungal particles, it is likely that deposition can occur at all regions of the respiratory tract. Spore particles with aerodynamic diameter larger than 5 µm have been predicted to deposit in greater extent in the naso-pharynx and extra-thoracic regions, where they may trigger nasal and ocular disorders such as allergic rhinitis and allergic asthma (86). Particles smaller than 5 µm are more respirable and have been predicted to enter the alveoli where they can trigger allergic alveolitis or infections when viable (87, 88) (**Figure 7**). However, there is no absolute particle size delimitation associated to deposition sites in the respiratory tract. Particle

deposition in the lung is likely continuous and gradually increases with their increasing aerodynamic equivalent diameter (89).

Between 30 and 40% of spore particles from *Aspergillus* and *Penicillium* (aerodynamic diameter: 2 - 3 µm) have been predicted to deposit in the alveoli during nasal respiration versus 70% during oral respiration (86). It has been shown that 67% of inhaled spores from *Calvatia excipuliformis* (aerodynamic diameter of 3.1µm) by Syrian golden hamster were deposited in the alveoli while 32% and 0.5% were found in respiratory bronchioles and broncho-trachea, respectively (90). Furthermore, a computerized respiratory model has predicted that 17% and 19% of inhaled submicronic fragments from *A. versicolor* will deposit in the alveolar regions of an adult males and infants of three months, respectively. For spores from the same species, predicted alveolar depositions were 14% and 6% in adult and infant, respectively (35). The diversity in size, shape and source of inhaled fungal aerosol are thus of great importance for their fate in the respiratory system. Characterization of various arrays of particle types in the fungal aerosols is therefore believed to provide new insights on their physical properties. Such data may also be useful to better predict deposition site of fungal aerosols in the respiratory tract.

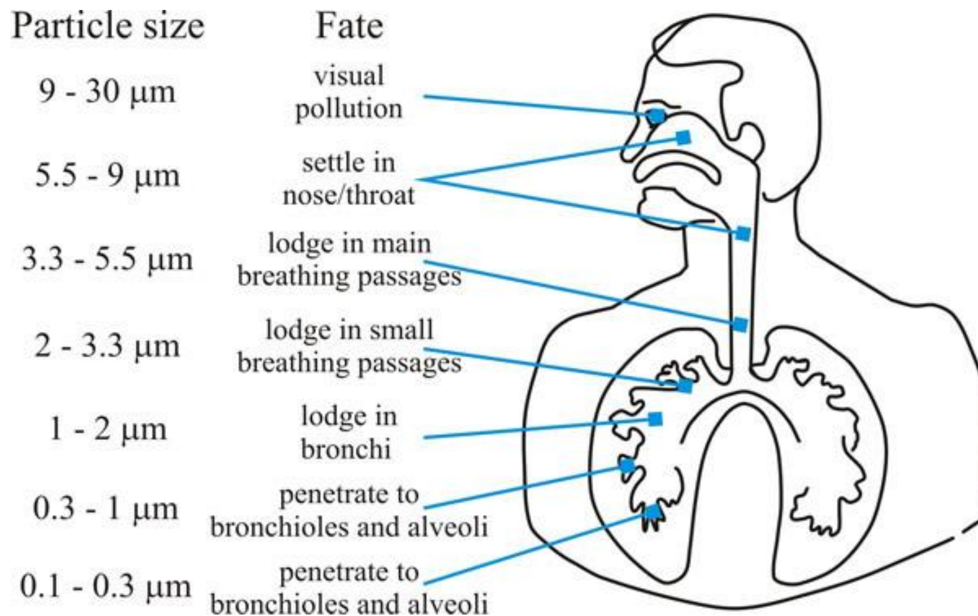

Figure 7: Illustration of human respiratory tract and approximate deposition sites of particles. (From http://www.davidmoore.org.uk/Assets/Clinical_groupings.htm; 4th mars 2015)

1.6.Sampling techniques of fungal aerosols in indoor air

The presence of fungal aerosols in the air can be assessed after collection with specific devices and subsequent analysis. Collection of airborne fungal particles can be performed by gravitational (passive) or volumetric (active) techniques (28, 91). The passive collection is done by gravitational sedimentation of airborne fungal spores and fragments onto sampling petri dishes loaded with nutrients (92–94) or dustfall collectors (95). This is an inexpensive technique with no volumetric measurement of polluted air and mimics well the natural sedimentation of particles. It is biased toward larger fungal aerosols with high terminal settling velocity compared to the volumetric sampling.

Volumetric sampling of airborne fungal particles is done by suction of air onto media or through porous filters at specific velocity and during a predefined period of time. Airborne spores and fragments can be captured by impaction onto nutrient media (semi-solid or liquid) or onto porous filter by filtration. Because the sampled air volume is known, the fungal aerosol load can be estimated as colony forming units per m^3 of air (CFU/m^3) or as fungal particle counts per m^3 of air. It can also be estimated as mass concentration of a relevant fungal biomarker per volume of air. The filtration samplers have advantages

compared to impaction samplers because of the possibility to perform various downstream analyses (28). Active volumetric sampling is rapid and accepted by most official national guidelines for air quality control. However it causes high dehydration stress on microorganisms (like bacteria) with subsequent loss of viability. As a consequence, viable fungal aerosol load can be underestimated (91, 96).

1.7. Analytical methods for detection and characterization of fungal aerosols

Fungal particles in indoor air samples are traditionally assessed by culture and microscopic methods. However, their biomass can also be estimated from biomarkers such as ergosterol, phospholipid fatty acids, beta-glucans, fungal alcohols and genomic DNA.

1.7.1. Culture method

For detection of viable fungal aerosols, indoor air samples can be collected onto dishes loaded with nutrient media that favor colony growth. Air samples can also be collected into aqueous media or onto filters and further transferred onto growing media by dilution plating. Following incubation for a few days at appropriate temperature, viable spores and fragments can germinate into visible colonies that are quantified. The results are reported as colony forming units (or CFU) per volume of air sampled (97, 98). The advantage of culture method is the possibility to assess phenotypic characteristics of fungal species for identification. Yet, it is limited to detection and quantification of fungal spores and fragments that could germinate on synthetic media. One CFU may contain many viable cells. The total fungal bioaerosol is underestimated and un-germinated fungal particles are overlooked, although these may be potential carriers of allergens, antigens or toxins. The sensitivity of the culture method has been estimated to be below 10 CFU per m^3 of air samples (99).

1.7.2. Microscopic methods

Microscopic analysis is a method that allows detection, morphological characterization and enumeration of fungal particles, regardless of their viability. There are three major microscopic techniques commonly used in the detection and characterization of fungal aerosols: light microscopy, fluorescence microscopy and scanning electron microscopy (100)

Light microscopy

Light microscopy is routinely used for detection and quantification of fungal aerosols. Fungal spores present in indoor or outdoor air samples can be recognized and quantified in light microscope, but staining with fuchsin acid improves visibility of hyaline spores (101). Large hyphal fragments have been detected in outdoor air samples using light microscopy (29, 30). However small spores from *Aspergillus* and *Penicillium* species are difficult to distinguish from each other and are often counted as one class (97). Shapes, sizes, colors and surface ornamentation of spores can be described to some extent, but classification to species level is difficult to achieve.

Fluorescence/Confocal microscopy

Fluorescence microscopy can also be used for identification and quantification of fungal aerosols after staining with dyes or fluorochromes. The incorporation of dye can be done either by mean of specific proteins like antibodies or lectins or directly into the particles. Acridine orange is commonly used to non-specifically stain nucleic acids in airborne microorganisms including fungi (99, 102, 103). Other dyes like ethidium bromide and 4', 6-diamidino-2-phenyl-indole (DAPI) that readily penetrate the cell membrane have also been used to stain DNA (102), and fluorescein diacetate (FDA) to stain proteins. However many fungal spores do not favor incorporation of dyes or if they do, the fluorescence from the dye can be masked by their natural pigmentation (101).

Scanning electron microscopy

Scanning electron microscopy (SEM) has been used for identification and quantification of spores and spore aggregates (46, 100, 104, 105) and hyphal fragments (61). It has also been used to confirm the occurrence of submicronic fragments in *in vitro* aerosolization experiments (32, 72). Small particles such as spores from actinomycetes have been successfully detected by SEM (97). Detailed characteristics of surface morphology, shape and size can be revealed by SEM. However species recognition is not possible.

Direct and indirect immunolabelling for microscopic detection of fungal aerosols

Fungal particles can be detected with the fluorescence or confocal microscope after direct staining with specific antibody (polyclonal or monoclonal) conjugated with fluorochromes or other visualization probes. Particles can also be indirectly stained by using secondary antibody conjugated with visualization probe (106–108). The halogen immuno-assay (HIA)

for microscopic detection uses IgE from patients to stain conidia or hyphal fragments that contain allergens against which the subjects have been sensitized (59, 108, 109). However, this technique is limited by the microscopic resolution and could not reveal particles smaller than 1µm (59).

Immunolabelling of surface antigens for microscopic visualization is an efficient technique for detection and characterization of cells or microorganisms in a complex matrix. Microscopic immunodetection of fungal spores and other microorganisms is a well established technique for studying and characterizing their surface antigens. Monoclonal or polyclonal primary antibodies and secondary antibodies with detection probes are required in indirect immunostaining of fungal or other microbial surface antigens (110).

Monoclonal antibodies are specific to a single epitope and are therefore used when high specificity level is wished. In contrast, polyclonal antibodies favor staining of a broad range of epitopes and maximize therefore binding efficiency on the surface of microorganisms or fungal particles as compared to monoclonal antibodies. However, high cross reactivity associated with polyclonal antibodies has been reported for fungal spores even with monoclonal antibodies. Indeed monoclonal antibodies against *Aspergillus versicolor* have shown cross-reactivity toward closely related genera like *Penicillium*, but also to distant genera like *Paecylomyces* (106). The choice of detection probe is dependent on the type of microscope. For fluorescence and confocal microscopy, fluorochromes are used where excitation, emission wave length and bleaching characteristics of these compounds are important. Various fluorochromes have been tested on spores and large hyphal fragments with success (59, 111). For detection at high resolution in electron microscopy, electron-dense probes such as gold and silver are required (112).

Identification of fungal particles in a complex matrix of field samples requires specific staining that allows visualization of targeted particles in SEM. Indeed, spores from *Alternaria* sp. *Cladosporium herbarum, A. versicolor* and *P. chrysogenum* have been successfully stained with antibodies and gold particles that were resolved in SEM (113, 114). This indicates a great potential in developing a HIA-like method for SEM detection.

Detection limits of microscopic methods

Detection limit for microscopic methods is dependent on the magnification, the size of the view field, the number of counted fields and exposed filter area (for filter sampling).

Further, the volume of air sampled is decisive for the level of the detection limit. With SEM, the lowest detectable number was estimated to 2×10^4 spores when 50 fields were counted at 2000× magnification for 8h filter sampling at 2 $Lmin^{-1}$ (115). However microscopy is one of the best techniques to describe morphological characteristics of particles (116).

1.7.3. Flow cytometry

Flow cytometry (FCM) has also been successfully used for quantification of fungal spores following appropriate staining with fluorochrome or dyes (114, 117–119). As an example, the presence of approximately 11% fungal spores in outdoor air samples (Beijing China) was successfully demonstrated using FCM following staining by Calcofluor-White M2R fluorochrome (118). However, FCM does not reveal the shape characteristics and the fungal source of quantified particles.

1.7.4. Chemical and biochemical methods

Different fungal constituents have been used as biomarkers for detection of fungal aerosols in indoor or outdoor air. Use of membrane constituents such as ergosterol, phospholipid fatty acids (PLFA) and beta- glucans have been reported. Also fungal allergens, antigens, fungal alcohols (mannitol and arabitol) and mycotoxins have been used to demonstrate occurrence of airborne fungal particles. Moreover molecular techniques based on quantitative polymerase chain reaction (QPCR) have also been used to reveal the presence of fungi in indoor bioaerosols. These methods are suitable for quantification of fungal biomass in complex matrix but do not provide any information on fungal particle morphology.

Ergosterol

Ergosterol is the main sterol of the fungal membrane and is considered as a robust indicator of total fungal biomass (98). Ergosterol has been used as biomarker of fungal biomass in settled house dust (120), in materials from moldy buildings (121) and in outdoor particulate matter with size <2.5µm ($PM_{2.5}$) and particulate matter with size between 2.5 and 10µm ($PM_{2.5\text{-}10}$) samples (73, 122). Analysis of ergosterol requires high performance liquid chromatography (HPLC) (limit of detection: 500 pg/sample) or gas chromatography/tandem mass spectrometry (GC/MS) (detection limit: 10 pg/sample) (123, 124). The average range of 0.68-1.89 pg $spore^{-1}$ versus 1µg mg^{-1} mycelia have been

reported (73, 124). From indoor environments, Axelson et al reported ergosterol concentrations of 6 – 45 µg g^{-1} from house settled dust and 0.2 – 0.3 ng L^{-1} from air samples in swine barn (123). Although ergosterol is a highly specific estimator of fungal biomass, it is labile and quickly degrades photo-chemically after cell death (125), suggesting that non-viable and old fungal fragments will be underestimated.

Fungal phospholipid fatty acids

There are two membrane phospholipid fatty acids (PLFAs) (18:2ω6 and 18:3ω3) that are specific for fungi (126). The 18:2ω6 has been used for viable fungal biomass quantification in complex matrix like soil (127). Both PLFAs have been quantified in air samples as surrogate for fungal biomass (75). Approximate concentration in *Cladosporium* spp. ranged between 0.15 and 1.75 pg $spore^{-1}$. The use of PLFA is limited to detection of fresh or viable fungal biomass due to their fast degradation after fungal cell death (127).

Beta- glucans

Beta-glucans are polymers of glucose molecules linked through β-(1→3); β-(1→4) and β-(1→6) linkages (128). They can be quantified in environmental samples by Limulus amoebocyte lysates (LAL) or antibody based assays (129, 130). In environmental samples, the LAL assay is more efficient with detection limit of 20 pg ml^{-1} (131, 132) as compared to the antibody based assay (800 pg mL^{-1} and 42600 pg mL^{-1} with monoclonal and polyclonal antibodies, respectively) (129, 133)

From mold contaminated indoor environments, airborne (1→3)-β-D-glucan measured in different particle size fractions with LAL assay was in the range 0.09 – 12.9 ng m^{-3} for assumed spore size fraction, 0.02 – 4.1 ng m^{-3} for a mixture of spores and fragments fraction and 0.02 – 0.7 ng m^{-3} for the submicronic fraction (64). In working environments with high level of airborne fungal particles, the level of beta-glucans in PM_1 fraction was in the range 0.68 – 27 ng m^{-3} (78). Recently, Rivera-Mariani et al reported a HIA method adapted to microscopic quantification of (1→3)-β-D-glucan-bearing airborne particles (134).

The presence of (1→3)-β-D-glucans in plant materials, algae and bacteria reduces their specificity as biomarkers of fungal biomass (135). However, beta-glucans independently from their origin have been shown to be potent activators of different immune cells including macrophages and neutrophils (136, 137).

Other fungal alcohols

Arabitol and mannitol have been suggested and used as specific tracers for fungal spores in environmental samples (122, 138). Mean values of 1.2 (range: 0.8 – 1.8) and 1.7 (range: 1.2 – 2.4) pg were estimated per spore for arabitol and mannitol, respectively. Good correlations were found between the concentration of these tracers in outdoor air and flow cytometric quantification of fungal spores: 9.1±5.2%; 11.3±5.2% and 10.8±10.7% as average proportion based on arabitol, mannitol and flow cytometry, respectively (118). However, there is no report, to our knowledge, on specific detection of fungal fragments by means of these tracers.

Immunoassay for enzymes, antigens and allergens

Different enzymes have been used as molecular tracers for airborne fungal biomass. The quantification of N-acetyl-beta-D-glucosaminidase (NAGAse) and N-acetyl-hexosaminidase (NAHA) have been associated with the presence of fungal biomass in airborne or settled dust in moldy indoor environments (78, 82, 139–143). This detection technique is commercially available as Mycometer Air™. For antigen and allergen detection, there are numbers of immunoassay based on monoclonal or polyclonal antibodies raised against specific antigens or allergens present in the fungal particles. Usually, antigens or allergens are extracted from the sampling medium and detected by means of enzyme-linked immunostaining assay (ELISA) techniques (144).

Polymerase chain reaction (PCR) based techniques

The polymerase chain reaction (PCR) is a molecular technique based on the extraction of fungal genomic DNA followed by exponential amplification of a given fragment *in vitro* using polymerase chain reaction. Fungal species can be identified when appropriate primers targeting the internal transcribed spacer (ITS) region of the nuclear ribosomal coding cistron are used. This region has been proposed as an universal DNA barcode for fungi (145) and has been sequenced for characterization of the profile of fungal species in indoor airborne or settled dust (146). Specifically, two consensus primers (ITS4 and ITS5) have been proposed and used to amplify the region between 3'18S-like and 5'28S-like genes including the 5.8S gene and 2 internal transcribed spacers (147). Other species specific primers have be also used for similar purpose (148).

Furthermore, fungal aerosols containing genomic DNA can be quantified using quantitative PCR (QPCR) of spores from targeted species. QPCR uses a fluorescence reporter whose signal increases proportionally with the quantity of the PCR products. To date, there have been developed mold specific QPCR assays at EPA (United States Environmental Protection Agency) with probes and primers for about 100 fungal species (149). Results are reported as equivalent DNA per spore unit in bioaerosol samples. The advantage of this method is the identification to species level which is important for species profile in indoor air. However, the fungal genomic DNA extraction is challenging because of varying efficiency and number of nuclei per fungal cell/spores. With submicronic fragments, it is unknown whether they contain genomic DNA.

2. Aims of the study

The overall aim of this thesis is to obtain more knowledge on the composition and morphological characteristics of fungal aerosols and to develop a detection and enumeration method for fungal fragments in environmental air samples.

Based on this main objective, following specific aims were defined:

1. Profile and morphological characterization of *in vitro* aerosolized fungal particles from pure fungal cultures (Paper I)

2. Origin and importance of submicronic fragments aerosolized from pure fungal cultures (Paper II)

3. Development of an indirect immunoassay for detection of submicronic fungal fragments in indoor air (Paper III).

3. List of the papers

Paper I:

Komlavi Anani Afanou, Anne Straumfors, Asbjørn Skogstad, Ida Skaar, Linda Hjeljord, Øivind Skare, Brett James Green, Arne Tronsmo and Wijnand Eduard. **Profile and morphology of fungal aerosols characterized by field emission scanning electron microscopy (FESEM)**; *Journal of Aerosol Science and Technology*, in press.

Paper II:

Komlavi Anani Afanou, Anne Straufors, Asbjørn Skogstad, Terje Nilsen, Ole Synnes, Ida Skaar, Linda Hjeljord, Arne Tronsmo, Brett J. Green, Wijnand Eduard. **Submicronic fungal bioaerosol: High resolution microscopic characterization and quantification** *Applied and Environmental Microbiology* Volume 80; 22: 7122–7130, 2014

Paper III:

Komlavi Anani Afanou, Anne Straumfors, Asbjørn Skogstad, Ajay P. Nayak, Ida Skaar, Linda Hjeljord, Arne Tronsmo, Wijnand Eduard, Brett James Green. **Indirect immunodetection of fungal fragments by field emission scanning electron microscopy** (*Applied and Environmental Microbiology* Submitted)

4. Summary of the papers

Paper I: Profile and morphology of fungal aerosols characterized by field emission scanning electron microscopy (FESEM)

We investigated in this paper the composition, the size and shape characteristics of fungal aerosols generated from pure cultures of *Aspergillus fumigatus, A. versicolor* and *Penicillium chrysogenum* using two generators (SPG and FSSST) at 12 and 20 $Lmin^{-1}$. Five types of spore particles and four types of fragment particles were quantified and characterized by size (length and width) and shape (aspect ratio) using field emission scanning electron microscope (FESEM). The spore particles were identified by the number of spore units per aggregate and comprise single spore aggregates of 2, 3, 4 and ≥5 spores. The fragment particles were classified by length as submicronic fragments (0.2-1µm), 1-2µm fragments, 2- 3.5µm fragments and >3.5µm fragments. The effects of the generators and airflow rates on the profile were also investigated.

The mean sizes and shape of spore particles irrespective of fungal isolate were in the ranges 2.42 – 15.04µm, 2.02 – 4.26µm and 1.18 – 4.19 for the length, width and aspect ratio, respectively. For the fragment particles, these values were 0.52 – 12.8µm, 0.33 – 5.10µm and 1.78 – 4.68, respectively. Based on the aspect ratios, single spores were near spherical (aspect ratio: 1:1 – 1.5:1) while spore aggregates and fragments were oblong (aspect ratio: 1.5:1 – 3:1) or fiber-like (aspect ratio: >3:1) in shape.

Composition analysis showed dominance of single spores in the aerosol from *A. fumigatus* (median: 53%) while submicronic fragments dominated the aerosol from *A. versicolor* (median: 34%) and *P. chrysogenum* (median: 31%). Furthermore the spore fraction from *A. versicolor* showed dominance of larger spore aggregates (≥5 spores, median: 32%) while single spores dominated similar fractions from *A. fumigatus* (median: 70%) and *P. chrysogenum* (median: 28%). The fraction of fragment particles was dominated by submicronic fragments (>60%) for all tested fungal isolates. Based on shape, the aerosol from *A. fumigatus* was dominated by near- spherical particles (median: 53%) while oblong particles were dominant in the aerosol from *A. versicolor* (68%) and *P. chrysogenum*

(55%). Fiber-like particles represented 21% and 24% of the aerosol from *A. versicolor* and *P. chrysogenum,* respectively.

The aerosol profile was significantly affected by the generator and airflow used. Submicronic fragments from all tested fungal isolates were the most affected particle type. Single spores, aggregates of 2 spores and large fragments (≥3.5μm) were also affected, but to lower extent. The physical size measurement revealed that a fraction of spore particles and fragment particles has similar lengths, suggesting the difficulties to discriminate these particle types using particle size. Submicronic fragments were the major component of the fragment fraction for all species. Furthermore the profile of the fungal aerosol is complex with particles of different morphologies and source, supporting the need to include a broader range of mold particles in exposure assessments. Significant effects of the generators and airflows on the aerosol composition indicate that comparison of characterization results from different research groups using different aerosolization chambers will likely be difficult.

Paper II: Submicronic fungal bioaerosol: high-resolution microscopic characterization and quantification

In this study, the importance of submicronic fungal particles derived from 2 and 8 week-old *Aspergillus fumigatus, A. versicolor* and *Penicillium chrysogenum* cultures grown on agar and gypsum board was investigated using field emission scanning electron microscopy (FESEM). The number of submicronic particles released in a novel aerosol generator chamber: Stami particle generator (SPG) developed at National Institute for Occupational Health (NIOH) was compared to the Fungal Spores Source Strength Tester (FSSST) at 12 and 20 L min^{-1} airflow. The effects of age and media on the number of released submicronic fragments were also assessed. The overall median numbers of aerosolized submicronic particles were 2×10^{5} cm^{-2}, 2.6×10^{3} cm^{-2} and 0.9×10^{3} cm^{-2} for *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. *A. fumigatus* released significantly more submicronic particles ($p<0.001$) than *A. versicolor* and *P. chrysogenum* irrespective of generators, airflow, media or age. The ratios of the submicronic fragments to larger particles, regardless of generator, airflow and media types, were 1:3, 5:1 and 1:2 for *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. The generators showed very diverse effects on the number of aerosolized submicronic fragments. However, consistently higher numbers of submicronic fragments were generated at 12 $Lmin^{-1}$ for all tested isolates in FSSST although the differences were not significant, except for *P. chrysogenum*. In the SPG, the numbers of submicronic fragments showed no clear trend at the tested airflows. The overall results indicate that higher numbers of submicronic fragments were aerosolized from the older cultures of the three isolates. Moreover, spore fragments, identified by the presence of rodlets, amounted to 13%, 2% and 0% of the submicronic particles released from *A. fumigatus, A. versicolor* and *P. chrysogenum,* respectively. Submicronic particles with and without rodlets were also aerosolized from cultures grown on cellophane-covered media, indirectly confirming their fungal origin. Both hyphae and conidia could therefore fragment into submicronic particles and aerosolize *in vitro*. These findings highlight the contribution of fungal fragments to bioaerosols, with regard to their potential effects on human health.

Paper III: Indirect immunodetection and enumeration of fungal fragments by field emission scanning electron microscopy

We report in this paper a novel indirect immunodetection method using polyclonal antibodies raised in chicken (IgY) against *Aspergillus versicolor* and high resolution field emission scanning electron microscopy (FESEM) for enumeration. The particles subjected to immunolabelling were initially immobilized onto polycarbonate membrane coated by poly-L-lysine followed by vapor fixation with glutaraldehyde. Immobilization of the fragments onto polycarbonate filter is an extraction-free approach that effectively enables staining and detection of different types of fungal fragments impacted onto the sampling filter.

The efficiency of the staining method based on a positive control revealed that 84% of submicronic fragments of *A. versicolor* mycelia were positively labeled as compared to 89 - 100% of the larger fragments. Cross-reactivity of the new antibody toward 23 fungal isolates, four bacteria and two plant materials was also investigated. Only four fungal isolates did not cross-react with the chicken IgY against *A. versicolor*. One actinomycete tested also cross reacted with the antibody. The most interesting thing was the plant derived materials that showed no cross-reactivity, confirming the possibility to use this antibody on field samples containing plant materials.

