Molecular profiling of indoor microbial communities in moisture damaged and non-damaged buildings

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II **Pitkäranta, M**., Meklin, T., Hyvärinen, A., Paulin, L., Auvinen, P., Nevalainen, A. and Rintala, H. 2008. Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Applied and Environmental Microbiology 74:233-244.

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I MP participated in study design, did the cloning and sequencing, performed data-analysis and drafted the manuscript.

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ABSTRACT

Epidemiological studies have shown an elevation in the incidence of asthma, allergic symptoms and respiratory infections among people living or working in buildings with moisture and mould problems. Microbial growth is suspected to have a key role, since the severity of microbial contamination and symptoms show a positive correlation, while the removal of contaminated materials relieves the symptoms. However, the cause-and-effect relationship has not been well established and knowledge of the causative agents is incomplete. The present consensus of indoor microbes relies on culture-based methods. Microbial cultivation and identification is known to provide qualitatively and quantitatively biased results, which is suspected to be one of the reasons behind the often inconsistent findings between objectively measured microbiological attributes and health.

In the present study the indoor microbial communities were assessed using culture-independent, DNA based methods. Fungal and bacterial diversity was determined by amplifying and sequencing the nucITS- and16S-gene regions, correspondingly. In addition, the cell equivalent numbers of 69 mould species or groups were determined by quantitative PCR (qPCR). The results from molecular analyses were compared with results obtained using traditional plate cultivation for fungi.

Using DNA-based tools, the indoor microbial diversity was found to be consistently higher and taxonomically wider than viable diversity. The dominant sequence types of fungi, and also of bacteria were mainly affiliated with well-known microbial species. However, in each building they were accompanied by various rare, uncultivable and unknown species. In both moisture-damaged and undamaged buildings the dominant fungal sequence phylotypes were affiliated with the classes Dothideomycetes (mould-like filamentous ascomycetes); Agaricomycetes (mushroom- and polypore-like filamentous basidiomycetes); Urediniomycetes (rust-like basidiomycetes); Tremellomycetes and the family Malasseziales (both yeast-like basidiomycetes). The most probable source for the majority of fungal types was the outdoor environment. In contrast, the dominant bacterial phylotypes in both damaged and undamaged buildings were affiliated with human-associated members within the phyla Actinobacteria and Firmicutes.

Indications of elevated fungal diversity within potentially moisture-damage-associated fungal groups were recorded in two of the damaged buildings, while one of the buildings was characterized by an abundance of members of the *Penicillium chrysogenum* and *P. commune* species complexes. However, due to the small sample number and strong normal variation firm conclusions concerning the effect of moisture damage on the species diversity could not be made. The fungal communities in dust samples showed seasonal variation, which reflected the seasonal fluctuation of outdoor fungi. Seasonal variation of bacterial communities was less clear but to some extent attributable to the outdoor sources as well.

The comparison of methods showed that clone library sequencing was a feasible method for describing the total microbial diversity, indicated a moderate quantitative correlation between sequencing and qPCR results and confirmed that culture based methods give both a qualitative and quantitative underestimate of microbial diversity in the indoor environment. However, certain important indoor fungi such as *Penicillium* spp. were clearly underrepresented in the sequence material, probably due to their physiological and genetic properties. Species specific qPCR was a more efficient and sensitive method for detecting and quantitating individual species than sequencing, but in order to exploit the full advantage of the method in building investigations more information is needed about the microbial species growing on damaged materials.

In the present study, a new method was also developed for enhanced screening of the marker gene clone libraries. The suitability of the screening method to different kinds of microbial environments including biowaste compost material and indoor settled dusts was evaluated. The usability was found to be restricted to environments that support the growth and subsequent dominance of a small number microbial species, such as compost material.

TIIVISTELMÄ (Abstract in Finnish)

Kosteusvaurioiden aiheuttamalla huonolla sisäilmalla tiedetään olevan epidemiologinen yhteys mm. astman, allergisten oireiden ja hengitystieinfektioiden esiintyvyyteen. Mikrobikasvulla epäillään olevan tärkeä rooli ilmiön aiheuttajana, sillä havaitun ”home”kasvun laajuuden ja oireiden vakavuuden välillä on positiivinen yhteys ja toisaalta homeisten materiaalien poisto vähentää oireita. Tämänhetkinen tieto oireita aiheuttavista tekijöistä ja oireiden syntymekanismeista on kuitenkin vajavaista. Sisäympäristöjen mikrobilajiston tuntemus perustuu suurelta osin viljelypohjaisilla menetelmillä saatuun tietoon. Viljelymenetelmien kuitenkin tiedetään antavan laadullisesti ja määrällisesti vääristyneen kuvan mikrobistosta, minkä epäillään olevan yhtenä syynä siihen, että sisäympäristöistä mitattujen mikrobistojen ja terveysongelmien välillä ei aina havaita johdonmukaisia yhteyksiä.

Tässä työssä tutkittiin sisäympäristöjen mikrobistoja viljelystä riippumattomin, DNA-pohjaisin menetelmin. Sieni- ja bakteerilajiston kartoittamiseen käytettiin ribosomaalisten DNA-merkkijaksojen (ITS- ja 16S -geenialueet) monistusta ja sekvensointia. 69 homelajin solumäärät määritettiin lisäksi kvantitatiivisella PCR-menetelmällä (qPCR). Saatuja tuloksia verrattiin samoista näytteistä viljelymenetelmin saatuihin tuloksiin.

Sisätilojen mikrobidiversiteetin havaittiin olevan DNA-pohjaisin menetelmin merkittävästi viljelymenetelmin todettua monimuotoisempaa ja lajirikkaampaa. Yleisimmät sekvenssityypit olivat peräisin tunnetuista lajeista mutta kaikista tutkituista rakennuksista löydettiin myös uudentyyppisiä DNA-merkkisekvenssejä, joista osa saattaa edustaa aiemmin tuntemattomia mikrobilajeja. Sekä kosteusvaurio- että verrokkirakennuksissa yleisimmät sienten sekvenssityypit vastasivat kaariin Ascomycetes ja Basidiomycetes (kanta- ja kotelosienet) kuuluvien luokkien Dothideomycetes, Agaricomycetes, Urediniomycetes ja Tremellomycetes, sekä heimon Malasseziales lajien DNA-sekvenssejä. Ko. ryhmiin lukeutuu home-, lakkisieni-, kääpä-, ruoste- ja hiivalajeja. Suurin osa sienilajistosta oli todennäköisimmin peräisin ulkoympäristöstä. Sitä vastoin bakteerisekvenssien enemmistö vastasi ihmisperäisten, pääjaksoihin Actinomycetes ja Firmicutes kuuluvien lajien merkkijaksoja. Mikrobiryhmien esiintymisessä kosteusvaurio- ja verrokkirakennuksissa havaittiin eroja; kahdessa tutkitusta vauriokohteesta havaittiin verrokkia korkeampaa diversiteettiä rakennusperäisiä lajeja sisältävissä sieniryhmissä, kun taas yhden vauriokohteen sekvenssiaineistossa havaittiin poikkeuksellisen runsaasti *Penicillium chrysogenum*- ja *P. commune* –lajiryhmittymiin kuuluvia merkkijaksoja. Pienestä näytemäärästä ja lajiston voimakkaasta normaalivaihtelusta johtuen luotettavia johtopäätöksiä kosteusvaurioiden osuudesta lajiston vaihteluun ei kuitenkaan kyetty tekemään. Sienilajistosta kuvattiin vuodenaikaisvaihtelua, joka vastaa lajiston vaihtelua ulkoympäristössä. Bakteerilajiston vuodenaikaisvaihtelu ei ollut yhtä selkeää, mutta eräiden ryhmien osalta vaihtelu oli yhdistettävissä ulkoympäristön bakteerikulkeuman vaihteluun.

Menetelmien vertailu osoitti sekvensoinnin toimivuuden kokonaislajiston kuvauksessa, osoitti kohtuullisen kvantitatiivisen korrelaation sekvensoinnin ja qPCR:n antamien tulosten välillä ja

vahvisti aiemmat havainnot siitä, että viljelymenetelmä antaa sekä määrällisen että laadullisen aliarvion lajistosta. Toisaalta eräiden merkittävien sisäilmahomeiden kuten *Penicillium*:in ja sen sukulaisten todettiin olevan aliedustettuja sekvenssiaineistoissa, todennäköisesti lajien fysiologisista ja geneettisistä ominaisuuksista johtuen. Lajispesifisen qPCR:n katsottiin olevan herkkä ja tehokas menetelmä lajiston määrälliseen tutkimiseen, mutta menetelmän hyödyntämiseksi tarvitaan kattavampaa tietoa kosteusvauriomateriaaleilla esiintyvistä mikrobeista.

Työssä kehitettiin lisäksi menetelmä mikrobilajistojen sekvensointipohjaisessa kartoittamisessa tarvittavien kloonikirjastojen käsittelyn tehostamiseksi, sekä arvioitiin menetelmän toimivuutta komposti- ja huonepölynäytteillä. Menetelmän hyödynnettävyyden todettiin rajoittuvan ympäristöihin joissa olosuhteet suosivat harvojen mikrobilajien voimakasta lisääntymistä ja joissa siten on selkeästi dominoitu mikrobiyhteisörakenne, esimerkkinä kehittynyt kompostimassa.

ABBREVIATIONS

ABPA	Allergic bronchopulmonary aspergillosis
ARDRA	Amplified ribosomal DNA restriction analysis
DG18	Dichloran-glycerol agar
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EPS	Extracellular polysaccharide
HDM	House dust mite
HPLC	High-pressure liquid chromatography
INSD	International nucleotide sequence database
LPS	Lipopolysaccharide
MAC	*Mycobacterium avium* complex
MEA	Malt extract agar
MS-GC	Mass spectrometry-gas chromatography
MVOC	Microbial volatile organic compound
nucITS	Nuclear internal transcribed spacer (commonly also ITS)
ODTS	Organic dust toxic syndrome
OTU	Operational taxonomic unit; here synonymous to "phylotype"
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
rDNA	Ribosomal DNA; the genomic region of DNA containing the rRNA coding genes and intervening spacers including nucITS
RFLP	Restriction fragment length polymorphism
RH	Relative humidity
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT	Respiratory tract
RTI	Respiratory tract infection
sp.	Species
spp.	Species, plural
SSCP	Single strand -conformation polymorphism
SM	Storage mite
TGGE	Temperature gradient gel electrophoresis
tRFLP	Terminal restriction fragment length polymorphism

1. INTRODUCTION

1.1 The indoor microbiome and human health

People in the modern world spend circa 90% of their time in various indoor environments (Schwab 1992). Thus human exposure to microbes concentrates on the species present in these environments, in practice, mainly on food- and indoor airborne bacteria, fungi and viruses. Environmental microbes have both beneficial and harmful effects on health. Microbial exposure starting at birth leads to the development of the commensal human microbiome which has an elementary role on various vital functions, ranging from food digestion and pathogen resistance to the proper development and maintenance of immune functions (Jarchum and Pamer 2011). For example, the exposure to diverse micro-organisms is believed to be a critical factor explaining the lower incidence of allergic diseases in children in farming environments compared to urban environments (Ege *et al.* 2011). Microbial health risks of indoor environment in turn are often associated with low indoor air quality (IAQ) in the presence of excess moisture and mould contamination in a building (Mendell *et al.* 2011). This phenomenon has been of concern at least the past 3500 years (Leviticus, ch. 14, v. 33-48) but is very acute today. The indoor environment may also serve as a reservoir for pathogens. The rate of opportunistic infections caused by a variety of fungal and bacterial species of low virulence has increased due to the growing proportion of immunocompromised and chronically ill population (Groll and Walsh 2001, Liu 2011).

1.1.1 Building moisture, microbes and human illness

A higher prevalence of morbidity, especially in respiratory illness has been reported in water damaged, damp and mouldy houses compared to undamaged ones (IOM 2004). The significance of this phenomenon is striking; according to a recent estimate twenty percent of current asthma cases in the United States – altogether 4,6 million cases – may be attributable to residential dampness and mould (Mudarri and Fisk 2007). In Finland, exposure to moisture damaged building conditions is recorded as the most significant individual cause of occupational asthma (Piipari and Keskinen 2005). A recent meta-analysis of relevant epidemiologic literature by Mendell *et al.* (2011) concluded that dampness or mould had – globally – consistent positive associations with several allergic and respiratory outcomes. Besides development and exacerbation of asthma, associated conditions included dyspnea and wheeze without asthma diagnosis, cough, allergic rhinitis and eczema. Sufficient evidence of association was also found for increased occurrence of upper respiratory infections and bronchitis (Mendell *et al.* 2011). Besides these, there are other conditions that have been empirically associated with indoor mould exposure, but for which the epidemiological evidence has been seen inconclusive (Mendell *et al.* 2011). Such diseases include hypersensitivity pneumonitis (HP, also known as extrinsic allergic alveolitis) and the organic toxic dust syndrome (ODTS), which have originally been described as occupational lung diseases (Husman 1996, IOM 2004, Mendell *et al.* 2011). HP is an inflammatory lung illness occurring in susceptible people after inhalation of high quantities of

specific microbial antigens. ODTS is a non-allergic illness associated with occupational microbial exposure in agricultural environments. Besides pulmonary dysfunctions, these conditions involve non-specific symptoms such as fever, cough, nausea, fatigue and headache, which are also commonly reported among occupants of moisture-damaged buildings (IOM 2004).

Microbial growth and emissions are hypothesized to play a key role in the development of building-related illnesses (Figure 1). This idea is supported by the fact that microbial growth is more or less an inevitable result of extended wetting of building surfaces, and observations of "dampness" and "mould" are most easily interpreted as visual and olfactory signs of microbial growth. The hypothesis is also supported by the health effects of verified microbial exposure in occupational settings mentioned above, as well as by the knowledge obtained from toxicological studies; spores, fragments and metabolic compounds released from several microbial strains isolated from contaminated building sites have toxic, inflammatory and immunomodulatory effects on mammalian cells and tissues *in vitro* and *in vivo* (WHO 2009, Mendell *et al.* 2011).

Despite the consistent epidemiological association between dampness, the causality, the causative agents and disease mechanisms are poorly understood. During the last 25 years, the correlation between the presence of moisture damage and microbial attributes (i.e. the how moisture damage alters the indoor microbiomes) and between microbial attributes and adverse health findings (i.e. the probability that the observed health effects occur due to microbial exposure) have been assessed in tens of studies (as summarized in e.g. IOM 2004, WHO 2009 and Mendell *et al.* 2011). However, based on the currently available information, Mendell *et al.* (2011) recently concluded that any quantitative microbial measure does not provide a more reliable indicator of potential health risks than a careful examination of the presence of dampness, water damage, visible mould or mould odor or a history of water damage in a building. The poor performance of objectively measurable indicators in epidemiological studies may be explained by the complex and compound nature of indoor exposures, synergistic effects of microbial and non-microbial pollutants, and the varying extent of the population susceptibility. Such complexity makes finding associations difficult even using large data sets (Nevalainen and Seuri 2005).

Besides the complexity of the exposing agent, the microbial exposure assessment has been complicated by the deficiencies in the traditional methods used to identify and enumerate microbial agents. The major problems relate to selectivity and low resolution of such methods. By traditional plate cultivation only species that grow and produce characteristic morphological structures in laboratory conditions can be identified. Direct microscopy and measurement of proxies for fungal and bacterial biomass (such as ergosterol, β-D-glucans, extracellular polysaccharides [EPSs], endotoxin and muramic acid) can reveal also unculturable material, but the capacity to distinguish between microbial taxa is more or less limited (Pasanen 2001). For exposure assessment, however, an unselective, specific identification and enumeration of microbes would be necessary; the potential health-effects of microbes can be species- or strain-specific and independent of cell viability (Flannigan *et al.* 2011). The fast advances in the development of DNA-based methods for microbial identification may offer solutions for these problems. These methods are further discussed below in chapter 1.4.

1.1.2 The indoor environment as a reservoir for opportunistic human pathogens

People with decreased immunocompetence may be susceptible to normally harmless environmental microbes. Severe forms of immunodeficiency are caused by cancer radiation- and chemotherapy, transplantation medication and progressed HIV-infection. Other susceptible groups include premature infants, trauma patients and people with severe forms of common chronic conditions such as cardiovascular disease or diabetes mellitus. While the proportion of populations with increased susceptibility is expanding, the diversity of microbial species associated with infections is also growing; besides well known nosocomial agents such as *Candida albicans*, *Staphylococcus aureus* or *Pseudomonas aeruginosa*, atypical microbes such as various saprotrophic fungi are emerging as causative agents of opportunistic infections (Groll and Walsh 2001). Certain fungi such as *Aspergillus fumigatus* are of concern both due to their pathogenicity as well as their allergenicity in the indoor environment.

The information about the potential reservoirs and natural habitats of rarely encountered opportunists is often scarce, and little is known about their occurrence in the normal living environment (Liu 2011). Recent efforts to map the indoor microbiomes have revealed that specific indoor niches can maintain opportunistic microbes; for example, Zalar *et al.* (2011) found that thermophilic members of the genus *Exophiala* – including species which are increasingly associated with human infections - commonly inhabit household dishwashers. Related fungi were also often found in other humid indoor environments in the study of Lian and de Hoog (2010). Nishiuchi *et al.* (2009) in turn investigated the homes of patients suffering from *Mycobacterium avium* complex (MAC) pulmonary infections. The authors demonstrated that bathtub inlets and showerheads in the case homes were commonly colonized by these bacteria, and may have served as sources of inoculum for the recurrent infections typical of the patients with MAC infections (Nishiuchi *et al.* 2009).
Moisture damaged buildings in which species capable of producing immunomodulatory mycotoxins occur in parallel with opportunistic species form an interesting environment with respect to human health. As mentioned in the previous chapter, lowered resistance agains upper respiratory infections as well as an increased prevalence of sequelae diseases such as otitis media have been associated with mouldy buildings (Mendell *et al.* 2011). In healthy individuals, building related microbes themselves generally establish their harmful effects via allergy or irritation/inflammation instead of an invasive infection. However, in some conditions colonization by a specific agent takes place. The most significant of such diseases is probably the allergic bronchopulmonary aspergillosis (ABPA). This chronic disease involves superficial growth of *Aspergillus* (*A. fumigatus* being the most commonly detected species) in RT mucus, which causes allergic inflammation of the epithelia and may lead into lung tissue scarring over time. The role of other fungi in related conditions is less well known (Gore 2010).

1.2 House dust

1.2.1 Airborne particles and dust sampling

The major route of human exposure to airborne microbes is via inhalation. However, direct measurement of airborne particles, especially of microbial ones, has shown to be

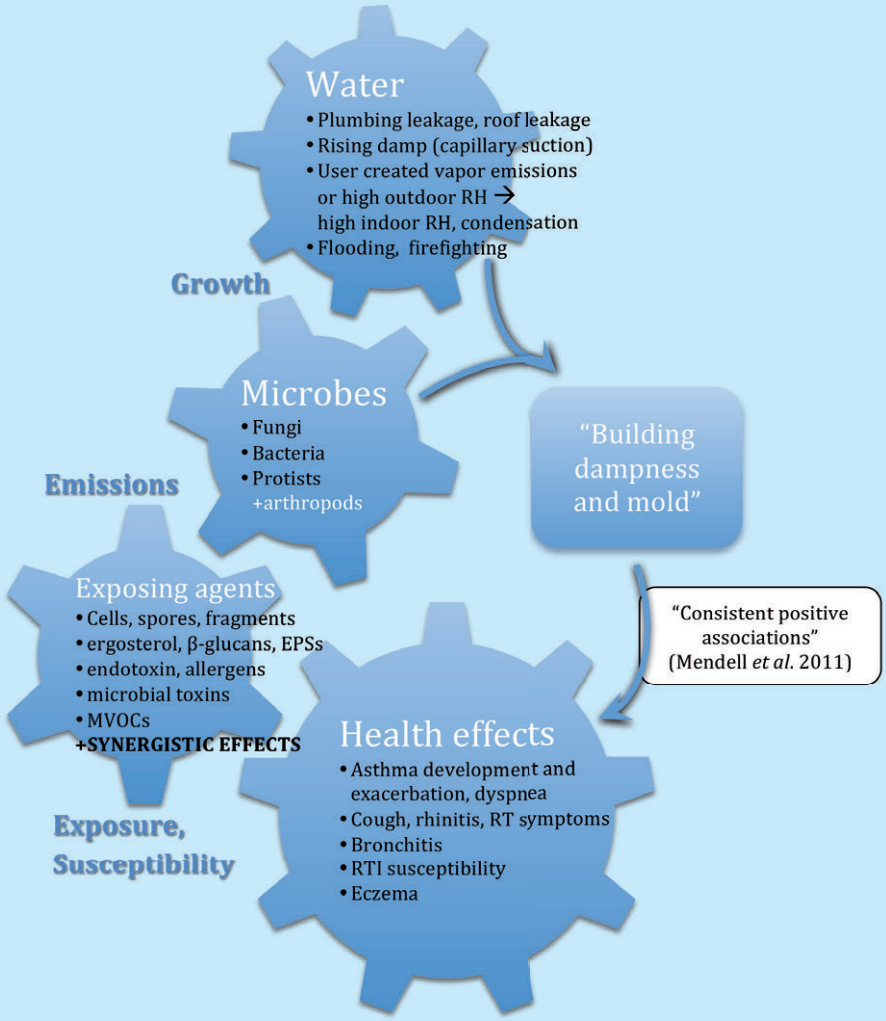

Figure 1. Summary of the hypothesized relationship between water damage, microbial growth and adverse health effects.

"Building dampness and mould" are known to be associated with adverse health effects in building users (right side of the figure). The left side of the figure elucidates the complexity of the involved agents and exposure dynamics. Water is the limiting factor for microbial growth in the indoor environment. Micro-organisms are ubiquitous in the environment and their growth generally initiates within days on wetted building materials. Saprotrophic bacteria, fungi and protists (e.g. amoebas) proliferate on building materials and numbers of Arthropods (eg. mites and insects) may also increase. The species diversity depends on the inoculum, substrate type and microbial succession takes place if water is available under a long period (Flannigan and Miller 2011).

Microbial growth may produce spores, hyphae, yeast- and bacterial cells as well as fragmented cell material (Green *et al.* 2005); mites contribute to the dispersal of fungal spores (Colloff 2009) while amoebas may aid in the dispersal of bacteria (Yli-Pirilä *et al.* 2006). Microbial material may contain bioactive compounds such as allergens and microbial toxins (Brasel *et al.* 2005a, Green *et al.* 2006, Polizzi *et al.* 2009, Täubel *et al.* 2011). The amounts and types of produced substances vary depending on the species and growth conditions, including substrate, water availability and co-occurring species (Murtoniemi *et al.* 2002 and 2005, Nielsen *et al.* 2003, Hirvonen *et al.* 2005). Microbial volatile- and particle-bound compounds may become airborne and spread along air currents to the living space (Górny *et al.* 2001, Green *et al.* 2006). Exposure to microbial compounds takes place mainly by inhalation, but to lesser extent also by skin contact and ingestion of dust (infants) (WHO 2009, Roberts *et al.* 2009). Part of the inhaled particles is deposited in the airways and small particles with diameter under 2.5 µm may end up in the alveoli (Górny 1999). Microbial compounds that become into contact with epithelial and immune cells may launch inflammatory allergic or non-allergic signaling or have toxic or immunosuppressive effects (WHO 2009). Putative candidates for the causative agents include eg. EPS, endotoxin, allergenic enzymes and other proteins, microbial toxins and volatile metabolites (WHO 2009). Immunomodulatory and toxic effects of these compounds may be responsible for the observed symptoms and allergic sensitization alone or in combination with other environmental pollutants such as tobacco smoke, traffic exhausts or non-microbial chemical emissions released from water affected building materials or other sources. Moreover, simultaneous exposure to multiple agents may have synergistic effects (WHO 2009). Typically only a part of equally exposed population develops symptoms. This variation in susceptibility may be attributed to various mechanisms such as individual differences in the capacity to tolerate, degrade and metabolize harmful substances (Wu *et al.* 2010) and genetic variation in the tendency to develop allergic responses in antigen contact (Kelada *et al.* 2003).