In a proof of concept experiment, the method was tested on three volumetric samples from a mold contaminated indoor environments during renovation work. Despite the small sample size (n=3), the novel method revealed that 6% of total collected submicronic fragments have fungal antigens. For the large fragments in the sizes range 1 – 2µm, 2 – 3.5µm and >3.5µm, these equivalent mean values were 44%, 73% and 82%, respectively. The fungal fraction represented 13% of the total aerosols and comprised 39%, 40%, 21% and 2% submicronic fragments, >1µm fragments, single spores and spore aggregates. The novel method is suitable for identification and enumeration of fungal fragments including submicronic fragments in a complex environmental matrix although the fraction of submicronic fragments will be underestimated. Identification of fungal species and discrimination of fungal fragments from actinomycete fragments is not possible. The extraction-free approach adopted in the present method was of great importance for the

aerosol composition characterization as it causes minor disturbance to the particles impacted on the filters and minor non-specific labeling.

5. Main results and discussion

5.1.Methodological considerations

5.1.1. Fungal isolates, cultures media and age

There are 89 fungal species reported as common and important fungi in indoor environments, including *Aspergillus versicolor* Tirobashi 1908 and *Penicillium chrysogenum* Thom 1910 (150). These species have been tested in many *in vitro* aerosolization experiments (31–33, 35, 37, 40, 108). *A. fumigatus* Fresenius 1863 is also found in indoor environments and can become an opportunistic pathogen causing aspergilliosis in subjects with reduced immune defense (151). Isolates from these three fungal species were chosen for *in vitro* aerosolization experiments (Paper I and II) because of their ability to grow on indoor materials under high humidity conditions and consequently release their bioaerosols into the indoor air (39, 57, 151, 152). The release of spores from these species is favored by air currents (38, 153). *A. versicolor* and *P. chrysogenum* have also been shown to release submicronic fragments from contaminated materials in many laboratory experiments (31–37, 40, 72, 154).

In the aerosol characterization studies (Paper I and II), *A. fumigatus*, *A. versicolor* and *P. chrysogenum* were grown on gypsum board because this material is commonly used as indoor building material. Gypsum board material represents for us the closest model for indoor environment materials. In paper II, malt extract agar media has also been tested because it supports massive fungal biomass growth (155) as well as agar media with cellophane cover in order to prevent aerosolization of substrate fragments as suggested by Scheermeyer et al (71). The age of the cultures has been reported to positively affect the number of submicronic fragments aerosolized from *A. versicolor* and *Stachybotrys chartarum* when fungal particles were size- separated by cyclones and analyzed for glucans and counted by automatic counter (37).

5.1.2. In vitro aerosolization of fungal particles

In vitro aerosolization of particles from fungal cultures has been used in studying different parameters that affect the aerosolization of fungal particles from pure or mixed cultures (31–37, 40, 41, 72, 154, 156). Technically, a mold contaminated substrate is subjected to air jets, with or without vibration. Released particles from the samples are transported by

air stream to a measuring instrument or collected onto filters for further analyses. Parameters such as air flow, relative humidity, and temperature are controlled and their influence on the fungal bioaerosol can be studied. Released fungal aerosols, including submicronic fragments and other fungal particles, have been enumerated by automatic particle counting or sizing instruments. However, the shape, size, source characteristics of various particles types and the aerosol composition require further investigation.

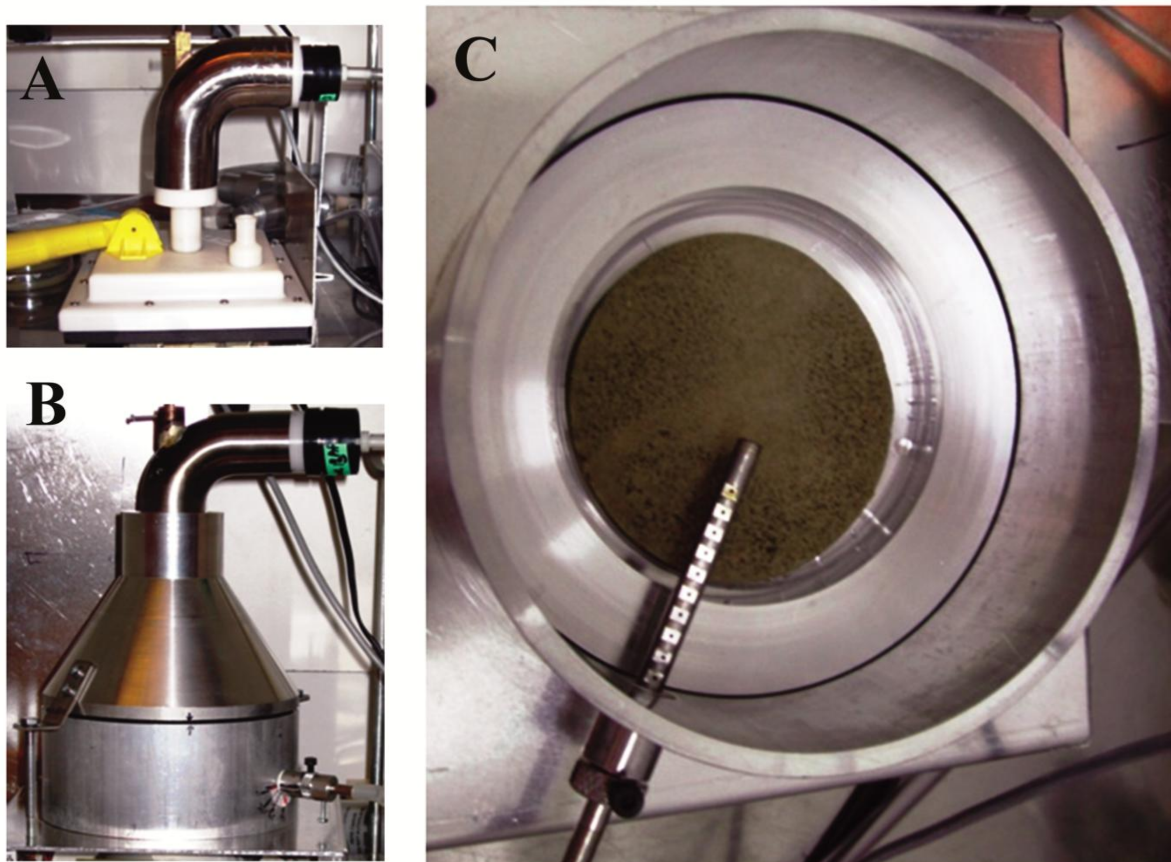

Figure 8: Photos of the aerosolization chambers: FSSST (A) and SPG (B). View of the air jet tube with orifices and the rotating plate inside SPG (C). (*Photo by Anani k Afanou*)

To study these characteristics in *in vitro* generated fungal aerosols, we built an in-house aerosolization chamber named Stami particle generator (SPG: Figure 8) that was compared to the well-known Fungal Spore Source Strength Tester (FSSST: Figure 8) (157)(Paper I and II). Both chambers were tested at 12 and 20 $Lmin^{-1}$. At these airflows, the equivalent mean velocities at the orifices in the aerosolization chambers were theoretically calculated to 18 and 29 m s^{-1} in SPG and 14 and 23 m s^{-1} in FSSST. The differences between the

velocities are likely to influence the release of particles from the culture. However, the real velocity of the air jet at the culture surface is not known. In addition, numerous parameters like electrostatic charging and air turbulence are likely to interfere with the particle aerosolization but are difficult to monitor.

5.1.3. High resolution field emission scanning electron microscopy (FESEM)

Scanning electron microscopy (SEM) has been previously used to enumerate fungal spores and large hyphal fragments in a number of environmental studies (39, 47, 61, 104, 105, 158). SEM was also used to confirm the presence of submicronic fragments (32, 72) and type of spore particles (31, 71) in *in vitro* aerosolized fungal aerosols but particles were not enumerated in these studies. In comparison to light or fluorescence microscopy, FESEM provides high resolution that improves data on morphological characteristics and identification of different types of particles, including the submicronic particles. However, enumeration at high resolution leads to lower counts of detectable particles in filter samples and subsequently higher detection limits. In this thesis, FESEM was chosen for fungal bioaerosol characterization and immunodetection because it offers good resolution for description of sizes, shapes, and surface structures (rodlets). Surface structures such as rodlets have been claimed to be specific to spores (159, 160) and have been used to discriminate between submicronic fragments from spores and hyphal fragmentation of *Aspergillus* and *Penicillium* isolates. Furthermore, the detection of immunolabelled submicronic fragments requires high resolution for visualization of immunogold labels.

5.1.4. Indirect immunolabeling using polyclonal chicken IgY antibody

Any immunodetection method is dependent on the quality and the quantity of the antibodies used. The choice of polyclonal or monoclonal antibodies is depend on the specificity level and the availability (161). The primarily goal in this thesis is to label fungal fragments in a complex matrix and such purpose can be achieved with polyclonal antibodies such as polyclonal IgY. Polyclonal IgY is the predominant serum immunoglobulin in birds, reptiles and amphibians. This antibody was chosen for the immunolabeling method (Paper III) because of high yield (up to 12mg/egg), non-invasive collection, high functional affinity and titer, low cost production process and without animal bleeding (162–164). Polyclonal IgY antibodies have been used to develop immunodetection methods for *Candida* spp. (165) and other microorganisms (166, 167).

Moreover, polyclonal antibodies were considered instead of monoclonal antibodies because of the need to maximize binding efficiency toward fungal fragments. In this regard, cross-reactivity toward non-fungal materials was tested. This was decisive for the utilization of the polyclonal IgY anti *A. versicolor* for detection of fungal fragments in complex matrix. In fact, 2 materials derived from plant and 4 bacteria were tested for potential cross-reactivity toward the antibody.

5.1.5. Cross reactivity of the anti *Aspergillus versicolor* polyclonal IgY

The absence of cross reactivity of the polyclonal antibody to plant materials was crucial for it application in the detection of fungal fragments in environmental samples. Cross reactivity of the anti *A. versicolor* polyclonal IgY toward many fungal species limit the detection of fragments to species level. Similarly, high level of cross reactivity has been reported in different studies whether monoclonal or polyclonal antibodies were used (106, 168, 169). Evolutionarly highly conserved proteins have been suggested to explain such high frequence of cross reactivity among fungi (169, 170). However, high cross reactivity to many fungal species in the present immunolabeling method together with spore count will permit the detection of total fungal aerosols load including the actinomycete.

5.1.6. Immobilization and vapor fixation of particles on polycarbonate filter

Fixation and labelling procedures immunodetection assay are conducted in aqueous phase. Soluble antigens on the surface of particles are likely to be displaced or even lost during the immunostaining process (171). To circumvent this problem, Grote reported staining techniques for detection of birch pollen allergen Bet v1 by electron microscopy of filter samples using vapor fixation. Similarly, fungal particles to be immunolabelled were vapor fixed with glutaraldehyde following immobilization onto polycarbonate filter by means of poly-L-lysine. Poly-L-Lysine is commonly used in cell culture to increase cell adherence to solid surfaces (172). Furthermore, various particles, especially spore aggregates, could be analyzed as deposited onto the sampling filters, favoring therefore composition assessment with minor disturbance.

5.2.Characteristics of in vitro generated fungal aerosols

5.2.1. Diversity of the fungal aerosol composition (Paper I and II)

The discovery of submicronic fragments during *in vitro* aerosolization changed the focus on spores, considered for decades as the major fungal aerosol component both in indoor and outdoor environments. Indeed submicronic fragments outnumber spores released in *in vitro* studies with *Aspergillus versicolor* (Paper II) and *Stachybotrys chartarum* (32, 35). This underscores the need for a detailed characterization of the fungal aerosol taking into account the physical characteristics of submicronic fragments and other particles. The *in vitro* aerosolization experiments revealed that the fungal aerosol is complex and comprises spore particles and fragment particles, supporting therefore previous findings on *A. versicolor, Penicillium melinii, Cladosporium cladosporioides, Botrytis cinerea, Trichoderma harzianum, Ulocladium* sp. and *S. chartarum* (31–33, 35–37, 40, 41, 154). The spore particles are composed of single spores and aggregates of various sizes and shapes. The mean physical length of spore particles varied between 2.41 and 15.04 µm while the shape, assessed as aspect ratio, revealed near spherical single spores (1:1 – 1.5:1) and spore aggregates with oblong (1.5:1 – 3:1) to more fiber-like (>3:1) shape. The physical size and shape characteristics of the spore particles are in agreement with previous characterization reports on fungal spores (46, 86, 173). Similarly, fragment particles comprise both fragments of various mean sizes varying between 0.52 and 12.8 µm in length and the three groups of shapes. The presences of various arrays of particles in fungal aerosols will likely favor deposition at different sites in the respiratory tract depending on size and shape and could simultaneously modulate a variety of toxic reactions due to the particle origin (**Figure 7**).

5.2.2. Diversity of fungal aerosol composition and potential toxicological responses

Exposure of the lungs to viable and non-viable fungal spores from *A. fumigatus and P. chrysogenum* induces lung inflammation with increased numbers of lung neutrophils and eosinophils in mice (174, 175). The type of inflammation seems to vary depending on whether spores are viable and germinating or non-viable. The non-viable spores have been shown to induce non-allergic inflammatory responses, in contrast to viable spores which mediate allergic inflammatory response. Similarly hyphae from germinating spores have been associated with allergic inflammation (176, 177). Moreover, data on the diversity of

particle source, size and shape as revealed in generated fungal aerosols (Paper I) suggests that various mechanisms of clearance, involving different immune cells may be induced upon inhalation of fungal particles. Large and fibrous-like particles may cause emergence of frustrated phagocytosis by macrophages, but induce mechanisms of neutrophils extracellular traps "NETosis" by neutrophils with their extracellular release of reactive oxidative species (ROS) while single spores are likely to be effectively phagocytized by macrophages (178–181). This encourages a detailed characterization of fungal bioaerosols and targeted toxicological assessment of different types of particles in order to improve our understanding of the role of fungal aerosols in adverse health outcomes observed in moldy indoor environments.

5.2.3. Fungal aerosol composition and deposition in the respiratory tract

We described a broader range of fungal bioaerosols with various sizes, shape, surface structures and their origin (Paper I and II). Size, shape, surface ornamentation, density and origins of the fungal particles have been reported to influence their aerodynamic behavior (28, 182). Hence, various particles will have different aerodynamic behavior and may deposit at different regions in the human respiratory tract (**Figure 7**).

The presence of submicronic fragments, fragments in the size range of 1- 3.5 μm, single spores, spore aggregates and large fragments (>3.5 μm) indicates that inhaled fungal aerosols will deposit in the three main regions of the respiratory track (**Figure 6**): the nasopharyngeal region, the tracheo-bronchial region and the alveolar region (86). Such dissemination of fungal particles may induce immune and toxic responses throughout the whole respiratory tract. Theoretically, most submicronic fragments released from *A. versicolor* and *S. chartarum* will deposit in the alveolar region as demonstrated by Cho et al. (2005). Also single spores as well as the fragments (<3.5 μm) may reach the alveoli (86, 183). The fate of spore aggregates (>3spores) and larger fragments (>3.5 μm) in the respiratory tract is more difficult to predict because of their aerodynamic sizes that varies whether they are clustered or chained for the same number of spore units (86). However, more large fragments and aggregates will probably be retained in the nasopharyngeal region, although a small fraction could eventually reach the trachea-bronchial region. Moreover, the antigen mass of particles will also be proportional to their size, meaning that larger spore aggregates will contain more antigens than small submicronic fragments (44).

Based on the aspect ratio (shape), the generated bioaerosols comprise three main groups: spherical particles represented by single spores; oblong particles that comprise the submicronic, larger fragments and spore aggregates and fibrous particles such as spore aggregates and larger fragments (Paper I). The presence of fiber-like particles in the fungal aerosols gives concern, since their deposition site is more difficult to predict (44, 184). Overall the diverse composition of the fungal aerosols is likely to favor penetration and deposition at different regions in the respiratory tract.

5.2.4. Fungal aerosol composition and size fractionation sampling of fungal aerosols

Interestingly, the complexity of the fungal aerosol profile is likely to limit the efficiency to collect pure samples of specific particle types using size fractionation equipments. The approach based on size fractionation of aerosolized particles in conjunction with quantification of biomarkers such as ergosterol, β-glucans or enzymes has been used for detection of submicronic fungal fragments in environmental samples. Further, the use of biomarker mass from size separated fractions (from cyclones or multistage impactors) is unlikely to provide complete information on the real composition of the fungal aerosols. Given the fact that single spores have been found in cyclone fractionated submicronic fragments (34, 83) because of spore bounce phenomenon, the biomass estimation of the fungal submicronic particles in the cyclone separated fraction could be questioned. Moreover, we found an overlap in length size between spore aggregates and larger fragments (>2µm) (Paper I) and this may reduce the possibility to generate pure particle fractions by size fractionation or to use particle counters or sizer for characterization of fungal aerosols.

5.2.5. Origin of fungal fragments

In previous studies, the number of submicronic fragments collected from *A. versicolor* and *S. chartarum* were over 300 times the number of spores, but their origin remained unclear. Mycelial and spore fragmentation have been suggested as the origin of submicronic fragments. Indeed Madsen et al (34) induced autolysis in 40 days old *Chaetomium globosum* cultures with carbonate buffer (pH 10) and reported significantly increased number of micro-particles (aerodynamic diameter <1.3 um) as compared to reference cultures. In contrast, Kanaani et al (70) demonstrated increasing fluorescence associated with submicronic fragment numbers simultaneously with decreased numbers in the spore

size range of *Aspergillus* and *Penicillium* spp. The authors attributed these observations to spore fragmentation. Similarly, Gorny et al (32) concluded after ELISA assay based on monoclonal antibodies against *Aspergillus* and *Penicillium* that submicronic fragments share similar antigens as spores. Since the immune response toward fungal particles varies depending on whether the particle is a spore or a hyphal fragment (176, 177), it is important to determine their origin. We demonstrated in Paper II that submicronic fragments originated mainly from hyphal biomass of the three isolates, although a small portion was from spores of the *Aspergillus* isolates. The rodlet structures as observed at high resolution on spore surface were used to discriminate between fragments of spore origin from the mycelial ones, at least from the *Aspergillus* species.

The process behind the spore fragmentation is not known, however, we can speculate on the breakage of spiny formations on the spore surface at high air velocity as reported by Kanaani et al (70). This explanation can be strengthened by the absence of submicronic fragments with rodlets from *P chrysogenum* isolate that has smooth spore surface. The orientation of the submicronic fragments on the collection filter could also hide the outer spore surface with rodlets, since the inner side of the membrane is lacking rodlets. Moreover, the mechanical force of spores from collisions during aerosolization can also explain the source of fragments as spores in spore aggregates were smashed into small fragments (**Figure 9**).

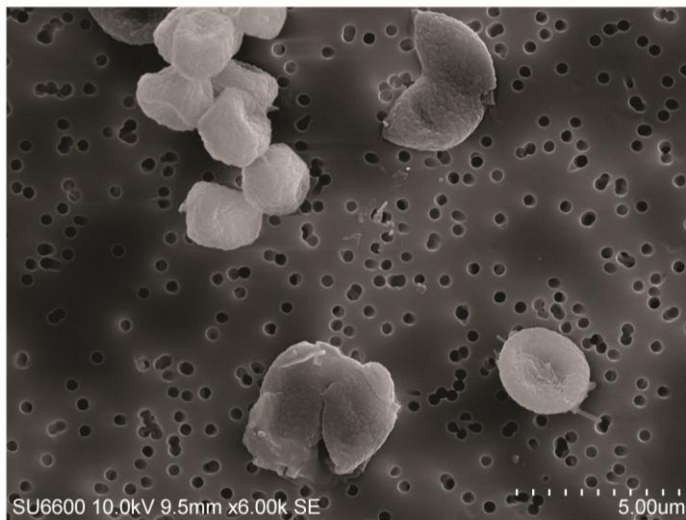

Figure 9: Micrographs of broken spores from *P chrysogenum*.

5.3. Detection of fungal fragments in mold contaminated indoor air samples

5.3.1. Detection of fungal submicronic fragments

There is to date, to our knowledge, no method that allows detection and enumeration of submicronic fragments of fungal origin in air samples. The novel immunodetection assay presented in this paper was used in a proof of principle study in a moldy environment to enumerate fungal fragments, including submicronic fragments. Samples were collected from a mold-contaminated building under renovation. The site was chosen to improve the likelihood that airborne fungal fragments could be collected. The efficiency of the immunostaining assay was estimated to 84% for submicronic fragments versus 89 – 100% for larger fragments using pure fragmented mycelia from *A. versicolor*. The presence of fungal submicronic fragments in the tested environment was confirmed by the novel method. Our study revealed that 6% of total collected submicronic particles (9.8×10^6 m^{-3}were of fungal origin as they contain fungal antigens. Thus fungal fragments with antigens on their surface were stained and could be visualized. However this fraction is likely to be underestimated, since 84% of labelling efficiency of particle <1µm could be achieved. Furthermore, the ratio of submicronic fungal fragments to spore particles was 1:2, which seems comparable to the *in vitro* results considering the likely differences between the fungal biota. Although the sampled environment in this proof of principle study is not representative for normal dwelling places with and without water damage, we believe that the novel method will permit extending fungal exposure assessment to fungal fragments including submicronic fragments.

5.3.2. Detection of large submicronic fragments and spores

The novel method contributed also to the detection of larger fungal fragments and a greater proportion of fragments collected in moldy environment was immunostained (44 - 82%). Spores in the aerosol were not detected by the antibody. The reason might be masking of spore antigens by hydrophobins on the outer spore wall (185, 186). However spores were easily recognized by their characterstic morphologies.

5.3.3. The composition of fungal aerosols in mold contaminated indoor air samples

Despite the small size of the field samples analyzed, 13% of the total aerosol collected were identified as fungal aerosols. The composition of the fungal aerosol was diverse and comprised various types of particles. The mean fractions of submicronic fragments, large

fragments (>1µm fragments), single spores and spore aggregates were 39%, 40%, 19% and 2% of fungal particles collected, respectively. Spore particles were mainly detected as single spores. The fraction of spore aggregates was relatively low. Similar results on spore aggregates have been reported by Eduard and Aalen who analyzed 5 field samples from sawmills (46). However, the level of antigens or allergens in this relatively small fraction of spore aggregates may be as high as in the single spore fraction. Further investigation is required for a more representative assessment of fungal bioaerosol composition in indoor environments.

6. Conclusions and future perspective

The following conclusions can be drawn from the present thesis:

- *In vitro* aerosolized fungal aerosol profiles are complex and comprise particles of various shapes, sizes and sources.
- The bioaerosol composition is influenced by the aerosolization chamber and airflow used.
- Submicronic fragments originate mainly from mycelial fragmentation, but a small fraction could also originate from spore fragmentation.
- The numbers of aerosolized submicronic fragments vary between species and are positively influenced by the culture age. Submicronic fragments outnumber other particles in the bioaerosols from *A. versicolor*.
- The aerosolization chamber and airflow showed no significant effects on the aerosolization of submicronic fragments except in case of *P. chrysogenum* which revealed negative effect of airflow in FSSST and the opposite effect in SPG.
- A novel method has been developed for the quantification of fungal fragmented particles including submicronic fragments in a complex environmental matrix.
- Most fragments could be immobilized and immunolabelled on polycarbonate filters.
- The immunodetection method is not species specific but seems specific for fragments.
- Spores could not be labeled, but recognized by morphology.
- The composition of fungal bioaerosols could be enumerated by combining immunolabelling and microscopic counting.

For the future work:

- Optimization of the method for automatic analysis in FESEM to improve the applicability and the efficiency.
- Test of monoclonal antibodies is needed to see if the species specificity can be improved.
- Combine antigen staining techniques with molecular based techniques such as QPCR or sequencing to improve spore quantification and species identification.

- Assessment of fungal fragments in indoor and occupational environments in order to study their exposure levels and health effects.

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8. Appendix

PAPER I - III

PAPER I

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Profile and morphology of fungal aerosols characterized by field emission scanning electron microscopy (FESEM)

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Abstract

Fungal aerosols consist of spores and fragments with diverse array of morphologies; however, the size, shape and origin of the constituents require further characterization. In this study, we characterize the profile of aerosols generated from *Aspergillus fumigatus*, *A. versicolor,* and *Penicillium chrysogenum* grown for 8 weeks on gypsum boards. Fungal particles were aerosolized at 12 and 20 $Lmin^{-1}$ using the Fungal Spore Source Strength Tester (FSSST) and the Stami particle generator (SPG). Collected particles were analyzed with FESEM. We observed spore particle fraction consisted of single spores and spore aggregates in four size categories, and a fragment fraction that contained submicronic fragments and three size categories of larger fragments. Single spores dominated the aerosols from *A. fumigatus* (median: 53%), while the submicronic fragment fraction was the highest in the aerosols collected from *A. versicolor* (median: 34%) and *P. chrysogenum* (median: 31%). Morphological characteristics showed near spherical particles that were only single spores, oblong particles that comprise some spore aggregates and fragments (<3.5µm), and fiber-like particles that regroup chained spore aggregates and fragments (>3.5 µm). Further, the near spherical particles dominated the aerosols from *A. fumigatus* (median: 53%), while oblong particles were dominant in the aerosols from *A. versicolor* (68%) and *P. chrysogenum* (55%). Fiber-like particles represented 21% and 24% of the aerosols from *A. versicolor* and *P. chrysogenum,* respectively. This study shows that fungal particles of various size, shape and origin are aerosolized, and supports the need to include a broader range of particle types in fungal exposure assessment.