The diagram shows that the connection between excess moisture and ill health effects is complex and dependent on the realization of several independent phenomena: sufficient microbial growth needs to occur; harmful substances need to be produced and they need to be emitted and spread to the living environment in sufficient quantities; susceptible people need to be exposed to them and the duration or frequency of exposure needs to be sufficient to launch the symptoms.

problematic (Pasanen 2001). Airborne concentrations of microbes show significant spatial and temporal variability (Verhoeff *et al.* 1990 and 1992, Nevalainen *et al.* 1992, Law *et al.* 2001), which makes determining the "representative airborne microbial level" of a building difficult. Indoor levels usually reflect those of outdoor air, where diurnal variation, and variation due to meteorological conditions are considerable (Li and Kendrick 1995a, 1995b, 1995c). Human activity levels affect the resuspension of settled particles and move-

ment of airborne particles from room to room (Law *et al.* 2001, Ferro *et al.* 2004). Moreover, activities such as opening of a cellar door, cleaning, cooking and handling firewood and entrance of people and pets from outdoors may raise microbial levels significantly in a temporary manner (Lehtonen *et al.* 1993). Changes in ventilation, whether mechanical or natural, affect the air movements and transportation of particles and volatiles within the building, and may also alter the routes and quality of intake air. For example, the use of equipments such as central vacuum cleaners, local exhaust fans for cloth dryers and bathrooms, and even furnaces that underpressurise the room space may lead into temporary periods of altered entrance of replacing air. In the absence of proper intake air ducts, the intake air may infiltrate trough the building envelope from contaminated structures such as crawl spaces, funnels or water-damaged sites and increase the airborne concentrations of contaminants. In combination these phenomena cause significant temporal and spatial variation to indoor airborne microbial levels. To overcome this variability and to obtain a representative sample from a building, sampling over long time periods, preferably days to weeks has been evaluated to be necessary (Hyvärinen *et al.* 2001). However, long-term air sampling has several technical limitations. Depending on the used sampling device, overloading of agar plates and impaction slides, or blocking of filter membranes used to collect particles takes place and limits the collection time. Significant desiccation and subsequent loss of viability of microbial particles is a problem in extended forced air sampling (Wang *et al.* 2001). If short-term samples are used, multiple samples are needed to gain adequate representativeness of microbial levels in a building (Hyvärinen *et al.* 2001).

The collection of settled, once airborne dust is an alternative to air sampling (Flannigan 1997, Dillon *et al.* 1999). Dust can be allowed to accumulate on surfaces for several weeks or months prior to collection. The long collection period acts as a buffer against variable airborne concentrations and makes dust a long term, time-integrate sample of airborne material (Portnoy *et al.* 2004, Egeghy *et al.* 2005). Dust samples have been used in the assessment of indoor exposure to environmental pollutants such as heavy metals, pesticides, phthalates and other chemicals (Roberts *et al.* 2009), as well as to biologicals such as mites and pet allergens (Roberts *et al.* 2009, Colloff 2009).

Viable microbes have been measured from dust in numerous studies (Verhoeff 1994a, Verhoeff and Burge 1997, Dillon *et al.* 1999, Chao *et al.* 2002, Chew *et al.* 2003, Horner *et al.* 2004, see also Appendix I table). Yet the results have sometimes been concluded to be poorly representative of airborne microbial levels due to their low correlation with short-term air samples (Ren *et al.* 1999). One problem with dust is the great difficulty of enumerating fungal spores by direct microscopy due to the abundant background debris (Pasanen 2001). For this, viable cultivation has traditionally been the only feasible method for the identification and enumeration of dustborne microbial communities. While the long term accumulation period is the main advantage of dust as a sample type, it is also its weakness when measuring analytes with varying stabilities. If significant degradation or inactivation of the measured substance takes place during the collection, the results can be expected to be underestimates of the "fresh" airborne concentrations. This is

a valid concern in the case of cultivation, since the persistence of microbial species in indoor conditions varies significantly (Sussman 1968, Verhoeff *et al.* 1994a). Contrasting to cultivation methods, direct DNA-based microbial detection methods do not suffer from this problem since they are independent of cell viability. Microbial DNA also persists well in indoor conditions (Fierer *et al.* 2010, Lauber *et al.* 2010).

The methods used to collect settled dust vary greatly between studies, ranging from vacuum-collection from carpets, chairs, mattresses of smooth horizontal surfaces to sieving the fine dust fraction directly from the dust bag of the residential vacuum cleaner (Macher 2001). The diversity of sample types undermines between-study comparisons, since different dust types are prone to reflect different aspects of indoor exposures. For example, mattress and chair dust may be heavily contributed by human microbial flora (Täubel *et al.* 2009), while floor dust content may be significantly affected by coarse debris, which has not necessarily been airborne (Lewis *et al.* 1999). Dust collected from floors is most commonly used in large epidemiological studies due to its ease and low costs of collection, yet dust on elevated surfaces could be considered to represent better the inhalable fraction of airborne dust. Sampling devices, for example electrostatic collectors, have recently been developed for standardized sampling of settled dust (Noss *et al.* 2008), and airborne dust (Nilsson *et al.* 2004). With such collectors the sampling time and area/volume can be standardized.

1.2.2 Microbiological composition of dust

Dust is a complex mixture of organic and inorganic material. In general, the composition of dust varies depending of building type and use, and major particle sources. For example, the composition of office dust differs from home dust, while dust in apartments differs from that in houses, and further, dust in rural houses differs from dust in urban houses (Macher 2001, Chew *et al.* 2003, Mølhave *et al.* 2007, Pakarinen *et al.* 2008, Ege *et al.* 2011). The study of Mølhave *et al.* (2007) exemplifies the crude content of office dust. A large composite sample of 11 kg of floor dust was collected from seven large Danish office buildings (area 12 751 m^2). The organic fraction was 33%, total concentration of microorganisms 130.000 ± 20.000 CFU g^{-1} and concentration of viable fungi 71.000 ± 10.000 CFU g^{-1}. The dust also contained human and animal skin fragments, hairs, paper and textile fibers, glass wool, wood and metal particles, as well as unknown organic and inorganic particles. The size fraction of < 10 µm, i.e. the size class of most fungal and bacterial spores/cells, accounted for < 1.5 % of total mass.

House dust components of microbial origin may include viable and non-viable intact fungal conidia, spores and spore clumps; fragments of spores, hyphae, sclerotia, lichen soredia and fruiting bodies; and bacterial cells, endospores and fragmented cells (Piecková *et al.* 2004, Green *et al.* 2006, Alenius *et al.* 2009). The size and shape of intact fungal spores varies from tiny, round to ovoid 2 - 5 µm conidia of *Aspergillus*, *Penicillium* and other trichocomaceous moulds to large, oblong ≥ 50µm conidia of *Alternaria* and *Helminthosporium*. Fungal fragments vary in size from sub-micrometer (< 1 µm) particles to larger hyphal parts of the length of tens to hundreds micrometers (Górny 2004, Green *et al.* 2006). Microbial particles carry many structural compounds that can be measured as crude proxies for microbial biomass; such

include ergosterol and (1→3)-beta-D-glucan (for fungi) and lipopolysaccharide (LPS, or endotoxin) and N-acetylmuramic acid (for gram negative and –positive bacteria, correspondingly). Enzymes and other proteins are also present, as well as non-volatile metabolic products such as microbial toxins and volatile organic compounds (MVOCs) like alcohols, terpenes and aldehydes, whose production is usually restricted to few individual fungal species or strains (Korpi *et al.* 1997, Górny 2004, Cho *et al.* 2005, Green *et al.* 2006, Bloom *et al.* 2009, Täubel *et al.* 2011).

1.2.3 Development and dynamics of microbial populations in dust

Viable microbial populations in dust may be either of autochthonous or allochthonous nature or a combination of these two (Bronswijk 1981). An autochthonous population (a true population) develops by active growth and proliferation on site. Allochthonous populations (pseudopopulations), in contrast, develop by mechanisms other than proliferation, i.e. by passive accumulation of particles from the surroundings. For example, air-circulating filtering appliances like house hold vacuum cleaners and HVAC systems without an appropriate high-efficiency particulate air (HEPA) filtration may shape indoor microbial assemblages by inefficient removal and even enrichment of small spores and fragments which may pass through the filters and return into the indoor air (Scott *et al.* 2004, Cheong 2005). Viable communities may be further shaped by differential longevity of individual species, which results in enrichment, and proportional increase of persistent species (Scott *et al.* 1999).

Sources. Bacteria and fungi are ubiquitous in the outdoor air, indoor air and also in settled dust. The main sources of indoor microbes are the outdoor environment (soil, decomposing plant litter and the phylloplane [surfaces and tissues of living plants]), humans, pets, house plants and raw or spoiled materials like vegetables, fruit, mouldy bread and firewood (Hunter *et al.* 1988, Lehtonen *et al.*, 1993 Wouters *et al.* 2000, Scott 2001, Glushakova *et al.* 2004, Aydogdu *et al.* 2010). The transfer of microbial particles from outdoors takes place by airborne transmission through open doors and windows, ventilation ducts and leakages in the building envelope. Microbes are also carried indoors along with soil, plant debris and other particles attached to shoe soles, clothes and pet fur (Pasanen *et al.* 1989, Lehtonen *et al.* 1993, Law *et al.* 2001). In addition to these "background" microbial sources, a potentially very significant indoor contributor can be active microbial proliferation in building surfaces and constructions (Green *et al.* 2003). It is notable that each of the major natural fungal habitats (food, phylloplane, soil) harbours species that may actively proliferate on indoor materials and finishes in the presence of excess moisture. Thus it may be difficult to distinguish between the contribution of "normal" microbial sources vs. inappropriate mould growth due to water damage in building (WHO 2009, Lawton *et al.* 1998, Fahlgren *et al.* 2010).

Deposition and resuspension. Dust acts as both a sink and a source for airborne particles. Yet, the common finding that the microbes in indoor air resemble more those in outdoor air than those in dust, suggests that resuspension is partial (Ren *et al.* 1999, Chew *et al.* 2003, Shelton *et al.* 2002). Size and shape variation among microbial particles contributes to their differential dispersal, deposition and resuspension in indoor spaces. In practise, due to

ventilation and human activities indoor air is in continuous movement and deposition and resuspension happen all the time in parallel. Smaller particles tend to mix more efficiently to the entire room space and stay longer airborne compared to bigger particles (Carlile et al. 2001, Li *et al.* 2005, Oberoi *et al.* 2010). On the other hand, fine particles (<2.5 μm) have been reported to resuspend less efficiently by human activity than larger particles (Thatcher and Layton 1995, Chen and Hildemann 2009). The sample type and sampling location may have an effect on the observed dustborne microbiomes. Ren *et al.* (1999) found more small-spored species such as *Aspergillus* and *Penicillium*, and less large-spored species such as *Mucor*, *Wallemia* and *Alternaria* in indoor air than in vacuumed floor dust. Baudisch *et al.* (2009) in turn reported significantly higher concentrations of viable *Penicillium*, *Aspergillus* and *Eurotium* in dust collected from top of shelves than in floor dust.

Dormancy and longevity. In long-term sample types such as settled house dust the differential longevity of deposited organisms may greatly affect the culture-based measure of the sample's microbial content. In some studies, the viable fungal composition of dust sample has been suspected to be significantly associated with its age (Baudisch *et al.* 2009). The major environmental factors that affect the survival of fungal propagules include temperature, relative humidity (RH), radiation and predation by other organisms such as mites. In indoor conditions the longevity of fungal propagules varies greatly between species and also between different spore- and particle types within one species. In general, hyphal fragments lose their viability, i.e. the ability to start new growth first. Dispersal spores persist longer, and survival structures may stay viable over decades (Sussman 1968). Properties associated with extended spore longevity include spherical cell form, thick cell walls, melanin and other pigments in cell walls, low spore water content, and high trehalose content (Sussman 1968, Carlile *et al.* 2001). A majority of the fungal types most commonly cultivated from dust samples persist very long times in indoor conditions. For example, the spores of *Aspergillus*, *Penicillium* and *Fusarium* spp. may retain their viability over ten years or longer. Much shorter survival times have been reported for others, e.g. 2-6 months for many basidiospores and only days or even hours for some plant pathogens (Sussman 1968). As for common phylloplane fungi, apart from *Aureobasidium pullulans* they survive much shorter times compared to eg. *Penicillium* spp. (Scott 2001, Baudisch *et al.* 2009). Flannigan and Miller (2011) suggested that the relatively high abundance of *Aureobasidium pullulans, Alternaria alternata* and *Epicoccum nigrum* in indoor vs. outdoor air compared to *Cladosporium cladosporioides*, *C. herbarum* and *C. sphaerospermum* could be explained by superior longevity of these species in indoor conditions (Flannigan and Miller 2011).

Microbial growth in house dust. Active microbial proliferation, i.e. the development of autochthonous populations in dust can be expected to alter the microbial community content significantly from the original proportions of microbes in the settled material. Using such dust as a representative of the microbial load of larger indoor spaces would in turn lead into severely biased view of the microbiological status of the building.

Deposited viable microbial particles may germinate, grow and proliferate if minimum requirements for temperature, substrate availability and water activity are met. The pres-

ence of organic debris in house dust generally provides sufficient nutrients for growth of saprotrophic fungi and bacteria and the limiting factor for growth becomes the availability of water (Korpi *et al.* 1997). Dust is usually hygroscopic and its water activity [a_w] reaches equilibrium with the RH of the air in the surrounding microenvironment. In the room temperature the minimum a_w required by the most xerophilic fungi is ca. 0.70-0.80 (Grant *et al.* 1989, Flannigan and Miller 2011). Fungal species capable of slow proliferation in such conditions include certain members of Eurotiomycetes like *Eurotium repens*, *Aspergillus penicilloides*, *Penicillium chrysogenum*, *P. brevicompactum* and a few other species, e.g. *Wallemia sebi* (Lustgraaf 1977, Hay *et al.* 1992, Kalliokoski *et al.* 1996). Most of the mould types common in outdoor air, for example *Cladosporium* and *Alternaria* are unable to proliferate in normal indoor conditions but need a liquid water source or near 100% RH to grow (Grant *et al.* 1989). Bacteria require considerably higher water activities, at least 0.90-0.95 for growth (Brown *et al.* 1976).

In dry dust the microbial metabolic activity and growth are negligible and the resulting populations develop through allochthonous processes. However, there are indications that local conditions able to support some level of fungal proliferation may regularly develop even in normal houses. Xerophilic fungi have been shown to form unnoticeable microcolonies on temporarily wetted surfaces (Pasanen *et al.* 1992). Microcolonies provide spores into the surroundings and may explain the low but stable levels of xerophilic fungi commonly measured indoors in cool climates during winter when the outdoor air is not a source of these species (Pasanen *et al.* 1992). Besides bathrooms and other living areas with obvious occasional moisture burden, indoor microbial proliferation may take place also in "dry" room areas in principally safe and stable indoor humidity levels due to a local increase in RH (Harriman 2011). The water activity of dust or other material may increase significantly if the surface temperature is lower than that of the surrounding air. Condensation of water is an extreme example of this phenomenon, but water activities sufficient to support microbial growth do not require condensation to occur. For example a local decrease in temperature by 5°C from 20 to 15°C increases the RH near the cool surface from 60% to 80%, a level sufficient to support slow but steady growth of xerophilic fungi. Such conditions may prevail nearby leaking building corners and seals, cold bridges and insufficiently insulated outer layers of the building envelope. Locally, another significant source of moisture concerns upholstered furniture, especially mattresses. The regular use of this furniture may lead into significant uptake of moisture generated by the users, and subsequently into microbial proliferation in the bound dust (van Reenen-Hoekstra *et al.* 1993, Beguin *et al.* 1995). Mattress dust is often collected for the evaluation of personal microbial exposure in home environment, but it must be remembered that due to the potential local amplification of adapted microbes this sample type may emphasize flora distinct from the remaining building.

Few laboratory studies report the effects of elongated storage in slightly elevated RH on real-life dust microbial communities. Korpi *et al.* (1997) observed significant proliferation of fungi in house dust after 25 days of incubation in 84-86% RH. The proliferation rates differed markedly between species, as over thousand-fold amplification was measured for viable *Aspergillus* but less than hundredfold increases for most other fungi. Incuba-

tion in 75% RH has also been shown to significantly alter the community composition; a ten-week incubation of dust in 75% RH in the room temperature was reported to result in a ten-fold increase in the levels of xerophilic *Aspergillus, Penicillium* and *Wallemia sebi*. In contrast, *Aureobasidium, Fusarium, Geotrichum, Monilia* and *Mucor,* which were present in fresh dust, were lost within four weeks of incubation. After ten weeks the fungal diversity had dropped significantly in all analysed samples and mainly xerophilic taxa *Aspergillus penicilloides*, *Penicillium brevicompactum* and *W. sebi* could be isolated (Hay *et al.* 1992). Even in conditions that do not support fungal proliferation, the mere survival times of microbes affect the dust viable composition, as described in the previous chapter. These reports show that dust may not be a stable reservoir for viable fungi, but instead, the communities may be severely affected by local conditions over time.

1.2.4 Fungal diversity in house dust

***History*.** The first studies on fungal levels and diversity in house dust date back to the 1940s and 1950s. By that time, the major outdoor air spora had already been characterized and associations between fungi and allergic diseases such as asthma symptoms and rhinitis had been detected (for an early review, see Morrow and Lowe 1943). The early studies explored the occurrence of fungi in pillows, furniture stuffings and -covers as well as house dust in relation to asthmatic reactions and allergic sensitization (Conant *et al.* 1936, Flood 1931, Wallace *et al.* 1950, Swaebly and Christensen 1952). Among the first studies to explore dusts collected from the indoor environment, Swaebly and Christensen (1952) reported viable fungal levels of $1x10^4$ - $5x10^5$ cfu g^{-1} of house dust, consisting mainly of *Penicillium* and *Aspergillus*, while other fungi, mainly *Alternaria* and *Cladosporium* were reported to dominate in outdoor air. Significantly higher counts of fungi and bacteria were found in old and used furniture materials compared to new ones, which the authors reported to indicate active growth and sporulation in and on the stuffing over time (Swaebly and Christensen 1952). The authors noted that the fungal levels and types in house dust often differed significantly from those in both indoor and outdoor air. They also reported significant temporal fluctuation in the indoor air mould levels during the day and associated the variation with human activities. These early findings concerning the viable fungal levels; the prevalent indoor and outdoor taxa; the strong variation on fungi in indoor air, and the tendency of indoor materials to accumulate fungal spores over time have since been verified by numerous other studies (Flannigan and Miller 2011).

***Major indoor taxa*.**
Table 1 lists the dominant viable microbial genera in indoor air and dust samples according to the literature. Appendix 1 table gives a detailed list of the fungal species commonly isolated from house and office dust samples using culture methods. Based on a myriad number of culture-dependent studies (see footnote in Appendix 1 for references) supported by a so far limited number of culture-independent reports (Amend *et al.* 2010a, Noris *et al.* 2011) the indoor environment is dominated by a restricted number of globally occurring fungal taxa.

Circa twenty fungal genera and about 200 individual fungal species are commonly isolated from dust by standard cultivation methods (Table 1). Sterile isolates ("*mycelia sterilia*" or "non-sporulating isolates") are also commonly cultivated from dust samples, and

Table 1. Fungal and bacterial genera commonly isolated from indoor samples using culture-based methods

Fungi[a]		Bacteria[b]	
Filamentous, Ascomycota		**Actinobacteria (gram$^+$)**	
Acremonium	*Fusarium*	*Arthrobacter* [1, 2, 3]	*Mycobacterium* [2, 3]
Alternaria	*Pithomyces* *	*Corynebacterium* [1, 2, 3, 4]	*Nocardia* [3, 6]
Aspergillus	*Penicillium*	*Kocuria* [3, 5]	*Rhodococcus* [1, 3]
Aureobasidium	*Phoma*	*Micrococcus* [1, 2, 3, 4, 5, 6]	*Streptomyces* [1, 3]
Chaetomium	*Scopulariopsis*	**Firmicutes (gram$^+$)**	
Cladosporium	*Trichoderma*	*Aerococcus* [2, 3, 5]	*Staphylococcus* [1, 2, 3, 4, 5, 6]
Eurotium	*Ulocladium*	*Bacillus* [1, 2, 3, 4]	*Stomatococcus* [5, 6]
Filamentous, Basidiomycota		*Enterococcus* [3, 4, 5]	*Streptococcus* [2, 3, 4, 6]
Wallemia		**α-Proteobacteria (gram$^-$)**	
Filamentous, Mucoromycotina**		*Agrobacterium* [2, 3]	
Mucor	*Rhizopus*	**γ-Proteobacteria (gram$^-$)**	
Yeasts, Asco- and Basidiomycota		*Acinetobacter* [1, 2, 3, 4, 5, 6]	*Klebsiella* [1, 2]
Candida	*Saccharomyces*	*Aeromonas* [2, 3]	*Moraxella* [1, 2, 3, 4, 6]
Cryptococcus	*Sporobolomyces*	*Chryseomonas* [1, 3]	*Pantoea* [1, 3, 5]
Rhodotorula		*Enterobacter* [1, 2, 3, 5, 6]	*Pseudomonas* [2, 3, 4, 5, 6]
		Erwinia [3, 5]	*Serratia* [3, 5]
		Flavimonas [3, 5]	

[a)] for references, see footnote in Appendix 1. [b)] references: [1)] Andersson *et al.* 1999, [2)] Flannigan *et al.* 1999, [3)] Górny *et al.* 2002a, [4)] Fleischer *et al.* 2003 [5)] Bouillard *et al.* 2005, [6)] Aydogdu *et al.* 2010. Genera occurring in two or more of the listed studies are included in the table. *Syn. *Leptosphaerulina*. **previously: phylum Zygomycetes; Hibbett *et al.* 2007.

form a significant proportion of total counts in many studies (Beguin *et al.* 1999, Chao *et al.* 2002, Hicks *et al.* 2005). Sterile isolates consist of both ascomycetous and basidiomycetous colonies that do not form characteristic spore-forming structures on culture and are thus morphologically unidentifiable. Species common in outdoor and ambient air (*Cladosporium*, *Alternaria*, *Epicoccum*) are prevalent also in dust, but, depending on the collection site, taxa with extended longevity (*Aureobasidium*, *Eurotium*, *Penicillium*, yeasts), large spore/particle size (*Mucor*, *Alternaria*) and/or outdoor soil/debris origin (*Penicillium*, *Fusarium,* yeasts) may be accentuated compared to the ambient air (Hyvärinen *et al.* 1993, Ren *et al.*1999, Chao *et al.* 2002, Chew *et al.* 2003, Horner *et al.* 2004).

1.2.5 Bacterial diversity in house dust

Bacterial genera commonly isolated from indoor air and dust samples in a selection of culture-based studies are listed in Table 1. Contrasting to fungi, the major source of bacteria in indoor environment is humans; the human normal flora constitutes mainly of bacteria, which cover all human body sur-

faces and are shed to the environment alone or in fomites (Sciple *et al.* 1967). Accordingly, indoor bacterial levels have been shown to be significantly increased by the number of occupants (Bischof *et al.* 2002, Giovannangelo *et al.* 2007). The majority of dustborne bacteria consists of members of gram-positive phyla Actinobacteria and Firmicutes. Gram-negative bacteria, mainly Proteobacteria are also present. Moreover, recent culture-independent studies have indicated the presence of members of the Bacteroidetes and other groups, which occur in lower numbers (Pakarinen *et al.* 2008, Täubel *et al.* 2009, Noris *et al.* 2011). Actinobacteria, Firmicutes and Bacteroidetes dominate on the human skin (Grice *et al.* 2009). The recent study by Täubel *et al.* (2009) confirmed that the vast majority of 16S marker sequences obtained from matress dust corresponded with those of the users' skin flora. A considerable, yet weaker contribution of human-associated bacteria was seen in floor dusts. The dominant human-associated genera in dust included *Corynebacterium*, *Propionibacterium, Staphylococcus, Lactobacillus* and *Streptococcus* (Täubel *et al.* 2009). In addition to humans, pets may be major sources or carriers of bacteria indoors; Fujimura *et al.* (2010) reported a dog-associated increase in bacterial diversity, majority of which was putatively associated with an increased import of bacteria from outdoors.