Keywords: fungal aerosols, shape, size, FESEM

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Introduction

Indoor environments with a high moisture content often result in saprophytic fungal colonization of structural building materials. Fungal colonization of these indoor environments in combination with indoor dampness has been associated with adverse health outcomes (Institute of Medicine 2004; World Health Organization 2009). However, epidemiological and exposure studies including the assessment of fungal spore exposure in indoor air could not confirm such an association (Eduard 2009). Epidemiological studies based on traditional methods of identification and quantification of fungal spores or fungal colony forming units have not provided insight into health effects from other airborne fungal particles such as fragments of spores and hyphae.

The composition of fungal aerosols is diverse and comprises particles of various sizes, shapes and origin (spores and mycelia), thus with varying aerodynamic behavior (Lacey 1991). Single spores from many molds encountered in indoor environments are globose or ellipsoidal with smooth or ornamented surface (Reponen et al. 2001) while chained spore aggregates are often elongated and fiber-like. Moreover, hyphal fragments have various morphologies: they can be tubular, elongate or fiber-like and irregular.

The aerodynamic diameter of particles is commonly used in the prediction of particle motion and deposition in human respiratory tract. Shape also influences motion and deposition of particles in human respiratory tract (Yeh et al. 1976) and aerosol samplers. For non spherical or irregular particles, aerodynamic size is dependent on the particle shape and surface structures (Cox 1995) therefore one-dimensional size measurement poorly describes irregular or fibrous shaped particles. To date, little is known about the shape of various particle types reported as fungal aerosols except for the distribution of single spores and aggregates. Moreover, detailed characteristics of the fungal aerosol profile are unknown.

The morphology and size of spores, and hyphal and spore fragments have been assessed by microscopy in attempts to describe the fungal aerosols (Afanou et al. 2014; Eduard et al. 1988, 1990; Green et al. 2005; Halstensen et al. 2007; Heikkila et al. 1988; Karlsson and Malmberg 1989; Pady and Gregory 1963; Pady and Kramer 1960; Vestlund et al. 2014). Quantification and size-characterization of fungal particles have also been performed by optical measurement of the aerodynamic diameter of aerosol particles using automatic particle counters or sizers. For this purpose, a fairly narrow size range has been used to discriminate spores from fragments in aerosols generated from fungal cultures (Górny and Ławniczek-Wałczyk 2012; Górny et al. 2002; Kanaani et al. 2009; Kildesø et al. 2000, 2003; Sivasubramani et al. 2004). This approach led to the discovery of submicronic fragments. Fungal biomarkers such as ergosterol (Lau et al. 2006; Menetrez et al. 2009; Rao et al. 2005), phospholipid fatty acids (Womiloju et al. 2003) and β-glucans (Adhikari et al. 2009; Frankel et al. 2013; Madsen et al. 2009; Rao et al. 2005; Reponen et al. 2007; Seo et al. 2009, 2014; Singh et al. 2011) have also been used to demonstrate the presence of fungal biomass in various sizes of fungal aerosols. Further, enzymes, e.g.: chitinases (Madsen 2012; Madsen et al. 2005, 2009), antigens (Górny et al. 2002) and allergens

(Menetrez et al. 2001) as well as mycotoxins (Brasel et al. 2005; Sorenson et al. 1987) have been used to demonstrate allergenicity and toxicity potential of various size fractions of the fungal aerosols. Actually, the cut-off characteristics of the size-selective samplers are decisive for the separation efficiency.

The toxicological and immunological properties of allergen-bearing fine particles have been found to be strongly correlated with the particle number and overall surface than the particle mass (Nygaard et al. 2004). Thus, it can be hypothesized that the number of fungal particles with aerodynamic diameter $< 2.5\mu m$ may show stronger correlations with observed health outcomes, than with mass. Moreover, hyphae have been shown to elicit stronger allergic inflammation as compared to single spores that induced non-allergic inflammatory responses (Bozza et al. 2002; Hohl et al. 2005). Also, Branzk et al. showed that neutrophils with mechanisms of neutrophils extracellular traps (NETosis) and reactive oxidative species (ROS) were involved in clearance of fiber-like particles and large spore aggregates (Branzk et al. 2014). A detailed description of the fungal aerosol by simultaneous assessment of physical size, shape and particle numbers is only possible by microscopy (Cox 1995).

The present study was therefore intended to provide more knowledge on the size, shape and origin characteristics of various fungal particle types, as well as the profile of the fungal aerosols generated from pure fungal cultures. The effect of generators and airflows used on the whole aerosol composition was also investigated.

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Materials and Methods

Fungal culture

Isolates of *Aspergillus fumigatus* Fresenius 1863 (strain A1258 FGSC) obtained from the Fungal Genetics Stock Center (University of Missouri, Kansas City, KS), *Aspergillus versicolor* Tirobaschi 1908 (strain VI 03554), and *Penicillium chrysogenum* Thom 1910 (strain VI 04528) obtained from the Section of Mycology at the Norwegian Veterinary Institute (Oslo, Norway) were used. The characteristics of these fungal isolates and the procedure for inoculum preparation have been previously described elsewhere (Afanou et al. 2014). Briefly, spores from 2-week-old cultures grown on malt extract agar (MEA) (Samson et al. 2004) were harvested by gently scraping cultures submerged in 20 mL phosphate buffered saline (PBS) containing 0.1% Tween 20 using a sterile inoculation loop. The spore suspension was transferred into 50 mL centrifuge tubes and resuspended by vortexing for 2×30 seconds followed by sonication for 3 minutes in an ultrasonic bath at a frequency of 35 kHz (Sonorex RK 510H, Bandalin Electric, Berlin, Germany). The suspension was filtered through a 10 µm nylon mesh filter (Millipore, Tullagreen Cork, Ireland), centrifuged at 1500×*g* for 5 minutes (Sigma 4k15, Osterode, Germany) and the pellet re-suspended in 30 mL sterile milliQ water containing 10% glycerol. The spore concentration was determined by filtration of 0.1 mL of 10 fold diluted suspension through a 25 mm diameter polycarbonate filter with 0.4 µm pore size (Millipore, Tullagreen Cork, Ireland) and subsequent enumeration of deposited spores by field emission scanning electron microscopy (FESEM). One milliliter (containing ca. 10^8 spores) of this spore suspension was used to inoculate gypsum board (GB) (Lafarge Gips Dekoform 6 AK, Oberursel, Germany) commonly used as indoor building material in Norwegian residential environments. The GB was cut into 80 mm diameter circular plates that were prepared following the procedure previously described (Afanou et al. 2014). Following 8 weeks of incubation at 25±1°C and 45±5% relative humidity (in the incubator), the GB plates were utilized for aerosolization experiments.

Aerosolization and collection of fungal aerosols

The fungal cultures were aerosolized with the Fungal Spore Source Strength Tester (FSSST) (Sivasubramani et al. 2004), and Stami Particle Generator (SPG) (Afanou et al. 2014). The FSSST was built in polyvinyl chloride with a square internal area of 144 cm^2, and 112 orifices of 0.4 mm diameter that directed air jets perpendicularly toward the culture plates. The SPG was built in aluminum and included a rotating plate of 156 cm^2 as support for the culture plates. A 6 mm diameter tube outfitted with ten orifices of 1.2 mm diameter served to direct air jets perpendicularly toward the culture plate. Schematic illustration for both generators and the experimental setup are shown in **Figure 1**. The aerosolization experiments were performed at airflow rates of 12 and 20 L min^{-1}. At these airflows, the air velocities at the orifices were 18 and 29 m s^{-1} in the SPG, and 14 and 23 m s^{-1} in the FSSST, respectively. During aerosolization the GB plates were situated at 14 mm below the orifices. Fungal aerosols were liberated by air

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currents provided through the orifices. Each aerosolization experiment was run for 120 sec while rotating the plate supporting *A. versicolor* and *P. chrysogenum* culture at 0.5 rpm in the SPG. In FSSST, the culture dishes were aerosolized without rotation. In the experiment with *A. fumigatus*, a modified approach was used to prevent bioaerosol overload on the sampling filter. Briefly the *A. fumigatus* culture surface area was covered with a plate that contained a 1 cm diameter central hole. *A. fumigatus* particles were liberated using the same airflow rate as described above. However, the sampling interval was 60 sec while rotating at 1 rpm in SPG. Released particles from the culture were transported by the air currents within each system onto a 0.4 µm pore sized polycarbonate filter membrane (Isopore, Millipore, Ireland) and contained in open 37 mm cassettes (SKC Inc., Eighty Four, PA). All experiments were conducted in a Bio Safe Grade II laminar flow hood to prevent external particle contamination and to protect laboratory workers from fungal exposure. The generator chamber was cleaned with 70% ethanol and purged for 10 min with HEPA filtered air prior to each experiment. Each experimental treatment was run in triplicate. Sixteen blank experiments were performed using cleaned empty chamber for background adjustment. Blanks with GB plates were disregarded because the substrate material after fungal bio-deterioration is different from the substrate at start (Sánchez 2009). Four blank experiments for each generator×flow rate combination were run for 120 sec.

Sample preparation and analysis

Briefly, a segment (ca. 2.4 cm^2) was cut from the collection filter, mounted on a 25 mm aluminum pin stub (Agar Scientific Ldt, Stansted Essex, UK) using double-sided carbon adhesive discs (Ted Pella inc. Redding, CA, USA), and coated with platinum in a Balzers SDC 050 sputter coater (Balzers, Liechtenstein) as previously described by Afanou and colleges (Afanou et al. 2014). Samples were analyzed using FESEM (SU 6600 Hitachi, Ibaraki-ken, Japan) in the secondary electron imaging (SEI) mode. The microscope was operated at 15 keV acceleration voltage, 1.8 kV extraction voltage and a working distance of 10 mm. Particles were quantified using the counting criteria described by Eduard and Aalen (Eduard and Aalen 1988). The particles were considered to be homogenously distributed on the filter because an electrically conducting filter holder had been used to reduce electrostatic charging of the filter holder (Eduard et al. 1990).

Identification and classification of the fungal particles

Spore particles were identified and classified by their morphological features while other indiscernible particles were defined as fragment particles and classified by length (**Figure 2**). Spore particles were categorized into five classes according to spore units per aggregate: single spores, and aggregates of 2, 3, 4, and ≥5 spores. Fragment particles were grouped in four classes based on their length: 0.2 – 1µm fragments, 1-2µm fragments, 2-3.5µm fragments, and ≥3.5µm fragments. Four hundred particles or a maximum of 100 fields were counted at 3,000 – 6,000× magnification depending on the particle density on the filter and type of particle. The lowest

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3 detectable number of particles was 8×10^3 and 2×10^4 particles/filter at 3,000× and 6,000×
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5 magnification, respectively. The background numbers of each particle type by cm^2 surface of the
6 supporting culture plate were in the range: 0–200 for single spores, 0 for aggregates of 2 spores,
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8 0–45 for aggregates of 3 spores, 0 for aggregates of 4 spores, 0–40 for aggregates of 5 spores or
9 more, 160–4000 for submicronic fragments, 55–800 for 1 – 2µm fragments, 0 for 2 – 3.5µm
10 fragments and 0–160 for ≥ 3.5µm fragments.
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13 *Size and shape measurements*
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15 The largest width and length were additionally measured on 12 randomly selected spore particles
16 (single spores and aggregates of 2- 6 spores). Similar measurements were performed on each
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18 type of fragment particles (submicronic and larger fragments) counted in 200 randomly selected
19 fields at 3,000× magnification. Measurements were performed at 3,000 – 40,000× magnification
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21 depending on particle type and size. Shape of each particle was described by their aspect ratio
22 calculated as length to width ratio.
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24 *Statistical analysis*
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26 Data from 12 experiments of each fungal species were available for statistical analysis. All
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28 counts were adjusted for blank prior to the estimation of the number of particles per cm^2 culture.
29 Negative or zero counts after background adjustment, were arbitrarily assigned 0.1 in order to
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31 enable ratio calculations and log ratio transformations for statistical analysis. Primary results
32 were reported as median numbers, 25th and 75th percentiles of particles of each type per exposed
33 culture area. The median numbers of particle types were compared between the fungal species
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35 using the nonparametric Kruskal-Wallis (K-W) test for multiple categories followed by *post hoc*
36 Wilcoxon-rank sum test (Mann-Whitney U tests) and Bonferroni adjusted p-values (significant p
37 values <0.017).
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39 Proportions of each particle type were calculated as the ratio of particle type counted to the total
40 number of particles and were also reported as medians and percentiles (25th and 75th). The effects
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42 of the generators and airflows on the particle composition of the fungal aerosols were statistically
43 assessed by applying linear mixed model on centered log ratio (CLR) transformed particle ratios
44 (Aitchison 2003; Pawlowsky-Glahn and Egozcue 2006). CLR transformation was performed
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46 with CoDaPack version 2.01.15 (Department of Computer Science and Applied Mathematics,
47 University of Girona, Girona Spain) by dividing each count ratio by the geometric mean of count
48 ratios for each treatment. This transformation has been described to remove the constant sum
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50 constraint on compositional data (Aitchison 2003; Pawlowsky-Glahn and Egozcue 2006) prior to
51 analysis.
52 Schematically the models were:
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54 Generator: *CLR=constant + Type + Type×Generator + experiments (random) + residuals*
55 Airflow rates: *CLR=constant + Type + Type×Flow + experiments (random) + residuals*
56 (CLR: centered log ratio; *Type* represent the nine types of particles; *Generator* represents the two generators used;
57 *Flow* represents the two airflows rate used)
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To detect significant changes in the fungal aerosol composition between generators or airflows, we tested the significance of the two interaction terms ($Type \times Generator$) and ($Type \times Flow$). This was done by likelihood ratio tests between models with and without the interaction terms. Here, the models without the interaction terms included the two variables of the interaction term as single fixed effects (Fitzmaurice et al. 2011; Jaeger 2008). Furthermore, particle fractions that significantly changed between generators or airflows were pinpointed by the sign and significance of the individual coefficients of these two interaction terms. Significant p-values ($p<0.05$) were adjusted for multiple comparisons using the false discovery rate method (Benjamini and Hochberg 1995; Benjamini 2010). Statistical analysis was performed in Stata SE 13 (Statacorp LP, College Station, TX, USA).

Results

Size and shape characteristics of spores and fragment particles

All counted particles were classified according to their physical length measured by FESEM. The average sizes for spore particles were in the range 2.41 – 15.04µm and 2.02 – 4.26µm for lengths and widths, respectively (**Table 1**). The corresponding sizes for fragment particles were 0.52 – 12.80µm and 0.33 – 5.10µm (**Table 2**). Three shape groups were recognized from the aspect ratios (**Table 3**). Spherical or near-spherical particles with aspect ratio in the range 1:1 – 1.5:1 group specifically the single spores irrespective to species. The median proportions of this group were 53%, 7% and 6% of total aerosolized particles from *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. A second group of oblong shaped particles has aspect ratios in the range 1.5:1 – 3:1 and represents 45%, 68% and 55% (median) of the aerosolized particles from *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. Further, this group comprises 0.2 – 1µm fragments, 1 – 3.5µm fragments, and spore aggregates except the largest aggregates. The third group has elongate and fiber-like shape with aspect ratio >3:1. Larger fragments (>3.5µm) and larger spore aggregates belong to this group which represents 1%, 21% and 24% (median) of total particles aerosolized from *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. The overall average lengths (widths) were in the ranges 2.41 – 2.97µm (2.02 – 2.53µm) for near spherical particles, 0.52 – 8.55µm (0.33 – 3.65µm) for oblong particles and 3.5 – 15.04µm (2.22 – 5.10µm) for the elongate and fiber- like particles. The micrographs of various particle types observed are shown in **Figure 2.**

Number of spores and fragment particles

The total median numbers of aerosolized particles were 4.8×10^5 per cm^{-2} culture of *A. fumigatus,* 5.6×10^3 for *A. versicolor* and 8.9×10^3 for *P. chrysogenum.* Median sum spore particle numbers were 2.9×10^5, 0.8×10^3 and 0.9×10^3 per cm^{-2} culture, while the corresponding numbers for fragment particles were 1.9×10^4, 4.2×10^3 and 8.6×10^3 for per cm^{-2} culture for *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively. The median number of spore particles released from *A. fumigatus* cultures differed significantly from *A. versicolor* and *P. chrysogenum* cultures

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(**Table 4**). *A. fumigatus* cultures released over 300 times the median number from *A. versicolor* (p <0.001) and *P. chrysogenum* (p <0.001). The number of fragment particles did not differ significantly between the tested species (p= 0.2).

Fungal aerosol composition:

The bioaerosols collected from *A. fumigatus* was predominantly composed of single spores (median 53% of total particles) while submicronic fragment fractions were the highest in the aerosols from *A. versicolor* (submicronic fragments, median 34%) and *P. chrysogenum* (submicronic fragments, median 31%). Within the spore particle fraction from *A. fumigatus* and *P. chrysogenum*, single spores were most common: medians 70% and 28%, respectively, whereas the aerosols from *A. versicolor* were mainly composed of larger spore aggregates (aggregates of ≥5 spores, median 32%) **(Table 4).** Moreover, the submicronic fragments were dominant in the fragment particle fraction from all three isolates: medians 62%, 70% and 63% of total fragments from *A. fumigatus*, *A. versicolor* and *P. chrysogenum*, respectively.

The combined proportions of larger fragments (medians of 1 – 2µm fragments, 2 – 3.5µm and ≥3.5µm) amounted to median 3%, 9% and 8% of all particles collected from *A. fumigatus, A. versicolor* and *P. chrysogenum*, respectively (**Table 4)**. The median proportions of single spores were 53%, 7% and 6% while the medians of all spore aggregates combined were 26%, 23% and 10% of all particles from *A. fumigatus, A. versicolor* and *P. chrysogenum* cultures, respectively (**Table 4)**.

Effect of generator types on the fungal aerosol composition

Significant changes in the aerosol composition related to the type of generator were demonstrated trough CLR regression models and likelihood ratio tests of the interaction terms "*Generator×Type*" (**Tables** 5 Supplement). The test was significant for *A. fumigatus* cultures aerosolized at 12 Lmin^{-1} (p= 0.005), for the *A. versicolor* and *P. chrysogenum* cultures both aerosolized at 12 and 20 Lmin^{-1} (p= <0.001- 0.007). The fraction of submicronic fragments was mainly affected when changes in the profile were observed between SPG and FSSST at 12 and 20 Lmin^{-1}. Further, the fraction of submicronic fragments was significantly higher in SPG (p= 0.001) as compared to FSSST with *A. fumigatus* cultures aerosolized at 12 Lmin^{-1} **(Figure 3A)** while this fraction was lower in SPG as compared to FSSST at airflow 12 Lmin^{-1} for *A. versicolor* (p<0.001) **(Figure 3C)** and *P. chrysogenum* (p= 0.007) **(Figure 3E).** In addition, the single spore fraction was significantly larger in SPG versus FSSST with *P. chrysogenum* cultures (p<0.001). At 20 Lmin^{-1}, no significant difference was observed between SPG and FSSST in the bioaerosol composition from *A. fumigatus* cultures **(Figure 3B)**. But with *A. versicolor* cultures, the 2 – 3.5µm fragments fraction was significantly lower in SPG compared to FSSST (p= 0.001) **(Figure 3D).** Furthermore, the submicronic fragment fraction from *P. chrysogenum* cultures was significantly larger in SPG versus FSSST (p= 0.002) **(Figure 3F)**.

Effect of airflow rates on the fungal aerosol composition

Fungal cultures subjected to air flow rates of 12 $Lmin^{-1}$ and 20 $Lmin^{-1}$ showed significantly different aerosol profiles. The likelihood ratio test of the interaction term "*Flow×Type*" was significant with both SPG and FSSST for all tested fungal isolates (p= <0.001 - 0.003) (**Tables 6** Supplement). For *A. versicolor,* the fraction of spore particles was the largest at 20 $Lmin^{-1}$ in both generators (p= 0.004 – 0.02) whereas the proportion of >1µm fragment particles was highest at 12 $Lmin^{-1}$ (p= <0.001 – 0.03) in FSSST (**Figure 4C** and **4D**). Similar results were observed for *A. fumigatus*, but only in the SPG; the fraction of aggregates of ≥5spores increased with increasing airflow (p= 0.012), while ≥3.5µm fragments fraction (p <0.001) and the submicronic fragments fraction decrease with increasing airflow (p= <0.001– 0.002) (**Figure 4A**). In the FSSST, the difference between the single spore fractions at 12 and 20 $Lmin^{-1}$ was closed to significance (p= 0.006) with the largest fraction obtained at 20 $Lmin^{-1}$ (**Figure 4B)**. Fractions of single spore and aggregates of ≥5spores of *P. chrysogenum* were higher at 20 $Lmin^{-1}$ than at 12 $Lmin^{-1}$ (p= 0.008) in the FSSST but not in SPG (**Figure 4E** and **4F**). The ≥3.5µm fragments fraction increased with increasing airflow in both generators (p= <0.001 – 0.013) (**Figure 4E** and **4F**) as opposed to the findings for the *Aspergillus* isolates. The submicronic fragment fraction decreased with increasing airflow rate in both FSSST (p <0.001) and SPG (not significant: p=0.07).

Discussion

With FESEM, the aerosols generated from fungal cultures revealed diverse arrays of particles with different size, shape and origin. The composition varied substantially between species and aerosolization conditions. The aspect ratio revealed three major particle shapes: near spherical, oblong and fiber-like. All single spores were fitted in the group of near spherical particles which is in close agreement with morphology described for spores from these species (Samson et al. 2004).

The aerodynamic diameter of these particles could therefore be a good predictor of their behavior in the respiratory tract as suggested by Reponen et al (1995) assuming that surface ornamentation has negligible effects. Further, most submicronic fragments were oblong while most of spores aggregates and larger fragments were elongate and fiber-like in shape. It is therefore likely that motion and deposition models of these particles in the respiratory tract will have to consider their shape characteristics in addition to aerodynamic equivalent diameter as reviewed by Lacey (1991).

Based on the physical sizes of various particles (microscopic length and width), many large fragments were found in the same size range as single spores and spore aggregates. Obviously, the heterogeneity of size and shape of fragment particles will confound the recognition of spore particles by large fragments when automatic counters or sizers are used. For example, Gorny et al used optical diameter of 1.6 µm (a size delimitation) to discriminate between small fragments

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and spores from *Aspergillus versicolor*, *Penicillium melinii*, *P. chrysogenum* and *Cladosporium cladosporioides* culture (Górny and Ławniczek-Wałczyk 2012; Górny et al. 2002). Similarly, Madsen described the composition of aerosols from *Botrytis cinerea* by allocating specific aerodynamic diameter ranges to specific particles types such as fragments (<1.6 µm), microconidia (1.8 – 3.3µm) and macroconidia (3.5 – 10.4 µm) (Madsen 2012). In these studies, the spore or conidia fractions are likely to comprise both spore aggregates and large fragments.

Using aerosol size fractionation samplers, spores and fragment fractions have been reported likewise as studies that used automatic particle counters. Further quantification of fungal biomarker in the specific fractions allows the application of this approach in field settings for quantification of fungal submicronic fraction. For example the mass of β-glucans was estimated in different size fractions of air samples collected by cyclones from indoor environments with molds contamination. The fungal aerosol profile was thus described as submicronic fragments fraction (<1 µm), a mixture fraction of spores and fragments (1.05 – 2.25 µm) and a spore fraction (>2.25 µm) (Reponen et al. 2007; Seo et al. 2009, 2014). Moreover, multi-stage impactors (Cho et al. 2005; Górny et al. 2002) as well as filters with different pore size in series (Brasel et al. 2005) have been used to disclose the fungal aerosol composition. However, the problem of spore bounce associated with cyclone sampling (Lindsley et al. 2006; Madsen et al. 2009) as well as the cut-off characteristics of cyclones suggest possible contamination of the submicronic fragment fraction by larger particles.

In any case whether fungal aerosols are studied with automatic counter or size fractionation coupled to biomarkers, characterization of the aerosol composition revealed only a one-dimensional size description of spores and fragment particles. In contrary, two dimensional size measurements revealing shapes and sizes characteristics of various constituents present in the fungal aerosol was achieved in the present study using field emission scanning electron microscopy. Such visualization of particles favors their classification whether as spore or fragments. The present microscopic approach provides therefore more detailed characteristics of the fungal aerosols as compared to other approaches.

The total numbers of spore particles aerosolized from *A. fumigatus* were significantly higher as compared to *A. versicolor* and *P. chrysogenum* ($p<0.001$), but no species difference was observed for fragment numbers ($p=0.2$). We found no significant difference between *A. versicolor* and *P. chrysogenum* either for spore or fragment particles. This is in contradiction to results reported by Gorny et al (2012) who also conducted aerosolization experiments on these species and observed significantly higher number of spores and fragments from *A. versicolor* as compared to *P. chrysogenum*. We can only speculate about what causes the deviating results: differences in experimental parameters such as generator, airflow and cultures age and the variation between isolates from same fungal species. Nevertheless, the dominance of the submicronic particle fraction in the aerosols from *A. versicolor* or *P. chrysogenum* is in agreement with previously reported studies based on automatic particle counters or sizers (Cho et

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al. 2005; Górny and Ławniczek-Wałczyk 2012; Górny et al. 2002; Madsen et al. 2005; Seo et al. 2009).