In addition to the inhabitants, outdoor air is a significant source of bacteria. The recent study by Noris *et al.* (2011) indicated that especially gram-negative bacteria in house dust were of non-human, putatively outdoor origin. This was supported by the fact that the proportion of gram-negative bacteria was substantially higher in an unoccupied test house than in occupied houses, where the amount of gram-positive bacteria was significant (Noris *et al.* 2011). The dominance of gram-negative bacteria, especially of proteobacterial classes in outdoor air has been demonstrated in several studies (Fierer *et al.* 2008, Brodie *et al.* 2007, Fahlgren *et al.* 2010).

To our knowledge, the presence or diversity of Archaea in indoor environments has not been studied. Since archaea occur in soils, aquatic environments and also in the human gut, they would probably be found also in the indoor environment using suitable methods. In the investigation by Brodie *et al.* 2007, 307 archaeal taxa were detected in urban outdoor air using DNA-based methods. The majority of the detected arhaea were members of Euryarchaeota (Brodie *et al.* 2007).

1.2.6 Interactions between fungi and mites in the indoor environment

Moist indoor substrates, especially building materials that are constantly wet due to water damage may maintain diverse ecosystems with various microbes and e.g. mites and amoebae. The predominant mites found in buildings are house dust mites (HDMs) and storage mites (SM). The dominant HDM species *Dermatophagoides pteronyssinus*, *D. farinae* and *Euroglyphus maynei* belong to the family Pyroglyphidae ("pyroglyphid mites"), while most SM species, ie. *Glycyphagus domesticus*, *Tyrophagus putrescentieae* and *Acarus siro* are members of Glycyphaginae and Acaridae. In addition, tens of other, less frequent mite species belonging to these and other families within the subclass of Acari are found in the indoor environment (van Asselt 1999). Allergic sensitization to both dust- and storage mites is common in both rural and urban environments (Arias-Irigoyen 2007, Pennanen 2002). HDMs, especially *D. pteronyssinus* are often considered to be the major sources of domestic mite allergens due to their

predominance in house dust samples (Arlian *et al.* 2002). However, in cold climate regions, including Scandinavia, HDMs are rare due to the low indoor air RH. In such environments SMs may be of more importance, especially in the context of water damaged problem buildings (Warner *et al.* 1999, Charpin *et al.* 2010).

HDMs thrive in dark and warm sites where protein-rich substrate such as human dander is available. HDMs are thus the dominant mite type found in mattresses and padded furnitures. They are able to grow in lower RH than fungi (RH >55% in RT). In contrast, SM species require RH levels similar to fungi (RH >80%) and thrive in the same microenvironments. High numbers of storage mites, especially *G. domesticus* have been found from cellars and other home areas with high humidity, as well as from stored foods (Ishii *et al.* 1979, Mehl 1998, van Asselt 1999). Recently, mouldy interior wall surfaces colonized by *Cladosporium*, *Aspergillus*, *Ulocladium*, *Alternaria*, *Penicillium* and *Acremonium* were reported to be commonly co-infested by storage mites (Charpin *et al.* 2010). Dense mite populations may also develop on e.g. gypsum board in moist wall cavities, where the mites graze on fungal growth, e.g. *Stachybotrys chartarum* and *Acremonium* spp. (Scott 2001). Mites are known to feed on fungal mycelium and spores, yet the dietary preferences for different fungi may vary between mite species. Fungal spores are partially digested by mites and excreted in the mite faeces. Intact spores may germinate in the excreted faecal pellet, which provides nutrients for growth even in the absence of other substrate. Thus mites contribute to the dispersal and proliferation of indoor fungi (Colloff 2009).

1.3 Moisture damage and indoor microbial communities

1.3.1 Microbial findings associated with moisture damage

The effect of moisture damage on dust- and airborne microbial communities has been assessed in several studies in order to identify probable causative agents for observed health impacts for research purposes, or to detect useful indicators of moisture problems for building diagnostics. To summarize, the effect of water damage varies greatly. In culture-dependent studies, correlations seem to be observed more often for air than dust samples (e.g. Hyvärinen *et al.* 1993), but culture-independent methods have revealed strong correlations for dust samples (e.g. Lignell *et al.* 2008, see chapter 1.4.4). In general, large areas of visible mould usually cause significant changes in viable indoor microbiota and the airborne microbial levels may clearly correlate with the severity of the damage (Green *et al.* 2003, Lignell *et al.* 2008). In contrast, damage hidden inside the building cavities may not raise the viable fungal levels notably (Miller *et al.* 2000). In large data sets the mean concentrations of airborne viable fungi are often higher in moisture damaged than in undamaged buildings (Pasanen *et al.* 1992, Verhoeff *et al.* 1992, Li and Kendrick 1995a, Garrett *et al.* 1998, Lawton *et al.* 1998, Hyvärinen *et al.* 2001, Green *et al.* 2003). However, the concentration distributions of damaged and undamaged buildings are often largely overlapping (Nevalainen *et al.* 1991, Hyvärinen *et al.* 2001) and in some studies no association between damage and viable fungal concentrations has been found (Strachan *et al.* 1990, Chew *et al.* 2003). Instead, increased diversity and/or changes in the microbial types have been found in some studies (Miller *et*

al. 2000, Nilsson *et al.* 2004, Baudisch *et al.* 2009). However, in some cases, no detectable associations between damage and microbial concentrations or types have been seen (Ren *et al.* 2001, Chew et a. 2003, Piecková *et al.* 2004). Despite the variable findings, detection of certain species that are seen to occur rarely in normal indoor environment in culture has been concluded to be highly indicative of moisture problems in most cases (Samson *et al.* 1994). The first moisture damage indicator list was compiled in an international workshop of microbiologists (Samson *et al.* 1994) and included the following species or genera: *Aspergillus fumigatus*, *A. versicolor*, *Exophiala*, *Eurotium*, *Fusarium*, *Penicillium* spp. (e.g. *P. chrysogenum* and *P. aurantiogriseum*) *Phialophora*, *Rhodotorula*, *Stachybotrys*, *Trichoderma*, *Ulocladium* and *Wallemia*. This list, sometimes supplemented by additional genera such as *Botrytis*, *Chaetomium*, *Paecilomyces* and *Rhinocladiella* is commonly used as the "field guide" for evaluating the microbiological status of a building using air sampling.

Besides fungi, the occurrence of certain bacteria has been observed to be indicative of moisture problems (Rintala *et al.* 2004). Members of the Actinobacteria, especially genera *Streptomyces*, *Pseudonocardia* and *Nocardiopsis*, but also some *Mycobacterium* spp. (see also discussion on bacterial findings in dust), commonly grow on moist building materials (Rintala *et al.* 2002, Torvinen *et al.* 2006, Schäfer *et al.* 2009, Suihko *et al.* 2009). Many of these genera include known producers of various bioactive secondary metabolites and VOCs and are also capable of releasing aerosolizeable spores and mycelial fragments to the surrounding air (Schöller *et al.* 2002, Górny et a. 2003) and may thus be of importance with respect to occupant health. Schäfer *et al.* (2009) detected matching phylotypes of genera *Brevibacterium*, *Streptomyces*, *Nocardia*, *Nocardiopsis* and *Micrococcus* in parallel material and bioaerosol samples collected from moisture damaged buildings. However, Actinobacteria are also common in soil and plant material, and despite their status as indicator microbes for moisture damage (Samson *et al.* 1994), their abundance indoors may also represent other sources (Johansson *et al.* 2011).

In recent years, increasing attention has been paid to fragmented spore and vegetative cell material released from fungi and filamentous actinomycetes growing on indoors materials (e.g. Górny 2004). Since fragmented material loses its viability faster than intact spores, it is less efficiently detected by cultivation and may be largely overlooked. However, the mass of released fragments may be comparable to that of released spores, and fragments may outnumber spores by several orders of magnitude due to their small size (Reponen *et al.* 2007). Fine fragments of < 2.5 µm have been shown to be released from microbial growth on indoor materials and to be present in moisture damaged indoor environments (Górny *et al.* 2002b and 2003, Brasel *et al.* 2005b, Cho *et al.* 2005, Reponen *et al.* 2007). Like spores, fragmented cell material may carry toxic and antigenic compounds and, due to the small size, may be efficiently deposited in the human airways (Górny *et al.* 2002b, Brasel *et al.* 2005b, Green *et al.* 2005, Cho *et al.* 2005). Fungal fragments have been assessed by the measurement of N-acetyl-β-D-glucosaminidase (NAGase) and (1 → 3)-β-D-glucans in the fine particle fraction (Madsen *et al.* 2009, Reponen *et al.* 2007). Microbial fragments have also been shown to contain nucleic acids (Madsen *et al.* 2009), which makes them feasible targets for PCR based detection methods.

1.3.2 Seasonal variation and indoor sampling

In the subarctic climate the effect of outdoor microbial sources on indoor microbial assemblages is at their minimum during winter due to frozen ground and snow-cover. The indoor airborne concentrations of viable fungi have been shown to be very low then, typically between 10^1 - 10^2 cfu/m^3. Due to low background, unnormal intramural microbial sources are most visible and most easily detected then; thus, restricting air sampling to winter months is strongly recommended (Reponen *et al.* 1992). However, if long term samples are collected and analysed using culture-independent techniques, microbial material persisting from past seasons may be detected even in winter samples, which may undermine the detection of fresher material emitted from intramural sources. Textile-covered furniture are known to accumulate and maintain even viable microbes over long time periods, but little is known about the persistence and seasonality of non-viable microbial materials in indoor matrices such as dust over extended time periods. The recent study by Kaarakainen *et al.* (2009) using qPCR indeed suggested that differential seasonal loads of fungi are better represented in floor than rug dust.

1.4 Molecular methods in microbial biodiversity studies

The advances in molecular technologies have revolutionized the research of both fungal and bacterial ecology during the last two decades. The key steps have been the establishment of protocols for universal amplification and sequencing of phylogenetically informative gene regions, mainly within ribosomal DNA (eg. Edwards *et al.* 1989, White *et al.* 1990, Jürgens *et al.* 1997); the development of high-throughput capillary sequencers and upscaled pipelines and equipments for processing large DNA clone libraries; and recently, the advent of highly parallel "next-generation" sequencing methods. The latter enable the sequencing of hundreds of thousands of DNA fragments without previous separation by cloning (Margulies *et al.* 2005).

Based on the comparison of direct microscopy analysis and cultivation of microbes, it has been long known that a vast majority of microbial cells are uncultivable in laboratory conditions (Staley and Konopka 1985, Rappe and Giovannoni 2003). This "uncultivable majority" may include a) dead and dormant cells or spores of well-known, cultivable species, b) cells of microbial species that require specific enrichment and isolation techniques to be detected and c) species whose growth is inhibited by competing species on culture plates (Amann *et al.* 1995). Recently, direct DNA-targeting methods that circumvent the requirement of monoculture isolation for characterization have made each of these uncultivable categories accessible for phylogenetic analysis. Thus it is no wonder that molecular, cultivation-independent techniques have become routine tools for microbial community characterization and monitoring. Today the list of explored environments is very long, extending from soils (Fierer *et al.* 2007, Urich *et al.* 2008) and plant ecosystems (Neubert *et al.* 2006) to the atmosphere (Fröhlich-Nowoisky *et al.* 2009); and from the human skin (Costello *et al.* 2009) to domestic environments such as shower curtains, drains and even toilet rims (McBain *et al.* 2003, Kelley *et al.* 2004, Egert *et al.* 2009).

Each of these studies has been efficient in providing information about the diversity

and structure of microbial communities present. In most cases, the observed diversity has greatly exceeded the known viable diversity. For example, using an RNA-based metatranscriptomic approach to assess soil biodiversity Urich *et al.* (2008) showed that nineteen out of 24 validly described bacterial phyla were present in a soil sample collected from a single site. Fierer *et al.* (2007) in turn reported that the level of fungal diversity in soils was comparable to the bacterial diversity in soil. They estimated that the total number of fungal phylotypes could exceed 10^4 in some soil types. Using PCR and ribosomal DNA sequencing O'Brien *et al.* (2005) recorded a high number (412) of fungal phylotypes in two 1000 m^2 forest plots at a single sampling. The diversity is in line with that of an equally sized forest area previously recorded by a twelve-year sampling and identification of 71,222 fruiting bodies (O'Brien *et al.* 2005).

In addition to elucidating the habitats and ecological roles of previously cultured organisms, culture-independent studies have revealed the existence of previously unknown phylotypes and even entirely novel microbial lineages, some of which have been later isolated in culture. The most famous example may be the alphaproteobacterial group *SAR11* which constitutes up to 50% of the bacterioplankton in open-ocean surface waters and has been estimated to be the most abundant bacterial taxon on Earth (Morris *et al.* 2002). This wide-spread clade was first detected by direct 16S rDNA amplification and sequencing in the beginning of 1990s from the Sargasso Sea (Giovannoni *et al.* 1990) but the first member of this cluster was not isolated in culture until a decade later (Rappe *et al.* 2002). Similarly, a globally distributed root-associated fungal clade known as the *Soil Clone Group 1* (SCG1) was long known solely from molecular data (Schadt *et al.* 2003). Only very recently, the first cultivated member of the clade was described. Based on characterization of the isolates by their morphology and multilocus gene sequencing and subsequent clustering with environmental sequences, an entire novel ascomycete class Archaeorhizomycetes was proposed (Rosling *et al.* 2011). These fungi are slow growing, have inconspicuous morphology and lack sporulation in culture but are ubiquitous in various soils. These examples show that ecologically significant uncultivable or difficult to culture microorganisms are found from common and non-specific habitats such as sea water or soil.

As described, molecular techniques utilizing the direct analysis of phylogenetically informative sequences from total environmental nucleic acid extracts without prior microbial isolation have proven to be more efficient and much less selective methods compared to traditional cultivation methods. However, molecular methods have their own limitations and sources of bias, which can affect the results. The limitations applicable to the present work are reviewed in chapter 1.4.2.

1.4.1 Methods for DNA-based microbial community analysis

Principles. The phylogenetic and functional diversity of microbial communities can be efficiently assessed through the analysis of amplified marker gene fragments (amplicons) obtained by PCR from total community DNA extracts. The targeted genes can be either phylogenetically informative regions of the genome, such as ribosomal genes, or functionally (and often also phylogenetically) informative genes that code proteins linked to specific metabolic functions (Purkhold *et al.* 2000). *Universal* PCR primers, i.e. primers designed

to amplify the target region from all or most divisions within the kingdom of interest (fungi/bacteria/archaea) are often preferrable for total community analysis, yet also group-specific primer sets can be used (e.g. Gardes and Bruns 1993). In practice, universal primers target highly conserved regions flanking more variable stretches, which in turn contain sufficient amount of phylogenetic information for identification and classification purposes. The present consensus regions for phylogenetic purposes are the 16S rRNA gene for bacteria (Edwards *et al.* 1989, Woese *et al.* 1990), and the nuclear Internal Transcribed Spacer (ITS or nucITS) region for fungi (White 1990, Schoch *et al.* 2011).

PCR amplicon analysis. The obtained PCR amplicons are typically analysed either by fingerprinting- or sequencing techniques, as discussed below in more detail. Also, specific micro- or macroarrays may be set up to enable hybridization-based detection of DNA signatures at species level or at a higher taxonomic level (Wu *et al.* 2003, Brodie *et al.* 2007, Hultman *et al.* 2008, Jakobs *et al.* 2010). Another related solution for amplicon characterization is the hierarchical oligonucleotide primer extension method (HOPE), basically a multiplexed minisequencing technique coupled to fluorescent capillary electrophoresis, which enables the semiquantitative detection of multiple target groups from environmental samples (Wu and Liu 2007). Probing-based methods can be very sensitive and fast, but can only detect a limited number of pre-determined species or higher groups, for which specific probes need to be designed and tested for specificity.

Fingerprinting methods. Fingerprinting methods, including temperature- and denaturing gradient gel electrophoresis (TGGE and DGGE), terminal fragment length polymorphism (tRFLP) and single-strand conformational polymorphisms (SSCP) analysis offer fast and cost-efficient ways to perform rough comparisons of microbial communities in multiple samples in parallel (Widjojoatmodjo *et al.* 1994, Marsh *et al.* 1999, Muyzer *et al.* 1999, Anderson *et al.* 2004). The main drawback of fingerprinting methods is their low detection threshold or, more precisely, their low dynamic range. Fingerprinting methods also lack resolution, which may lead into further underestimates of species richness. Due to the lack of resolution, the identity of detected DNA fragments (bands or peaks) should also be verified by sequencing if phylogenetic community data is desired. Fragment extraction from DGGE gels and sequencing is sensitive to contamination and the quality of obtained sequences is often reduced (Anderson *et al.* 2004). Some of these problems can be overcome by combinatory approaches: for example, parallelization of DGGE and clone library sequencing makes it possible to affiliate phylogenetic information from long, good quality sequences to individual DGGE fragments (Liang *et al.* 2008). Recently, a novel technique that combines HPLC-based separation of PCR-amplicons with DNA fraction collection and sequencing was successfully applied for the characterization of wood decay fungi (Maurice *et al.* 2011). This method shows promise in facilitating fragment sequencing, but it remains to be seen how well this approach works on highly diverse communities. Generally, fingerprinting methods are suitable for monitoring major changes in microbial community composition, but are not ideal for detailed *de novo*-characterization of environmental samples (Brodie *et al.* 2003).

Sequencing methods. The full phylogenetic information carried in the amplified marker genes can be obtained by DNA sequencing. The analytical sensitivity of sequencing methods in detecting individual species within a community depends on the number of obtained sequences, and naturally also on the diversity and structure of the target community. For communities with low species richness and high evenness a small number of sequences is sufficient to provide coverage over the majority of species. In contrast, for the characterization of diverse communities with uneven species distribution more sequences are needed. Until the very recently introduced "next generation" sequencing methods (see below), the PCR amplicon sequencing has been done by cloning the PCR amplified fragments into an *E. coli* plasmid library. While the contruction of large plasmid clone libraries is relatively inexpensive, obtaining sequence data from the clones using the common Sanger chemistry (Sanger *et al.* 1977) and capillary electrophoresis is costly and time-consuming. Since many natural microbial communities have long-tailed rank abundance distribution of species (i.e., there are only few abundant species, but a large number of rare species; Gans *et al.* 2005) obtaining a reasonable coverage over the rare species necessitates the repeated sequencing of the abundant types. To avoid this, methods for pre-sequencing screening of identical clones have been needed. Fingerprinting methods, mainly amplified ribosomal DNA restriction analysis (ARDRA), have been applied to cluster clones into phylotypes prior to sequencing (e.g. Fröhlich-Nowoisky *et al.* 2009, Fujimura *et al.* 2010). However, as mentioned above, fingerprinting methods suffer from the lack of resolution, ending up clustering unrelated sequences together (Sklarz *et al.* 2009). Moreover, they easily also end up being time-consuming and expensive. If the dominant members of a community are known, an alternative is to design specific oligonucleotide probes for them and remove them from the amplicon pool by subtractive hybridization. However, this method does not provide information about the original abundance of the subtracted sequence types in relation to other sequence types.

"Next-gen" sequencing methods. At present, the "next-generation" sequencing methods, especially the 454 pyrosequencing method (Margulies *et al.* 2005) offer an appealing alternative to Sanger sequencing and are becoming increasingly popular. The 454 pyrosequencing method originally suffered from short (100 bp) sequence read lengths which were insufficient for phylogenetic purposes. Sequencing errors have also been of more concern in the pyrosequencing method (Kunin *et al.* 2010, Tedersoo *et al.* 2010). Recent amendments have significantly improved the usability of the methodology in microbial diversity studies. The 2008 update to the "Titanium" chemistry yields longer (~400bp) read lengths and tag-sequence based multiplexing makes it possible to analyse multiple samples in one run. Improved algorithms for clearing out the ambiguous sequences have improved the quality of the data (Huse *et al.* 2010, Quince *et al.* 2011). With the present Roche 454 GS FLX System, ca. 100-200 samples can be conveniently processed within one sequencing run, producing ca. 5 000 - 10 000 reads of 400 bp from each sample. While the enormous throughput and speed are the clear advances of the method, one disadvantage of pyrosequencing compared to clone library sequencing is that the sequenced DNA itself is not stored for later use in e.g. the design and testing of qPCR or probing methods. Concern-

ing highly parallel pyrosequencing it also is notable that typical pyrosequencing data sets cannot be handled with the traditional desktop tools for sequence analyses but, instead, require significant computational power and efficient bioinformatics tools (e.g. Schloss *et al.* 2009).

Quantitative PCR. Quantitative PCR is a popular and highly accurate and reliable method for the quantification of gene copy number or gene expression (Giulietti *et al.* 2001). The quantitativity is obtained by monitoring the accumulation of fluorescently labeled PCR product in real time and comparing it with a standard curve obtained from a series of control amplifications of known amounts of the target gene. While qPCR is highly sensitive and fast, the number of species targetable in parallel is limited, and untargeted species are not detected. The specificity depends on the used method (e.g. the commonly used SybrGreen and TaqMan methods differ in their specificity) and each assay needs to be tested in vitro with target and nontarget sequences. The detection limit (cells/spores per reaction) of qPCR varies between sample types and assayed species due to e.g. the differential extractability of DNA and the presence of PCR inhibitors. Detection limits ranging from less than one to several hundreds of cells/spores per reaction have been reported (Haugland *et al.* 2004, Kärkkäinen *et al.* 2010).

1.4.2 Limitations and challenges of DNA-based methods in community characterization

In the optimal case, sequencing results would fully reproduce the original species frequencies in the analysed sample. However, in practice the results from whole community analyses incorporating a PCR amplification step are semiquantitative in nature; due to an unequal amplification of sequences that differ in length, nucleotide composition and/or GC content, the proportions of amplification products do not always mirror the original species frequencies in the sample (Suzuki and Giovannoni 1996, Polz and Cavanaugh 1998, Amend *et al.* 2010b). These biases may affect more severely the analysis of fungal ITS sequences compared to bacterial 16S sequences due to the stronger length- and sequence variation of the former (Huber *et al.* 2009, Amend *et al.* 2010b). The results may also be affected by primer mismatches and the differential gene copy number (von Wintzingerode *et al.* 1997). The latter concerns especially ribosomal genes, which are located in repeated operons whose number may vary up to five-to-ten-fold between species in both bacteria and fungi (Herrera *et al.* 2009, Rastogi *et al.* 2009). Thus phylotype frequency results from PCR based analyses cannot be directly translated to relative species frequencies, but instead, should only be treated as rough indications of relative phylotype abundances. However, in spite of potential biases, the bias introduced by PCR and other steps of molecular community analysis has been considered to be much smaller than the selective bias typical of cultivation-based methods (Prosser *et al.* 2010).

The quality of ribosomal libraries can also be affected by artificially formed DNA sequences (Jumpponen *et al.* 2007). Such artifacts include chimeras and sequencing errors, which are formed during the PCR amplification and also to some extent during the cloning and transformation process (von Wintzingerode 1997, Thompson *et al.* 2002). Chimeras have been found to constitute significant proportions of some environmental clone libraries (Jumpponen *et al.* 2007), and

have aso been found to accumulate in public DNA databases (Hugenholz and Huber 2003, Tedersoo *et al.* 2011).

The annotation of environmental sequences itself is a critical step in the molecular identification process of microbes, and may easily produce misleading results for various reasons. Thus far, the taxonomic annotation of marker gene sequences has relied on either pairwise queries against nucleotide databases using local or global alignment tools (such as Blast and Fasta; Pearson and Lipman 1988, Altschul *et al.* 1990), and subsequent identification of the nearest relative based on sequence similarity. Another method, which is presently available for microbial ribosomal genes but not for the intervening hypervariable spacer regions or protein coding genes, is based on a hierarchical Bayesian classifier, which compares the occurrence of 8-mer subsequences between the query sequence and sets of classified sequences (The RDP Classifier; Wang *et al.* 2007). The methods relying on pairwise similarity searches are severely challenged by the presence of mis-annotated reference sequences in the International Nucleotide Sequence Database (INSD) (Nilsson *et al.* 2006). Moreover, concerning especially the fungal identification, the substantial variation in the intra- and inter-species variability within the nucITS region, plus the complex taxonomy of Fungi pose some additional challenges; hundreds of fungal species have multiple names for genetically identical morphotypes within species (which may represent the sexually reproducing teleomorhp form of the species and/or one or more asexually reproducing anamorph forms). Parallel names may also represent historical synonymes that cannot be solved due to the absence of type strains. In general, fungal taxonomy has gone through major revisions after the recent advances in molecular taxonomy (Hibbett *et al.* 2007) and various phyla have been re-classified and renamed.