The presence of large spore aggregates in the aerosols is of concern because they carry much more antigenic substances into the respiratory tract despite their smaller number in the generated aerosols. The long chained aggregates have fiber-like shape with aerodynamic properties that may increase their chance to reach the trachea-bronchial region. It has been recently demonstrated that neutrophils sense the microbe size and selectively release neutrophils extracellular traps (NETs) in response to large particles including fungal hyphal fragments and spore aggregates, but not in response to single spores (Branzk et al. 2014). It is therefore likely that different response mechanisms with substantially different cellular pathology will be induced upon inhalation and deposition of fungal particles in the respiratory tract. Large spore aggregates are likely to induce NETosis, whereas single spores are phagocytized. This size dependent response should be emphasized in future exposure-response studies, and it will be important to include particle size determination in the characterization of fungal aerosols. The present study provides important information of fungal bioaerosols in this respect. The composition of aerosols generated from *A. fumigatus* including submicronic fragments has, to our knowledge, not been previously reported. Single spore outnumbered other particles suggesting that there is higher probability to detect spores from this species if growing in indoor environments as compared to *A. versicolor* and *P. chrysogenum* with aerosols dominated by fragments.

The present study revealed also significant changes in the bioaerosol composition related to the generators and airflows used for all tested isolates. For example, with *A. fumigatus* cultures the bioaerosol profile changed significantly to relatively fewer single spores and more submicronic fragments in SPG compared to FSSST when the airflow was 12 $Lmin^{-1}$. Similarly the proportions of submicronic fragments and ≥3.5μm fragments increased at 12 $Lmin^{-1}$ compared to 20 $Lmin^{-1}$ when SPG was used. Gorny and co-authors (Górny and Ławniczek-Wałczyk 2012) reported that generation factors such as air jet velocity, direction and movements have significant effects on the number of small fragments (<1.6 μm) and spores released from fungal cultures of *A. versicolor* and *P. chrysogenum*. Different physical parameters such as the level of turbulence inside the generator, impaction of particles onto the substrate and mycelia, and electrostatic charges on the particles and generator surfaces may collectively influence the release, the transportation, and loss of particles during *in vitro* aerosolization. These factors may even differ between particle types. The particle types (spores, spore aggregates or fragments) that are mostly affected are likely of great importance for how the final composition of the bioaerosol may look.

The fraction of submicronic fragments was mostly influenced by the generator and airflow used. The most striking change was observed with *P. chrysogenum* aerosolized in the FSSST where the submicronic fragment fraction was 93% at a flow rate of 12 $Lmin^{-1}$ which was reduced to 6% at a flow rate of 20 liters min^{-1}. Only the results for *A. versicolor* seemed fairly consistent, showing increased spore fractions and lower fragment fractions at higher flow rate in both

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generators. The other fungal isolates showed more diverse results. Thus, the composition of fungal aerosols in these *in vitro* experiments was greatly influenced by generation conditions. As changes in the fungal profiles often were in opposite directions, extrapolation of our results to environmental conditions appears difficult. Direct measurements in the environment are therefore required in order to assess exposure to fungal particle types different from spores.

Although detailed characteristics of fungal particles were obtained with FESEM, this microscopic method has been criticized for introducing two biases. Firstly, the imaging that is performed in a vacuum chamber may cause distortion, collapse or disintegration of spore aggregates (Vestlund et al. 2014). Nevertheless, the morphological characteristics and the size measurements found are quite consistent with the literature (Frisvad and Samson 2004). In SEM, naturally collapsed spores have similar size (length and width) as compared to whole spores (Beckett et al. 1984). Furthermore, spores will desiccate when the relative humidity is below 100%, and desiccated spores are likely to occur naturally in the environment (Beckett et al. 1984). Secondly, the analyzed filter area is a small fraction (0.1-1%) of the exposed filter area. Although the distribution of particles in filter samples collected in electrically conducting cassettes is fairly homogenous (Eduard and Aalen 1988) the enumeration at higher resolution (3000-6000×) may lead to very low numbers or zero counted particles leading to highly Poisson distributed particle counts. However, the high resolution microscopic method is the only approach that permits classification and size and shape characterization of a broader range of fungal particles that include submicronic fragments, large fragments and spore particles.

Conclusions

The high resolution microscopic characterization of fungal aerosols from *A. fumigatus*, *A. versicolor* and *P. chrysogenum* revealed a complex profiles with different shape, origin and size. Fungal spores were aerosolized as single spores, chained or clustered spore aggregates with shape and size that favor their deposition in different regions in the respiratory systems. Released fragments were mainly submicronic fragments but also included larger fragments with sizes that partly overlap those of spore particles. The FESEM method used in the present study was satisfactory for assessing shape and size as well as the source of fungal particles aerosolized from pure cultures grown on gypsum board.

The composition of the experimentally generated fungal aerosols was significantly influenced by the generator or the airflow rate used. Changes in the profiles were too diverse to enable extrapolation to real conditions. The complexity and variability of the bioaerosol composition and the dominance of fragments in the fungal aerosol from *A. versicolor* and *P. chrysogenum* supports previous recommendations on detecting broader range of particles types during fungal exposure assessment studies.

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Acknowledgments

The present study is a part of the project "Fungal particles in indoor air" that is financially supported by the Norwegian Research Council: Grant number NFR196130/H10. The technical support provided by the Norwegian Veterinary Institute is gratefully acknowledged. The findings and the conclusions in this report are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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Figure 1: Schematic illustration of the aerosolization chambers: Stami Particle Generator (SPG), Fungal Spore Source Strength Tester (FSSST) and the experimental set up. One aerosolization chamber is connected to the system set up. Flow at the inlet and outlet of the chamber is measured by Flowmwter 1and 2, respectively. Hepa filter 1 and 2 filter the air to and from the pump, respectively. Constant relative humidity is maintained by the dryer (in line tube with silica beads). P2021 ionizer reduces elctrostatic charges. Reproduced with permission from American Society of Microbiology: Afanou et al. 2014; Applied and Environmental Microbiology, Vol 80, Number 22, 7122 – 7130.

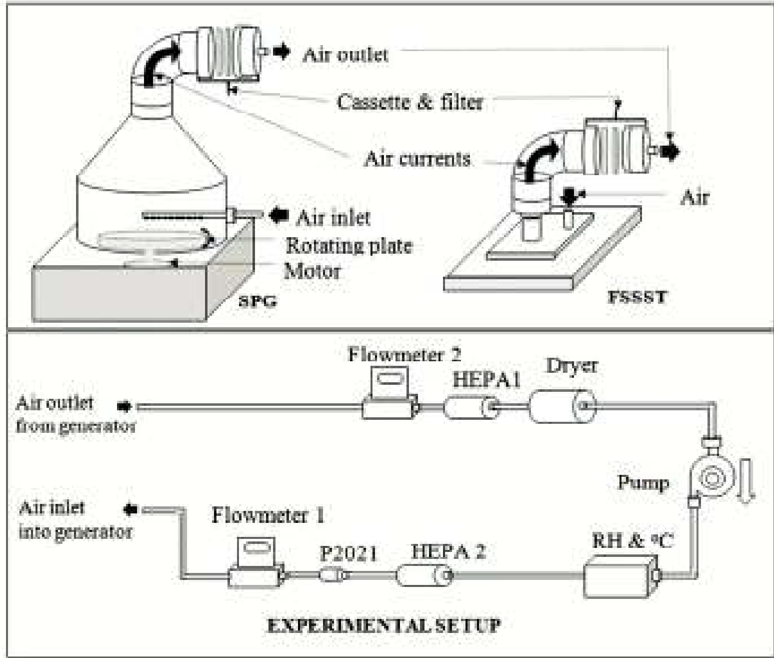
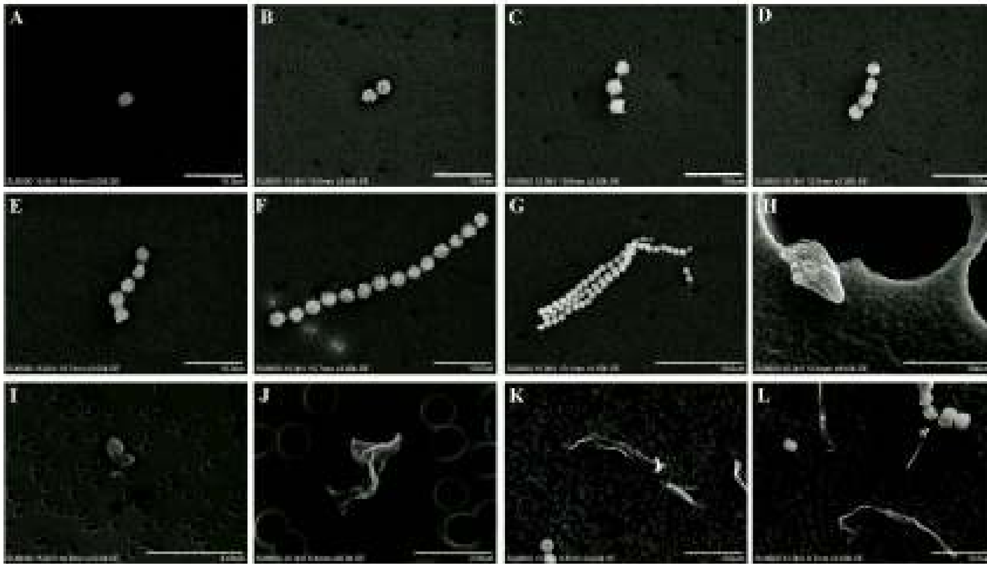
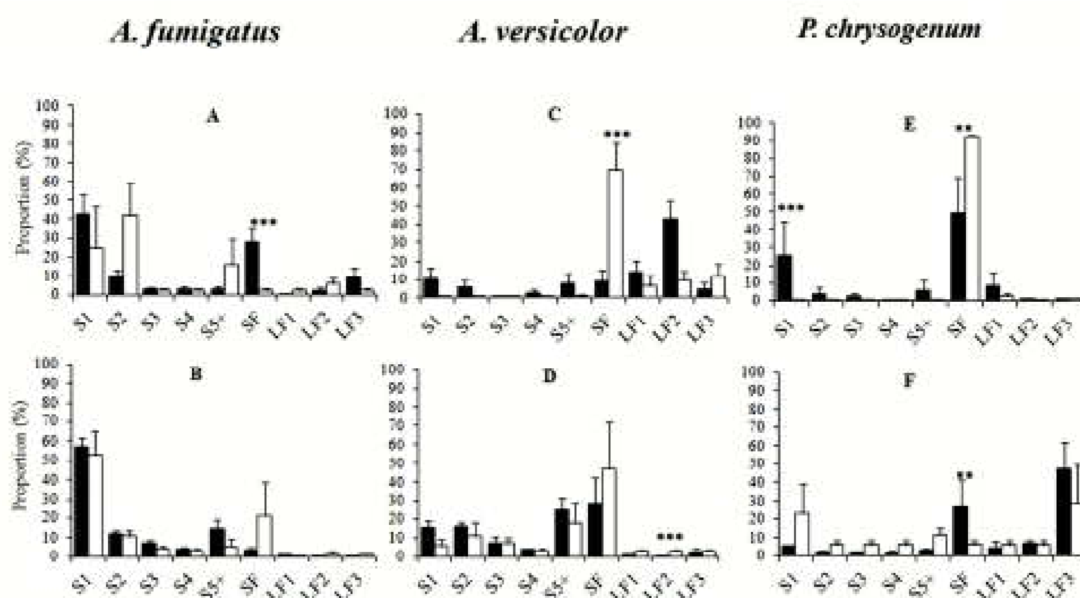


Figure 2: Micrographs of various fungal aerosolized particles



Spore particles (A-F); A: Single spores B-G: Aggregate of 2, 3, 4, 5 and >5 spores from *A. versicolor*. Fragment particles (H-L) ; H: Submicronic fragments: <1µm ; H-L: Large fragments; I: 1 – 2µm fragments; J: 2 – 3.5µm fragments, K-L: >3.5µm fragments. Scale bar: 10µm (A-F and K-L); 30µm (G); 0.5µm (H); 5µm (I); 2µm (J).

Figure 3: Distribution of mean proportions of particle types aerosolized from *A. fumigatus*, *A. versicolor* and *P. chrysogenum* cultures; SPG (black columns) and FSSST (white columns) at 12 $Lmin^{-1}$ (A, C and E) and at 20 $Lmin^{-1}$ (B, D and F).



S1: Single spores; Aggregates of 2 (S2), 3 (S3), 4 (S4), ≥5 (S5) spores. SF: Submicronic fragments, LF1: 1-2µm fragments, LF2: 2-3.5µm fragments and LF3: ≥3.5µm fragments. * p≤0.05, ** p≤0.01, *** p≤0.001 (t-test of interaction coefficients between generator and particle types in the centered log-ratio mixed effect regression model of the proportions of particles. All p-values were adjusted for multiple comparisons by the false discovery rate method). Error bars represent standard errors.

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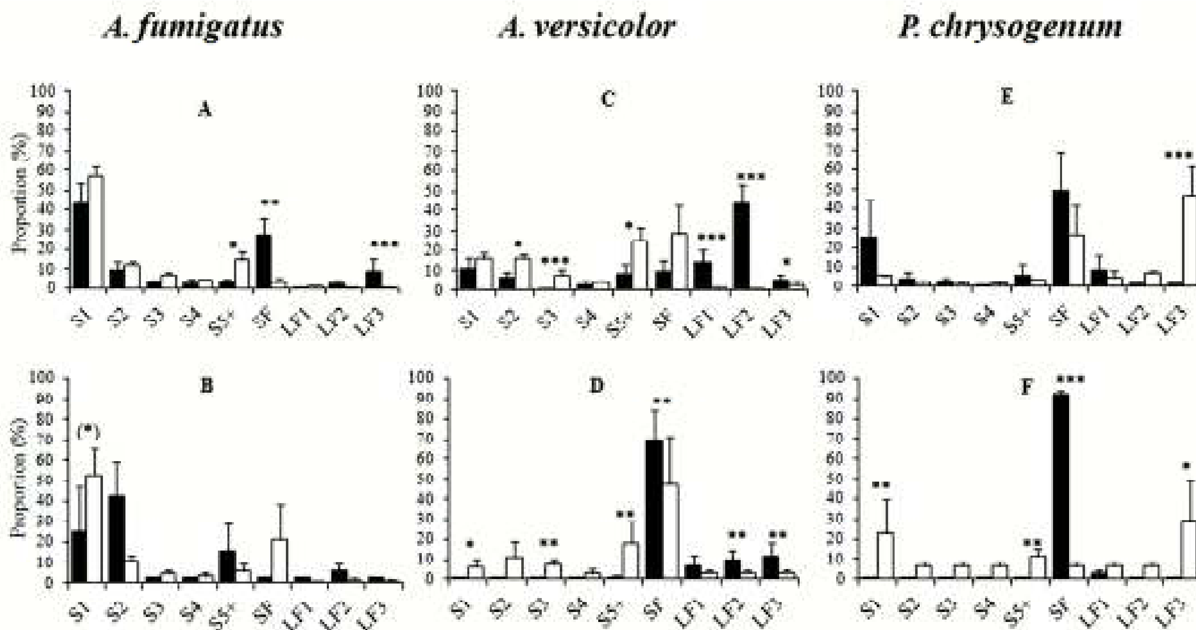
Figure 4: Distribution of mean proportions of particle types aerosolized from *A. fumigatus*, *A. versicolor* and *P. chrysogenum* cultures. Airflow 12 Lmin^{-1} (black columns) and 20 Lmin^{-1} (white columns) with SPG (A, C and E) and FSSST (B, D and F).



S1: Single spores; Aggregates of 2 (S2), 3 (S3), 4 (S4), ≥5 (S5) spores. SF: Submicronic fragments, LF1: 1-2µm fragments, LF2: 2-3.5µm fragments and LF3: ≥3.5µm fragments. * p≤0.05, ** p≤0.01, *** p≤0.001 (t-test of interaction coefficients between airflow and particle types in the centered log-ratio mixed effect regression model of the proportions of particles. All p-values were adjusted for multiple comparisons by the false discovery rate method). Error bars represent standard errors.

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Table 1: Size and aspect ratio of spore particles.

	A. fumigatus						*A. versicolor*						*P. chrysogenum*					
	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4	S5	S6	S1	S2	S3	S4	S5	S6
N	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12	12
AM																		
Length	2.68	4.77	6.42	6.83	8.55	12.26	2.97	5.87	8.00	10.61	12.51	15.04	2.41	4.32	6.77	8.88	11.45	12.28
Width	2.25	2.61	3.20	3.64	3.65	4.26	2.53	2.66	3.20	3.65	3.75	4.25	2.02	2.07	2.22	2.78	2.88	3.34
Aspect ratio	1.20	1.83	2.05	1.97	2.45	3.14	1.18	2.22	2.57	3.10	3.70	3.88	1.21	2.16	3.11	3.45	4.19	4.16
SD																		
Length	0.27	0.61	1.61	1.15	1.03	2.48	0.26	0.78	1.15	1.82	2.30	3.17	0.28	0.48	1.10	2.03	1.93	2.11
Width	0.30	0.19	0.46	0.85	0.94	1.08	0.19	0.21	0.50	0.97	1.09	1.10	0.35	0.38	0.31	0.62	0.74	1.13
Aspect ratio	0.15	0.23	0.59	0.56	0.58	1.26	0.20	0.34	0.56	0.94	1.44	1.63	0.20	0.46	0.63	1.33	1.04	1.63

AM: arithmetic mean; SD: standard deviation; Length and Width in µm.
S1: Single spores; Aggregates of 2 (S2), 3 (S3), 4 (S4), 5 (S5) and 6 (S6) spores

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49

Table 2: Size and aspect ratio of fragment particles

	A fumigatus				*A versicolor*				*P chrysogenum*			
	<1 µm	1-2 µm	2-3.5 µm	>3.5 µm	<1 µm	1-2 µm	2-3.5 µm	>3.5 µm	<1 µm	1-2 µm	2-3.5 µm	>3.5 µm
N	113	41	27	19	102	44	25	29	67	36	30	68
AM												
Length	0.53	1.38	2.59	11.20	0.52	1.40	2.49	12.80	0.63	1.44	2.59	10.6
Width	0.33	0.77	1.41	4.69	0.34	0.76	1.46	5.10	0.37	0.75	1.42	3.34
Aspect ratio	1.78	2.12	2.26	3.34	1.78	2.19	1.89	3.01	2.00	2.17	2.08	4.68
SD												
Length	0.20	0.27	0.44	10.50	0.21	0.28	0.35	11.77	0.20	0.30	0.43	9,50
Width	0.14	0.34	0.91	5.10	0.22	0.35	0.40	4.53	0.16	0.28	0.64	2.50
Aspect ratio	0.77	1.00	0.87	4.15	0.86	1.04	0.83	3.25	1.47	0.94	0.72	5.82

AM: arithmetic mean; SD: Standard deviation; Length and width in µm

<1µm: submicronic fragments (SF); 1 - 2µm fragments (LF1); 2 – 3.5µm fragments (LF2) > 3.5µm fragments (LF3)

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49

Table 3: Shape characteristics of fungal bioaerosols based on aspect ratio. AM arithmetic means.

Range of aspect ratio (AM)	*A. fumigatus*			*A. versicolor*			*P. chrysogenum*		
	Spore particles	Fragment particles	Median (%)	Spore particles	Fragment particles	Median (%)	Spore particles	Fragment particles	Median (%)
1:1 – 1.5:1 (near spherical)	Single spores	-	53	Single spores	-	7	Single spores	-	6
1.5:1 – 3:1 (oblong)	Aggregates of 2, 3, 4 and 5 spores	<1µm, 1 – 2 µm and 2 – 3.5 µm	45	Aggregates of 2 and 3 spores	<1µm, 1 – 2 µm and 2 – 3.5 µm	68	Aggregates of 2 spores	<1µm, 1 – 2 µm and 2 – 3.5 µm	55
>3:1 (Fiber -like)	Aggregates of 6 spores	>3.5 µm	1	Aggregates of 4, 5 and 6 spores	>3.5 µm	21	Aggregates of 3, 4, 5 and 6 spores	>3.5 µm	24

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Table 4: Particle types aerosolized from *A. fumigatus A. versicolor* and *P. chrysogenum* (all experiments combined).

	A. fumigatus (N=12)		***A. versicolor*** (N=12)		***P. chrysogenum*** (N=12)		
Particle types	Number of particles [ab]	% of total [b]	Number of particles [ab]	% of total [b]	Number of particles [ab]	% of total [b]	K-W test p-value
Spores							
S1	170 (7.0—1400)	53 (26—62)	0.3 (0.02—1.4)	7 (0.4—14)	0.4 (0.06—2.4)	6 (1—10)	***0.001***
S2	47 (2.0—240)	12 (8—17)	0.2 (0.02—1.8)	7 (0.3—15)	0.06(0.06—0.40)	1(0.6—5)	***<0.001***
S3	20 (0.4—107)	4 (2—5)	0.09 (0.02—0.7)	1 (0.3—6)	0.06 (0.06—0.3)	2 (0.7—4)	***0.001***
S4	11 (0.06—118)	3 (2—4)	0.02 (0.02—0.4)	1 (0.3—4)	0.06 (0.06—0.1)	1(0.6—3)	***0.005***
≥S5	26 (0.6—140)	5 (2—15)	0.3 (0.09—2.1)	9 (1—19)	0.06(0.06—0.6)	3(0.7—8)	***0.01***
Sum	**290 (9.0—1950)**	**88 (75—94)**	**0.9 (0.3—7.0)**	**39 (5—58)**	**1.0 (0.3—3.4)**	**16 (8—42)**	***<0.001***
Fragments							
SF	13.0 (1.0—300)	5 (1—21)	2.0 (0.6—5.0)	34 (7—62)	8.0 (0.6—10)	31 (10—89)	*0.2*
LF1	2.0 (0.06—4.1)	1 (0.2—1)	0.06(0.06—0.5)	2 (1—8)	0.06 (0.06—0.6)	2 (1—8)	*0.2*
LF2	2.7 (0.06—4.1)	1(0.2—4)	0.06 (0.06—0.6)	4 (0.5—22)	0.06 (0.06—0.7)	2 (0.7s—6)	*0.07*
LF3	2.0 (0.06—4.1)	1 (0.1—3)	0.06 (0.06—0.2)	3 (1—8)	0.06 (0.06—3.0)	4 (1—38)	*0.2*
Sum	**19.0 (1.8—350)**	**12 (6—25)**	**4.0 (1.0—6.0)**	**61(42—95)**	**9.0 (2.0—15.0)**	**84 (58—92)**	*0.2*
TOTAL	**480 (17—2659)**		**5.6 (2.4—14)**		**8.9 (3—16.5)**		

[a] Data represent 10^3 particles cm^{-2} of culture media. [b] Medians, 25th and 75th percentiles (in parentheses); K-W= Kruskal Wallis test for the three fungal species. The *post-hoc* Wilcoxon rank sum test of differences between species for median numbers with *A. fumigatus* significantly higher than *A. versicolor* and *P. chrysogenum*. Significance level ($p \leq 0.017$). S1: Single spores; Aggregates of 2 (S2), 3 (S3), 4 (S4) and ≥5 (S5) spores. SF: Submicronic fragments, LF1: 1-2µm fragments, LF2: 2-3.5µm fragments and LF3: ≥3.5µm fragments

Table 5: Distribution models for *A. fumigatus. A. versicolor* and *P. chrysogenum.* CLR mixed effect regression stratified by airflows and with generator particle types as fixed effects and repeated experiments as random effect. CLR: centered log ratio.

	A. fumigatus						*A. versicolor*						*P. chrysogenum*					
	SPG versus FSSST						SPG versus FSSST						SPG versus FSSST					
	Flow =12Lmin^{-1}			Flow =20 Lmin^{-1}			Flow =12 Lmin^{-1}			Flow =20 Lmin^{-1}			Flow =12 Lmin^{-1}			Flow =20 Lmin^{-1}		
	B	SE	P-value	B	SE	P-value	B	SE	P	B	SE	P-value	B	SE	P	B	SE	P-value
Intercept	-0.65	0.63	0.3	-2.69	0.62	**<0.001**	0.73	0.50	0.1	-0.61	0.47	0.2	0.57	0.41	0.2	-0.31	0.42	0.5
Particles																		
LF1	reference						reference						reference					
LF2	0.77	0.88	0.4	0.46	0.88	0.6	0.58	0.70	0.4	0.00	0.67	1	-1.34	0.58	0.02	0.00	0.59	1
LF3	0.00	0.88	1.0	0.23	0.88	0.8	0.66	0.70	0.3	0.00	0.67	1	-0.81	0.58	0.2	1.23	0.59	0.04
SF	0.00	0.88	1.0	4.04	0.88	**<0.001**	3.03	0.70	**<0.001**	2.57	0.67	**<0.001**	3.76	0.58	**<0.001**	0.00	0.59	1
S1	1.35	0.88	0.1	5.94	0.88	**<0.001**	-2.32	0.70	**0.001**	0.63	0.67	0.3	-1.34	0.58	0.02	0.77	0.59	0.2
S2	2.76	0.88	**0.002**	4.35	0.88	**<0.001**	-2.32	0.70	**0.001**	0.40	0.67	0.5	-1.34	0.58	0.02	0.00	0.59	1
S3	0.00	0.88	1	3.36	0.88	**<0.001**	-2.32	0.70	**0.001**	0.94	0.67	0.2	-1.34	0.58	0.02	0.00	0.59	1
S4	0.00	0.88	1	3.46	0.88	**0.005**	-2.32	0.70	**0.001**	-0.60	0.67	0.4	-1.34	0.58	0.02	0.00	0.59	1
S5+	0.99	0.88	0.30	3.34	0.88	**<0.001**	-1.55	0.70	0.03	1.54	0.67	0.02	-1.34	0.58	0.02	0.77	0.59	0.2
Interaction																		
SPG×LF1	-1.73	0.88	0.05	1.47	0.88	0.1	0.24	0.70	0.7	-1.14	0.67	0.09	-0.66	0.58	0.3	-0.53	0.59	0.4
SPG×LF2	-1.73	0.88	0.05	0.20	0.88	0.8	0.96	0.70	0.2	-2.21	0.67	**0.001**	-0.08	0.58	0.9	0.78	0.59	0.2
SPG×LF3	0.75	0.88	0.4	-0.56	0.88	0.5	-1.64	0.70	0.02	-1.21	0.67	0.07	-0.62	0.58	0.3	1.33	0.59	0.03
SPG×SF	2.90	0.88	**0.001**	-1.67	0.88	0.06	-3.37	0.70	**<0.001**	-0.29	0.67	0.7	-1.57	0.58	**0.007**	1.81	0.59	**0.002**
SPG×S1	2.00	0.88	0.02	-0.36	0.88	0.7	1.57	0.70	0.03	1.28	0.67	0.06	2.44	0.58	**<0.001**	-0.14	0.59	0.8
SPG×S2	-1.00	0.88	0.3	-0.32	0.88	0.7	1.20	0.70	0.09	1.56	0.67	0.02	0.23	0.58	0.7	-0.51	0.59	0.4
SPG×S3	-0.02	0.88	1.0	0.05	0.88	1.0	-0.34	0.70	0.6	0.11	0.67	0.9	0.23	0.58	0.7	-0.48	0.59	0.4
SPG×S4	-0.12	0.88	0.9	0.46	0.88	0.6	0.43	0.70	0.5	1.13	0.67	0.09	-0.54	0.58	0.4	-1.41	0.59	0.02
SPG×S5+	-1.06	0.88	0.2	0.73	0.88	0.4	0.95	0.70	0.2	0.79	0.67	0.2	0.57	0.58	0.3	-0.88	0.59	0.2
LR test																		
df	8			8			8			8			8			8		
Chi^2	**0.005**			0.5			**<0.001**			**0.002**			**0.003**			**0.007**		

B: regression coefficient. SE: standard error. P-values: significant values in bold. df: degree of freedom. LR: likelihood ratio test. S1: Single spores; Aggregates of 2 (S2), 3 (S3), 4 (S4), ≥5 (S5) spores. SF: Submicronic fragments, LF1: 1-2µm fragments, LF2: 2-3.5µm fragments and LF3: ≥3.5µm fragments.

PAPER II

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Submicronic Fungal Bioaerosols: High-Resolution Microscopic Characterization and Quantification

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Submicronic particles released from fungal cultures have been suggested to be additional sources of personal exposure in mold-contaminated buildings. *In vitro* generation of these particles has been studied with particle counters, eventually supplemented by autofluorescence, that recognize fragments by size and discriminate biotic from abiotic particles. However, the fungal origin of submicronic particles remains unclear. In this study, submicronic fungal particles derived from *Aspergillus fumigatus*, *A. versicolor*, and *Penicillium chrysogenum* cultures grown on agar and gypsum board were aerosolized and enumerated using field emission scanning electron microscopy (FESEM). A novel bioaerosol generator and a fungal spores source strength tester were compared at 12 and 20 liters min^{-1} airflow. The overall median numbers of aerosolized submicronic particles were 2 × 10^{5} cm^{-2}, 2.6 × 10^{3} cm^{-2}, and 0.9 × 10^{3} cm^{-2} for *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. *A. fumigatus* released significantly ($P < 0.001$) more particles than *A. versicolor* and *P. chrysogenum*. The ratios of submicronic fragments to larger particles, regardless of media type, were 1:3, 5:1, and 1:2 for *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. Spore fragments identified by the presence of rodlets amounted to 13%, 2%, and 0% of the submicronic particles released from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*, respectively. Submicronic particles with and without rodlets were also aerosolized from cultures grown on cellophane-covered media, indirectly confirming their fungal origin. Both hyphae and conidia could fragment into submicronic particles and aerosolize *in vitro*. These findings further highlight the potential contribution of fungal fragments to personal fungal exposure.

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Some saprophytic fungi can colonize building materials and other materials in indoor environments with high humidity. Such invasion is commonly associated with poor indoor air quality and health complaints among exposed people (1, 2). Inhaled fungal spores have been recognized as irritants and allergens and may elicit lung function changes, hypersensitivity pneumonitis, organic dust syndrome, and chronic bronchitis, asthma, and rhinitis (3, 4). However, estimated indoor levels of fungal spores reported in the peer-reviewed literature appear to be too low to explain adverse health effects (4).

Experiments using automatic particle counters have revealed the aerosolization of particles smaller than conidia when agar or building materials colonized by cultures of *Aspergillus versicolor*, *Trichoderma harzianum*, *Ulocladium* spp., *Penicillium melinii*, *Cladosporium cladosporioides*, *Stachybotrys chartarum*, *Botrytis cinerea*, or *Rhizopus* sp. were subjected to air jets under controlled conditions (5–15). These particles have been suggested to be fungal fragments and important sources of allergens (16–18), antigens (7), (1→3)-beta-D-glucans (19–25), and mycotoxins (26, 27). Exposure to fungal submicronic particles may therefore provide an explanation for health effects observed in moldy indoor environments (28).

Aerosolization of fine particles from mold-contaminated building materials by air jets or from cultures grown on agar media has been reported using a variety of automatic particle counters (5, 7, 8, 11, 13, 21–23). Automatic particle counters are based on particle acceleration, electric static charge pulses, or light scattering techniques to count particles as small as 6 nm in diameter. With these instruments, fungal spores could be discriminated from other particles by their limited size range. Furthermore, some instruments apply autofluorescence to separate abiotic particles from biotic particles. However, they cannot morphologically distinguish spores, spore aggregates (SA), hyphal fragments, and fragmented spores.

Alternative approaches to characterizing fungal fragments have used the detection of fungal biomarkers, including (1→3)-beta-D-glucans (10, 19, 21, 23, 25, 29), N-acetyl-glucosaminase (23), fungal antigens (7), and mycotoxins (26, 27), in the submicronic particle fractions (21, 22, 25, 30, 31). Some of these studies were confounded by imperfect size separation of the systems utilized since the presence of larger particles such as spores has been demonstrated in the assumed submicronic fraction (10, 23, 29). Furthermore, (1→3)-beta-D-glucans in environmental samples could derive from bacteria and plants, including algae (32, 33). Although detection of fungal biomarkers may provide more-direct evidence of airborne fungal fragments, it is also possible that these particles originate from the substrate as well as from the fungus (12). Moreover, fungal enzymes, including N-acetyl-glucosaminase, fungal antigens, and mycotoxins, may be released into and lead to the deterioration of the substrate (34) and therefore to the release of fragments. These limitations emphasize the

Received 26 May 2014 Accepted 5 September 2014
Published ahead of print 12 September 2014
Editor: A. A. Brakhage
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doi:10.1128/AEM.01740-14

need for an alternative approach for identification and quantification of submicronic particles (35, 36).

Large hyphal fragments have been observed in indoor air samples by microscopy following immunostaining (4, 17, 18, 36). Scanning electron microscopy (SEM) has been used to resolve and quantify fungal and actinomycete spores (38–40, 56) as well as large hyphal fragments (41). SEM has also been used to qualitatively assess submicronic particles (7, 13, 26). However, those studies did not enumerate or determine the fungal origin of the submicronic particles. Recognition of the fungal origin of these particles is essential to improve our understanding of the potential adverse health effects associated with exposure to these particles, as experimental studies have shown that fungal hyphae and spores have different toxic properties (4, 42, 43).

Therefore, the present study was initiated to characterize submicronic particles aerosolized from fungal cultures by air jets using field emission SEM (FESEM). Furthermore, submicronic particles were classified among larger fungal particles and enumerated with respect to experimental parameters, including biotic factors (species, growth medium, and culture age) and abiotic factors (generator and airflow).

MATERIALS AND METHODS

Fungal cultures. Fungal isolates evaluated in this study included *A. versicolor* (Vuillenium) Tirobaschi 1908 (strain VI 03554), *A. fumigatus* Fresenius 1863 (strain A1258 FGSC), and *P. chrysogenum* Thom 1910 (strain VI 04528). *A. versicolor* and *P. chrysogenum* were chosen because these species are common in the indoor environment (44). *A. fumigatus* was chosen because this species has been studied extensively as a causal agent of aspergilliosis. *A. versicolor* and *P. chrysogenum* strains were kindly provided by the Section of Mycology at the National Veterinary Institute of Norway. The *A. fumigatus* strain was purchased from Fungal Genetic Stock Center (University of Missouri, Kansas City, KS).

Fungal inocula were prepared by gently scraping 2-week-old cultures grown on 2% malt extract agar (MEA) medium (45) and were submerged in 20 ml sterile phosphate-buffered saline (PBS) containing 0.1% Tween 20. The spore suspensions were collected into 50-ml centrifuge tubes and resuspended by vortex mixing 2 times for 30 s each time followed by sonication for 3 min in an ultrasonic bath (Sonorex RK 510H; Bandalin Electric, Berlin, Germany) at 35 kHz. The suspension was filtered through a 10-μm-pore-size nylon mesh filter (Millipore, Tullagreen Cork, Ireland) and centrifuged (model 4k15; Sigma, Osterode, Germany) (at 1,500 × *g* for 5 min), and the pellet was resuspended in 30 ml sterile Milli-Q water containing 10% glycerol. The spore suspension was kept at 4 ± 1°C until inoculation. The concentration of spores was determined by filtration of 0.1 ml of the suspension through a 25-mm-diameter polycarbonate filter (Millipore, Tullagreen Cork, Ireland) (0.4 μm pore size) and enumeration by FESEM.

For aerosolization experiments, fungal cultures were grown on agar medium or on gypsum board discs (GB). Agar media included 2% MEA, which was chosen because it supports increased biomass production as previously described in several other reports (8, 13, 14). MEAC, i.e., MEA (2%) covered with a cellophane membrane (Visella Oy, Valkeakoski, Finland), was designed as an internal control to prevent potential aerosolization of colonized growth medium and was developed for pure fungal biomass production (46, 47). Gypsum boards (common indoor building material; Coop Bygg, Vinterbro, Norway) were cut into 80-mm-diameter discs, soaked with water for 16 h, autoclaved at 132°C for 1 h, and placed in 90-mm-diameter sterile petri dishes (10). MEA dishes were inoculated with 0.1 ml of the spore suspension containing approximately 1×10^6 spores and gypsum boards with 1 ml of the same inoculum. All inoculated petri dishes were sealed with parafilm and incubated at 25 ± 1°C and

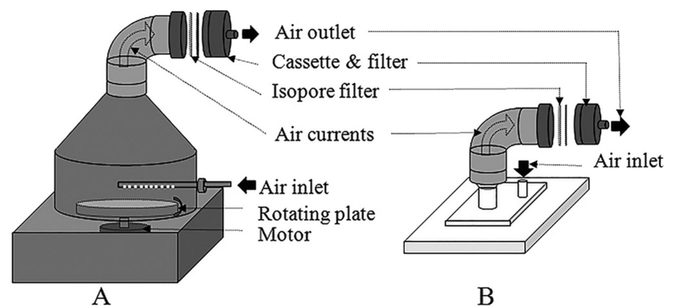

FIG 1 Instruments used for aerosolization and collection of particles from fungal cultures. (A) Stami particle generator (SPG). (B) Fungal spores source strength tester (FSSST).

45% ± 5% relative humidity (RH). MEA and MEAC were incubated for 2 and 8 weeks and GB for 8 weeks.

Instrumentation for generation of fungal particles. Two particle generators were evaluated in this study. The fungal spore source strength tester (FSSST) is a portable bioaerosol generator developed at the University of Cincinnati (48) and has been previously described elsewhere (10, 11, 21, 27). The second generator is a novel generator fabricated in-house termed the Stami particle generator (SPG) that included design features from the generators described by Kildesø et al. (5), Scheermeyer and Agranovski (12), and Lee et al. (13).

The SPG (Fig. 1A) was fabricated at the National Institute of Occupational Health, Norway (NIOH), out of aluminum with smooth joints in order to minimize particle loss from static charging and turbulent flow. The internal cross-sectional area is 156 cm^2. Particles were aerosolized by air jets from a tube outfitted with 10 nozzles of 1.2 mm diameter mounted 16 mm above the culture surface. Air jets were directed perpendicularly to the culture plates. During aerosolization, culture plates were rotated by means of a time-regulated direct current (DC) motor with angular velocity adjustable from 0.05 to 2 rpm. The aerosol was directed through a 90° stainless steel bend onto an in-line 37-mm-diameter polycarbonate filter (Isopore; Millipore, Ireland) with 0.4 μm pore size in an open-face standard aerosol cassette made out of conductive polypropylene (SKC Inc., Eighty Four, PA).

The FSSST (Fig. 1B) is constructed out of polyvinyl chloride with a square internal area of 144 cm^2. Particles were liberated by air jets through 112 orifices of 0.4 mm diameter situated at a distance of 16 mm above and directed perpendicularly to the culture surface. Released particles were aerosolized onto a polycarbonate filter as described above.

The experimental setup is shown in Fig. 2. Airflow was provided by a rotary vane pump (model G24/27; Gardner Denver Thomas, Bandhagen, Sweden) and controlled by a computer system developed at NIOH. Inlet and outlet air was filtered using HEPA filtration (Hepa Versapor capsules; Pall Corporation, Port Washington, NY), and flow rates were monitored by two mass flow meters (822 Top-Trak; Sierra Instruments, Monterey, CA) mounted before and after the generator. Electrostatic charging was reduced by an in-line alpha ionizer (model P-2021; NRD LLC, Grand Island, NY). Air temperature and RH were monitored in real time using a Picolog 1261 sensor (model AV095/086; Picotech, Tyler, TX). The RH was maintained at 18.8 ± 0.4% using a silica gel in-line dryer (model L144; Air Sentry, Rockwall, TX).

Aerosolization and collection of fungal particles. Experiments were conducted on 2- and 8-week-old cultures grown on agar media (MEA and MEAC). In contrast, only 8-week-old GB cultures were evaluated as fungal growth was not observed on GB after 2 weeks. Flow rates of 12 and 20 liters min^{-1} were applied to each set of culture plates. The tested airflows at the orifices were theoretically equivalent to air velocities of 18 and 29 m s^{-1} in the SPG and 14 and 23 m s^{-1} in the FSSST, respectively. Particles were aerosolized for 120 s at 0.5 rpm from *A. versicolor* and *P. chrysogenum* cultures. To avoid filter overloading in *A. fumigatus* experiments, the

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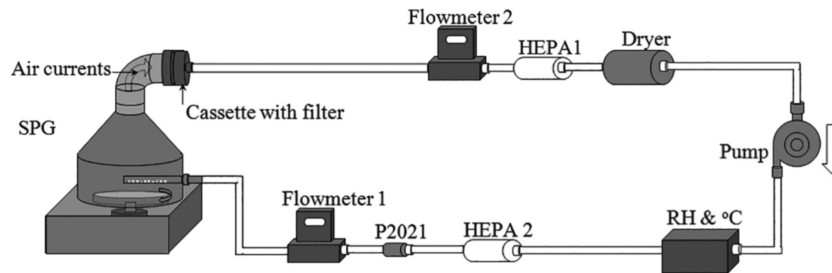

FIG 2 Experimental setup. The generator (SPG) with mounted cassette is connected to the system setup. Flowmeters 1 and 2 measure the airflows at the inlet and outlet of the generator, respectively. HEPA 1 and HEPA 2 filter the airflows from the outlet and to the inlet, respectively. The dryer (in-line tube containing silica gel) maintains a constant relative humidity in the air at the inlet, and the in-line P2021 ionizer reduces electrostatic charges.

aerosolized culture surface was reduced by the use of a cover plate with a central hole of 1 cm diameter, and cultures were aerosolized for 60 s at 1 rpm. The FSSST was operated without rotation of the culture plates. In both generators, culture plates were placed 16 mm below the orifices. All experiments were conducted in a Bio Safe Grade II laminar flow hood (Scanlaf Mars Labogene, Lynge, Denmark). The generator chamber was cleaned with 70% ethanol and purged with HEPA-filtered air prior to each experiment. A total of 16 blank experiments were performed at 12 and 20 liters min^{-1} in an empty chamber during 120 s. The blank experiments were designed to correct for background particles prior to aerosolization experiments.

Sample preparation and analysis by high-resolution FESEM. A quarter-segment (ca. 2.4 cm^2) of the filter specimen was cut from the collection filter and mounted on a 25-mm-diameter aluminum pin stub (Agar Scientific Ltd., Stansted Essex, United Kingdom) using double-sided carbon adhesive discs (Ted Pella Inc., Redding, CA) in a sterile laminar airflow cabinet. Samples were coated with platinum during 20 to 30 s with 40 mA current in a 4×10^{-1} mbar vacuum using a Balzers SDC 050 sputter coater (Balzers, Liechtenstein). The coating time corresponds to a 6-to-10-nm-thick platinum layer according to the calibration curves provided by the manufacturer.

Samples were viewed using a SU 6600 FESEM (Hitachi, Ibaraki-ken, Japan) in the secondary electron imaging (SEI) mode. For the enumeration, the microscope was operated at an acceleration voltage of 15 keV, an extraction voltage of 1.8 kV, and a working distance of 10 mm. Particles were quantified using the counting criteria described by Eduard and Aalen (37). Particles were assumed to be homogenously distributed on the filter, as that has been previously demonstrated for fungal spores collected in electrically conducting filter holders (49).

Particles were recognized by their morphological features as fragments, spores, and spore aggregates, sized by length, and further classified as submicronic particles (0.2 to 1 μm diameter) and larger particles (>1 μm diameter). Four hundred particles or a maximum of 100 fields were counted at ×3,000 to ×10,000 magnification depending on the particle density on the filter and type of particle. The number of particles per culture area (cm^2) was calculated by dividing the total number of particles on the filter by the exposed culture area. The minimal detectable particle numbers were 8×10^3 and 9×10^4 particles per exposed filter at ×3,000 magnification and ×10,000 magnification, respectively.

The outer cell wall rodlet layer is characteristic of hydrophobins found on the fungal conidial surface (50, 51) and was used to confirm the fungal spore origin of submicronic particles. For classification of particles originating from hyphal biomass, the morphology of freeze-dried mycelial biomass was used for comparison. Morphological classification of 100 randomly selected submicronic particles was conducted at high magnification (×100,000 to ×300,000), an acceleration voltage of 25 keV, and a working distance of 5 to 6 mm.

Experimental design and data analysis. For each fungal species, comparative experiments were run on 2- and 8-week cultures grown on MEA and MEAC and on 8-week cultures grown on GB. The two generators and two airflows were compared in a total of 178 aerosolization experiments. Each combination of these factors was run in triplicate. Particle counts were adjusted for blanks and are described by medians and 25th and 75th percentiles because the data were not normally distributed. Negative values after blank adjustment were assigned a value of zero. Blank counts for submicronic fragments (SF) ranged from 160 to 4,300 particles per cm^2. The effects of experimental factors, including generator, flow, and culture age, on the number of submicronic fragments were compared using the nonparametric Wilcoxon-rank sum test (Mann-Whitney U tests). The effects of species and media were tested by the Kruskal-Wallis test for multiple categories followed by *post hoc* Wilcoxon rank sum tests. The biotic factors (medium and culture age) and abiotic factors (generator and airflow) were individually compared because of the factorial design. A two-sided P value of 0.05 was regarded as statistically significant. For multiple *post hoc* comparisons, the P value of 0.05 was Bonferroni corrected (significance level for 3 comparisons = 0.017). STATA SE 12 (Statacorp LP, College Station, TX) was used for statistical analysis.

RESULTS

Morphology of fungal particles. Figure 3 shows the particle types aerosolized from fungal cultures, including single spores and spore aggregates (Fig. 3A, B, and C), a submicronic particle (Fig. 3A, arrow a), and a larger particle (Fig. 3D, arrow b). Disrupted spores were observed in some of the experiments, indicating that abiotic forces on spores could have been large enough to shear conidia into smaller fragments during aerosolization (Fig. 3D). A spore with typical rodlet structure is shown in Fig. 3E. Submicronic particles with rodlet structures were observed and regarded as being specific for spore fragments (Fig. 4A and B). Fragments without rodlet structures (Fig. 4C, D, and E) may have derived from hyphae, to which they were morphologically similar, or they may have derived from the substrate, as no fungal phenotypic features were observed.

Aerosolized spores and fragments. Collected spores and fragments were identified and classified into four groups: single spores (SS), spore aggregates (SA), larger fragments (>1 μm) (LF), and submicronic fragments (0.2 to 1 μm) (SF). The overall numbers of all particle types with respect to species are summarized in Table 1. The median number of particles per cm^2 ranged between 0.93×10^3 cm^{-2} and 2.0×10^5 cm^{-2} for SF; 0 and 42×10^3 cm^{-2} for LF; 0 and 330×10^3 cm^{-2} for SS; and 0.094×10^3 cm^{-2} and 200×10^3 cm^{-2} for SA. Significant differences between species were observed for all particle types ($P < 0.001$), showing that these particles were aerosolized in much higher numbers (median, 42×10^3 to 200×10^3 cm^{-2}) from *A. fumigatus* cultures than from *A. versicolor* cultures (median, 0 to 2.6×10^3 cm^{-2}) and *P. chrysogenum* cultures (median, 0 to 1.2×10^3 cm^{-2}). Significantly more

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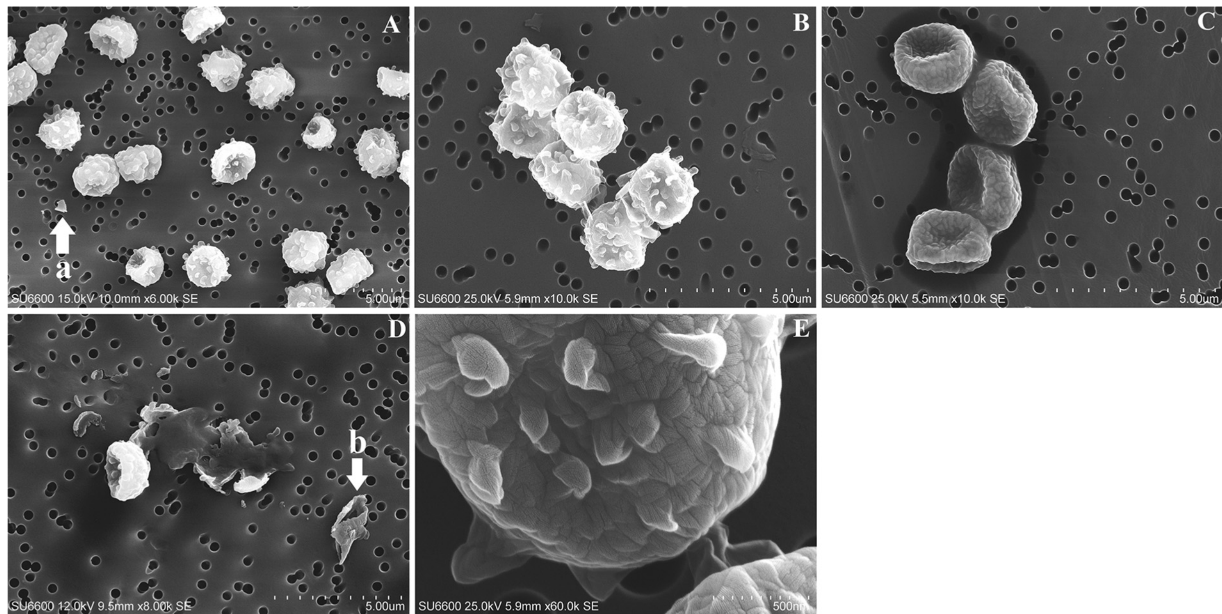

FIG 3 Micrographs of aerosolized particles. (A) Warted to spiny spores from *A. fumigatus* (8-week-old cultures grown on GB and aerosolized by the use of the FSSST at 12 liters min^{-1}). (B) Spiny spores from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). (C) Rugose spores from *P. chrysogenum* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). (D) Shattered spores of *A. fumigatus* (8-week-old cultures grown on GB and aerosolized by the use of the FSSST at 12 liters min^{-1}). (E) Spore with rodlet structure from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}). Arrows shows submicronic (a) and larger (b) particles on the filter membrane. SE, secondary electron.

single spores and spore aggregates were aerosolized from *P. chrysogenum* than from *A. versicolor* ($P < 0.001$). Due to the major differences observed between species, further analyses were performed separately for each species.