Furthermore, it is notable that the concept of "species" among clonally reproducing organisms such as bacteria and many anamorphic fungi is questionable since "species" can no longer be determined as "a group of individuals capable of interbreeding" as for sexually reproducing organisms. Instead, "high level of genetic similarity between the individuals" is used as a proxy for conspecificity. In bacteria, an arbitrary limit of 97% 16S sequence similarity is often used to delineate species level operational taxonomical groups (OTUs). However, when compared with a more reliable determinant, such as the overall DNA similarity between two species, it has become apparent that these two determinants do not necessarily correlate well (Stackebrandt *et al.* 2006). Also in Fungi the sequence similarity threshold is problematic; in some genera, the intraspecies variability can be several percentages on the nucITS region, while some genera, such as *Penicillium*, harbour clusters of related species that contain identical or nearly identical ITS sequences. Moreover, observations of intragenomic rDNA sequence variation in fungi have been recently made, which may lead into inflated diversity estimates and false conclusions of the presence of "cryptic species" in DNA based microbial community studies (Lindner and Banik 2011). The described phenomena call for both similarity and phylogeny based annotation of environmental sequences, if both resolution and sensitivity are wanted. Recently, novel bioinformatics solutions have become available, which use the identification of the "lowest common ancestor" among the blast-hits, and show promise in facilitating the dynamic, automated analysis of environmental sequences (e.g. "the FungalITSPipeline",

MEGAN, CLOTU; Nilsson *et al.* 2009, Huson *et al.* 2009, Kumar *et al.* 2011). Furthermore, recent efforts towards parsing large sets of publicly available reference sequences into curated databases will greatly improve the reliability of the annotation results (Tedersoo *et al.* 2011).

1.4.3 DNA-based methods in indoor microbial community analyses

Molecular methods were first applied in indoor research in the late 1990's. In a series of cases, a toxigenic fungal species *Stachybotrys chartarum* was associated with severe forms of building related illness (Dearborn *et al.* 1999). Culture-based detection of this species was found to be problematic, but it could be enumerated from samples in a sensitive and reproducible manner using qPCR (Haugland *et al.* 1999b). Since then, qPCR assays have been designed for the enumeration of other indoor fungi, and qPCR is practically the only DNA based method that has been widely used in indoor studies. A large set of qPCR assays has been designed by the US Environmental Protection Agency (e.g. Haugland *et al.* 2004, Meklin *et al.* 2004). At present, the assays cover over 100 mould species commonly encountered in indoor environments (see: http://www.epa.gov/nerlcwww/moldtech.htm). In addition to fungi, protocols for detecting indoor relevant bacteria, including *Mycobacterium tuberculosis* and *Legionella* spp. (Pascual *et al.* 2001), environmental mycobacteria (Torvinen *et al.* 2010) and *Streptomyces* spp. (Rintala *et al.* 2004) have been published. In order to assess the total concentration of fungi and bacteria, universal qPCR methods have been designed (Haugland and Vesper 2002b, Kärkkäinen 2010, Yamamoto 2010, Chemidlin Prévost-Bouré *et al.* 2011). However, the concept of "universal quantitative PCR" is somewhat ambiguous since the assay quantitativity may be questionable if the target DNA contains variable regions that amplify unequally between species, or if the target copy number differs between species (Haugland *et al.* 1999a).

QPCR has been successfully used to elucidate the total counts of specific fungi and streptomycetes in e.g. indoor dusts (Meklin *et al.* 2004, Rintala *et al.* 2004, Lignell *et al.* 2008, Kaarakainen *et al.* 2009) and building material samples (Pietarinen *et al.* 2008), as well as to compare mould concentrations in indoor and outdoor air (Meklin *et al.* 2007). The differences between microbial levels in farming and non-farming homes have also been studied (Kärkkäinen *et al.* 2010). In general, the studies have indicated higher prevalences of occurrence for individual species, and 2-3 orders of magnitude higher total cell concentrations than viable methods, showing that viable methods underestimate both diversity and concentration of microbes. Using qPCR, several studies have also reported elevated fungal or bacterial cell counts in dust in association with water damage in buildings, yet the species in question have varied between studies. Lignell *et al.* (2008) reported a significant correlation between the extent of visible moisture damage and cell counts of specific fungi, including *Penicillium brevicompactum*, *Wallemia sebi* and *Trichoderma viride* group in dust samples. Bellanger *et al.* (2009) in turn observed significantly higher concentrations of *Cladosporium sphaerospermum* in the air and surface samples collected from moisture damaged homes than reference homes.

In their study of homes of infants who had developed a severe, potentially building-associated adverse lung condition, Vesper at al. (2004) observed several times higher counts of several mould species than in con-

trol homes, including *Trichoderma viride*, *Aspergillus fumigatus*, *A. ochraceus* and *S. chartarum*. The homes of the children were repored to be severely water damaged (Etzel *et al.* 1998, Dearborn *et al.* 1999). Meklin *et al.* (2004) studied a larger set of homes with or without moisture damage in the same geographic region, and found elevated concentrations of some of the fungal species observed by Vesper *et al.* (2004), yet in the differences between damaged and undamaged houses were less profound than those observed by Vesper *et al.* Based on the results obtained by Vesper *et al.* (2004), a qPCR-based index, the "Environmental Relative Moldiness Index", (ERMI) was developed to describe the indoor fungal burden from putatively indoor-contamination indicating fungal taxa for US homes (Vesper *et al.* 2007). The index was designed to document increases in both the diversity and the concentrations of "moisture indicator" taxa. However, despite the elegant design of the index, the correlation between ERMI and the extent of moisture damage (Reponen *et al.* 2010) and between ERMI and various health effects (Mendell 2011) remains unclear. Recently, however, Reponen and colleagues (2011) reported a significant positive association between elevated ERMI values and the development of asthma. These studies show that qPCR may be a highly efficient tool for detecting elevated microbial concentrations indoors. However, in order to use qPCR in "building diagnostics", more robust information is needed on the sources and local occurrence of different microbial species and their associations with moisture damage. It is also notable that the assayed species themselves have been selected based on information obtained almost solely from cultivation studies. Based on information obtained from culture-independent studies from other environments, it is possible that significant indoor species exist, which have not been identified as such due to their poor culturability. The existence of such species can be assessed by studying indoor samples in detail using unselective DNA based methods such as direct sequencing approaches.

1.4.4 Biomarker analyses used in indoor microbial studies

Several structural compounds typical of bacterial and fungal cells ("biomarkers") have been used as proxies for microbial biomass in the assessment of indoor microbial exposure (Pasanen 2001). Biomarkers for fungi include ergosterol and (1-3)-β-D-glucan the main component of fungal cell membrane and -wall (Pasanen 2001, Gehring *et al.* 2007). As for bacteria, lipopolysaccharide (LPS), the main component of gram-negative cell wall is a common biomakrker for this bacterial group, while the measurement of N-acetylmuramic acid is used to evaluate the abundance of gram-positive bacteria (Sebastian and Larsson 2003). Especially LPS and (1-3)-β-D-glucan have been seen to reflect the relevant part of indoor microbial exposure since these compounds launch inflammatory responses in humans (Pasanen 2001). However, while biomarker analyses are suitable tools for a crude assessment of microbial concentrations, they do not differentiate between microbial species, which is crucial information e.g. in the evaluation of the sources or potential health effects of the studied microbes.

2 AIMS OF THE STUDY

The main aims of this study were:

1. **To perform a detailed characterization of indoor fungal and bacterial communities in Finnish office buildings using cultivation-independent methodology on long-term samples of settled particulate material.** Based on previous studies from other environments, molecular methods can reveal a substantially wider proportion of the total microbial diversity than culture based methods.

2. **To characterize the seasonal variation occurring in these communities using molecular methods.** Viable concentrations of fungi are known to vary seasonally, which limits the use of culture methods in building investigations during the growth season. However, no such information is available for uncultivable microbial particles.

3. **To investigate the variation in molecular communities within and between sets of moisture damaged and undamaged buildings.** Moisture damage does not always manifest itself in altered cultivable microbial communities but adverse heath effecs are nevertheless experienced. Major changes in total microbial communities typical of damaged buildings were sought. Offices were chosen since the effect of human activities on microbiomes is smaller in offices than e.g. homes.

4. **To compare the results from microbial community analysis obtained using culture dependent and culture-independent methods.** Three methods: (a) a traditional method (plate cultivation), (b) an unselective, semiquantitative DNA-based method (clone library sequencing) and (c) a quantitative, species-specific DNA-based method (qPCR) were used and compared.

5. **To develop a method for enhanced identification and sequencing of rare phylotypes in environmental clone libraries.** Such method would enable faster and more cost-efficient sequencing of large environmental clone libraries.

3 MATERIALS AND METHODS

3.1 Buildings and samples

For the characterization of indoor microbiomes, samples were collected from six small office buildings in three locations in central Finland (Table 1). Three of the targets were moisture- and mould damaged buildings and the remaining three buildings were age-, building frame-, ventilation type- and usage matched, paired controls that were not moisture damaged. Long-term samples of settled dust were collected from mainly uncarpeted floors (II, III) or elevated surfaces (I). Dust samples were collected by vacuuming several m^2 of smooth surfaces twice a week over ca. 1-2 months in order to gain a representative long-term sample of the studied space. A room from the complaint area, yet without visible signs of mould, in the moisture damaged buildings was selected for sampling, and a room matching the type of use was sampled in the reference building. Samples representing each of the four seasons were collected over one year from one building pair (II, III), while the effect of moisture damage renovation was followed in two building pairs by collecting samples pre- and post-remediation (I). Building material samples were collected from the two damaged buildings during their renovation (I).

For the development and testing a macroarray hybridization method for enhanced clone library screening (IV), microbial communities in biowaste compost samples were used as study material. Five separate compost samples representing two different composting scales (pilot and full) and two facility types (drum and tunnel) were collected from two municipal composting plants.

Table 2. Buildings and samples studied for the characterization of indoor microbial communities. Detailed description of the buildings and sampling methods are given in corresponding studies.

Building	Frame type, condition	Location	Samples (n)	Study
B1 (1, In)[1]	brick, moisture damaged	3	floor dust (4)	II, III
B2 (2, Re)[1]	brick, undamaged	3	floor dust (4)	II, III
B3 (In1)[1]	wood, moisture damaged	1	surface dust (2), building material (7)	I
B4 (Re1)[1]	wood, undamaged	1	surface dust (2)	I
B5 (In2)[1]	concrete, moisture damaged	2	surface dust (2), building material (9)	I
B6 (Re2)[1]	concrete, undamaged	2	surface dust (2)	I

[1]The name used to refer to the building in the corresponding study/studies.

3.2 Experimental methods

An overview of the methods applied in this study is given in Table 3. A detailed description of the microbial community analysis protocols is shown in Figure 2.

3.2.1 Microbial community characterization

The microbial communities in dust samples (I, II, III) were determined, enumerated and compared using culture-dependent and culture-independent methods as described in Figure 2. Fungal communities in building material samples (I) were studied using plate cultivation and clone library sequencing as described in Figure 2.

3.2.2 Development and evaluation of a negative macroarray hybridization method for enhanced screening of fungal diversity in environmental samples (IV and unpublished results)

The principle of the macroarray method for detecting, enumerating and negative selection of abundant phylotypes in clone libraries is presented in Figure 3. The suitability of this method for the analysis of indoor samples was assessed using sequence data obtained from study II as follows: analogous to the protocol presented in study IV, the most abundant phylotypes in indoor dust samples were identified and their summed proportions were calculated for each sample and for the combined data. The expected savings in time and expenses were then evaluated based on the estimated reduction in numbers of sequenced clones.

Table 3. An overview on the experimental methods used in this thesis. Detailed descriptions of the methods are given in corresponding studies.

Method	Study
Fungal community characterization by nucITS clone library sequencing	I, II, IV
Bacterial community characterization by 16S rRNA gene clone library sequencing	III
Species-specific quantitative PCR	I, II
Viable fungal community characterization by plate cultivation	I, II
Fungal biomass measurement by ergosterol analysis	I, II
Macroarray probe design	IV
Macroarray hybridisation	IV

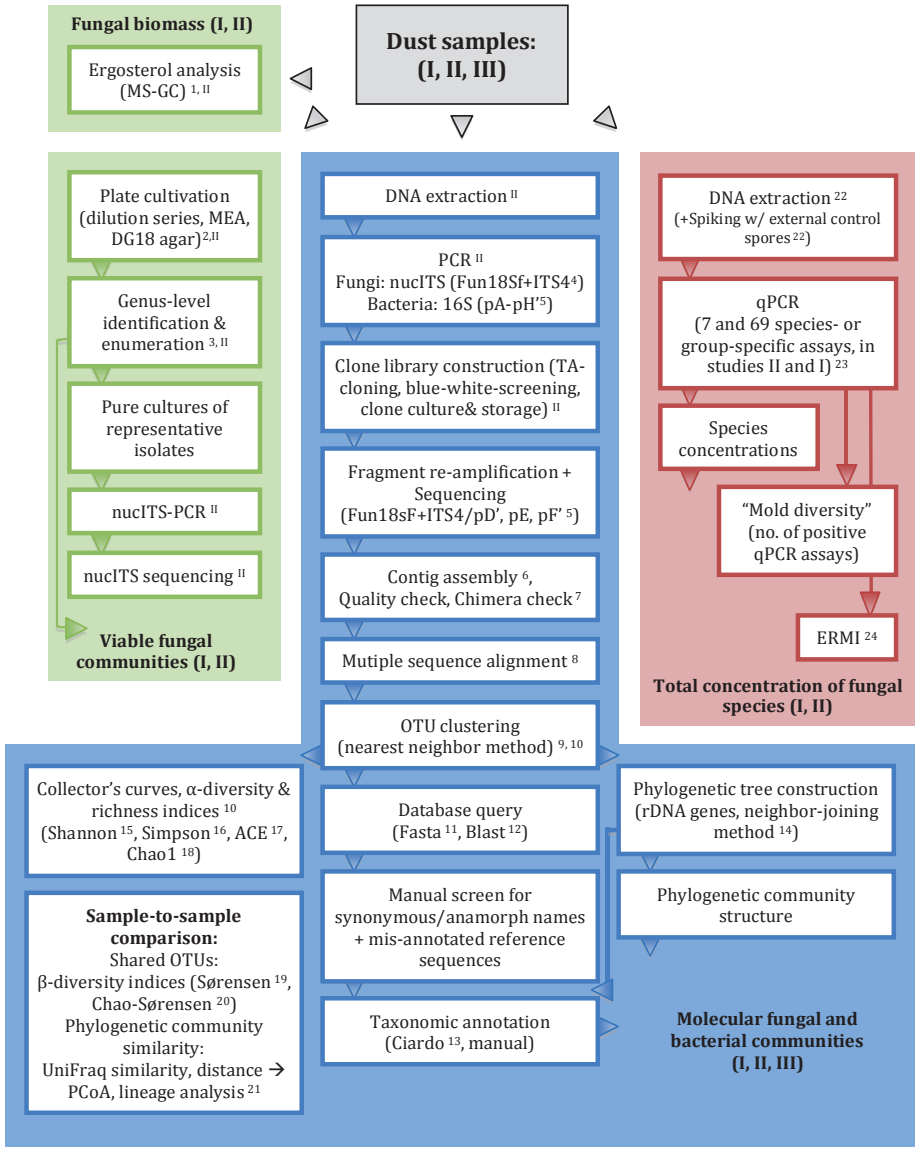

Figure 2. A flowchart showing the laboratory protocols and bioinformatic methods used in the present study for characterizing microbial communities in dust samples. Methods for analysis of viable fungi and fungal biomass are shown in green. Cultivation-independent methods for community analysis are shown in blue (clone library sequencing-based) and red (qPCR-based). References to methods: 1. Sebastian and Larsson 2003. 2. Samson *et al.* 1994. 3. Samson *et al.* 1996. 4. White *et al.* 1990. 5. Edwards *et al.* 1989. 6. Staden *et al.* 2000. 7. Huber *et al.* 2004. 8. Thompson *et al.* 1994. 9. Felsenstein 2005. 10. Schloss *et al.* 2005 and Schloss *et al.* 2009. 11. Pearson and Lipman 1988. 12. Altschul *et al.* 1990. 13. Ciardo *et al.* 2007. 14. Saitou and Nei 1987. 15. Krebs 1989. 16. Simpson 1949. 17. Chao *et al.* 1993. 18. Chao 1984. 19. Sørensen 1948. 20. Chao *et al.* 2005. 21. Lozupone 2006. 22. Brinkman *et al.* 2003. 23. Haugland *et al.* 2002a. 24. Vesper *et al.* 2007.

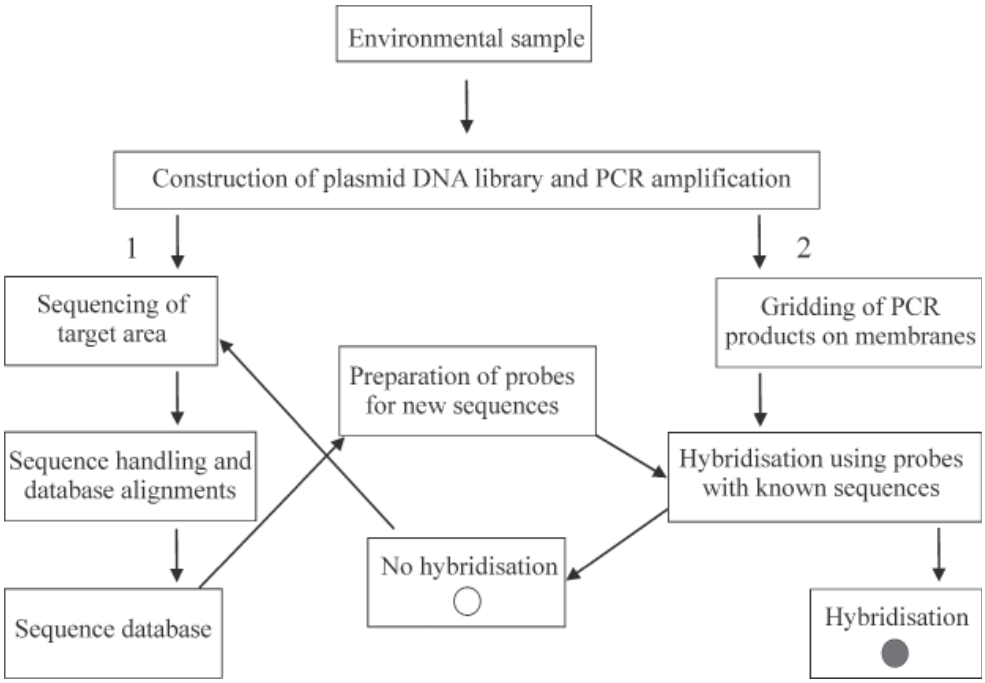

Figure 3. The principle of the macroarray hybridization method for detecting, enumerating and negative selection of abundant phylotypes in clone libraries. The left side (1) of the figure shows the clone library sequencing protocol for identifying the abundant phylotypes in the sample. The right side (2) describes the protocol for preparing library macroarrays on nylon membranes and the hybridization step with specific oligonucleotide probes. Probes are designed for the abundant phylotypes and hybridized on the membrane. Only unhybridized clones are picked for sequencing, which reduces the number of sequenced clones.

4. RESULTS AND DISCUSSION

Indoor microbial exposure may have important implications on health (Mendell *et al.* 2011), but the indoor microbial determinants are not fully understood. The aim of this study was to gain a deeper understanding of these determinants by using culture-independent DNA-based methods to characterize fungal and bacterial communities. The study focused on a selection of moisture damaged and undamaged buildings that represent typical Scandinavian office-type working environments.

4.1 Microbial diversity in dust (I, II, III)

Microbial diversity was assessed by sequencing microbial ribosomal marker genes from 16 long-term, composite samples of floor (II, III) and above floor level (I) settled dust from six office buildings. Large surface areas were sampled in order to avoid sampling bias due to spatial heterogeneity of surface dust. Carpets or textile covered surfaces were not sampled in order to avoid collecting old particle material.

4.1.1 Clone libraries and sequence data (I, II, III)

Fungal and bacterial communities in dust- and building material samples were characterized by means of PCR-amplification, cloning and sequencing of full-length nucITS region (fungi) and the 16S Ribosomal RNA gene (bacteria). Based on our previous work on clone libraries (Pitkäranta *et al.* 2005) and the observations by other authors (Wang and Wang 1996, Jumpponen *et al.* 2007), we developed an optimized PCR protocol that combines a low PCR cycle number with a high number of replicate reactions to minimize the effect of PCR drift and the number of artefactual PCR products, especially chimeric molecules (II). The results indicated that this improvement was feasible as only 0.02% (II) and 0.3% (I) of the sequences resulting from the optimized protocol were chimeric, compared to 8.7% (II) and ≥30% (Wang and Wang 1996, Pitkäranta *et al.* 2005, Jumpponen 2007) using conventional PCR protocol with a high thermocycle number.

After parsing the raw data for chimeras, low-quality sequences and non-target sequences, a total of 2421 fungal nucITS and 776 bacterial 16S sequences of full-length were obtained (Table 4). Apart from one sample (sample no. 4 in Table 4), a minimum of 100 fungal, and 76 bacterial sequences were obtained from each library.

Table 4. The sequence material. Number of good quality clone library sequences obtained and used in downstream analyses in studies I-III.

Sample	Source (*name*[1])	I, II[2]	III[3]
	Location 1		
1	B3 – pre-remediation (*In1a*)	225	-
2	B4 – pre-remediation (*Re1a*)	207	-
3	B3 – post-remediation (*In1b*)	100	-
4	B4 – post-remediation (*Re1b*)*	26	-
	Location 2		
5	B5 – pre-remediation (*In2a*)	100	-
6	B6 – pre-remediation (*Re2a*)	167	-
7	B5 – post-remediation (*In2b*)	119	-
8	B6 – post-remediation (*Re2b*)	137	-
	Location 3		
9	B1 – winter (*1W*/*Winter D*)	141	102
10	B2 – winter (*2W*/ *Winter R*)	174	159
11	B1 – spring (*1Sp*/*Spring D*)	180	76
12	B2 – spring (*2Sp*/*Spring R*)	152	109
13	B1 – summer (*1Su*/*Summer D*)	225	144
14	B2 – summer (*2Su* /*Summer R*)	170	82
15	B1 – fall (*1F*/*Fall D*)	141	104
16	B2 – fall (*2F*/*Fall R*)	157	117
	Combined data	2421	776

[1] The name used to refer to the sample in the corresponding study/studies
[2] Full-length fungal nucITS-sequences
[3] Full-length bacterial 16S-sequences
*The clone library was small due to low starting amount of fungal biomass (as indicated by ergosterol analysis).

4.1.2 Microbial diversity and richness in dust samples (I, II, III)

Our results show a high diversity of fungi and bacteria in dust. A total of 606 fungal and 283 bacterial OTUs were detected using sequencing. The theoretical fungal richness calculated by using nonparametric ACE-estimator was about 100 to 400 OTUs per composite dust sample (Table 5). This richness is comparable to that found in soil and plant tissue samples using the same methodology (O'Brien *et al.* 2005, Neubert *et al.* 2006). The total number of fungal genera identified from the six buildings was 166. This level of diversity is significantly higher than that usually obtained using culture; eg. the study of Verhoeff *et al.* (1994a), which is one of the most thorough investigations of culturable house dust fungi,

Table 5. Observed and estimated microbial community OTU richness in dust samples.