Effects of biotic parameters on the emission of submicronic particles. The effects of culture media and age are summarized in Table 2. These experiments revealed that the number of SF collected from cultures grown on MEA and MEAC were not statistically different when cultures of the three isolates were 2 weeks old. However, significant differences were revealed for older (8-week) cultures of *A. fumigatus* and *A. versicolor* (P values, <0.01 and <0.001, respectively). For *A. fumigatus*, the number of SF generated from MEAC (median, 570×10^3 cm^{-2}) was significantly greater than the number generated from MEA (median, 1.2×10^3 cm^{-2}) ($P < 0.01$). The difference between GB results (median, 21×10^3 cm^{-2}) and MEAC results was also significant ($P < 0.01$),

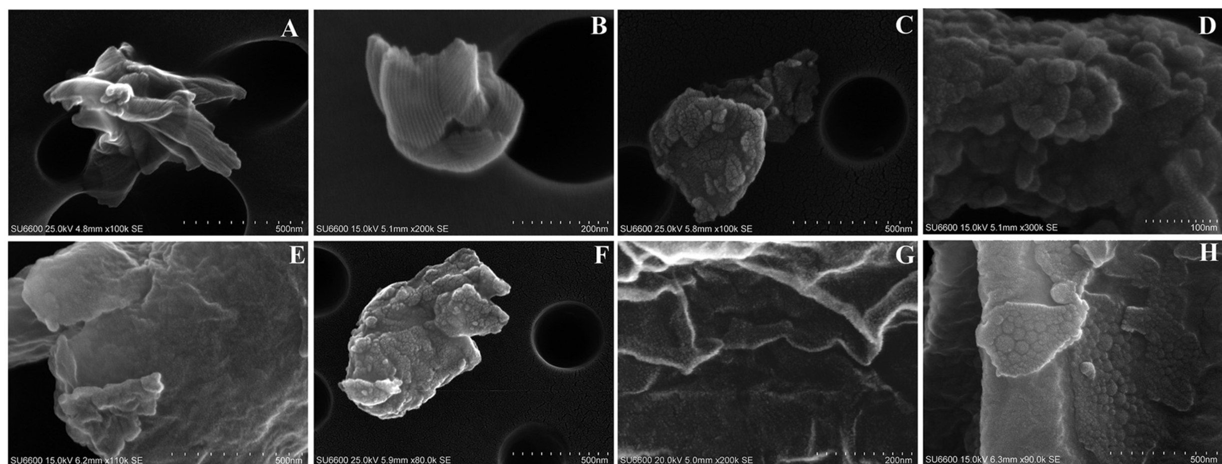

FIG 4 (A to E) Surface structures of submicronic fragments with rodlets from *A. versicolor* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 12 liters min^{-1}) (A) and *A. fumigatus* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 20 liters min^{-1}) (B) and surface structures of submicronic fragments without rodlets from *A. versicolor* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}) (C), *A. fumigatus* (2-week-old cultures grown on MEA and aerosolized by the use of the SPG at 12 liters min^{-1}) (D), and *P. chrysogenum* (2-week-old cultures grown on MEAC and aerosolized by the use of the SPG at 12 liters min^{-1}) (E). (F to H) Control micrographs of freeze-dried hyphal fragments without rodlets from *A. versicolor* (F), *A. fumigatus* (G), and *P. chrysogenum* (H).

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TABLE 1 Particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum* cultures differentiated by particle type[a]

		Submicronic fragments			Large fragments			Single spores			Spore aggregates		
Species	*n*	Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value	Median no.	25th and 75th percentiles	K-W test *P* value
A. fumigatus	59	200[ab]	7.4 and 410	<0.001	42[cd]	0.00 and 85	<0.001	330[ef]	21 and 1,200	<0.001	200[hi]	11 and 640	<0.001
A. versicolor	60	2.6[a]	0.60 and 5.0		0.40[c]	0.00 and 1.0		0.00[eg]	0.00 and 0.3		0.090[hj]	0.00 and 0.50	
P. chrysogenum	59	0.93[b]	0.00 and 3.0		0.00[d]	0.00 and 0.90		0.96[fg]	0.00 and 5.5		1.2[ij]	0.10 and 11	

[a] Data represent 10^3 particles cm^{-2}. Results represent combined data from experiments performed under different media, culture age, airflow, and generator conditions. n = number of repeated experiments grouped by fungal species; K-W, Kruskal-Wallis. Superscript roman letters a to j indicate the results of *post hoc* Wilcoxon rank sum tests of differences between species: medians with same letter are significantly different ($P \leq 0.001$).

but the difference between GB and MEA results was not. For *A. versicolor*, the highest SF numbers were released from MEA and GB (medians, 5.3×10^3 cm^{-2} and 4.7×10^3 cm^{-2}, respectively) compared to MEAC (0.74×10^3 cm^{-2}). Comparisons between MEAC and MEA and between MEAC and GB revealed significant differences ($P \leq 0.001$).

The effects of culture age were statistically significant for *A. versicolor* grown on MEA (median, 1.3×10^3 cm^{-2} for 2 weeks of growth versus 5.3×10^3 cm^{-2} for 8 weeks of growth; $P < 0.05$) and for *P. chrysogenum* (median, 0.5×10^3 cm^{-2} for 2 weeks of growth versus 2.8×10^3 cm^{-2} for 8 weeks of growth; $P < 0.05$). For *A. fumigatus* grown on MEAC, significantly higher numbers of SF were obtained from 8-week-old cultures (median, 570×10^3 cm^{-2}) than from 2-week-old cultures (130×10^3 cm^{-2}) ($P < 0.01$).

Effects of abiotic factors on the emission of submicronic particles. The effects of generator and airflow are shown in Table 3. No significantly different numbers of SF aerosolized from *Aspergillus* cultures were observed. The *P. chrysogenum* cultures were significantly affected by the generator and airflows used. Higher numbers of SF were released in the FSSST (median, 1.8×10^3 cm^{-2}) than from the SPG (median, 0.1×10^3 cm^{-2}) at 12 liters min^{-1} ($P = 0.01$). At 20 liters min^{-1}, the opposite trend was observed, with higher numbers of SF from SPG (median, 2.1×10^3 cm^{-2}) than from FSSST (median, 0.0 cm^{-2}) ($P < 0.01$). Increased airflow generated more SF in the SPG (median, 0.1×10^3 cm^{-2} at 12 liters min^{-1} versus 2.1×10^3 cm^{-2} at 20 liters min^{-1}) ($P < 0.01$), while fewer SF were observed in the FSSST (median, 1.8×10^3 cm^{-2} at 12 liters min^{-1} versus 0.0×10^3 cm^{-2} at 20 liters min^{-1}) ($P = 0.001$).

Origin of submicronic particles. High-resolution FESEM of SF revealed that most submicronic particles had a surface structure similar to that of the vegetative biomass (Fig. 4F, G, and H). Thirteen percent of the SF released from *A. fumigatus* showed rodlet structure, confirming a conidial origin (Fig. 4B). This proportion was only 2% for *A. versicolor* cultures, while no submicronic fragments with rodlet structure were observed for *P. chrysogenum*.

DISCUSSION

The detection of aerosolized fragments that contained ornamentation of conidium walls provides direct evidence of the presence of fungal fragments among the submicronic particles. The results further suggest that submicronic particles mainly originate from the hyphal biomass and not from the substrate. These data provide new insights into the aerosolization of fungal bioaerosols, as studies using automatic particle counters or detection of biomarkers could not reveal the nature of submicronic particles. Our findings additionally confirm assumptions of researchers in previous studies that submicronic particles released from fungal cultures originate from the fungal biomass and not from the inoculated substrate (5–8, 11, 13–15, 21, 23, 26), but we cannot exclude the possibility that the colonized medium also contributes, as suggested by Scheermeyer and Agranovski (12).

A number of studies have suggested that submicronic particles originate from conidial and hyphal fragmentation. Madsen and coworkers hypothesized that these particles were derived from mycelial autolysis (23), while Kanaani and colleagues associated their origin with spore fragmentation (11). The observation of submicronic particles with rodlet structures indicates that some of

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TABLE 2 Effects of culture media and age on submicronic particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum*[a]

Biotic factors	*A. fumigatus* n	Median	25th and 75th percentiles	K-W test P value	*A. versicolor* n	Median	25th and 75th percentiles	K-W test P value	*P. chrysogenum* n	Median	25th and 75th percentiles	K-W test P value
Age and medium												
2 wks												
MEA	12	270	130 and 450	0.2	12	1.3	0.0 and 4.3	0.6	11	0.50	0.01 and 1.20	0.5
MEAC	11	130	81 and 240		12	2.5	0.8 and 3.4		12	0.097	0.00 and 0.80	
8 wks												
GB	12	21[b]	0.40 and 380	**0.003**	12	4.7[d]	2.5 and 8.7	**0.001**	12	2.8	1.1 and 11	0.3
MEA	12	1.2[a]	0.02 and 320		12	5.3[c]	3.0 and 8.1		11	2.8	0.80 and 4.7	
MEAC	12	570[ab]	260 and 1,500		12	0.74[cd]	0.06 and 2.0		12	0.57	0.00 and 86	
Medium and age												
MEA												
2 wks	12	270	130 and 450	0.06	12	1.3	0.0 and 4.3	**0.03**	12	0.5	0.014 and 1.2	**0.01**
8 wks	12	1.2	0.02 and 320		12	5.3	3.0 and 8.1		11	2.8	0.9 and 4.7	
MEAC												
2 wks	11	130	81 and 240	**0.003**	12	2.5	0.8 and 3.4	0.07	12	0.097	0.0 and 0.8	0.5
8 wks	12	570	260 and 1,500		12	1.7	0.06 and 2.0		12	0.6	0.0 and 86	

[a] Data represent 10^3 particles cm^{-2}. n = number of repeated experiments; K-W, Kruskal-Wallis. P values representing significant differences are indicated in bold. Superscript roman letters a and b indicate the results of *post hoc* Wilcoxon rank sum tests for differences between media performed with *A. fumigatus*; medians with same letter are significantly different ($P \leq 0.01$). Superscript roman letters c and d indicate the results of *post hoc* Wilcoxon rank sum tests for differences between media performed with *A. fumigatus*; medians with same letter are significantly different ($P \leq 0.001$).

these particles originate from conidia. Rodlet structures have been reported to be specific to the surface of reproductive structures, especially asexual conidia (52, 53). Rodlets were observed on the spore surface of the three tested isolates. However, only a minor proportion of the submicronic particles contained rodlet structures. It is possible that the orientation of particles on the filter can obscure the outer spore wall surface with rodlets and that such a condition could lead to underestimation of submicronic fragment numbers from spores. These findings further our understanding of conidium fragmentation associated with *in vitro* experiments of particle generation from fungal cultures by air jets. The strong air currents in the generator may cause particles to impact on internal surfaces and induce shear forces that may lead to particle fragmentation. Conidium fragmentation, visualized as submicronic fragments with rodlet structures, was observed with *Aspergillus* isolates and not with *P. chrysogenum*, indicating that conidium fragmentation was not common with the tested isolate. The warted and spiny outer-wall morphology of spores derived from the *A. fumigatus* and *A. versicolor* isolates may result in easier shearing into submicronic fragments than the smooth spore characteristics associated with *P. chrysogenum* isolates. It is not clear, however, whether spore fragmentation also occurs under environmental conditions or whether this is an artifact of extreme experimental conditions. The absence of submicronic fragments with a rodlet structure from *P. chrysogenum* seems in contradiction with the results reported by Kanaani and coworkers (11), who reported that most spore fragmentation occurred with *Penicillium* species. Since those authors did not specify the *Penicillium* species,

TABLE 3 Effects of generator and airflow on submicronic particles generated from *A. fumigatus*, *A. versicolor*, and *P. chrysogenum* cultures[a]

Abiotic factors	*A. fumigatus* n	Median	25th and 75th percentiles	K-W test P value	*A. versicolor* n	Median	25th and 75th percentiles	K-W test P value	*P. chrysogenum* n	Median	25th and 75th percentiles	K-W test P value
Airflow and generator												
12 liters/min												
FSSST	15	126	0.0 and 380	0.06	15	2.6	1.6 and 5.7	0.8	15	1.8	0.9 and 8.7	**0.01**
SPG	15	340	130 and 510		15	2.5	1.3 and 5.4		15	0.097	0.0 and 1.8	
20 liters/min												
FSSST	15	81	9.8 and 340	0.8	15	0.3	0.0 and 4.0	0.1	14	0.0	0.0 and 1.1	**0.002**
SPG	14	174	0.9 and 340		15	2.9	0.9 and 6.4		15	2.1	0.9 and 4.4	
Generator and airflow												
FSSST												
12 liters/min	15	126	0.0 and 380	0.1	15	2.6	1.6 and 5.7	0.8	15	1.8	0.9 and 8.7	**0.001**
20 liters/min	15	81	9.8 and 340		15	0.3	0.0 and 4.0		14	0.0	0.0 and 1.1	
SPG												
12 liters/min	15	340	130 and 510	1	15	2.5	1.3 and 5.4	0.1	15	0.097	0.0 and 1.8	**0.004**
20 liters/min	14	174	0.9 and 340		15	2.9	0.9 and 6.4		15	2.1	0.9 and 4.4	

[a] Data represent 10^3 particles cm^{-2}. n = number of repeated experiments; K-W, Kruskal-Wallis. P values representing significant differences are indicated in bold.

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we can only speculate that they used a strain that produces conidia with protrusions, as many species within this genus have spores with surface ornamentation (54). Furthermore, it seems unlikely that those authors were able to discriminate between fragments from spores and those from hyphae using automatic particle counting based on aerodynamic size and autofluorescence measurements.

The main proportion of submicronic particles observed in the present study had surface structures similar to those of the vegetative mycelial biomass (hyphae). However, the morphology of hyphal fragments lacked characteristic features suitable for discriminating them from possible fragments from the growth medium. In order to prevent particle aerosolization from the nutrient medium, we studied the aerosolization of submicronic particles from cultures grown on cellophane-covered medium (MEAC). It has been reported that cellophane membranes are permeable only to molecules 90 kDa in diameter or smaller (55), and this cover has been shown to better separate the mycelial biomass from the substrate (46, 47, 57). It is therefore likely that submicronic particles liberated from MEAC are of fungal origin. The observation of similar numbers of submicronic particles aerosolized from 2-week-old MEA and MEAC cultures further suggests that the submicronic particles aerosolized from young cultures grown on MEA were mainly derived from hyphal structures and not from agar (Table 2). This comparison assumes that the characteristics of mycelial growth on MEA and MEAC are similar.

Significantly higher numbers of submicronic fragments were released from 8-week-old cultures of *A. versicolor* grown on MEA and GB than from those grown on MEAC. Similar results were observed for *P. chrysogenum*, although the differences were not significant. This may indicate that some of the submicronic particles were released from the media, as they were morphologically indiscernible. The release of substrate fragments has been suggested by Scheermeyer and Agranovski (12), who ascribed this to desiccation and weakening of the MEA substrate surface during prolonged incubation times. However, this hypothesis is not supported by our results for *A. fumigatus*. Surprisingly, the highest numbers of submicronic particles from *A. fumigatus* were from cellophane-covered MEA rather than from uncovered MEA or GB.

We observed highly significant differences between the three isolates when data from all experiments were combined. The highest numbers were obtained from *A. fumigatus* (median, 200×10^3 cm^{-2}), while *A. versicolor* and *P. chrysogenum* released fewer fragments (median, 2.6×10^3 cm^{-2} and 0.9×10^3 cm^{-2}, respectively). Our results are difficult to compare to those of previous studies due to differences between criteria for fungal fragments and the use of partly different species, different strains, and different aerosolization methods. However, relative differences observed between species within studies are more reliable. We can then compare our results obtained with *A. versicolor* and *P. chrysogenum* to results from the studies by Kildesø et al. (5) and Górny and Ławniczek-Wałczyk (14), who found that *A. versicolor* released more small particles than *P. chrysogenum*, which is qualitatively similar to our findings (7, 11, 14).

Significantly higher numbers of submicronic particles were obtained from older cultures of all isolates. As for the *Aspergillus* species, these differences (obtained on MEAC for *A. fumigatus* and on MEA for *A. versicolor*) have been attributed to desiccation stress (12) and mycelial autolysis (23). This effect was also observed with *P. chrysogenum* cultures grown on MEA and can be explained likewise (58). In addition, the presence of liquid exudate on 2-week-old cultures is a characteristic of this species and may further reduce the emission of fungal fragments (54, 59). In contrast, the abiotic variables, including the type of generator and airflow, showed no significant effect on the aerosolized number of submicronic particles from the *Aspergillus* species. With *P. chrysogenum*, we found significantly more SF with the FSSST at the lowest flow rate, while with the SPG, SF counts were highest at the highest flow rate. Furthermore, at 12 liters min^{-1}, the number of SF was higher with the FSSST than with the SPG, but at 20 liters min^{-1}, the opposite trend was observed. Differences in jet dimensions, laminar or turbulent airflows, and electrostatic properties of the construction material of the generators combined with the structural characteristics of fungal cultures may all play important roles in the aerosolization of SF as suggested by Kanaani et al. (11) and Górny and Ławniczek-Wałczyk (14).

An important aspect for evaluation of indoor fungal exposure is the ratio of submicronic particles to other particles, including spores and larger fragments, which has been reported in several studies. These values were approximately 1:3 for *A. fumigatus*, 5:1 for *A. versicolor*, and 1:2 for *P. chrysogenum* in the present study and are within the range of ratios reported in previous studies (7, 8, 11, 14). High fragment-to-spore ratios are of concern because the exposure to fungal agents is underestimated unless fragments are quantified, which currently is possible only by the detection of biomarkers in the submicronic aerosol fraction. Although these biomarkers may present health risks of their own, they document only indirectly the occurrence of submicronic fragments and not their enumeration.

The fact that submicronic fungal fragments are generated shows that an additional burden of respirable particles can be produced which may contribute to personal exposure to fungi but which is overlooked using current methods of exposure assessment.

This report demonstrates the contribution of spore wall fragments (containing the rodlet layer) to personal fungal exposure, which to our knowledge had not been previously identified. The observation that most fungal fragments originate from the hyphae may also have implications for health risks associated with fungal exposure. Hyphae have been shown to induce allergic inflammation in experimental studies, whereas spores induce mainly nonspecific inflammation (4).

Conclusions. The FESEM enumeration of similar numbers of submicronic fragments from 2-week-old cultures grown on MEA and MEAC indicated that these fragments were of hyphal or conidial origin and were not from the agar. Conidial fragments were observed in experiments performed with *A. fumigatus* and *A. versicolor* but not in those performed with *P. chrysogenum*. It is not clear whether conidial fragmentation is a natural process. Although the fungal origin of submicronic particles generated from fungal cultures was demonstrated under controlled conditions in this study, it still remains unclear whether submicronic fungal fragments occur in sufficient numbers in the indoor environment to represent an additional fungal burden following personal exposure. Future studies should be designed to confirm and enumerate the presence of submicronic fungal fragments in environmental samples.

ACKNOWLEDGMENTS

This work was financially supported by the Norwegian Research Council (grant NFR196130/H10).

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The technical support provided by the Norwegian Veterinary Institute is gratefully acknowledged.

The findings and the conclusions in this report are ours and do not necessarily represent the views of the National Institute for Occupational Safety and Health.

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PAPER III

1 ***Indirect immunodetection and enumeration of fungal fragments by field emission scanning***
2 ***electron microscopy***

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16

Abstract

Submicronic fungal fragments have been observed in *in vitro* aerosolization experiments. The occurrence of these particles has therefore been suggested to contribute to respiratory health problems observed in mold contaminated indoor environments. However, the role of submicronic fragments in exacerbating adverse health effects has remained unclear due to limitations associated with detection methods. In the present study, we report the development of an indirect immunodetection assay that utilizes chicken polyclonal antibodies developed against spores from *Aspergillus versicolor* and high resolution field emission scanning electron microscopy (FESEM). Immunolabeling was performed with *A. versicolor* fragments immobilized and fixed onto poly-L-lysine coated polycarbonate filter. Eighty four percents of submicronic fragments compared to 100% of larger fragments generated from pure freeze dried mycelial fragments of *A. versicolor* were positively labeled. In proof of concept experiments, air samples collected from moldy indoor environments were evaluated using the immunolabeling technique. Fungal fragments including submicronic fragments were definitely labeled with gold. Overall, 13% of total collected particles were derived from fungi. This fraction comprises 79% and 21% fragments and spore particles, respectively. The methods reported in this study, enable the enumeration of fungal particles including submicronic fragments in a complex heterogeneous environmental sample.

Keyword: Immunodetection, immunogold labeling, fungal aerosols, Scanning electron microscope

Introduction

Personal exposure to fungal aerosols in damp buildings has been associated with respiratory morbidity (1, 2). Fungal spore exposure levels in residential indoor environments seem too low to explain such an association (3). Submicronic fungal fragments observed *in vitro* during aerosolization experiments with fungal cultures is believed to contribute to the respiratory health problems observed in moldy indoor environments (4, 5). However, this role of submicronic fragments has remained unclear due to limitations associated with their quantification.

Airborne fungal particles have been shown to include spores in addition to larger and smaller (submicronic) fragments of spores and hyphae. These fragments may constitute a significant reservoir for antigens, allergens and toxins in addition to spores. To date, the quantification of submicronic fungal fragments has remained technically challenging in environmental samples due to the lack of adequate detection and enumeration methods (6, 7). In this regard, the burden of fungal submicronic fragments in fungal contaminated environments has remained limited. *In vitro* studies that have evaluated the release of submicronic fragments have provided insight into the aerodynamic characteristics as well as the abiotic factors that influence the release of these particles. These laboratory studies of common indoor fungal isolates have shown the need to include the enumeration of submicronic fragments, in addition to spores and larger fragments during exposure assessment of mold-contaminated environments (5, 8, 9).

Methodological advances have been made in a number of studies by using fungal membrane constituents such as ergosterol, phospholipid fatty acids and (1→3)-β-D-glucans to demonstrate the presence of fungal biomass in size fractionated fungal aerosols (9–16). Further, sugar alcohols (arabitol and mannitol) (17), enzymes (N-acetyl hexosaminidase and N-acetyl-D-glucosaminidase) (18–22), antigens, allergens (23–26) and DNA (27–29) have been used as

63 proxies for total fungal exposure or occurrence of airborne fungal particles. However, none of
64 these detection approaches enabled the detection and enumeration of fungal particles in the
65 submicrometer size range. Quantifying particles in this size range will provide the more accurate
66 assessment of fungal exposure due to the toxicological properties of very fine particles ($<2.5\mu m$).
67 In this regard, the toxicological properties of allergen containing fine particles has been shown to
68 be more strongly correlated to their number and overall surface area than to their mass (30).

69 The immunostaining of allergens and surface antigens for microscopic visualization has
70 enabled detection and quantification of large fungal particles ($>1\mu m$) including spores and
71 fragments (23, 31–33). However, the detection of submicronic fragments by this technique has
72 not been possible due the methodological limitations associated with microscopic resolution. The
73 adaptation of this technique for scanning electron microscopy (SEM) has contributed to an
74 improved microscopic resolution and has enabled the detection of immune labeled particles in the
75 submicrometer size (34, 35).

76 In the present study, we describe a novel indirect immunostaining technique that utilizes
77 FESEM to resolve and identify fungal fragments in the submicrometer size range. This method
78 was further tested in proof of principle experiment with indoor air samples from a mold
79 contaminated school building.

80

81 **Materials and Methods**

82 *Preparation of fungal material for immunization*

83 An isolate of *Aspergillus versicolor* (VI03554) was provided by the Section of Mycology,
84 Norwegian Veterinary Institute. *A. versicolor* was selected because this species is a common
85 contaminant of water infiltrated building materials in indoor environments (36). The frozen
86 isolate stock was revitalized on 2% malt extract agar (MEA) and allowed to grow for 14 days at

87 25°C. Conidia were collected by submerging the cultures in phosphate buffer saline (pH 7.4;
88 Sigma Aldrich GmbH, Schnelldorf, Germany) containing 0.05% (v/v) Tween 20 (PBST) for 5
89 min. Spores were then gently scraped into the buffer solution. To dissociate aggregates, the
90 conidia suspension was vortexed for 30s followed by sonication (Sonorex RK 510H; Bandalin
91 Electric, Berlin, Germany) at 35 kHz for 5 min. After filtration through a 10 µm mesh using
92 steriflip (Millipore), the filtrate was washed three times in PBS by centrifugation at 4100×*g*. The
93 washing step was introduced to remove soluble antigens on the conidial surface.

94

95 *Production of polyclonal chicken antibodies (pIgY)*

96 A suspension of *A. versicolor* suspension containing approximately 10^7 mL^{-1} conidia was
97 sent to Norwegian Antibodies (Kroer, Aas, Norway) for the production of custom polyclonal IgY
98 (pIgY). Briefly, Lemon race hens (Oraug Killing Oppdrett, Askim Norway) were immunized five
99 times with *A. versicolor* conidia between the ages 11 to 30 weeks old. The eggs were collected
100 and pIgY antibodies were extracted from egg yolks and purified using ammonium sulfate ($(NH_4)_2$
101 SO_4) precipitation and centrifugation according to the manufacturer's method (37). The final
102 pIgY concentration was 12 mg mL^{-1} in PBS (pH 7.4) containing 0.02% NaN_3 to restrict bacterial
103 or fungal contaminants. The pIgY was then used in subsequent development of the
104 immunolabeling assay. The pIgY was not affinity purified.