Sample (no. / name[a])	Fungal communities			Bacterial communities			Study
	S_{obs} [b]	S_{ACE} [c]	*Shannon (H')* [d]	S_{obs} [b]	S_{ACE} [c]	*Shannon (H')* [d]	
1 / B3_Pre	98	220	4.06	-	-	-	I
2 / B4_Pre	45	103	2.22	-	-	-	
3 / B3_Post	62	142	3.94	-	-	-	
4 / B4_Post	21	67	2.97	-	-	-	
5 / B5_Pre	37	77	2.73	-	-	-	
6 / B6_Pre	48	93	2.95	-	-	-	
7 / B5_Post	42	167	2.68	-	-	-	
8 / B6_Post	75	298	3.88	-	-	-	
9 / B1_Wi	50	109	3.44	30	147	1.88	II, III
10 / B2_Wi	47	79	3.45	53	282	3.18	
11 / B1_Sp	70	295	3.48	59	228	3.96	
12 / B2_Sp	61	194	3.58	65	188	3.85	
13 / B1_Su	81	327	3.51	60	176	3.47	
14 / B2_Su	69	333	3.52	45	223	3.28	
15 / B1_Fa	91	418	4.30	66	305	3.88	
16 / B2_Fa	92	393	4.24	55	171	3.27	

[a] Sample name abbreviations: Pre: pre-remediation sample, Post: post-remediation sample, wi: winter-time sample, Sp: spring-time sample, Su: summer-time sample, Fa: fall-time sample. [b] S_{obs}: number of observed OTUs (species level phylotypes); [c] S_{ACE}: number of estimated OTUs using the ACE richness estimator (Chao et al. 1993); [d] *H'*: Shannon biodiversity index, a measure of community richness and evenness, where a higher value indicates a higher diversity (Krebs 1989).

yielded 54 genera from 60 buildings using ten different cultivation protocols in parallel. Our efforts to characterize the viable communities indicated that the genus-level diversity was 1-13 genera per sample (I, II), i.e. typical of culturable studies assessing house dusts (e.g. Piecková *et al.* 2004). These results supported the hypothesis that the cultivable methods reveal a minority of the total diversity in house dust samples. It must be noted, however, that the results obtained by cultivation and by sequencing are not fully comparable; identification of e.g. yeasts on viable cultures was not attempted, and very rarely occurring genera were not identified and enumerated using cultivation. Phenotypic identification of yeasts would be possible by a combined morphological and biochemical approach but not by microscopy alone (Freydiere *et al.* 2001). Based on a comparison of Shannon diversity indices and the estimated total numbers of OTUs using the nonparametric ACE estimator, the level of fungal diversity in dust samples collected from elevated surfaces (Table 5, samples 1-8) seemed to be similar to that measured in floor dusts during the same season, i.e. winter (Table 5, samples 9-10). However, the variation between samples was significant and the number of analysed samples is too low to make conclusions concerning diversity differences between the sample types.

The numbers of observed and estimated fungal phylotypes in the seasonal study (II) leveled with those reported by Fröhlich-Nowoisky *et al.* in 84 outdoor air filter samples collected at a single location in the Central Europe over a period of one year (Fröhlich-Nowoisky *et al.* 2009). There were similarities also in the community structures between the two data sets, for example the diversity of decomposing agaric species was substantial and increased towards the fall in both studies. In addition to our observations on the multitude of other outdoor taxa in indoor samples, this similarity shows that long-term indoor dust samples may reflect relatively well the seasonally fluctuating fungal diversity from outdoor sources. The details of the community composition are presented below.

The level of bacterial diversity in dust samples was similar to that observed for fungi (Table 5, samples 9-16, III), apart from the summer and fall samples (samples 13-16) in which the fungal diversity was higher than bacterial. Similar bacterial diversity estimates have been found by others from surface and filter dusts (Noris *et al.* 2011) and from mattress and floor dusts (Täubel *et al.* 2009). Based on the observations on the accumulation of theoretical total OTU number (by ACE estimator) as a function of sampled clones, it was estimated that the total number of bacterial species in dust was roughly 500 per building per year (III).

In addition to microbial sequences, considerable amounts of plant-borne sequences were present in both fungal and bacterial libraries. Fungal and plant chromosomes contain orthologous rDNA operons with similar gene organization (Alvarez and Wendel 2003) and plant nucITS region may amplify with universal fungal primers if plant DNA is abundantly present and the primers are not fully specific. The latter was the case with our primer set (II), which explains plant sequences in our fungal ITS libraries. As for bacteria, universal bacterial primers may amplify plant DNA located in chloroplasts because of their cyanobacterial origin and orthologous ribosomal genes. Both nuclear rDNA operons and chloroplast DNAs are present in plant cells in high numbers. In the present study, the plant sequences could be affiliated to the source species by pairwise comparison of sequences against public nucleotide databases, which gave additional information on the types of biological particles in dust. The plant particles had originated from alimentary plants, house plants and tobacco as well as from trees and grasses growing outside the buildings. Sequences from *Betula alba* (birch) and *Acer platanoides* (norway maple) were most dominant. Plants were detected equally from floor- and above-floor level samples. The presence in the latter sample type suggests that the plant material either entered the building airborne or aerosolized and resuspended from floors. This suggests that a) the biological diversity of airborne dust, even wintertime office dust, is very wide, and b) the analysis of nucITS DNA sequences might be a feasible method for species specific assessment of environmental exposure to plant particles.

4.1.3 The fungal community composition in dust (I, II)

Overview. Sixteen composite dust samples representing six buildings were collected and their fungal content was analysed by nucITS clone library sequencing. The vast majority of fungal phylotypes were affiliated with two phyla: Basidiomycota and Ascomycota (Figure 4, A). The most diverse phylum was Basidiomycota (58% of OTUs), while Ascomycota was the most abundant phylum in clone num-

bers, accounting for 57% of sequences. Within Ascomycota, the classes Dothideomycetes, Eurotiomycetes and Leotiomycetes formed the majority of both diversity (OTUs) and clones (Figure 4, B). Within Basidiomycota, the classes Agaricomycetes, Tremellomycetes, Basidiomycetes incertae sedis and Pucciniomycetes were dominant. The details of the fungal community findings are described below. The species identified in the course of this study (non-singletons) are also listed in parallel with the fungal species identified from dust samples in previous studies (Appendix I). The full lists of detected phylotypes, their fre-

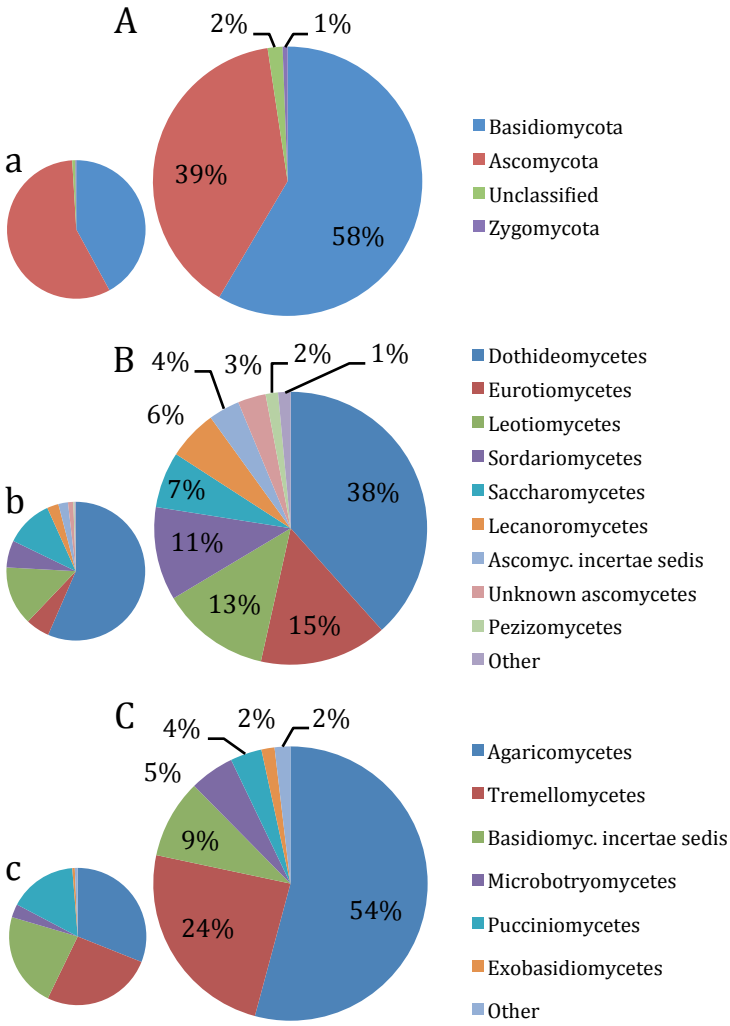

Figure 4. The taxonomic diversity of dust-borne fungi observed by sequencing: relative proportions of different phyla (A), different classes of Ascomycota (B), and different classes of Basidiomycota (C). Large circles (A-C) depict the relative OTU richness in each group. Small circles (a-c) depict the relative abundances of nucITS clones. Combined results for all dust samples in studies I and II are shown.

quency of occurrence across samples, annotation details and INDS identification numbers are listed in the supplementary materials of the studies I, II and III (published online).

The present distribution of diversity among fungal classes largely corresponded with that observed by Amend *et al.* (2010a) in 31 floor dust samples collected from various geographical locations in the temperate regions in the northern hemisphere. However, some major differences were seen; most importantly, Agaricomycetes and Ustilaginomycetes formed major groups in our data, but were minor constituents in the data set of Amend *et al.* In contrast, the relative diversity within Sordariomycetes and Eurotiomycetes was higher in their material than ours. Similar to the results by Amend *et al.* (2010a), Fujimura and colleagues (2010) who studied microbial diversity in urban house dusts, did not detect Agaricomycetes or Ustilaginomycetes among the dominant phylotypes. The higher abundance of these outdoor-related taxa in our material is most probably explained by the fact that the studied buildings were located nearby large forest areas, which are abundant sources of both Agaricomycetes (largely cap fungi) and Ustilaginomycetes (rusts). In our study, the buidings were relatively small and squat (1-2 floors) and thus the outdoor borne material may have transported indoors more efficiently than in the other studies. These results agree with previous observations in that the vegetation in the surrounding environment and well as the design and usage of the studied buildings contribute significantly to the indoor microbiomes (Li and Kendrick 1995c).

The proportion of pathogenic and opprotunistic fungi was low in the sequence material. We evaluated the BSL-classifications of the species affiliations of non-singleton OTUs (see the Appendix 1 table). Most of the species detected in indoor dust samples here for the first time were not BSL-classified. Two species of *Trichosporon*, *T. jirovecii* and *T. mucoides* (or *T. dermatis*) were detected. These are classified to BSL-2 due to their ability cause serious infections in immunocompromised patients. They are, however, often members of normal skin flora and may also derive from other habitats, e.g. soil (Liu 2011). As can be seen from the Appendix 1 table, BSL-1 classified species are commonly present in dust. Their occurrence as causative agents of opportunistic infections may relate to the thermotolerant nature of many of the fungi, but also to the overall abundance in the human living environment. An interesting finding was the almost entire lack of *Aspergillus* spp. in our sequence material. In contrast, several species of *Aspergillus* were detected to occur in house dust in concentrations of 10^3-10^5 CE/g. Among these was *A. fumigatus*, which is classified as BSL-2 organism. *Aspergillus* spp. occurred mainly in one of the locations, equally in both the moisture damaged index building and the undamaged reference building, and probably originated from outdoor air.

Normal variation. The sample-to-sample variation in molecular fungal assemblages observed in wintertime dust samples was assessed using the data from the undamaged reference buildings (Figure 5). The most prevalent and also dominant classes were Dothideomycetes, Tremellomycetes, Basidiomycetes incertae sedis, Agaricomycetes and Saccharomycetes. Most classes were present in all or all but one samples studied, but the relative abundances of different classes varied markedly between samples. Saccharomyces cerevisiae (Saccharomycetes) and Cladosporium sphaerospermum (Dothideomycetes)

occurred in high numbers in individual buildings but otherwise the proportion of individual species was ≤ 10

Filamentous ascomycetes. Filamentous ascomycetes usually form the majority of cultivable fungi in dust (Verhoeff *et al.* 1994a and 1994b, Koch 2000, Chao *et al.* 2002, Scott 2001, Chew *et al.* 2003, Piecková *et al.* 2004, Horner *et al.* 2004, Park *et al.* 2008, Hicks *et al.* 2005). In the present material OTUs affiliated with filamentous ascomycetes accounted for ca. 48% of the total clones. The dominant filamentous ascomycete phylotypes in our material were affiliated with (in the order of abundance) *Cladosporium herbarum*, *C. sphaerospermum*, *C. cladosporioides*, *Penicillium chrysogenum*, *Aureobasidium pullulans*, *Leptosphaerulina* (*Pithomyces*) *chartarum*, *L. trifolii*, *Sclerotinia sclerotiorum*, *P. commune* and two unknown members of the class Dothideomycetes; OTU423 shared 86% similarity with *Phaeotheca fissurella*, and OTU311 shared 96% similarity with *Macrophoma* sp. Together the abundant phylotypes accounted for 51 % of all ascomycete clones. Apart form the unknown types, most of the abundant species are typical findings in dust samples (see Appendix 1). The unknown OTU related to *Ph. fissurella* (OTU423; INSD id. FR682166) may be of significance since it was present in nearly all samples collected form the water damaged buildings, sometimes in considerable abundance, but was largely absent from the reference buildings (I, II). The pattern of occurrence may be coincidential but may also refer to moisture-damage-related- or other indoor sources for this species. While the closest relative *Ph. fissurella* is a phytopathogen, another member of the genus, an oligotrophic dematiaceous species *Ph. triangularis* has been isolated from HVAC humidifier walls in office buildings (de Hoog *et al.* 1997). However, since the sequence similarity between the

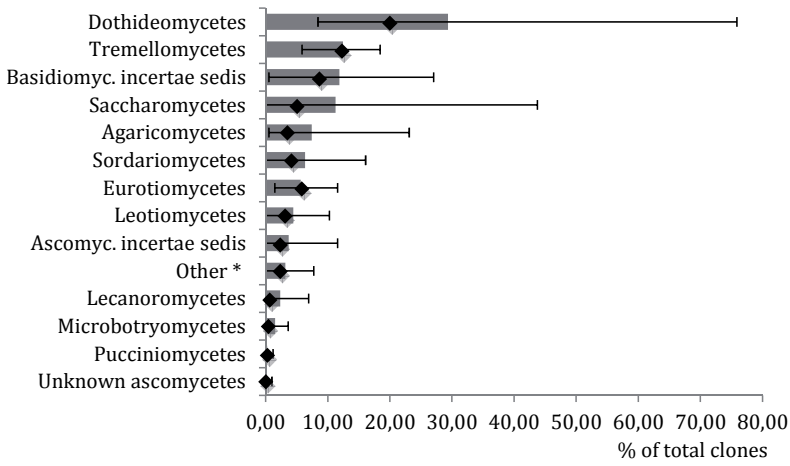

Figure 5. The relative abundances of fungal classes and their variation in dust samples collected from undamaged reference buildings (n=5). The average percentage clone frequencies (grey bars), minimum and maximum frequencies (error bars) and median values (black rhombuses) are shown. *Other includes: Agaricostilbomycetes, Arthoniomycetes, Cystobasidiomycetes, Exobasidiomycetes, Orbiliomycetes, Pezizomycetes, Taphrinomycetes, Ustilaginomycetes, Wallemiomycetes, Zygomycetes incertae sedis, unclassified fungi.

unknown phylotype and its known relatives was low, the potential ecological similarity between the species can only be guessed.

Other phylotypes were present in small numbers (<1% of total clones each) but their diversity was stunning; ca. 200 rare OTUs were affiliated with filamentous ascomycete species. These covered both cultivated and previously uncultivated species. In study I, previously unknown, verified phylotypes accounted for over 10% of all diversity. An interesting cluster of uncultured ascomycetes whose class affiliation could not be determined, occurred in several samples in the study (Cluster #3 in Figure 3 of study I; see Additional Table S1 of study I for details). Some of the sequences in the group shared high similarities with uncultured fungi detected in other environments but the cluster seems to lack cultivated, identified representatives. Another interesting cluster (Cluster #1 in Figure 3 of study I) which occurred almost exclusively in one of the damaged buildings, contained several unknown phylotypes which clustered with sequences of *Exophiala xenobiotica*, *Rhinocladiella atrovirens* and *Cladophialophora minutissima* isolated from the building materials in study I.

Our results demonstrate the dominance of previously known cultivable mould species in dust. However, these species are accompanied by a high diversity of rarer species, some of which represent unknown, uncultured and/or unsequenced fungi. Sterile, unidentifiable isolates are common in viable studies (Beguin *et al.* 1999, Chao *et al.* 2002, Hicks *et al.* 2005), and have been associated with moisture damage and/or respiratory symptoms in some studies (Strachan *et al.* 1990). Thus, the yet unidentified species may be of interest with respect to building related health effects and should be studied in more detail.

Filamentous basidiomycetes. The proportion of OTUs affiliated with filamentous basidiomycetes (mainly classes Agaricomycetes and Pucciniomycetes) was ca. 20% of all clones. Individual OTUs representing the class Agaricomycetes were present in low numbers; the most abundant types were identified as *Hypholoma capnoides*, *Armillaria borealis*, *Coprinus stercoreus*, *Botryobasidium subcoronatum* and *Antrodia sitchensis*, which are common mushrooms, decomposers and polypores outdoors in Scandinavia. Each of these phylotypes accounted for 0.5-1% of total clone numbers, and other OTUs were present in lower frequencies. Despite the rarity of individual OTUs, the summed proportion of Agaricomycete clones was substantial in some clone libraries. Few studies have explored the numbers and frequency of occurrence of basidiomycete spores and cell material in indoor environment. Basidiospores are often present in outdoor air, and are also known to be transported to indoor air, as observed by e.g. Li and Kendrick using direct microscopy (Li and Kendrick 1995c), but cannot be identified and enumerated using culture-based methods. On conventional growth media, basidiomycetes are easily overgrown by ascomycetous and zygomycetous moulds. Due to their inconspicuous morphology, basidiomycetes are usually included in colony counts of "sterile isolates" if present on culture plates. Our results suggest that cell material of basidiomycetous origin may be more abundant in dust than previously considered, especially in the autumn, reflecting the abundancy of this group outdoors (see results below concerning seasonal variation of dust fungi). Recently, basidiomycetous species diversity was shown to outnumber that of ascomycetes in outdoor air, especially in the summer and fall (Fröhlich-Nowoisky *et al.* 2008). Amend *et al.*

(2010a) reported a lower proportion (ca. 5%) of filamentous basidiomycete OTUs in their house dust samples collected from various geographical regions (Amend *et al.* 2010a).

Yeasts. Yeasts-like species are found from both Ascomycota and Basidiomycota. They are rarely identified in indoor cultivation-based studies, but may be classified as red, dark and white/unpigmented types, or may be identified on special culture media and by using biochemical tests (Freydiere *et al.* 2001). Yeasts are more prevalent in dust than in indoor air samples and occur often in concentrations similar or higher than filamentous fungi. In our material, yeasts were prevalent and yeast clones were dominant in some samples. Together yeasts accounted for 28% of all clones. Basidiomycetous yeasts were dominant; 76% of total yeast-like clones were affiliated with this phylum. Members of the class Tremellomycetes (mainly *Cryptococcus victoriae*, *C. wieringae*, *C. magnus*, *C. friedmannii*, *C. carnescens, Mrakia gelida* and *M. frigida*) accounted for almost 40% of all yeasts clones. The second important group of yeasts was of *Malassezia* spp. (class Basidiomycetes *incertae sedis*, 43% of yeasts clones); *M. restricta* was clearly the most common affiliation but also *M. sympodialis*, *M. slooffiae*, *M. globosa* and *M. japonica* were detected. *Malassezia* spp. colonize the skin of healthy human individuals, but several species are also associated with clinical skin conditions such as seborrheic dermatitis (dandruff) and *pityriasis versicolor* (Gupta *et al.* 2001). These results suggest that in addition to contributing significantly to the indoor bacterial assemblages (see below), the human occupants may be significant sources of indoor fungal material as well. This may be of importance in e.g. personal exposure studies using fungal biomarkers; *Malassezia* spp. may contribute to ergosterol concentrations, since ergosterol is the major sterol of *Malassezia* cell membrane (Gerla and Scheinfield 2008). In contrast, glucan assays may not be affected, since unlike many other fungi, the main cell wall carbohydrate of *Malassezia* has been observed to be (1→6)-β-d-glucan instead of the (1→3)-β-d-glucan typically targeted in biomarker assays (Kruppa *et al.* 2009). In addition to the known *Malassezia* spp., an unidentified member of the *Malasseziales* (BF-OTU429, INDS id. FR682172) was prevalent in our material (I, II). The OTU shares low (ca. 80%) similarity with any cultivated species, but the identical phylotype has been repeatedly encountered in molecular studies assessing soil and oceanic habitats in various geographic locations; including Singapore, the island of Reunion, Czech Republic, China and Hawaii (INDS id. HQ436049, JF691131 and GU327510, GU941385 and EU915323, correspondingly). Contrasting to the human-associated members of the Malasseziales in our data, the phylotype BF-OTU429 occurred with a location- rather than a building specific pattern. Based on these observations, the natural habitat and source of this potentially novel species was the outdoor environment rather than the human occupants.

OTUs affiliated with yeasts of Microbotryomycetes, another basidiomycetous class (incl. *Rhodotorula mucilaginosa*, *Sporobolomyces ruberrimus*, *R. slooffiae*, *R. glutinis*, *R. pinicola* and several related but unidentified yeasts) were present in dusts with a lower prevalence. The ascomycetous yeasts were solely members of the class Saccharomycetes, dominant species being *Saccharomyces cerevisiae* and *Debaryomyces hansenii*. These accounted for 18% and 2% of all yeast clones, correspondingly. Few clones of *Candida albicans* and *Pichia* spp. were also present.

Apart from the abundance of *Malassezia*, a species that does not grow well on common laboratory conditions, our results concerning major yeast genera were in agreement with previous culture-based studies. In the extensive survey of 60 homes in The Netherlands by Verhoeff *et al.* (1994a) reported *R. minuta*, *R. mucilaginosa*, *Cryptococcus albidus* and *C. laurentii* as the dominant yeasts. Glushakova *et al.* (2004) detected several members of *Candida*, *Cryptococcus*, *Pichia* and *Rhodotorula* in house dust. In that study, the majority of indoor yeasts were shown to originate from indoor plants and pot soil. Here, based on the high number of phylotypes occurring in low frequencies in our samples, their elevated diversity and abundance during spring and summer (see below), most *Cryptococcus* spp. and also other yeasts including *Rhodotorula* spp. probably originated mainly from outdoor sources.

4.1.4 The bacterial community composition in house dust (III)

Bacterial communities were followed over one year in two buildings (III). Throughout the year, the communities were dominated by gram-positive bacteria, especially members of the Firmicutes and Actinobacteria (Figure 6). The dominant phylotypes were present in both buildings and were affiliated with species associated with human skin and mucosa; *Staphylococcus epidermidis*, *Propionibacterium acnes*, *Lactococcus lactis*, *Corynebacterium* sp., *Streptococcus thermophilus*, *Lactobacillus* sp., *Streptococcus mitis*, *S. parasanguis* and *Gemella morbillorum* (in the order of abundance) were present in over half of the samples and together accounted for ca. 40% of total clones. *Serratia fonticola*, a soil/water-borne member of the Enterobacteriaceae, occurred in high numbers in one sample. All dominant phylotypes, and the majority of minor types shared

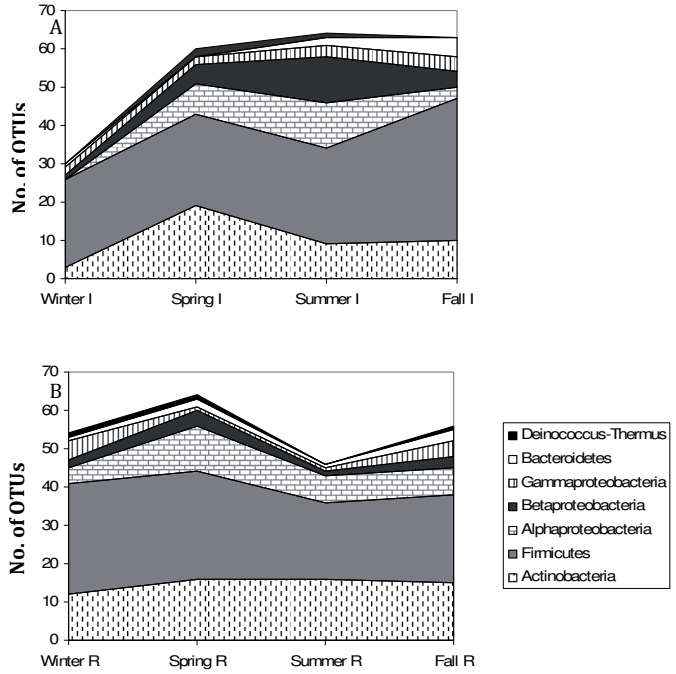

Figure 6. Bacterial community composition in dust (III). Seasonal variation of bacterial diversity on class level in two buildings (A and B) is shown.

a high (>97%) sequence similarity to known bacterial species.

4.2 Variation in microbial community composition

4.2.1 Seasonal variation (II, III)

***Fungi* (II).** Seasonal variation of indoor fungal communities was analysed from floor dust samples collected from two neighboring buildings with similar structure but different moisture damage status (II). Dust was collected solely from hard floor and above floor surfaces, since carpets and textile-covered surfaces can retain microbes and thus may not reflect the seasonal load but material accumulated over longer time periods.

The main seasonal trends of fungi are visible in Figure 7 and Figure 8, which show the seasonal variation in diversity on class level and examples of clone frequencies of selected genera or groups expressing clear seasonal pattern. The overall diversity of fungi increased markedly from the winter towards the fall (Figure 7, Table 5). The clearest seasonal trends followed the outdoor fungal succession:

- A diversification of decomposing and mycorrhizal basidiomycetes (class Agaricomycetes) towards fall.
- A high abundance and somewhat elevated diversity of several phylloplane species, including *Cladosporium cladosporioides*, *C. herbarum*, *Epicoccum nigrum*, *Aureobasidium pullulans* and *Leptosphaerulina* (*Pithomyces*) *chartarum* and related species (class *Dothideomycetes*) as well as *Cryptococcus* spp. (class Tremellomycetes) in the spring and summer.
- An abundance of rusts (Pucciniomycetes) in the spring, summer and fall samples.

Interestingly, one group of clear indoor origin also showed seasonal occurrence; human skin-associated yeasts (*Malassezia* spp., class Basidiomycetes *incertae sedis*) were most diverse and abundant in the cold seasons (fall and winter). Their abundance may relate to low indoor RH, which often causes drying of the skin and more excessive scaling of skin material.

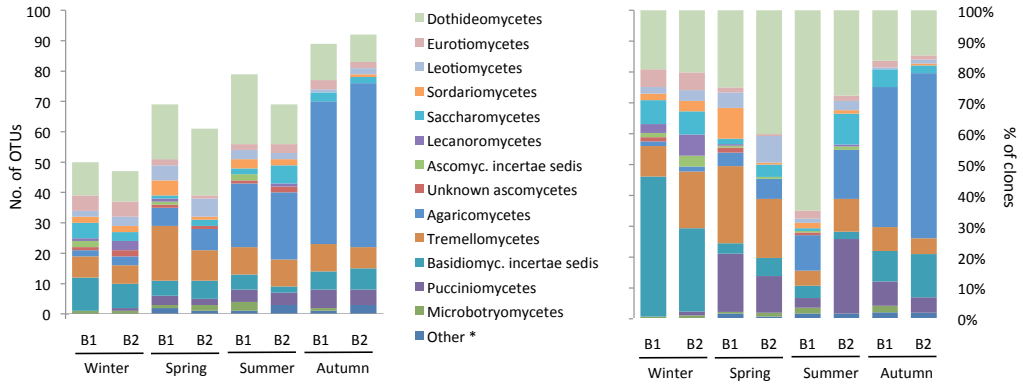

Figure 7. Seasonal variation in the OTU diversity (left) and the relative abundancies of fungal classes (right) in two buildings (B1-2). *Other includes: Agaricostilbomycetes, Arthoniomycetes, Cystobasidiomycetes, Exobasidiomycetes, Orbiliomycetes, Pezizomycetes, Taphrinomycetes, Ustilaginomycetes, Wallemiomycetes, Zygomycetes incertae sedis, unclassified fungi (II).

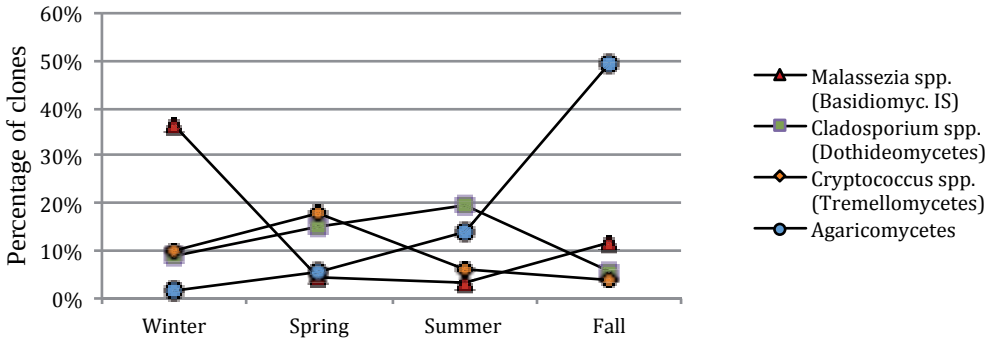

Figure 8. Seasonal variation in the relative abundancies of selected fungal genera. The average values of the two buildings (B1 and B2) were used (II).

Another interesting detail was the sporadic mass occurrence of certain rust species in dusts; *Melampsoridium betulinum* (the birch rust) that releases its spores in masses during the late summer and fall, occurred in high numbers in summer and fall samples, whereas *Thekopsora areolata* (the cherry-spruce rust) which releases its winter spores earlier in the spring, was observed in spring and summer samples.

Based on the observed numbers of shared OTUs between samples, we found that the fungal communities were most similar in the two studied buildings during the fall, even though the diversity was distinguishingly high then. In contrast, lowest similarity between buildings was seen in winter samples. The fungal communities also followed the seasonal succession of outdoor mycota in many aspects. These observations suggest that the effect of outdoor environment on indoor fungal assemblages was significant, especially during the growth season. Similar conclusions have been made by others using culture-dependent (Koch *et al.* 2000) and culture-independent methods (Amend *et al.* 2010a). However, viable communities in dust samples do not always vary according to season (Ren *et al.* 1999, Horner *et al.* 2004). Based on the generally low number of shared OTUs in samples samples from successive seasons, and the restricted occurrence of certain species during one or two seasons in accordance with their ecology, it seems that the studied sample type (floor dusts) reflected well the particle load typical of each season. Furthermore, it was estimated that the proportion of old particle material in samples collected from hard surfaces was low. Thus it is unprobable that e.g. the assessment of winter samples using DNA-based techniques would be severely affected by material pertaining from the previous season. Also Kaarakainen *et al.* (2009) observed in their qPCR based study that outdoor-borne taxa such as *A. pullulans* and *Cladosporium* spp. measured from floor (dust bag) dusts showed clear seasonal variation which was in accordance with their natural occurrence.

***Bacteria* (III).** Little is known about the seasonal variation of bacterial communities in house dust. In a study by Reponen *et al.* (1992), indoor air bacterial assemblages showed little seasonal variation whereas Moschandreas *et al.* (2003) observed that the seasonal variation was residence dependent without clear overall trends. In our material (III), the seasonal variation in bacterial com-

munities was less clear than fungal due to the dominance of human-associated bacterial phylotypes - largely members of the Actinobacteria and Firmicutes - that were relatively constantly present across seasons. In general, larger differences in the bacterial communities were detected between buildings than between seasons. However, some seasonal trends could be seen. Subsequent seasons tended to remind each other in their phylogenetic community composition. The diversity of alpha- and beta-proteobacteria varied according to season, being least diverse during fall and winter and most diverse during the spring and summer. Bacteria of putative outdoor origin (soil, plants) were identified among these groups, and also within Actinomycetes, including members of e.g. Frankiaceae, Nocardiaceae, Methylobacteriaceae, Rhizobiaceae and Oxalobacteraceae (III). Also house plants are a potential source of soil- and plant-associated bacteria. However, bacteria from such sources should be detectable throughout the year, but above mentioned families did not occur, or occurred only sporadically in winter samples, which supports their outdoor origin. The dominance of proteobacteria in outdoor air during the growing season has been observed in several studies (Brodie *et al.* 2007, Fierer *et al.* 2008, Fahlgren *et al.* 2010). These results suggest that outdoor sources determine the indoor bacterial levels and types to some extent, but the effect is considerably weaker than for fungi because the effect of the indoor sources (mainly occupants) is significantly stronger.

4.2.2 Moisture damage (I, II, III)

Mould and moisture problems may demonstrate in elevated levels (Dharmage *et al.* 1999, Klánová *et al.* 2000) and/or altered types of viable fungi in dust and air (Miller 2000), but changes are not always seen. Nevertheless, building related symptoms may prevail. In such situations, the exposing agents have been suspected to be transferred in non-viable or non-culturable particles, or to be emitted sporadically so that short-term samples do not "catch" them. Following this logic, long-term samples analysed with a culture-indpendent method might better reflect the actual exposures, at least if the exposing agents are carried in DNA-containg particles. In the present study, the effect of moisture damage on the fungal flora was studied by comparing the molecular diversity and community composition in three pairs of damaged (index) and reference buildings (studies I and II). The occurrence of bacteria was studied in one building pair (III). From the seasonal study (II), only winter samples were used to compare the buildings, since the effect of outdoor sources was obvious in other seasons (see previous chapter). The overall level of diversity, and diversity within fungal classes was addressed, as well as the occurrence of indicator fungi (Samson *et al.* 1994) and fungi known to commonly colonize building materials (Appendix I). Furthermore, in two of the buildings (B3 and B5), fungi growing on the moisture-damaged sites were identified from material samples collected during renovation, and the occurrence of these species in the dust samples was examined.

Comparison of microbial assemblages in damaged and reference buildings. Apart from a finding of high abundance of *Penicillium* spp. in one of the index buildings, no obvious indications of the moisture damage's effect on fungal or bacterial communities were seen (I, II, III). However, some observations were associated with the building condition. In two of the index buildings (B1 and B3) an

elevated diversity was observed compared to the paired reference buildings (B2 and B4); the numbers of observed and estimated OTUs (S_{obs}/S_{ACE}) were 50/109 and 47/79 for the first index-reference pair (B1 and B2, correspondingly), and 98/220 and 45/103 for the second pair (B3 and B4).

In the first building pair (B1 and B2, study II) fungal community structures were very similar on class level and the diversity increase in the index building was not clearly affiliated with any individual class. In the second pair of buildings (B3 and B4, study I), increased diversity in the index building occurred within several classes, including Dothideomycetes, Eurotiomycetes, Tremellomycetes and Agaricomycetes, which harbor species capable of growing on indoor substrates. However, also classes containing clearly outdoor and human associated fungi, including Lecanoromycetes (lichenized fungi), Pucciniomycetes (rusts) and Basidiomycetes incertae sedis (mainly *Malassezia* skin yeasts) were diversified, indicating that also other factors than building growth contributed to the increased fungal diversity in the building. Such factors may include e.g. differences in the cleaning routine. In the third pair (B5 and B6), as already mentioned, high numbers of *Penicillium chrysogenum* and *P. commune* occurred in the index building clone library. However, within the remaining clones, increased diversity in the classes Agaricomycetes and Dothideomycetes was seen similar to building B3 (I).

The high diversity of Agaricomycetes in winter samples is interesting, since in the seasonal study (II), this class was strongly seasonal occurring in significant numbers only in summer and fall samples. Certain basidiomycetes such as *Serpula lacrymans* have been long known to cause severe indoor wood decay and their spores can occur in indoor samples. Since basidiomycetes are easily overgrown by ascomycetous moulds, or are left unidentified due to lack of specific morphology in culture, their true frequency of occurrence on indoor materials and dust and air samples may be higher than understood thus far. Molecular methods have turned out to be suitable for their identification. A recent study using molecular methods indicated that fungal biodiversity in wood decay turned out to be far more significant and diverse than has hitherto been described (Maurice *et al.* 2011). In that study, several basidiomycetous species grew in parallel with each other and with ascomycetous mould species on damaged building materials. In our material the phylotypes characteristic of the two index buildings (I) were affiliated with *Clitocybe subditopoda*, *Gloeophyllum sepiarium*, *Hypholoma sublateritium*, *Serpula lacrymans*, *Thelephora terrestris* and *Trametes ochracea*. Of these, *G. sepiarium* and *S. lacrymans* are known indoor wood decay-causing fungi and may well have originated from the constructions of the wood-framed index building (I). *T. ochracea* is a common wood-decaying polypore, which to our knowledge has not been reported to be building-associated. A relative of *H. sublateritium*, *H. fasciculare*, has been detected in floor boards (Schmidt 2007). The remaining species are wood-decomposing, saprotrophic agarics that produce sporocarps on needle and wood litter under conifers and on logs and stumps. While the growth of these species in some layers of damaged buildings might be possible, they more probably originated from wood/needle debris carried indoors on shoes. Since they were detected in dust samples collected solely from elevated surfaces, they obviously were resuspended from the floor debris and transferred to the surfaces via the indoor air.

Table 6. Percentage frequencies of fungal genera in winter-time dust samples from the index and reference buildings. Percentage frequencies of total cfus (cultivation) and clones (sequencing) in a sample are listed. Genera with a frequency of 1% or higher are included (I, II)

Sample	Viable count (cfu)	Viable taxa (% of total cfus)	Molecular taxa (% of total nucITS clones in library)[a]
1 (B3_pre) (index)	96 295	*Penicillium* (61%), *Cladosporium* (9%), non-sporulating (9%), *Aureobasidium* (5%), *Verticillium* (5%), unknown (5%), Sphaeropsidales (4%), *Aspergillus* (1%), *Trichoderma* (1%), yeasts (1%)	Unidentified filamentous ascomycetes (15%), yeasts (12%), rusts (15%), *Cladosporium* (8%), *Aureobasidium* (5%), *Leptosphaerulina* (4%), *Epicoccum* (3%), *Phaeothecoidea* (3%), *Hormonema* (2%), *Botrytis* (2%), *Phoma* (1%), *Fusarium* (1%), *Penicillium* (1%)
2 (B4_pre) (ref.)	2 500 456	*Cladosporium* (93%), *Acremonium* (5%), yeasts (2%)	*Cladosporium* (65%), yeasts (10%), unid. fil. ascomyc. (7%), *Leptosphaerulina* (3%), *Hormonema* (3%), *Aureobasidium* (2%), *Phoma* (1%), *Ampelomyces* (1%)
3 (B3_post) (index)	5 729	*Penicillium* (29%), yeasts (29%), non-sporulating (29%), *Aureobasidium* (14%)	Yeasts (40%), *Cladosporium* (10%), basidiomycetes (10%), unid. fil. ascomyc. (7%), *Phoma* (6%), *Leptosphaerulina* (4%), *Aureobasidium* (3%), rusts (2%), *Mucor* (2%), *Penicillium* (1%), other 1%
4 (B4_post) (ref.)	136	*Eurotium* (33%), *Alternaria* (33%), non-sporulating (33%)	Yeast (31%), *Hormonema* (15%), *basidiomycetes* (12%), *Aureobasidium* (8%), unid. fil. ascomyc. (8%), *Rhizoctonia* (8%), *Phaeosphaeria* (4%)
5 (B5_pre) (index)	1 729 729	*Penicillium* (100%)	*Penicillium* (49%), yeasts (7%), *Cladosporium* (5%), *Botrytis* (4%), unid. fil. ascomyc. (3%), *Aureobasidium* (3%), basidiomycetes (6%), rusts (3%), *Hormonema* (2%), *Leptosphaerulina* (2%), *Epicoccum* (1%), *Mycosphaerella* (1%)
6 (B6_pre) (ref.)	139 963	*Penicillium* (89%), *Acremonium* (8%), yeasts (2%), non-sporulating (1%)	Yeast (63%), *Fusarium* (8%), unid. fil. ascomyc. (8%), *Penicilium* (3%), *Aureobasidium* (3%), *Phoma* (2%), *Cladosporium* (1%), Botrytis (1%), *Hormonema* (1%) etc. 1%
7 (B5_post) (index)	1 149 099	*Penicillium* (100%)	Yeast (15%), *Penicillium* (46%), *Cladosporium* (8%), unid. fil. ascomyc. (6%), *Phoma* (3%), *Aspergillus* (2%), *Eurotium* (2%), *Aureobasidium* (1%), *Hormonema* (15), *Botrytis* etc. 1%
8 (B6_post) (ref.)	270 716	*Penicillium* (90%), yeasts (3%), non-sporulating (3%), *Aureobasidium* (2%), unknown (1%)	Yeast (27%), *Aureobasidium* (15%), *Acremonium* (9%), unid. fil. ascomyc. (9%), *Cladosporium* (6%), *Penicillium* (4%), *Botrytis* (4%), *Phoma* (1%), *Hormonema* (1%), *Fusarium* (1%), *Paecilomyces* (1%), *Aspergillus* (1%), *Trichoderma* (1%), *Alternaria* (1%) etc. 1%
9 (B1_Wi)	25 630	Yeasts (32%), *Penicillium* (18%), Sphaeropsidales (14%), *Aspergillus* (13%), *Acremonium* (5%), non-sporulating (5%), *Aureobasidium* (3%), *Stachybotrys* (3%), *Exophiala* (2%), *Paecilomyces* (2%), *Scopulariopsis* (2%), 3 other genera (<0.5% in total)	Yeasts (63%), unid. fil. ascomyc. (11%), *Cladosporium* (10%), *Hypogymnia* (3%), *Phoma* (1%), *Aureobasidium* (1%), *Sclerotinia* (1%), *Phaeoseptoria* (1%), *Philophora* (1%), *Arthrobotrys* (1%), *Cephalotheca* (1%).
10 (B2_ Wi)	127 990	Yeasts (78%), *Penicillium* (7%), non-sporulating (5%), *Fusarium* (4%), *Aspergillus* (2%), 7 other genera (<5% in total)	Yeasts (55%), unid. fil. ascomyc. (14%), *Cladosporium* (7%), *Hypogymnia* (5%), *Capnobotryella* (3%), *Aureobasidium* (2%), *Fusarium* (2%), *Exophiala* (2%), *Pseudocladosporium* (2%), basidiomycetes (1%), *Melampsoridium* (1%), *Trimmatostroma* (1%), unid. fil. basidiomyc. (1%)

[a)] For an easier comparison of the cloning and culture results (see below) yeasts are clustered together in this table even though they represent phylogenetically very distant and diverse organisms. Samples 1-8 are from the study I and samples 9-10 from study II.

For a more convenient comparison of the community structures on higher phylogenetic level, OTUs were grouped into genus-level entities (Table 6, right column). In two of the damaged buildings (B1 and B3), a high proportion of clones were affiliated with "unidentified filamentous ascomycetes"; the phylotypes shared the highest sequence similarity with ascomycete species with typically hyphal growth, but the sequence similarities were too low to allow annotating these phylotypes into certain genus or species. These occurred in mainly low numbers, but as described in the previous chapter, some may have originated from indoor growth.

Occurrence of indicator microbes and material-associated fungi in dust samples (I). Microbial communities developing on moisture damaged materials are often diverse (Andersson *et al.*, 1997; Tuomi *et al.*, 2000, Hyvärinen *et al.*, 2002; Schmidt 2007); according to literature (Appendix I), a majority of the fungi common in dust have also been isolated from building materials. Excluding obvious outdoor taxa such as *A. pullulans*, *C. herbarum* and *C. cladosporioides* from the list of material-associated species, it was calculated that in the three pairs of index/reference buildings, material-associated taxa accounted for 2/6%, 18/63% and 56/18% of total clone in libraries, i.e. the proportion was higher in the index building in only one of the three building pairs. The analysis of so called indicator taxa (Samson *et al.* 1994) produced similar results (data not shown). To perform a more exact analysis of the occurrence of the material-associated fungi in dust, building material samples were collected from the damaged parts of the building during the renovation of the damaged buildings. From these samples, 45 fungal species-level phylotypes were detected either by clone library sequencing or by sequencing cultivated fungal isolates. Among these were species that were very common in most dust samples, such as *C. cladosporioides*, *C. herbarum*, *Phoma herbarum*, *Leptosphaerulina* (syn. *Pithomyces*) *chartarum*, but also species occurring rarely in the clone libraries, including *Penicillium* spp., *Trichoderma citrinoviride*, *T. atroviride* and *Rhinocladiella atrovirens*. Building-specific, material-associated taxa accounted for 13/14%, 28/72% and 60/19% of total clones in index/reference sample pairs. The clearly elevated percentage in the last index building was caused by the above-mentioned mass-occurrence of *Penicillium* spp. The genus was distinguishingly abundant also in culture plates from the building. Thus, based on these results, the effect of building sources was not better distinguishable using clone library sequencing than by using cultivation. On the other hand, since the characterization of material fungi relied largely on culture (there were technical problems in the clone library construction from material samples, see discussion in study I), it is possible that a larger proportion of dust-borne fungi may have been attributable to building sources than could be verified.

According to the literature, cultivable bacterial groups associated with moisture damaged building materials include mainly members of the class Actinobacteria, of which the genera *Amycolatopsis, Nocardiopsis, Nocardia, Promicromonospora, Pseudonocardia, Saccharomonospora, Saccharopolyspora, Streptomyces* and have been reported to be the most abundant (Suihko *et al*. 2009, Schäfer *et al.* 2010). Moreover, using culture-independent approach (PCR and clone library sequencing), Schäfer *et al.* (2009) detected additional genera in Actinobacteria to occur on materials,

of which *Arthrobacter*, *Jiangella* and *Nesterenkonia* were frequent. Also members of other classes, including low-G+C gram-positive bacteria (e.g. *Laceyella* (*Thermoactinomyces*) and *Bacillus* spp. in Firmicutes) and gram-negative bacteria (e.g. *Agrobacterium* spp.) have been isolated from wet building materials (Andersson *et al.* 1997, Schäfer *et al.* 2009). A range of previously unknown species and genera were recently isolated and described from building materials (Schäfer *et al.* 2009). The presence of *Streptomyces* spp. and other material-associated bacteria listed in the above-mentioned studies in the dust samples collected from the index and reference building was screened in the study III. Some taxa occurred sporadically on dust samples of both index and reference building but no tendency of occurrence with respect to the damage could be seen (III). *Bacillus cereus*, which has been suspected to be of importance in the indoor environment due to its production on emetic toxins, and its capability of growth on wet building materials (Andersson *et al.* 2010), was detected only in the index building.

These results suggested that the effect of water damage on microbial assemblages in dust was not obvious. However, certain issues undermined the detection of potentially building related microbes. First, our clone library sizes were limited and the abundantly present outdoor- and human-associated microbes potentially masked the presence of material-associated species. Second, the frequencies of certain important, potentially material-associated fungal taxa may have been severely underestimated in the sequence material (see next chapter). Third, our knowledge of the varieties of potentially material-associated microbes is based on cultivation and may thus be an underestimate of the true microbial diversity on materials. For these reasons, material-associated species may have actually been present in higher abundance in dust samples than could be verified here. In the future, a thorough culture-independent characterization of fungi and bacteria occurring on building materials is needed. After that, the identified species would be best quantified from settled dust- or long-term air samples in the occupied spaces using qPCR, which is a truly quantitative method unlike clone library sequencing which may both under- and overestimate the relative species abundances. From such results, the exposure to material-associated microbes could be better evaluated.

4.3 Methodological considerations

4.3.1 PCR/Clone library representativeness

Direct sequencing methods may show a biased view of the original template frequencies, yet the estimations of the severity of the problem vary (von Wintzingerode *et al.* 1997, Huber *et al.* 2009, Amend *et al.* 2010b). In our data the relative clone and OTU abundances within most classes seemed to reflect well the anticipated species abundance distributions in the environment. For example, the species richness in Agaricomycetes – the class harbouring mushrooms and polypores – followed a clear seasonal pattern being very high in the fall. Individual OTUs in this class occurred in low numbers, indicating that these species originated from sporadic, weak or distant sources, which is in agreement with their occurrence in the environment. Within some other classes, the clone abundance in relation to the OTU richness was high, e.g. for the classes Pucciniomycetes and Basidiomyce-

tes incertae sedis (Figure 4, C), which harbor rusts and human skin yeasts (Malasseziales). These species can be expected to accumulate in substantial numbers in dust; rusts for their massive leaf-surface colonization potential and ubiquitous presence in the immediate environment surrounding the buildings, and Malasseziales for their year-round presence in high numbers on human skin surface (Paulino *et al.* 2006). Also the prevalence and abundance of *Cladosporium* spp. was in line with their prevalence in the environment (e.g. Li and Kendrick 1995 c).