105

106 *Poly-L-lysine coating of polycarbonate filters*

107 Each 37 mm in diameter and 0.4 µm pore size polycarbonate filter of (Millipore, Tullagreen
108 Cork, Ireland) was placed in a 90 mm Ø petri dish and covered with 1 mL poly-L-lysine solution
109 (0.01%; 70-150,000 MW; Sigma Aldrich) for 15 min in a laminar flow hood at room temperature
110 (RT). The remaining poly-L-lysine solution was removed and the filter air dried (38). The poly-

111 L-lysine coated filters were then mounted in standard aerosol cassettes (SKC inc., Eighty Four,
112 PA) prior to sampling. For sampling, particles were vacuumed onto the coated face of the filter.

113 *Preparation of a positive control aerosol sample for immunostaining*

114 *A. versicolor* hyphal fragments (AVM) were prepared and used as a positive control for
115 pIgY-immunodetection experiments. Briefly, 4-5 day old cultures were grown on a cellophane
116 membrane covered on MEA (39). The samples were harvested by scraping the mycelial mass into
117 a 15 ml polypropylene centrifuge tube. The biomass was freeze dried using a Drywinner 1.0-6.0
118 (Heto, Denmark) connected to a RZ2 vacuum pump (Vacuubrand Gmbh, Wertheim, Germany)
119 for 22 hours. The dried biomass was then ground in a Retch MM 301 mixer mill (Retsch Gmbh,
120 Haan Germany) and the resulting mycelial powder was vacuumed through a high flow respirable-
121 thoracic cyclone (GK2.69 Cyclone, BGI Waltham MA, USA) at 4.2 $Lmin^{-1}$ onto the poly-L-
122 lysine coated polycarbonate filters.

123

124 *Preparation of a negative control aerosol samples for immunostaining*

125 Wood dust (pine dust: PD) was prepared from an artificially dried, planed and heat treated
126 *Pinus sylvestris* wood sample without visible staining (Bergene Holm AS, Kirkenær, Norway).
127 The wood dust was generated by sanding the wood piece with sand-paper (Mirox P120, Mirka
128 Finland) over a petri dish under sterile conditions. The dust was then vacuumed onto poly-L-
129 lysine coated filters as described above.

130

131 *Fungal particle immunolabeling procedure*

132 A 1/8 segment (ca. 1.2 cm^2) of the poly-L-lysine coated polycarbonate filter with samples
133 was cut and mounted onto a 25 mm double sided carbon tab (Agar Scientific Ltd, Stansted Essex,
134 UK), and glued onto a 25 mm metal grid (NIOH, Norway) was used as a support. The samples

135 were vapor-fixed with glutaraldehyde overnight at RT in a fume hood to prevent the loss of
136 soluble antigens (40). A sterile 9 mm Ø petri dish chamber containing a cellulose pad soaked with
137 1mL 25% glutaraldehyde, Sigma) was used during this procedure.

138 Following the fixation, samples were then placed in a sterile 6 well plate (VWR, Norway)
139 and free aldehyde sites were quenched for 2×5 min with 1 mL of 0.02M glycine (Sigma). The
140 samples were then blocked for 1h at RT in 2 mL tris-buffered saline (pH 8; Sigma) containing
141 0.05% Tween 20 and 5% skimmed milk (TBSTSM). Thereafter, the samples were incubated in 1
142 mL of anti *A. versicolor* pIgY diluted in TBSTSM (1:100 equivalents to 120 µg mL^{-1}). After
143 incubation for 1h, samples were washed in 2 ml TBSTSM for 3×5 min. Following this washing
144 step, samples were incubated, as above, in 1 mL of secondary antibody of goat anti-chicken H&L
145 conjugated with 25 nm gold particles (ABCAM, Cambridge UK) diluted in TBSTSM (1:10).
146 Reagent controls incubated with TBSTSM instead of primary antibodies were included in each
147 experiment. After washing in TBSTSM followed by rinsing in 2 ml of BPC grade water (Sigma)
148 for 3×5min at RT, samples were then silver enhanced for 30 min at RT using 1 ml of Aurion kit
149 (Aurion R-gent SE-LM, EMS Hatfield, PA USA). The enhancement was stopped by washing the
150 samples in 2 ml water (BPC grade, Sigma) for 3×3 min. Samples were then dried under sterile
151 conditions for approximately 1h at RT before being mounted on specimen mounting stubs (25
152 mm) that were sputter-coated with 5-6 nm platinum in a Cressington 208HR sputter coater
153 (Cressington Scientific Instruments Ltd, Watford, UK). During all incubation and washing steps,
154 samples were subjected to gentle agitation.

155 *Efficiency of fragment immobilization and immunolabeling*

156 In order to check for the possible loss of particles during the immune labeling procedure,
157 different sizes of *A. versicolor* mycelial fragments (positive controls) enumerated on three

158 unlabeled filters were compared to three labeled filters. In both cases, the particles were
159 vacuumed onto poly-L-lysine coated filters and vapor-fixed as described above.

160 Positive controls (3 experiments) were used to evaluate the efficiency of the
161 immunolabeling procedure and the number of gold particles per fragment type (41). The labeling
162 efficiency was defined as the percentage of fragments, in each size category, labeled in relation to
163 the total fragments present on the filter or per volume of air sampled. Fragments in the negative
164 control samples were similarly assessed in order to determine the maximal level of nonspecific
165 labeling.

166

167 *Crude protein extraction for cross reactivity test*

168 The anti *A. versicolor* pIgY was tested for cross reactivity toward 23 fungal species, 4
169 bacterial species, and 2 plant species (Table 2). The tests were performed on water extracts to
170 assess water soluble antigens, whereas the insoluble fraction was tested after lysis using Yeast
171 Protein Extraction Reagent (YPER; Thermo Fischer Scientific, Norway).

172 All fungal, and 3 bacterial isolates (*Pseudomonas lurida, Bacillus subtilus* and
173 *Streptomyces coelicolor*) were grown in 80 mm petri dishes containing yeast extract sucrose
174 (YES) media (39) covered with sterile 90 mm polycarbonate membrane (0.22 µm pores;
175 Millipore) as this technique eases the biomass collection. The petri dishes were inoculated each,
176 with 200 µl of inoculum containing approximately 10^7 spores or bacteria. The dishes were sealed
177 and incubated for 6 days at 25°C in humidified chambers (zip lock bag + sterile water saturated
178 paper). The filter membranes covered with fungal or bacterial biomass were then carefully
179 removed from the media and transferred into 50 ml centrifuge tubes. The water soluble proteins
180 were extracted following the method described by Bridge (42). In short, fungal or bacterial
181 biomass was separated from the filter by vortexing the filter membrane with 5 ml water (BPC

182 grade water, Sigma) containing 1% (v/v) protease inhibitor (Thermo Fischer Scientific) and 10
183 Retsch metal beads (5 mm) for 10 min at RT. Aliquots (1.5 mL) of the biomass suspension were
184 prepared and kept at -20 °C until extraction of crude proteins. For water soluble protein
185 extraction, 1ml of the aliquot was transferred into an Eppendorf tube with screw cap and
186 centrifuged at 13,000×*g* at 4 °C for 10 min. The supernatant was transferred into 1.8 ml cryo tube
187 vials (Nunc, Roskilde, Denmark) and kept at -20 °C until crude proteins were quantified.

188 For the cyanobacterium, *Artrospira platensis,* 200 mg (obtained as dry powder) was
189 transferred to an Eppendorf tube containing 300 mg borosilicate glass beads (Sigma) and 500 µl
190 water (BPC grade water, Sigma). Samples were then agitated in a Mini Beadbeater (Biospec
191 Products Inc., Barlesville, OK USA) for 5×1min. The obtained suspension was then centrifuged
192 as described above and the supernatant collected and stored at -20 °C until crude proteins
193 quantification.

194 For extracts derived from plants, water soluble crude proteins were extracted by grinding
195 600 mg (dry weight) whole grain wheat flour: *Triticum aestivum* (sammalt mel, Norges Møller,
196 Norway) or fresh needles from pine tree (*Pinus sylvestris*) in Retsch jars containing 5 ml water
197 (BPC grade water, Sigma) with 10 metal beads for 3×5 min including a one min break between
198 each 5 min cycle. The obtained suspension was transferred to an Eppendorf tube and centrifuged
199 as described above. The supernatant was kept at -20 °C for protein quantification.

200 The lysates with YPER were prepared according to the manufacturer's instructions
201 (Thermo Fischer Scientific). Briefly, ca 200 ml of borosilicate solid-glass beads of 2 mm (Sigma)
202 and 500 µl of YPER containing 1% protease inhibitor (v/v) were added to the remaining wet
203 pellet (100 -200 mg) after the water extracted fraction from fungal and bacterial materials or ca.
204 500 - 600 mg from plant materials. The tubes were treated by bead beating for 5×1min in a Mini
205 Beadbeater followed by 20 min treatment on Vibramax 110 (Heidolph Instruments, Schwabach

206 Germany). Samples were thereafter centrifuged at 13,000×g and 4 °C for 10 min and the
207 supernatant collected into cryo tube vials. The procedure was repeated once and the collected
208 supernatants were combined and kept at -20 °C until crude protein analysis.

209 The crude protein concentration in both water extracts and lysates were determined using
210 BCA assay kit (Thermo Fischer Scientific). Serial diluted bovine serum albumin (BSA) was used
211 as a standard following the procedure from the manufacturer. The results of the BCA assay were
212 used to dilute all extracts to a final crude protein concentration of 1mg mL^{-1} prior to the cross
213 reactivity test of the *A. versicolor* pIgY.

214

215 *Sandwich ELISA for cross reactivity determination*

216 Ninety six well Nunc Maxisorp microplates (Sigma Aldrich) were coated overnight with 100 µl
217 pIgY (12 µg mL^{-1}) in 0.1M sodium carbonate buffer pH 9.6 (Sigma) at 4 °C. The plates were
218 washed 3 times with 200 µl PBS containing 0.05% (v/v) Tween 20 (PBST with pH 7.4) and the
219 remaining free binding sites were blocked with PBST containing 5% skimmed milk (Sigma) for
220 1h at RT. Water extracts or lysates diluted in PBST/5%SM (final protein content: 10 µg in 100
221 µl) were added to each well, and incubated at 37 °C for 2h. Water (BPC grade, Sigma) and YPER
222 (100 µl) used for extract and lysate preparation were included as negative controls. After plate
223 washing as described above, 100 µl of alkaline phosphatase conjugated to pIgY (stock: 1mg mL^{-1}
224) (ABCAM, Cambridge UK) and diluted (1:100) in PBST with 5% SM was added, and plates
225 were further incubated for 2h at RT. After washing, as above, the wells were incubated with 100
226 µl p-nitrophenyl phosphate disodium (Sigma Aldrich) in 0.05M Na_2CO_3 for 30 min and the
227 absorbance was measured at OD 405 nm using Spectromax i3 equipped with SoftMax Pro 6
228 software (Molecular Devices, LLC, Sunnyvale, California USA). All cross reactivity experiments
229 were run in triplicate and repeated three times.

230

231 *Environmental samples analysis*

232 Environmental samples were collected from a school with water infiltration and visible
233 mold growth. Samples were collected during renovation work. Airborne particles were collected
234 onto poly-L-lysine coated polycarbonate filters in open 37 mm diameter standard SKC cassettes
235 made out of conductive polypropylene. During sampling, the cassettes were mounted 1.5 m
236 above ground level and were connected to a vacuum pump Gast Model D0A-P109-FD (Gast
237 Manufacturing Inc, Benton Harbor, Michigan USA). A mass flow meter (822 Top-Track, Sierra
238 Instruments, Montery CA) was placed between the cassette and the pump to determine the flow
239 measurement. The mean flow rates varied between 18.7 L min^{-1} at start to 17.2 L min^{-1} at the end
240 of sampling. Three samples were collected during 1h intervals. Collected particles were stored at
241 RT until immunostaining.

242

243 *FESEM analysis*

244 Samples were visualized using a SU 6600 FESEM (Hitachi, Ibaraki – Ken, Japan). The
245 FESEM was operated in low vacuum (25 Pa) and imaging was performed in the back scatter
246 mode. Acceleration voltage of 15 kV, extraction voltage of 2.10 kV and working distances of 6 –
247 7 mm were used. Size stratified counts of fragments were performed with both the poly-L-lysine
248 immobilization test and the immunolabeling efficiency estimation. In short, fragments defined as
249 submicronic fragments (0.2 – 0.5 μm fragments; 0.5 – 1 μm fragments) were counted at 3000×
250 magnification in 200 fields of 1064 μm^2 each. Larger fragments (1 – 2 μm fragments, 2 – 3.5 μm
251 fragments and >3.5 μm fragments) were also counted at the same magnification but, only in 100
252 fields. The lowest detectable number of particles on the filter was $2 – 4 \times 10^3$.

253 The number of gold particles per fragment was assessed by counting gold particles attached
254 to 50 randomly selected fragment types in the positive and negative controls. The visualization
255 and enumeration were performed under similar conditions as above at 15,000 – 25,000
256 magnification depending on the fragment type. The maximal number of gold particles on each
257 fragment type in the negative control was used as threshold for counting labeled fragments in the
258 environmental samples.
259 Total numbers of the four types of fragments (submicronic fragments, 1 – 2 µm fragments,
260 2 – 3.5 µm fragments and >3.5 µm fragments) as well as the gold labeled fungal fragments were
261 enumerated in each environmental sample. Spore particles (single spores: spore aggregates of 2,
262 3, 4 and ≥5 spores) recognized by their morphology were also counted. Enumeration in the
263 environmental samples was performed on 100 fields (265 μm^2 each) at 6000× magnification.
264 Particle distribution on the filter was homogeneous as confirmed by the Poisson distribution test
265 for each type of particles ($\mathbf{d} = \frac{\mathbf{SD^2}}{\mathbf{AM}} \sim 1$ where SD is the standard deviation and AM is the
266 arithmetic mean number of counts per field of all counted fields) (43). The lowest number of
267 detectable particles in field samples was 1.5×10^4 m^{-3}.
268
269 *Data analysis*
270 Student t-test was used for arithmetic mean (AM) absorbance comparison in the cross
271 reactivity experiments and for the AM particle number comparison in the fragment
272 immobilization experiments. STATA SE 13.1 (Statacorp LP, College Station, TX) was used for
273 statistical tests and Microsoft Office Excel 2007 for graphs.
274
275

276 **Results**

277 *Immunolabeling efficiency and fragments immobilization on filter*

278 The binding of pIgY to *A. versicolor* fragments was demonstrated by the immunogold
279 labeling of fungal fragments in positive controls samples. Overall the fungal fragments with
280 homogenously distributed gold particles on the surface of the fungal fragments were observed
281 (**Figure 1: A1 and A2**). These data indicated the presence of antigens that bind to pIgY.
282 The average numbers of gold particles on each fragment type in the positive control samples were
283 significantly higher when compared to the negative controls and the background (**Table 1**). Most
284 fragments from the negative control samples (**Figure 1 B1 and B2**) showed no gold particles
285 binding to the surface; however, nonspecific staining (maximum 3 gold particles) was observed
286 mainly with fragments ≥3.5 µm (8%)(**Figure 2A)**.

287 The efficiency of immunolabeling of the positive controls is shown in **Figure 2B**.
288 Approximately 84% of submicronic fragments (0.2 – 1 µm) were labeled while 89, 99 and 100%
289 of 1 – 2 µm fragments, 2 – 3.5 µm fragments and ≥3.5 µm fragments were labeled, respectively.
290 Minor nonspecific background labeling was additionally observed (**Table 1**).

291 The immunogold labeling procedure did not influence the number of immobilized
292 fragments (< 3.5 µm) on the filter. In this regard, the comparison of mean number of fragment
293 particles in the five size ranges on unlabeled filter to the mean number on labeled filters revealed
294 no significant difference for different types of fragments with length < 3.5 µm. However, the
295 number of ≥3.5 µm fragments was significantly higher on labeled filters compared to the
296 unlabeled filters (2.00×10^6 versus 1.78×10^6, p=0.03) (**Table 2**). The total number of all fragments
297 on the labeled filters was similar and not statistically different from the unlabeled filter (2.73×10^6
298 versus 2.49×10^6; p=0.1).

299

300 *pIgY cross reactivity*

301 The results from the cross reactivity experiments are summarized in **Table 3**. *A.*
302 *versicolor* and 19 of the other 23 fungal species reacted with the pIgY raised against *A.*
303 *versicolor.* The absorbance of the lysates from *Fomitopsis pinocola*, *Mucor mucedo*,
304 *Saccharomyces cereviceae* and *Phoma herbarum* did not vary from the control, while the water
305 extracts showed significant differences. Moreover, the lysates from *Eurotium amstelodami* and
306 *Stachybotrys chartarum* showed significantly higher absorbance in comparison to the control.

307 Both water extracts and lysates from *Verticillum lecanii* (Hypocreales), *Geotrichum*
308 *candidum* (Saccharomycetales), *Botrytis cinerea* (Helotiales), and *Acremonium strictum*
309 (Hypocreales), showed no significant difference when compared to the control samples. The
310 absorbance of the water extracts and the lysates derived from the plant materials (wheat flour and
311 pine needles) was similar to the controls and showed the absence of antigens that react with the
312 pIgY. Similarly the pIgY showed no reactivity to the tested bacteria except for the actinomycetes
313 species, *Streptomyces coelicolor*.

314

315 *Environmental sample analysis*

316 Based on the maximal number of gold particles on the background in the positive and
317 negative control samples, and on different fragments in the negative controls, a threshold of 2 and
318 3 gold particles was used to enumerate $<3.5\mu m$ fragments and $\geq 3.5\mu m$ fragments, respectively, as
319 gold labeled. Immunogold labeled fragments in environmental samples are shown in **Figure 3**.
320 The most abundant particle types in the environmental samples were fragments of different size
321 and shape with an arithmetic mean of 10.9×10^{6} m^{-3}. These particles represented in average 93%
322 of all particles while the spore particles constituted only 7% (arithmetic mean 1.1×10^{6} m^{-3}, **Table**
323 **4**).

324 In total, 1.2×10^6 m^{-3} fragments, out of 12×10^6 m^{-3} total particles, were labeled with gold
325 and represented about 10% of the total number of particles present on the filter. Regarding the
326 larger fragments, 44, 73 and 82% of 1 – 2 µm fragments, 2 – 3.5 µm fragments and ≥3.5 µm
327 fragments were immunolabeled, respectively (**Table 5**). Only 6% of all submicronic fragments
328 collected were labeled with gold. Overall, the immunogold labeling showed that 11% of the total
329 fragments was of fungal origin. The fungal fraction of the aerosols is composed of 39%, 40%,
330 19% and 2% of submicronic fragments (0.2 - 1µm), large fragments (>1µm), single spores and
331 spore aggregates, respectively (**Table 4**).
332
333 **Discussion**
334 The results show that the novel immunogold labeling method described here is valuable
335 for detection and enumeration of fungal fragments in FESEM. The development of this method
336 comprises four major steps: 1) the production of the egg yolk polyclonal antibodies IgY against
337 *A. versicolor*, 2) the immobilization of sampled particles onto polycarbonate filters coated with
338 poly-L-lysine, 3) the immunolabeling of of fungal derived particles with the pIgY and 4) the
339 detection and enumeration of immunolabeled particles using FESEM. The labeling ability of the
340 pIgY antibodies was confirmed by the immunogold labeling of positive controls (mycelia
341 fragments from *A. versicolor*). The specificity of the antibody was tested in cross reactivity
342 experiments with materials of various origins including plant, fungi and bacteria using a
343 developed sandwich ELISA. As proof-of-principle the new method was applied to environment
344 samples derived from built environment with characterized water infiltration and fungal
345 contamination. The results demonstrated that the immunolabeling approach enabled the
346 identification and enumeration of fungal particles other than spores in complex environmental
347 samples through the immunolabeling of exposed surface antigens. Submicronic fungal particles

348 were successfully labeled with the anti *A. versicolor* pIgY and detected by FESEM. To our
349 knowledge, the immunodetection and enumeration of submicronic fungal fragments have not
350 been possible using preexisting methods of assessing fungal exposure.

351 The results derived from positive control experiments confirmed that artificially generated
352 fungal fragments including submicronic fragments contain surface antigens that could be labeled
353 and detected by FESEM. Our finding is in agreement with Gorny et al. who used an ELISA to
354 demonstrate that *in vitro* aerosolized fungal fragments from *A. versicolor* and *Penicillium melinii*
355 contained antigens on fragments in the submicrometer size (4).

356 Visually, the gold labeled antigens were homogeneously distributed on the surface of
357 various types of fragments. The efficiency of this immunostaining approach was 84% and 89-
358 100% for submicronic fragments and larger fragments, respectively. The reason why 16% of the
359 submicronic fragments were not labeled is unclear. We speculate that this could be due to the
360 density of the accessible antigenic sites, so that the likelihood of particles without binding sites
361 on their surface increases with decreasing particle size. A further explanation could be that
362 glutaraldehyde vapor fixation may denature epitopes and decrease antibody binding events (44).

363 Negative control experiments showed that 8% of the larger fragments (>3.5 μm) had 1 – 3
364 gold particles bound to the surface. It is likely that this was due to non-specific labeling since the
365 labeling intensity on the negative controls was significantly lower compared to the positive
366 controls. There were no gold particles on most of the fragments derived from negative control
367 samples. Similarly, there was limited gold particle binding on the background. In contrast,
368 positive control particles showed abundant gold particles binding on the surface of the fragments.

369 The immobilization of fragment on a membrane has not, to our knowledge, been
370 previously reported. This methodological approach enabled the assessment of a broader range of
371 particle types to be immunolabeled without loss of the particles on the sampling filter. The
372 labeling without extraction of particles from the filter is believed to contribute to a better
373 characterization of the bioaerosol composition since disturbance of particles after collection is
374 limited. Still, the significant difference between labeled filters and unlabeled filters for larger
375 particles (≥3.5 µm fragments) indicates that aggregates of larger fragments may dissociate into
376 smaller units during the aqueous staining process. Further investigation is therefore required to
377 better understand how larger fragments are affected by the immunolabeling procedure.

378 The presence of airborne fungal fragments has been based on visualization of hyphae-like
379 fragments or fragments with melanization or septation using light or scanning electron
380 microscopy (45–47). The size of observed fragments ranged between 2.5 and 100 µm and
381 concentrations varied between 3 and 3×10^{6} m^{-3} in outdoor settings. In indoor environments,
382 proportions between 4 and 33% of the total particle count have been published (6). The
383 application of the halogen immunostaining assay (HIA) has permitted the enumeration of
384 allergen containing spores and fragments larger than 2.5 µm (33). To date, there is no fungal
385 exposure assessment methodology technique that has enabled the enumeration of fungal particles
386 smaller than 2.5 µm inclusive submicronic fragments to evaluate the fungal aerosols within
387 fungal indoor environments.

388 The application of this novel immunodetection assay to environmental samples has
389 revealed the presence of immunolabeled fungal fragments in various sizes and morphologies. In
390 the proof of concept experiments, our results showed that the fungal aerosols comprise higher
391 numbers of antigen containing fragments (79%) in comparison to spore particles (21%). The

392 overall quantified fungal aerosols reveal that 39%, 40%, 19% and 2% were submicronic
393 fragments, large fragments (>1µm), single spores and spore aggregates, respectively. In contrast
394 to earlier studies that utilize the HIA, 25% of larger fragments (>2.5 µm) contained fungal
395 allergen (33). In the present study, the immunogold assay labeled 73 – 82% of larger fragments
396 (>2 µm). This difference may be associated to the assay efficiency or the size range that was
397 wider in the present study. Other studies that focuses on the occurrence of submicronic fragments
398 in mold contaminated indoor or outdoor air used different methods to detect β-glucan (14, 16, 18,
399 48) and NAHA (21) as biomarkers of fungal fragments in size fractionated samples. However,
400 these studies were limited by the imprecise fractionation of particles due to confounding variables
401 such as spore bounce (18, 48, 49). Further, these studies could not predict the number of
402 submicronic fragments present in the air. The results from our proof of principle experiments are
403 the first, to our knowledge, that report the number of submicronic fragments in mold
404 contaminated air samples.

405 The pIgY raised against *A. versicolor* reacts with water extracts and lysates from 20 of 24 tested
406 fungal isolates. Similar results have been reported with pAbs raised against spores from *A.*
407 *versicolor* (50–52) or other fungal species (53) where high levels of cross reactivity were
408 observed. Even monoclonal antibodies developed after immunization of animals with
409 spores/conidia were found not to be species specific (50, 54). Common and evolutionary
410 conserved fungal proteins have been suggested to explain this high level of cross reactivity
411 between fungi. These proteins have been identified as: 1) glyceraldehydes-3-phosphate
412 dehydrogenase, 2) a putative sorbitol or xylose reductase, 3) catalase A, 4) enolase and 5) malate
413 dehydrogenase. These products have been localized both on the outer membrane and in the
414 cytoplasm of many fungi (52, 54). Given the solubility of many fungal antigens in water as

415 shown by the high reactivity of the water extracts, vapor fixation with glutaraldehyde seemed
416 efficient in the preservation of antigens for immunodetection (40). This approach also efficiently
417 prevented non-specific staining of the background which was a major problem with aqueous
418 fixation (results not shown).

419 Our primary goal in this study was to discriminate fungal fragments including submicronic
420 fragments from plant and bacterial sources, which was achieved except for the actinomycete *S.*
421 *coelicolor*. The observed cross-reactivity toward most tested fungal materials makes the
422 polyclonal antibody very useful for achieving this goal. However, the cross reactivity of the pIgY
423 antibodies prevents the identification of fungal fragments to species level.