There were also some species whose clone abundance did not follow the expected abundance in the environment. Such included for example *Penicillium*, *Eurotium* and *Aspergillus*, which are considered to be the most prevalent and abundant members of viable dust communities in most studies, including those performed in Finland (e.g. Hyvärinen 2002, Flannigan and Miller 2011). Apart from one exception (index building B5 with heavy contamination by *Penicillium chrysogenum* and *P. commune*), sequences affiliated with the above genera were very rare in our material – constituting only 0.7% of total clones. In contrast, substantial numbers of *Penicillium* and *Aspergillus* spp. were shown to be present in several samples using species specific qPCR and cultivation. This suggests that the abundance of these common moulds may be severely underestimated by sequencing methods. As discussed in paper I, this phenomenon has been previously reported in the literature in various environments (e.g. Hunt *et al.* 2004, O'Brien *et al.* 2005) but has to our knowledge not been explained. One probable reason for this discrepancy lies in the differential extraction efficiency and rDNA copy number between fungal species. Haugland *et al.* (1999a) have demonstrated that using the bead-beating-based DNA extraction method (which was applied also in the present study), the difference in numbers of extracted rDNA copies was up to three-fold between *Aspergillus versicolor* (lowest) and *P. chrysogenum* (higher), and over twenty-fold between *A. versicolor* and *Alternaria* alternata (highest yield/copy number per cell). Using less efficient DNA-extraction methods even larger differences were seen, e.g. over 300-fold between *A. versicolor* and *A. alternata* using liquid nitrogen grinding for DNA extraction (Haugland *et al.* 1999a). These findings may largely explain the rarity of *Penicillium* and other Trichocomaceae moulds in sequence data sets.

Another aspect that could explain the low proportion of *Penicillium* spp. and relatives was also observed. Among our data, the nucITS regions of *Aspergillus* and *Penicillium* spp. had the highest G+C-content of all obtained nucITS fragments (Figure 9). The G+C-content of these species was typically between 56-61%. High G+C-content may reduce both the PCR amplification and the sequencing reaction efficiency due to the formation of stable secondary structures which may not be fully resolved during the denaturation phase of PCR and sequencing reaction (von Wintzingerode *et al.* 1997). In a multi-template PCR this leads into underrepresentation of such species in the end product. The G+C-content of *Penicillium* and *Aspergillus* spp. not represented in our clone libraries but detected using qPCR in the samples were examined from type strain reference sequences and were observed to posses G+C-contents between 56-61% (data not shown). Lowered detection sensitivity and/or lower clone library frequency has also been observed for low-G+C gram-positive vs. gram-negative bacteria (Korthals *et al.* 2008) and high-G+C vs. low C+G-grampositive bacteria (Krogius-

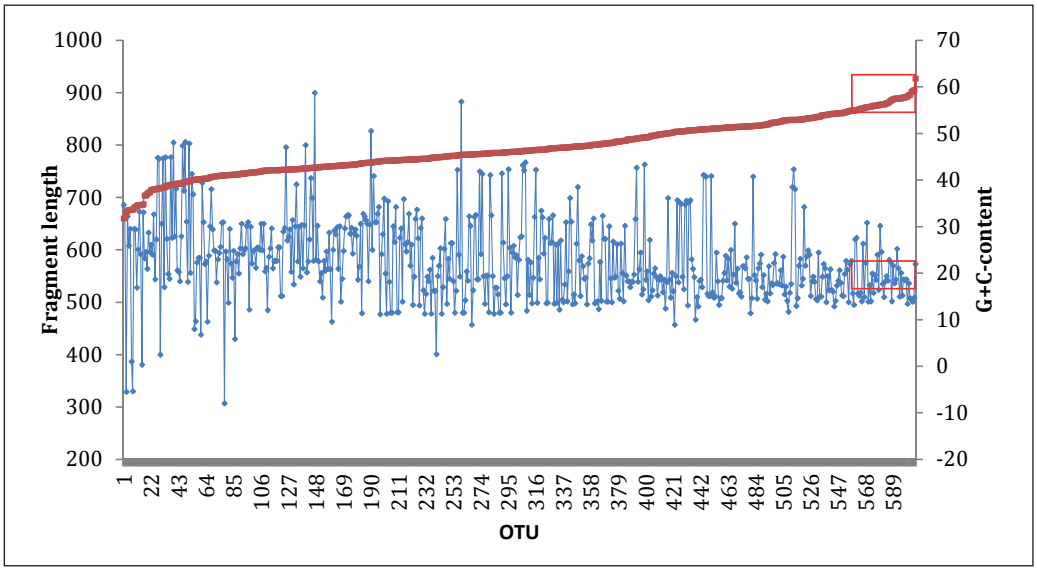

Figure 9. The G+C-contents (red line) and the fragment lengths (blue line) of the obtained nucITS OTU sequences. The OTUs are ordered by the G+C-content. The *Penicillium* and *Aspergillus* nucITS regions had high G+C-content (upper red square) but the fragment length was average (lower red square).

Kurikka *et al.* 2009). The nucITS fragment lengths were also examined, since fragment length may also affect the amplification efficiency (von Wintzingerode *et al.* 1997). Within our data, the ITS lengths of *Penicillium* and *Aspergillus* spp. represented the average length of all OTUs (Figure 9) and fragment length did not correlated with clone frequency (data not shown).

These observations indicate that apparently, at least in the case of certain fungi, the same characteristics that protect the organism against environmental stress and subsequently improve its longevity and dispersal (small, round cells, hardy cell walls and high G+C-content) seem to undermine the detection of these species by molecular methods in relation to less persistent species.

Affiliating the molecular data reliably with fungal species showed to be a challenging task. After the initial (automated) annotation of the representative nucITS sequences using either a simple 99% sequence similarity threshold for conspecificity (II), or the more flexible algorithm described by Ciardo *et al.* (2007), tens of sequence types were annotated into higher level groups (genus/class) instead of species despite high sequence similarities with fully annotated INSD reference sequences. This was due to equally high Blast matches with several distinct reference species. In some cases this was explained by a truly high similarity of the nucITS regions of two or more closely related species. However, in many cases database sequences from closely related species had been mis-annotated, or synonymous names for a single genetic species had been used. Moreover, the phylogeny of many fungi, including common indoor moulds such as *Cladosporium cladosporioides* and *C. herbarum* is unresolved, and the "species" have been observed to represent species

complexes rather than single, distinguishable entities (Bensch *et al*. 2011). Moreover, e.g. many *Penicillium* spp., which closely resemble each other in culture are also unresolvable by their nucITS sequences. In mentioned situations, an automated sequence clustering and annotation produces messy results. In general, we perceived that a thorough look into the matching species' taxonomy, and sometimes examining the correctness of the nearest reference sequences' annotations by additional Blast-searches was necessary to ensure the best annotation (I, II).

4.3.2 Comparison of the methods

The fungal abundance and diversity results obtained using culture, clone library sequencing and qPCR were compared (I, II). In study I qPCR was performed using a set of 69 species- or group specific assays for common fungal indoor and indicator species (Samson *et al.* 1994). The clone library sequencing method provided the most thorough view on fungal diversity in dust; the number of genera identified by sequencing was 140 whereas cultivation yielded 20 genera (Figure 10). Thus, both taxonomically richer and phylogenetically wider representation of fungi was obtained using clone library sequencing.

The relative sensitivities of sequencing, qPCR and cultivation were assessed in study I. Because the true species abundances in the samples were not known, percentage sensitivities could not be assigned to the techniques but the methods were only evaluated in relation to each other's performance. QPCR was found to be the most sensitive of the used techniques; in 92% of cases when a species was detected by sequencing it was also detected by qPCR from the same sample. In contrast, sequencing failed to detect 60% of cases when a species was shown to be present in a sample by qPCR. The species not detected by sequencing tended to be those with lower cell counts; their median cell equivalent count was 1.4×10^3 CE/g^{-1} while the median concentration of species detected by sequencing was 5.9×10^5. The Spearman rank correlation between sequencing and qPCR results was 0.59 ($p < 0.01$), meaning that the clone library frequencies tended to reflect the original species abundances in samples yet significant variation occurred (I). It was observed that phylotypes occurring as singletons in clone

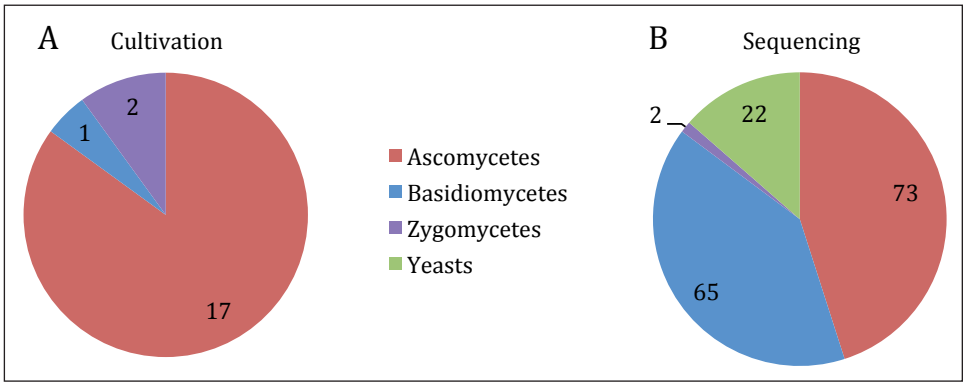

Figure 10. Phyla distribution of dust-borne fungal genera observed by cultivation (A) and by nucITS clone library sequencing (B). Number of identified genera is shown (I, II).

libraries were in some cases highly abundant in samples as measured by qPCR; their cell counts could be as high as 10^5 CE g^{-1} (I).

The comparison of culture- and qPCR results is somewhat ambiguous since culture isolates were only named to genus but most qPCR assays targeted species. The set of qPCR assays (69 assays) was also limited and did not cover all cultivated genera, e.g. *Phoma* (Sphaeropsidales) and *Verticillium*. However, in a majority of cases in the study I at least one representative of each cultivated genus was detected by qPCR in the corresponding sample. In such cases, the qPCR-assayed cell counts were ca. two orders of magnitude higher than the viable counts. This is comparable to the observations made by others on the percentage culturability of indoor fungi (Meklin *et al.* 2004, Lignell *et al.* 2008). In some cases nucITS sequence analysis of the isolates revealed that non-targeted species of the qPCR-targeted genera were present in samples. Such included *Acremonium alternatum*, *Alternaria citri*, *Aspergillus conicus* and *Wallemia muriae*.

To compare the "big pictures" obtained using cultivation and sequencing, fungi identified by sequencing were clustered into genus-level groups. Yeasts, filamentous basidiomycetes and unidentified ascomycetes were clustered into larger entities (Table 6). Viable communities were less diverse than molecular communities also using this approach, but similar trends were observed in the occurrence of many taxa, showing correspondence between the methods. The dominance of *Cladosporium* or yeasts in a sample was usually detected by both methods. The detection of Sphaeropsidales (in culture) and *Phoma* (by sequencing – *Phoma* is the major indoor member of the Sphaeropsidales group) usually correlated, yet in some samples it was not detected in culture, potentially due to overgrowth by *Penicillium*.

In accordance to our observations concerning the underrepresentation of *Penicillium* in sequence material, the comparison showed that the genus was detected by sequencing if it was clearly the dominant viable genus in the sample. Nevertheless, its proportion of clones was substantial only in samples with very high viable counts ($> 1 \times 10^6$). It was also seen that high viable numbers of *Penicillium* tended to cover the presence of other species. The occurrence of the less abundant species correlated poorly (Table 6).

4.4 Macroarray hybridization method for the enrichment of clone libraries

In study IV a macroarray method for enhanced screening of clone libraries was developed. The method utilizes the detection and negative selection of abundant fungal phylotypes in clone libraries by oligonucleotide probing, after which the unhybridizing clones representing rare phylotypes are picked for sequencing. The method was developed to speed up and lower the costs of sequencing environmental clone libraries. The principle was tested *in vitro* on fungal communities occurring in composting plants (IV). The usefulness of the principle for indoor samples was subsequently assessed *in silico* using sequence data obtained from indoor dust samples (unpublished data).

4.4.1 Study IV – compost fungal communities

Six oligonucleotide probes were designed for six fungal species that had been identified as the dominant members of fungal communities of composting facilities during the course of another study (Hultman *et al.* 2010. The hybrizidation method was used for negative

selection of these abundant phylotypes among 1536 clones gridded to a nylon membrane. Positive hybridization was detected for 59% of clones. The remaining 41% of the original clones were identified as rare, and were subsequently sequenced. The specificity of the method was calculated from a set of 384 clones, all of which were sequenced to verify their phylotypes. 91% of the hybridization signals corresponded to the sequencing result. A false negative hybridization was detected in the case of 20 clones (5.2% of total) and false positive in case of 15 clones (3.9% of total). These results demonstrated the efficiency of the method for enhanced analysis of fungal communities in compost samples.

4.4.2 Unpublished – dust fungal communities

Clone library sequence data obtained during the course of the study II was used to evaluate the feasibility of using the hybridization method on indoor material. Analogous to the original procedure, the six most abundant phylotypes were identified. Abundant phylotypes accounted for 26% of total clones (7 - 44% of clones in individual libraries) (Table 7). Thus, after the probing, an average of 74% of clones would have remained left to be characterized by sequencing.

Table 7. The observed diversity and proportion of abundant species in indoor dust samples.

Sample	S_{obs}	%Ab.
B1 winter	50	23%
B2 winter	47	20%
B1 spring	70	44%
B2 spring	61	41%
B1 summer	225	40%
B2 summer	170	13%
B1 fall	141	8%
B2 fall	157	7%
Total	345	26%

S_{obs}: number of observed phylotypes in sample.
%Ab.: summed percentage frequencies of the six most abundant phylotypes in sample.

Due to the different proportions of abundant phylotypes, the utility of the probing method differed significantly between the compost and dust samples. The advantage of screening out abundant phylotypes from compost nucITS libraries was evident, as only 41% of clones needed to be sequenced after probing. In contrast, such benefit was not seen with dust samples, since 74% of the clones belonged to rare or sporadically occurring species. Taking onto account the costs of materials and hands-in time required for gridding, hybridization and data-analysis in the hybridization step, the obtained savings in sequencing (26% of clones) were not seen high enough to make the inclusion of the hybridization step profitable. Thus the method was not taken into use in the sequencing analysis protocol of indoor dust samples. This conclusion was further supported our later observations on fungal community structure in other indoor samples (study I), which showed to be at least as diverse as the samples analysed in study II.

The hybridization and sequencing results suggested that the microbial community structure differed profoundly between the two environments; compared to the fungal communities of composts, which were dominated by few species, the assemblages occurring in indoor dust samples were markedly more diverse and the role of individual abundant species was less significant. This difference was also reflected in the distinct fungal community richness estimates for compost and

dust; the estimated number of phylotypes (S_{ACE}) ranged from 9 to 26 for the compost samples (Hultman *et al.* 2009) but from 79 to as high as 418 for dusts (study II).

The differential community structure reflects the profound differences of compost mass and indoor dust as microbial habitats. The higher water activity and substrate availability in compost mass supports the fast growth and dominance of a restricted number of adapted species. In contrast, the dry conditions in indoor dust make this environment a passive repository of settled material which is mainly shaped by allochthonous processes.

5. CONCLUSIONS

1. We hypothesized that the culture-based methods would reveal only a fraction of the true microbial diversity in indoor dust samples and that a wider diversity of fungi and bacteria would be seen by using DNA based methods. Our results supported this hypothesis; the well-known dominant microbial groups such as outdoor-borne phyllopllane fungi, yeasts and human-skin-associated bacteria were accompanied by a taxonomically wide and diverse array of known and unknown microbes that cannot be readily identified by routine cultivation. The number of fungal genera in each analysed sample greatly exceeded the viable diversity. Predominant uncultivated groups included unknown ascomycetes and decomposing agaric fungi, human skin-associated yeasts, and rusts. This demonstrates that people are exposed to a diverse array of microbes even in a normal office environment. However, since the sequencing method used here is semiquantitative in nature, the true abundances of the "novel" species are not known, and subsequently their relevance with respect to e.g. human health cannot be evaluated.

2. Viable fungal communities are known to vary according to season, which limits the use of culture methods in building investigations during the growth season. Little was known about the seasonal variation of bacteria, or about the variation of nonviable microbial particles over seasons. We observed that the seasonal variation in the sequence material, which covers both viable and nonviable microbes, was much more prominent than the variation in viable fungal communities. Bacterial communities showed milder seasonal variation due to a stronger contribution of human-associated taxa present throughout the year. Microbial communities were least diverse, and outdoor sources were weakest during winter, while various outdoor-borne microbes crowded other seasons' samples. The results from fungal analysis suggested that the studied sample type (floor dust), represents the seasonal load of microbes. Thus, as in culture-based building assessment, winter samples are preferable targets also for the molecular assessment of building sources.

3. We sought for major changes in total microbial communities in association with water damage. The effect of damage on dust communities was not evident, but instead, other sources were usually seen to dominate in both building types. Certain potentially material-associated species and groups were seen to occur in the damaged buildings, but these were mainly present in low numbers. However, since culture-independent reference data from building-material-associated microbes is scarce, and uncultivable species dominated in dust, our results were not well interpretable with this respect. Also the small sample number and deep normal variation of microbial communities between buildings undermined the assessment of the effect of building sources on dust communities. These results call for a) a culture-independent characterization of fungal and bacterial communities growing on building materials, and b) a subsequent assessment of a larger set of indoor bioaerosol samples to characterize the communities in damaged and undamaged buildings.

4. The comparison of clone library sequencing, qPCR and cultivation turned out to be somewhat irrelevant since the methods measure partly different things and are not fully comparable. However, the three methods largely agreed on the dominant fungal genera in the samples. qPCR was shown to be the most sensitive of the techniques for detecting individual species, yet a vast majority of species present in the samples were not detected due to the lack of a targeting qPCR assay. The overall quantitative correlation between qPCR and clone library results for qPCR-targeted species was moderate, but severe underestimating bias was observed in case of individual genera including *Penicillium* and related genera. Thus, while clone library sequencing is a good method for characterizing previously unknown microbial communities, it is not a suitable method for the quantitative analysis and comparison of samples.

5. In order to make clone library sequencing more efficient, a method for the negative selection of rare phylotypes was developed. This method was seen to be suitable for screening sample types that are heavily dominated by a limited number of species. In contrast, the method was not seen to be feasible for the characterization of highly diverse microbial communities with relatively even species distributions, such as indoor dust samples.

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Appendix 1. List of fungal species isolated from dust according to literature and the present study (I, II). Only studies reporting species-level identifications of fungi were included.

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Absidia corymbifera (syn. Lichtheimia corymbifera)	decaying vegetation, hay, soil	AF, CA	BSL2	2, 3, 7, 19, 23, 25
Acremonium butyri	soil		NC	4, 25
Acremonium murorum (syn. Gliomastix murorum)	decaying plant material, water, sewage, seeds, cereals, soil	M, P, W	NC	2, 3, 7, 23, 25
Acremonium sclerotigenum	soil		NC	4, 25
Acremonium strictum	humidifier water, soil	AF, CA, CO, GB, M, P, W, WO, WP	BSL1	2, 3, 4, 7, 13, 16, 22, 23, 25
Alternaria alternata	outdoor air, humidifier water, fruits, hay, soil	CF, I, M, P, UF, W, WP, GB	BSL1	3, 4, 7, 11, 12, 13, 18, 19, 22, 23, 25, 26
Alternaria citri	citrus fruits		NC	25, (I)
Apiospora montagnei (syn. *Apiospora arundinis*)	plant material		NC	2, 4, 25
Arthrinium phaeospermum	plant material, soil	M, WO	BSL1	1, 2, 3, 4, 6, 29
Armillaria borealis	soil		NC	(I, II)
Aspergillus candidus	hay, cereal, soil	CA, CO	BSL1	2, 3, 4, 7, 11, 14, 19, 23, 25
Aspergillus clavatus	cereal, flour, soil	AF, P	BSL1	7, 19, 23, 25
Aspergillus conicus	kernels		BSL1	(I)
Aspergillus flavipes	plant material, soil	CA, CF	BSL1	24, 25, 26
Aspergillus flavus	nuts, flour, stored food, clothes, soil	AF, CA, G, W, WP, UF	BSL2	1, 3, 7, 14, 15, 19, 20, 21, 22, 23, 25
Aspergillus fumigatus	decaying vegetation, hay, cereals, humidifier water, soil	AF, CA, CF, UF, WO	BSL2	2, 3, 4, 10, 11, 12, 14, 16, 19, 21, 22, 23, 25, 26
Aspergillus glaucus	plant material, soil		BSL1	14, 21, 24, 25
Aspergillus nidulans (teleom. *Emericella nidulans*)	seeds, cotton, hay, cereals, soil	AF, CA, CF, P, WO	BSL1	3, 2, 4, 7, 14, 18, 21, 23, 25, 26
Aspergillus niger	soil litter, dried plant products, cotton, cereals, hay, outdoor air	AF, CA, CF, CO, FI, GB, UF, W, P, PA, WO, WP	BSL1	1, 2, 3, 4, 5, 7, 10, 11, 12, 14, 18, 20, 21, 23, 24, 25, 26
Aspergillus niveus	soil	CF	BSL1	25, 26
Aspergillus ochraceus	grains, salted foodstuffs, soil	AF, CA, CO, CT, FI, GB, PA, WO, WP	BSL1	1, 2, 3, 4, 5, 7, 10, 11, 12, 14, 19, 21, 22, 24, 25, 26, (I)
Aspergillus oryzae	fermented soy products, soil	CF	BSL1	25, 26
Aspergillus penicilloides	dried foods, cereals, soil	CA, CO	NC	2, 3, 4, 7, 16, 19, 23, 25
Aspergillus restrictus	foods, fruit juice, cotton, cereals, soil	CA, P	BSL1	2, 3, 4, 7, 18, 19, 23, 24, 25
Aspergillus sydowii	cereals, flour, soil	AF, CA, CT, GB, P, UF, WP	BSL1	1, 2, 4, 7, 11, 12, 15, 18, 19, 22, 23, 24, 25
Aspergillus tamarii	soil	I	BSL1	2, 3, 19, 25
Aspergillus terreus	stored crops, cereal, hay, cotton, soil	CA, CF, P, WO, WP	BSL2	2, 7, 11, 19, 23, 24, 25, 26
Aspergillus unguis	seeds, soil		BSL1	2, 3, 12, 15
Aspergillus ustus	foodstuffs, soil	CA, CF, GB	BSL1	1, 2, 3, 7, 11, 15, 18, 22, 21, 25, 26
Aspergillus wentii	foodstuffs, soil	I	NC	4, 19, 25
Aspergillus versicolor	stored food, careal, hay, cotton, soil	AF, CA, CO, CT, FI, GB, P, PA, UF, W, WO, WP	BSL1	1, 2, 3, 4, 5, 7, 11, 12, 13, 14, 15, 16, 18, 19, 21, 22, 23, 24, 25, 27, 28

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Aureobasidium pullulans	foodstuffs, fruit, vegetables, cereals, humidifier water, soil	AF, CA, CF, P, PA, W, WO	BSL1	1, 2, 3, 4, 7, 12, 16, 20, 23, 25, 26, 27, 28, 29, (I, II)
Botrytis cinerea (teleom. *Botryotinia fuckeliana*)	plant material, hay, cereals, soil	CA	NC	2, 3, 4, 7, 21, 23, 25, 27, 29
Botryobasidium subcoronatum	dead wood (white rot)		NC	(II)
Cerrena unicolor	dead wood		NC	(II)
Chaetomium aureum	soil	CF	NC	23, 25, 26
Chaetomium funicola	soil	CF	BSL1	25, 26
Chaetomium globosum (syn. *C. cochliodes*)	soil	AF, CA, CF, GB, P, WP, WO	BSL1	2, 4, 7, 15, 18, 20, 21, 23, 25, 26, 29
Chaetomium indicum	hay, cereals, soil	CF	NC	2, 23, 26
Chondrostereum purpureum	wood (white rot)		NC	(II)
Chrysonilia sitophila	bakery goods ("bread mould")	I, CF	BSL1	2, 4, 19, 20, 23, 25, 26
Cladophialophora minutissima	phylloplane		NC	(I)
Cladosporium cladosporioides	cereals, foodstuffs, soil	CF, M, P, UF, W, WP	BSL1	2, 3, 7, 8, 10, 11, 12, 13, 14, 15, 16, 19, 20, 21, 23, 25, 26, 27, 28, (I, II)
Cladosporium herbarum	cereals, senescent plant material, soil	CA, CF, CO, CT, GB, P, PA, UF, WO	BSL1	1, 2, 3, 7, 10, 11, 19, 23, 25, 26, 27, 28, 20, (I, II)
Cladosporium macrocarpum	decaying plant material, soil		NC	10, 25
Cladosporium sphaerospermum	indoor surfaces, bread, soil	AF, CA, CO,CT, GB, P, PA, W, WO, WP	BSL1	1, 2, 3, 4, 7, 11, 12, 13, 14, 16, 18, 19, 20, 23, 25, 27, (I, II)
Clitocybe subditopoda	forest soil		NC	(I)
Coniothyrium fuckelii	plant material, soil		BSL1	2, 25
Coprinus stercoreus	dead wood		NC	(II)
Cordyceps sinensis	insects (entomopathogenic)		NC	(II)
Cortinarius armillatus	forest soil		NC	(I, II)
Cortinarius pholideus	forest soil		NC	(II)
Curvularia senegalensis	plant material, soil		BSL1	4, 25
Cylindrobasidium evolvens	dead wood		NC	(I, II)
Epicoccum nigrum	seeds, cereals, beans, soil	I, M, P, UF	NC	12, 18, 19, 23, 25, 27, 29, (I, II)
Epicoccum purpurascens	plant material, foodstuffs		NC	2, 3, 20
Eudarluca caricis	phylloplane (rust)		NC	(I, II)
Eurotium amstelodami	dried foods, cereals, nuts, fruit juices, hay, soil	AF, CA, CT, P	NC	2, 3, 4, 7, 12, 16, 18, 19, 23, 25
Eurotium chevalieri	dried foods, cereals, seeds, hay, soil	I	BSL1	2, 3, 19, 23, 25
Eurotium herbariorum	dried foods, dried meat, cereals, nuts, hay, soil	AF, CA, CO, CT, FI, GB, P	BSL1	7, 12, 15, 23, 25, 27, 28
Eurotium repens	plant material, foodstuffs, soil	CA, CO, GB, P	BSL1	2, 3, 4, 7, 17, 19
Eurotium rubrum	plant material, foodstuffs, soil	CA	NC	7, 19, 25
Exophiala jeanselmei	plant, decaying wood material, soil		BSL2	3, 25
Exophiala salmonis	salmon, leaf litter, wood		BSL1	(II)
Flammulina velutipes	dead wood, living trees		NC	(I)
Fusarium avenaceum (teleom. *Gibberella avenacea*)	woody plant material, important phytopathogen	CF	NC	22, 26, (I, II)

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Fusarium culmorum	grass, cereals, fruit, soil	I, W, CF	NC	2, 3, 4, 19, 23
Fusarium equiseti	plant material, soil	CF	NC	25, 26
Fusarium oxysporum	plant material, soil	CF	BSL2	3, 4, 25, 26, (I, II)
Fusarium solani	plant material, humidifier water, soil	I, CF, WO	BSL2	2, 3, 19, 20, 23, 26
Fusarium verticillioides (teleom. *Gibberella moniliformis*)	stagnant warm water, woody plant material, cotton, cereals, soil	W	BSL2	2, 18, 23
Geomyces pannorum	foodstuffs, paper	I, W	BSL1	2, 13, 19, 23, 25
Geotrichum candidum	seeds, fruits, soil	W	BSL1	3, 23, 25, 26
Gibberella fujikuroi	woody plant debris, soil	M	BSL1	18,
Gliocladium roseum (syn. *Clonostachys rosea*)	cereals	CA, UF	NC	2, 7, 23, 25
Gymnopus aquosus	forest soil		NC	(II)
Gymnopus dryophilus	forest soil		NC	(II)
Hormonema dematioides (teleom. *Sydowia polyspora*)	plant material, needles		BSL1	25, (I, II)
Hortaea werneckii (syn. *Exophiala werneckii*)	plant, decaying wood material, soil	I	BSL1	19, 25
Hypholoma capnoides	dead wood		NC	(I, II)
Hypholoma sublateritium	dead wood		NC	(I, II)
Hypogymnia physodes	dead wood		NC	(I, II)
Itersonilia perplexans	trees, rocks (lichen)		NC	(II)
Lecythophora hoffmannii	decaying wood, plant material	M	BSL1	18, 25
Leptosphaerulina/Pithomyces chartarum	live and decaying grass, soil	CA, CT, UF	NC	2, 4, 8, 12, 23, 25
Leptosphaerulina trifolii	live and decaying grass, soil		NC	(I, II)
Melampsora caprearum	larch-willow rust		NC	(I, II)
Melampsoridium betulinum	birch rust		NC	(I, II)
Melampsoridium hiratsukanum	alder rust		NC	(I, II)
Meria laricis	conifers (needle blight)		NC	(I)
Microdochium nivale	grass roots (snow mould)		BSL1	(I, II)
Microdochium phragmitis	poaceae (endophytic)		NC	(II)
Mucor circinelloides	plant material, soil		BSL1	2, 4, 3, 25
Mucor hiemalis	foodstuffs of plant origin, hay, soil		BSL1	2, 3, 23, 25
Mucor mucedo	soil		NC	3, 25
Mucor plumbeus	stored plant produce, hay, soil	I, M, WO	NC	8, 11, 18, 19, 20, 23, 25
Mucor racemosus	cereals, soil	CO, PA, WO, WP	BSL1	1, 2, 10, 19, 23, 25
Mucor spinosus	soil	M	NC	10, 11, 21
Mycosphaerella phacae-frigidae	decaying plant material		NC	(II)
Myrothecium roridum	plant material, soil	CF	NC	2, 3, 25, 26
Oidiodendrum rhodogenum	cork, straw	M	NC	23, 25
Panellus serotinus	decaying wood		NC	(I)
Paecilomyces lilacinus	humidifier water, stored foods, plastic, soil	M	BSL1	1, 11, 23
Paecilomyces variotii	compost, fat-containing foodstuffs, hay, grain, soil	CA, CO, CF, GB, P, PA, UF, W, WO	BSL2	1, 3, 7, 11, 13, 15, 18, 19, 21, 23, 25, 26, 29

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Parmelia sulcata	rocks, trees (shield lichen)		NC	(I)
Penicillium atramentosum	cheeses, walnuts, soil	CF	NC	25, 26
Penicillium aurantiogriseum	cereals, plant material, soil	AF, CA, CO, GB	BSL1	2, 3, 4, 7, 12, 15, 19, 25, 28
Penicillium brevicompactum	decaying vegetation, cotton, hay, cereals, soil	AF, CA, GB, P, UF, W	BSL1	1, 2, 3, 4, 7, 12, 13, 14, 15, 16, 18, 19, 23, 24, 25, 27, 28, (I)
Penicillium chrysogenum	foodstuffs, paper, soil	AF, CA, CO, CT, GB, UF, P, WP, PA, W, WO	BSL1	1, 2, 3, 4, 7, 11, 12, 15, 16, 18, 19, 22, 23, 24, 25, 27, (I)
Penicillium citreonigrum	decaying vegetation, cork, soil	M, WO	NC	12, 18, 23, 25
Penicillium citrinum	decaying vegetation, cotton, flour, soil	I, CF, GB, P	BSL1	4, 7, 12, 14, 15, 19, 23, 25, 26
Penicillium commune	foodstuffs, soil	AF, CA, CO, CT, GB, P	BSL1	7, 12, 15, 18, 19, 22, 23, 25, (I)
Penicillium corylophilum	decaying vegetation, foodstuffs, cereals, soil	AF, CA, GB, P, CO, CT, WO	NC	1, 2, 7, 12, 15, 18, 22, 23, 25, (I, II)
Penicillium crustosum	refrigerated foodstuffs, soil	AF, CA, WO	NC	1, 7, 12, 18, 22, 23, 25
Penicillium decumbens	seed, cereal crops, soil	AF, CA, CO, CT, P, UF, GB	BSL1	7, 12, 14, 15, 18, 25
Penicillium digitatum	mouldy citrus fruits, soil		NC	2, 3, 23, 25
Penicillium echinulatum	wood, lipids, cheese, soil		NC	4, 25
Penicillium expansum	pomeaceous fruits, cereals, flour, soil	M	BSL1	12, 14, 19, 20, 22, 23, 25
Penicillium funiculosum	foodstuffs, fruits, cereals, nuts, soil	CF	NC	23, 26, 25
Penicillium glabrum	soil	CA, UF, CF, GB, W	NC	7, 12, 13, 15, 18, 22, 23, 26, 27
Penicillium implicatum	fruits, soil	CA, CF, P	NC	7, 14, 25
Penicillium italicum	citrus fruits, soil	M	NC	2, 22, 25
Penicillium janczewskii	soil	CF, W	NC	4, 13, 26
Penicillium janthinellum	soil	AF, CA, UF	NC	7, 25
Penicillium miczynskii	soil	CF	NC	25, 26
Penicillium olsonii	water, straw, bread, jam, soil	AF, CA	NC	1, 3, 4, 7, 18, 22, 23
Penicillium oxalicum	seeds, cereals, greenhouse, tropical products, soil	CA, GB	NC	7, 14, 19, 25
Penicillium purpurogenum	cereals, soil	AF, CA, CF	BSL1	7, 12, 22, 26, 25
Penicillium roquefortii	blue cheese, foodstuffs		NC	(I)
Penicillium rugulosum	foodstuffs, soil	CA, P, W	BSL1	2, 3, 4, 7, 12, 14, 19, 23
Penicillium sclerotiorum	dried foods, peanuts, soil	CF	NC	12, 26
Penicillium simplicissimum	decaying vegetation, soil	AF, CA, CF	NC	7, 25, 26
Penicillium solitum	pomaceous fruits, (soil)	AF, CA	NC	1, 7, 12
Penicillium spinulosum	plant litter, soil	AF, CA, P	BSL1	2, 3, 7, 12, 25
Penicillium waksmanii	foodstuffs, soil	CF	NC	2, 14, 26
Penicillium variabile	soil	CA, GB, WO	NC	7, 12, 16, 23
Penicillium viridicatum	cereals, nuts, (soil)	AF, CA, CO, UF, GB, W	NC	7, 13, 14, 19, 25
Penicillium vulpinum	dung, insects, soil	M	NC	22, 25
Peniophora incarnata	plant material		NC	(II)
Phaeococcomyces nigricans	rock surface		NC	8, (I, II)
Phaeosphaeria herpotrichoides	plant material		NC	(II)
Phialophora fastigiata (syn. *Cadophora fastigiata*)	grass seeds, humidifier water, soil	WO	NC	23, 25

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Phlebia radiata	dead wood		NC	(I, II)
Phlebiopsis gigantea	dead wood		NC	(I, II)
Phoma eupyrena	plant material, (soil)		BSL1	3, 25
Phoma exigua	plant material, soil		NC	22, 25, (I, II)
Phoma glomerata	plant material, soil	WO, P	BSL1	4, 7, 18, 23, 25, (II)
Phoma herbarum	plant material, soil	W, P	BSL1	2, 3, 4, 7, 13, 18, 20, 25, (I, II)
Phoma macrostoma	plant material, soil		NC	(I, II)
Plectosphaerella cucumerina	plant material		BSL1	(II)
Polyporus tuberaster	dead wood		NC	(II)
Psathyrella candolleana	soil		NC	(II)
Pyrenophora teres	plant material		NC	(II)
Rhizoctonia cerealis	cereals, grasses		NC	(I)
Rhizopus oryzae	decaying fruit, vegetables, soil		BSL1	2, 4, 25
Rhizopus stolonifer (syn. *R. nigricans, Mucor stolonifer*)	decaying fruit, vegetables, soil	I	BSL1	2, 3, 4, 19, 20, 23, 24, 25, 28
Sclerotinia sclerotiorum	plant pathogen; vegetables, houseplants		NC	(I, II)
Scopulariopsis brevicaulis	plant and animal products, cereals, fruit, meat, cheese, butter, soil	CA, CT, P, WO, WP, W	BSL2	2, 4, 7, 13, 23, 25, 27, 29
Scopulariopsis brumptii	soil	P	BSL2	1, 3, 4, 7, 25
Scopulariopsis candida	plant matter, cereals, hay, soil	CT, P	NC	2, 3, 4, 7, 23, 25
Scopulariopsis chartarum	plant material, soil	WP	NC	4, 25
Scopulariopsis fusca	plant matter, foodstuffs, soil	M	BSL1	2, 22, 23, 25
Serpula lacrymans	decaying wood in buildings	M, WO	NC	7, 23
Sordaria fimicola	dung	CF	NC	26, 29
Stachybotrys chartarum (syn. *S. atra*)	cotton,straw, humidifier water, soil	AF, CA, CF, GB, P, UF, W, WP	NC	2, 3, 4, 7, 11, 13, 15, 16, 18, 19, 20, 23, 25, 26, 29, (II)
Steccherinum fimbriatum	bark, dead wood		NC	(I, II)
Suillus luteus	soil		NC	(II)
Syncephalastrum racemosum (syn. *S. verruculosum*)	plant material, dung, foodstuffs, cereals, cork, soil	CA	BSL2	2, 7, 12, 23, 25
Talaromyces flavus	plant material	AF, CA, CO, GB, P	NC	7, 25
Thamnidium elegans	dung		NC	4, 25
Thekopsora areolata	cherry-spruce rust		NC	(I, II)
Thelephora terrestris	dead wood		NC	(II)
Trichaptum abietinum	dead wood		NC	(II)
Trichoderma harzianum (syn. *Hypocrea lixii*)	decaying plant material, textiles, cereals, soil	AF, CA, GB, WO, UF	BSL1	2, 3, 4, 7, 12, 15, 18, 25, (I)
Trichoderma koningii	decaying plant material, compost, household waste, soil	CA, CF	BSL1	3, 4, 7, 23, 25, 26
Trichoderma viride	decaying plant material, cereals, foodstuffs of plant origin, soil	AF, CA, WO, GB, P, UF, CF	BSL1	2, 3, 7, 14, 16, 23, 25, 26, 29

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Tricholoma lascivum	ectomycorrhizal		NC	(I, II)
Tricholoma sejunctum	ectomycorrhizal		NC	(II)
Trichosporon cutaneum	skin, soil		BSL2	2, 3
Trichosporon jirovecii	skin, nails, soil		BSL2	(II)
Trichosporon mucoides / dermatis	skin, soil		BSL2	(I)
Trichosporon pullulans	pot soil, house plants		NC	3, 9, 29
Ulocladium botrytis	decaying plant material, soil		BSL1	2, 3, 4, 29
Ulocladium chartarum	decaying plant material, soil	AF, CA, CO, P, UF, W	BSL1	7, 11, 13, 19, 20, 22, 23, 25, 27, 28
Wallemia sebi	dried foodstuffs, cereals, salted fish, hay, soil	AF, CA, P	BSL1	2, 3, 4, 7, 14, 15, 16, 19, 22, 23, 24, 25, 27, 28
Yeasts				
Candida parapsilosis	domestic animals, soil		BSL1	2, 3
Candida tropicalis	humans, animals, soil		BSL2	4, 9
Candida zeylanoides	mucosa of humans and animals		NC	3, 4
Cryptococcus adeliensis	pigeon droppings		NC	(I)
Cryptococcus albidosimilis	soil		NC	(I, II)
Cryptococcus albidus	soil, plant surfaces, pot soil, house plants		BSL1	2, 4, 9, 27, 28, (I, II)
Cryptococcus carnescens	plant material		NC	8, (I, II)
Cryptococcus dimennae	plant material		NC	(II)
Cryptococcus festucosus	plant material		NC	(I, II)
Cryptococcus friedmannii	rock surface		NC	(I, II)
Cryptococcus laurentii	food, beverages, plants, soil		BSL1	2, 3, 23, 27, 28
Cryptococcus magnus	atmosphere		NC	(I, II)
Cryptococcus niccombsii	soil		NC	(II)
Cryptococcus oeirensis	leaf surface		NC	(I)
Cryptococcus terricola	forest soil		NC	(II)
Cryptococcus victoriae	soil		NC	(I, II)
Cryptococcus wieringae	flax		NC	(I, II)
Cystofilobasidium capitatum	sea water, soil		NC	(I, II)
Debaryomyces hansenii	pot soil, house plants, plant material, fruits, water, foodstuffs		BSL1	2, 3, 4, 9
Filobasidium uniguttulatum	skin, nails		BSL1	(I, II)
Malassezia globosa	human skin		BSL1	(I, II)
Malassezia restricta	human skin		BSL1	8, (I, II)
Malassezia slooffiae	human skin		BSL1	(II)
Malassezia sympodialis	human skin		BSL1	(I, II)
Mrakia frigida	soil		NC	(I, II)
Mrakia gelida	soil		NC	(I, II)
Rhodotorula glutinis	water, plants, soil		BSL1	4, 8, 9, 27, 28, (I)
Rhodotorula mucilaginosa (syn. *R. Rubra*)	foodstuffs, soil, epiphytic, house plants, soil		BSL1	2, 3, 4, 8, 9, 23, 27, 28, 29, (I, II)
Rhodotorula phylloplana	phylloplane		NC	(II)

Species	Common substrate/habitat	Building material substrate[A]	Pathog. [B]	Reference [C]
Hyphomycetes (filamentous fungi)				
Rhodotorula slooffiae	plant material		NC	(I, II)
Saccharomyces cerevisiae	fermented foodstuffs, fruits		GRAS	2, 3, 4, (I, II)
Sporobolomyces roseus	pot soil, house plants	W	NC	9, 23
Sporobolomyces ruberrimus	atmosphere		NC	(I, II)
Udeniomyces pannonicus	phylloplane		NC	(II)

The species identified in the present study (I, II) are indicated in bold. Phylotypes identified unambiguously on the species level, and present in dupletons or higher clone frequency are included.

[A] Building material abbreviations: AF: AHU filter; CA: carpets; CF: Cotton fabrics; CO: concrete; CT: ceramic tiles; FI: fiber insulations; G: glues; GB: gypsum board; I: indoor environment; M: materials (general); P: paint; PA: plaster; PL: plastic; UF: Urea-formaldehyde foam insulation; W: wall surface; WO: wood; WP: wall paper.

[B] Biosafety level class (BSL) according to de Hoog *et al.* (2009). NC: not classified, GRAS: generally regarded as safe.

Species reported in two or more studies were included in the table. Species encountered in one study included (reference): *Acremonium atrum* (29); *A. furcatum* (25); *A. kiliense* (25); *A. rutilum* (25); *Acrodontium salmoneum* (2); *Acrospeira mirabilis* (29) ; *Alternaria longipes* (8); *A. tenuissima* (25) ; *Aspergillus cervinus* (25); *A. ornatus* (25); *A. paradoxus* (25) ; *A. parasiticus* (25) ; *A. sclerotiorum* (25); *Blumeria graminis* (8); *Botryosphaeria sarmentorum* (8); *Botryosporium longibrachiatum* (4); *Botrytis allii* (25); *B. pilulifera* (25); *Candida catenulate* (9);*C. guilliermondii* (3); *C. haemulonii* (9); *C. kefyr* (teleom. *Kluyveromyces marxianus*) (2); *C. lusitaniae*, 4 ; *C. maltosa* (9); *C. pulcherrima* (syn. *Metschnikowia pulcherrima*) (3); *C. rugosa* (9); *Chaetomium circinatum* (25); *C. nozdrenkoae* (25); *C. piluliferum* (anam. *Botryotrichum piluliferum*) (3); *C. subspirale* (25); *Chrysonilia sitophila* (syn. *Monilia sitophila*) (3); *Cladosporium chlorocephalum* (25); *Cochliobolus sativus* (25); *Coniothyrium sporulosum* (25); *Cryptococcus diffluens* (9); *C. hungaricus* (4); *Curvularia geniculata* (teleom. *Cochliobolus geniculatus*) (25); *C. prasadii* (25); *C. protuberata* (25); *Davidiella macrospora* (8); *Diplococcium spicatum* (25); *Doratomyces microsporus* (25); *Drechslera biseptata* (25); *D. erythrospila* (2); *Emericella variecolor* (25); *Eupenicillium ochrosalmonium* (25); *Fusarium cinctum* (syn. *Myrothecium cinctum*) (25); *F. flocciferum* (25); *F. merismoides* (2); *F. nivale* (2); *F. proliferatum* (3); *Gilmaniella humicola* (25); *Gliomastix luzulae* (3); *Hainesia lythri* (25); *Humicola fuscoatra* (25); *H. grisea* (3); *Hyphopichia burtonii* (4); *Leptosphaerulina australis* (25); *Microsphaeropsis olivacea* (25); *Monascus ruber* (25); *Mortierella ramanniana* (25); *Neosartorya fischeri* (3); *Neurospora crassa* (21); *Nigrospora sphaerica* (25); *Ochroconis constricta* (syn. *Scolecobasidium constrictum*) (25); *Ophiostoma tenellum* (25); *Paecilomyces carneus* (3); *P. fulvus* (25); *P. fumosoroseus* (25); *P. inflatus* (25); *P. marquandii* (2); *Penicillium canescens* (25); *P. coprophilum* (25); *P. cyclopium* (14); *P. frequentans* (14); *P. glandicola* (25); *P. griseofulvum* (25); *P. hirsutum* (25); *P. islandicum* (25); *P. melinii* (25); *P. raistrickii* (25); *P. restrictum* (25); *P. roquefortii* (25); *P. verrucosum* (22); *Pestalotiopsis palustris* (25); *Phaeococcomyces exophialae* (2); *Phialophora radicicola* (2); *Phoma chrysanthemicola* (25); *P. fimeti* (25); *P. levellei* (25); *P. medicaginis* (25); *Pichia anomala* (syn. *Candida pelliculosa*) (4); *P. fermentans* (syn. *Candida lambica*) (4); *P. onychis* (9); *Pleospora herbarum* (2); *Podosphaera fusca* (8); *Rhizomucor variabilis* (18); *Rhodotorula graminis* (2); *R. minuta* (28); *Rollandina hyalinospora* (2); *Scedosporium inflatum* (4); *Sporotrichum pruinosum* (25); *Stachybotrys parvispora* (25); *Stemphylium botryosum* (25); *S. solani* (25); *Talaromyces trachyspermus* (25); *Torula herbarum* (25); *Torulaspora delbruekii* (4); *Trichocladium asperum* (25); *Trichoderma polysporum* (25); *T. pseudokoningii* (2); *T. roseum* (25); *T. virens* (syn. *Gliocladium virens*) (25); *Trichophyton mentagrophytes* (2); *T. terrestre* (3); *T. tonsurans* (25); *Trichosporon ovoides* (4); *Trichothecium roseum* (2); *Tripospermum myrti* (2); *Truncatella angustata* (25); *Ulocladium atrum* (25); *Wardomyces humicola* (25); *Verticillium luteoalbum* (4).

[C] References: 1: Andersen *et al.* 2011; 2: Beguin *et al.*1995; 3: Beguin *et al.* 1996; 4: Beguin *et al.* 1999 ; 5: Chew *et al.* 2003; 6: de Hoog *et al.* 2009; 7: Flannigan and Miller 2011; 8: Fujimura *et al.* 2010; 9: Glushakova *et al.* 2004; 10: Gravesen 1979; 11: Gravesen *et al.* 1999; 12: Horner *et al.* 2004; 13: Hunter and Bravery 1989; 14: Ishii *et al.* 1979; 15: Jarvis and Miller 2005; 16: Lignell *et al.* 2008; 17: Miller 1995; 18: Nilsson *et al.* 2009; 19: Northolt *et al.* 1995 ; 20: Park *et al.* 2008; 21: Piecková *et al.* 2004; 22: Polizzi *et al.* 2009; 23: Samson 2011; 24: Schober 1991; 25: Scott 2001; 26: Siu 1951; 27: Verhoeff 1994 a; 28: Verhoeff 1994 b; 29: Wickman *et al.* 1992. For habitat information, Mycobank (http://www.mycobank.org), INSD organism isolation source information, and culture collection data on the type strains was used in addition to the listed references.