424 **Conclusions**

425 The immunolabeling method and the enumeration of a broad range of airborne fungal particles
426 have the potential to selectively quantify fungal fragments including submicronic fragments in
427 complex heterogeneous environmental samples. Although identification to species level is not
428 possible, the present study demonstrated that submicronic and larger fragments with exogenous
429 fungal antigens could be labeled and enumerated in environmental samples using the novel
430 immuongold technique and FESEM. Spores were not labeled but could be recognized by
431 morphology. As a result, this methodological approach has the potential to further enables
432 detailed characterization of the fungal aerosol profile which has not been possible to date.

433

434

435 **Acknowledgment**

436 The present study is a part of the project “Fungal particles in indoor air” that is financially
437 supported by the Norwegian Research Council: Grant number NFR196130/H10. The technical
438 support provided by the Allergy and Clinical Immunology Branch (National Institute for
439 Occupational Safety and Health, Centre for Disease Control and Prevention, Morgantown WV),
440 Norwegian Veterinary Institute and the Environmental Microbiology Group at The Department
441 of Chemistry, Biotechnology and Food Science (Norwegian University of Life Science) is
442 gratefully acknowledged. The findings and the conclusions in this report are those of the authors
443 and do not necessarily represent the views of the National Institute for Occupational Safety and
444 Health.

445

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584 **Figures and Tables**

585 **Figure 1**: Micrographs of controls: Mycelial fragments from *Aspergillus versicolor* as
586 positive controls (A1 and A2) and pine dust as negative controls (B1 and B2). Arrows
587 showed immunolabeled submicronic fragments in the positive control. White spots
588 represent silver enhanced gold particles. Scale 5µm

589 **Figure 2**: Unspecific labeling with pine dust as negative control (A) and labeling efficiency
590 with fungal fragments from *A. versicolor* as positive control (B). Bars represent mean of
591 three repetitions. Error bars represent standard deviation.

592 **Figure 3**: Immunogold labelling of fragments from environmental airborne samples.
593 Micrographs show labeled submicronic fragments (A, B, D and F), unlabeled submicronic
594 fragment (B); labeled large fragments (C, G, H, I and J) and unlabeled larger fragments (A
595 and F) and unlabeled spores (G and I). White spots represent silver enhanced gold particles.
596 Scales: 2µm (A-H) and 5µm (I and J).

597 **Table 1**: Number of gold particles per fragment types in the positive and negative controls.

598 **Table 2**: Comparison of number of particles immobilized on poly-L-lysine coated filters
599 without and with immunolabelling procedure, N=3.

600 **Table 3**: list of materials tested for cross reactivity and the mean absorbance compared to

601 controls.

602 **Table 4**: Concentration of total particles, labeled fragments and spore particles in

603 environmental samples (10^6 m^{-3}, N=3)

604 **Table 5**: Proportion of immunolabeled fragments per fragment types in environmental

605 samples (10^6 m^{-3}, N=3

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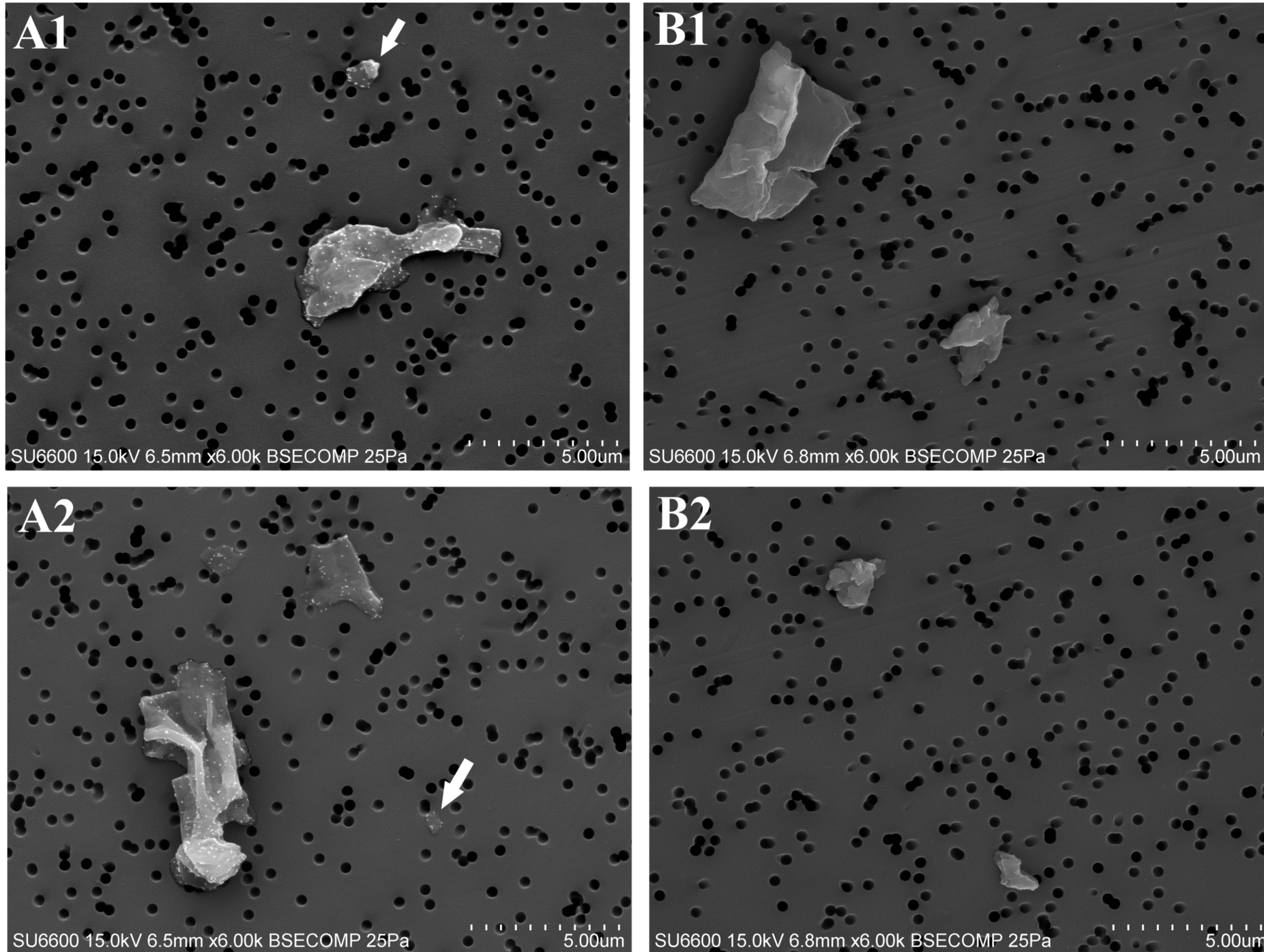
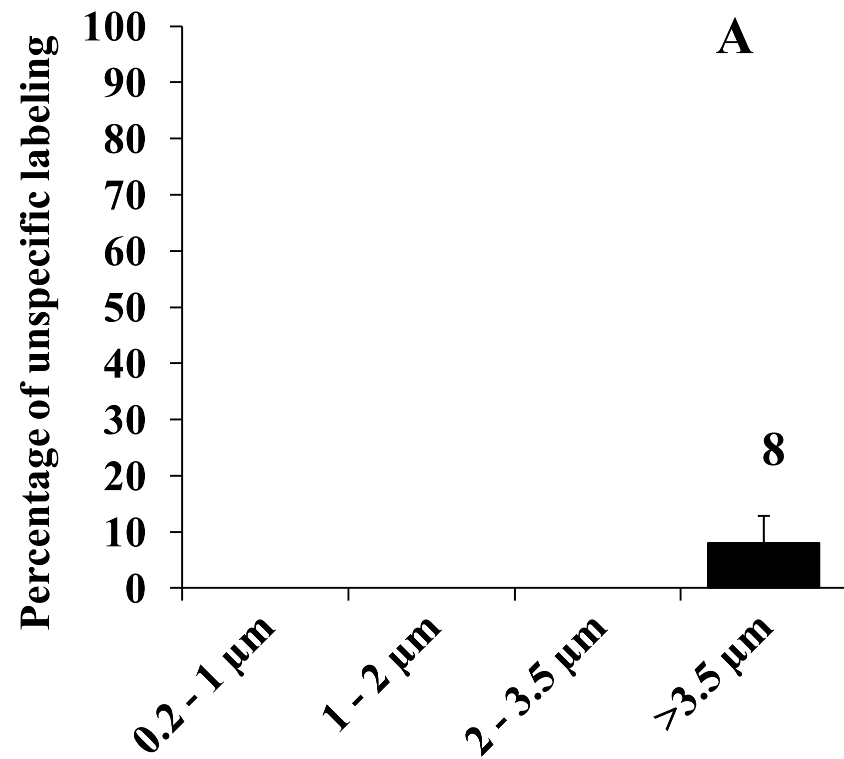


Figure 1: Micrographs of controls: Mycelial fragments from *Aspergillus versicolor* as positive controls (A1 and A2) and pine dust as negative controls (B1 and B2). Arrows showed immunolabeled submicronic fragments in the positive control. White spots represent silver enhanced gold particles. Scale 5µm



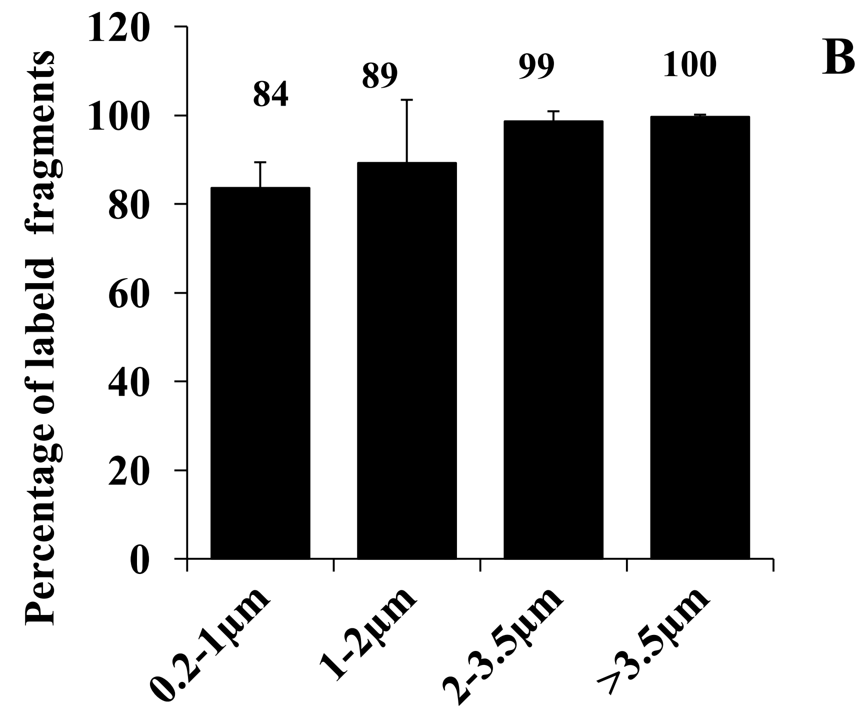


Figure 2: Unspecific labeling with pine dust as negative control (A) and labeling efficiency with fungal fragments from *A. versicolor* as positive control (B). Bars represent mean of three repetitions. Error bars represent standard deviation.

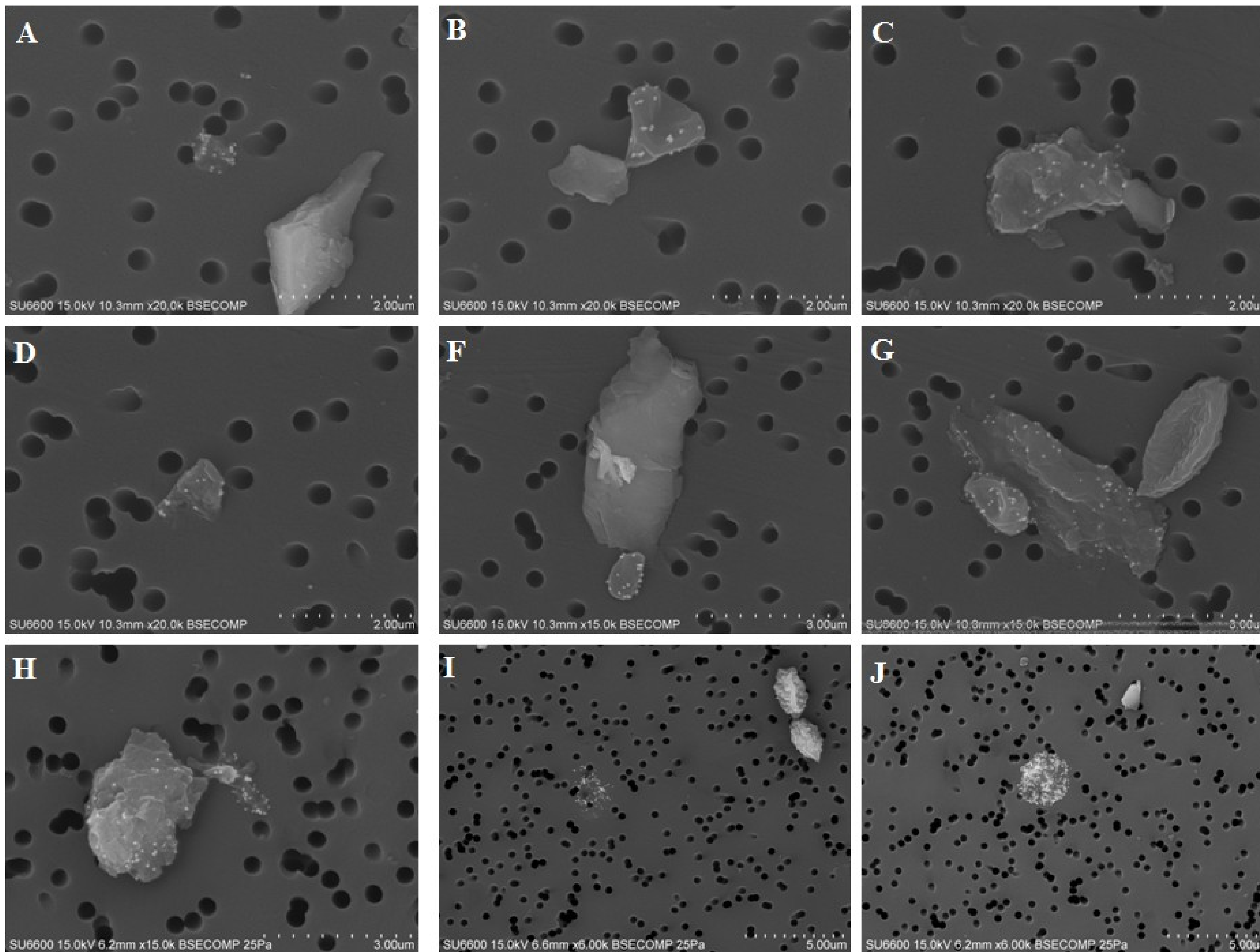


Figure 3: Immunogold labelling of fragments from environmental airborne samples. Micrographs show labeled submicronic fragments (A, B, D and F), unlabeled submicronic fragment (B); labeled large fragments (C, G, H, I and J) and unlabeled larger fragments (A and F) and unlabeled spores (G and I). White spots represent silver enhanced gold particles. Scales: 2µm (A-H) and 5µm (I and J).

Table 1: Number of gold particles per fragment types in the positive and negative controls.

		0.2 – 1µm fragments		1 – 2µm fragments		2 – 3.5µm fragments		>3.5µm fragments	
	Background (n=100)	Negative controls (n=50)	Positive controls (n=50)	Negative controls (n=50)	Positive controls (n=50)	Negative controls (n=50)	Positive controls (n=50)	Negative controls (n=50)	Positive controls (n=50)
AM	0.4	0	10.8	0.1	21.4	0.1	66.02	0.24	151.9
SD	0.64	0	8.5	0.3	14.4	0.3	24.2	0.69	73.3
Min	0	0	0	0	0	0	19	0	50
Max	2	0	30	1	47	1	124	3	405

AM: arithmetic means; SD: standard deviation

Table 2: Comparison of number of particles immobilized on poly-L-lysine coated filters without and with immunolabelling procedure, N=3.

Type of particles	Unlabeled filters, x10^4 particles/filter AM (SD)	Labeled filters, in x10^4 particles/filter AM (SD)	T-test p-values
0.2 – 0.5 µm Submicronic fragments	7.0 (0.8)	9.9 (2.8)	0.2
0.5 – 0.1 µm Submicronic fragments	9.9 (2.8)	8.3 (2.0)	0.5
1 – 2 µm fragments	14.4 (1.3)	14.8 (0.3)	0.6
2 – 3.5µm fragments	40 (8.3)	39.4 (6.2)	0.9
>3.5µm fragments	178 (8.2)	200 (9.4)	**0.03***
Total	249 (19)	272.7 (12)	0.1

AM: arithmetic means; SD: standard deviation; *p<0.05 after t –test.

Table 3: list of materials tested for cross reactivity and the mean absorbance compared to controls.

Species	Order	Water extract			YPER lysate		
		AM	SD	Test vs Controls; p-values	AM	SD	Test vs control; p-values
Controls		0.185	0.011		0.186	0.082	
Fungi							
Ascomycota							
Aspergillus versicolor VI03554	Eurotiales	0.725	0.234	**0.02**	0.469	0.125	**0.03**
Aspergillus niger VI06015	Eurotiales	1.096	0.451	**0.03**	0.668	0.051	**0.001**
Eurotium amstelodami VI05331	Eurotiales	0.648	0.279	0.05	0.533	0.056	**0.004**
Paecilomyces variotii UMB_AT01	Eurotiales	0.779	0.223	**0.01**	0.711	0.190	**0.01**
Penicillium chrysogenum VI04528	Eurotiales	1.100	0.421	**0.02**	0.843	0.043	**0.0003**
Aureobasidium pullulans VI05029	Dothideales	0.669	0.205	**0.02**	0.631	0.099	**0.004**
Botrytis cinerea UMB101BC	Helotiales	0.281	0.085	0.1	0.218	0.084	0.7
Chaetomium globosum VI05046	Sordariales	0.825	0.296	**0.02**	0.432	0.038	**0.009**
Cladosporium cladosporioides VI04541	Capnodiales	0.518	0.155	**0.02**	0.490	0.143	**0.03**
Epicoccum nigrum VI05728	Incertea sedis	0.433	0.144	**0.04**	0.404	0.106	0.05
Candida albicans VI05945	Saccharomycetales	0.496	0.127	**0.01**	0.296	0.089	0.2
Geotrichum candidum VI03865	Saccharomycetales	0.370	0.124	0.06	0.251	0.066	0.3
Saccharomyces cereviciae VI05951	Saccharomycetales	0.635	0.190	**0.01**	0.309	0.102	0.2
Phoma herbarum VI05764	Pleosporales	0.433	0.140	**0.04**	0.525	0.294	0.1
Ulocladium chartarum VI04844	Pleosporales	0.746	0.234	**0.01**	0.662	0.018	**0.001**
Alternaria alternata VI06044	Pleosporales	0.673	0.264	**0.03**	0.608	0.108	**0.006**
Stachybotrys chartarum VI	Hypocreales	0.754	0.342	0.05	0.511	0.113	**0.02**
Trichoderma harzianum VI04074	Hypocreales	0.422	0.097	**0.01**	0.457	0.128	**0.04**
Verticillium lecanii VI03418	Hypocreales	0.299	0.101	0.1	0.225	0.025	0.5
Acremonium strictum VI05921	Hypocreales	0.314	0.095	0.08	0.266	0,018	0.2
Zygomycota							
Mucor mucedo UMBM808	Mucorales	0.333	0.086	**0.04**	0.254	0.075	0.3
Rhizopus microsporus UMB_AT02	Mucorales	1.214	0.376	**0.009**	1.700	0.275	**0.001**
Basidiomycota							
Fomitopsis pinicola VI06099	Polyporales	0.439	0.108	**0.02**	0.274	0.104	0.3
Wallemia sebi UMB_AT03	Wallemiales	1.088	0.304	**0.007**	2.417	0.348	**0.0004**
Bacteria							
Proteobacteria							
Pseudomonas lurida UMBPSLH406	Pseudomonadales	0.265	0.061	0.09	0.230	0.076	0.5
Firmicutes							
Baccillus subtillus UMB15022013	Bacilliales	0.317	0.102	0.09	0.325	0.100	0.1
Cyanobacterium							
Artrospira platensis(Spirula Nutrex)	Oscillatoriales	0.180	0.040	0.9	0.234	0.099	0.6
Actinobacterium							
Streptomyces coelicolor UMB/ATCCBAA471	Actinomycetales	0.565	0.182	**0.02**	0.498	0.136	**0.03**
Plants							
Pinophyta							
Pinus sylveatris (Pine needles)	Pinales	0.261	0.083	0.2	0.224	0.087	0.6
Agiosperms							
Wheat flour (Triticum aestivum)	Poales	0.248	0.045	0.08	0.253	0.075	0.4

AM: Arithmetic means; SD Standard deviation; three experiments with triplicates for water extracts and YPER lysates. P-values after t-test of the AM of extracts or lysates to respective controls.

VI: Norwegian Veterinary Institute, Section of Mycology
UMB: Norwegian University of Life Science, Institute of Chemistry, Biotechnology and food science, Section of Environmental microbiology

Table 4: Concentration of total particles, labeled fragments and spore particles in environmental samples (10^6 m^{-3}, N=3)

Particle types	Total particles		Labeled fragments and spore particles	
	Concentration AM (min - max)	Mean percentage[a] %	Concentration AM (min - max)	Mean percentage[b] %
Fragment particles				
Submicronic fragments	9.8 (7.4 – 11.3)	84	0.5 (0.4 – 0.7)	39
1 – 2 µm fragments	0.6 (0.4 – 0.9)	5	0.2 (0.2 – 0.3)	17
3 – 3.5 µm fragments	0.2 (0.1 – 0.3)	2	0.2 (0.05 – 0.3)	12
≥3.5 µm fragments	0.3 (0.03 – 0.7)	2	0.2 (0.030 – 0.5)	11
All fragment particles	**10.9 (7.9 – 12.5)**	**93**	**1.2 (0.9 – 1.4)**	**79**
Spores particles				
Single spores	0.9 (0.090 – 2.1)	6	0.9 (0.09 – 2.1)	19
Aggregates of 2 spores	0.1 (0 – 0.4)	1	0.1 (0 – 0.4)	1
Aggregates of 3 spores	0.03 (0 – 0.07)	0.2	0.03 (0 – 0.07)	0
Aggregates of 4 spores	0.01 (0 – 0.04)	0.1	0.01 (0 – 0.04)	0.6
Aggregates of ≥5 spores	0.01 (0 – 0.04)	0.1	0.01 (0 – 0.04)	0.6
All spore particles	**1.1 (0.09 – 2.6)**	**7**	**1.1 (0.09 – 2.6)**	**21**
All particles	**12 (8 – 15.2)**		**1.6 (0.9 – 2)**	

AM: arithmetic mean

[a]Percentage of total collected particles

[b]Percentage of the immunolabeled fragments and spores (morphologically identified).

Table 5: Proportion of immunolabeled fragments per fragment types in environmental samples (10^6 m^{-3}, N=3)

Particle type	Percentage of labeled particles , % AM (min - max)
Fragment particles	
Submicronic fragments	5.6 (3.5 – 7.3)
1 – 2 µm fragments	44 (31 – 57)
3 – 3.5 µm fragments	73 (43 – 90)
≥3.5 µm fragments	82 (69 –100)
All fungal fragments	**10.9 (10.7 – 11.1)**

AM: arithmetic mean.

PhD Thesis:

Fungal aerosols: characterization and immunodetection of fungal fragments

By Komlavi Anani Afanou

Submitted the 23rd Mars 2015

Errata List submitted the 12th Mai 2015

Cover page:	«Characterization» changed to «characterization»; «sopppartikler» changed to «soppartikler»; «karakterisering» changed to «Karakterisering»
Page 3: Paragraph 1,	line 2: «Work Environments» changed to «Working Environment»
Paragraph 2,	line 4: «of life Science» added
Paragraph 6:	«time» removed
Page 5: Paragraph 1	line 7: «have been not» changed to «have not been»;
Paragraph 3	line 5: «fragnments» changed to «fragments»;
Paragraph 4	line 6: «behaviou» changed to «behaviour»
Page 7: Paragraph 2	line 3 and line 4: «og kvantifisert» moved from line 4 to line 3 line 8: «glutaraldehyde» changed to «glutaraldehyd»; line 8: «poly-L-lysine belagt » changed to «poly-L-lysin-belagt» line 9: «primære» changed to «primært»; line 9: «antistoffene» changed to «antistoff» line 10: «sekundære» changed to «sekundært»; line 10: «antistoffene» changed to «antistoff»
Paragraph 3	line 9: «observert» added

Thesis Number 2015:26; ISSN 1894-6402; ISBN 978-82-575-1277-4

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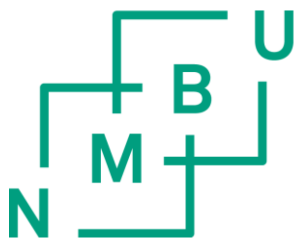

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