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Fungal DNA, Mould, Dampness and Allergens in Schools and Day Care Centers and Respiratory Health

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UPSALIENSIS
UPPSALA
2013

ISSN 1651-6206
ISBN 978-91-554-8788-1
urn:nbn:se:uu:diva-209597

Dissertation presented at Uppsala University to be publicly examined in Frödingsalen, Ulleråkersvägen 40, Uppsala, Friday, 6 December 2013 at 13:00 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in English. Faculty examiner: Nils Åberg (Astma & allergienheten, Drottning Silvias barn&ungdomssjukhus).

Abstract
Cai, G. 2013. Fungal DNA, Mould, Dampness and Allergens in Schools and Day Care Centers and Respiratory Health. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 942. 85 pp. Uppsala: Acta Universitatis Upsaliensis.
ISBN 978-91-554-8788-1.

Day care centers and schools are important environments for children, but few epidemiological studies exist from these environments. Mould, dampness, fungal DNA and allergens levels in these environments and respiratory health effects in school children were investigated in this thesis. In the day care centers studies, Allergen Avoidance Day care Centers (AADCs) and Ordinary Day care Centers were included. One third of the Swedish day care centers had a history of dampness or mould growth. Total fungal DNA levels were positively associated with risk construction buildings, reported dampness/moulds, rotating heat exchangers, linoleum floors and allergens (cat, dog, horse allergen) levels. The two school studies included secondary schools in Johor Bahru, Malaysia and elementary schools from five European countries (Italy, Denmark, Sweden, Norway, and France) (HESE-study). In Malaysia, 13 % of the pupils reported doctor-diagnosed asthma but only 4 % had asthma medication. The prevalence of wheeze in the last 12 months was 10 % in Malaysia and 13 % in the HESE-study. Cough and rhinitis were common among children in the HESE-study. There were associations between fungal DNA and reported dampness or mould growth. Fungal DNA levels and viable mould (VM) concentration in the classrooms were associated with respiratory symptoms (wheeze, rhinitis, cough, daytime breathlessness) in school children. In the HESE-study, associations were found between total fungal DNA, *Aspergillus/Penicillium* DNA and respiratory symptoms among children. Moreover, *Aspergillus versicolor* DNA and *Streptomyces* DNA were associated with respiratory symptoms in Malaysia and the HESE-study, as well as reduced lung function [forced vitality capacity (FVC) and forced expiratory volume in 1 second (FEV1)] among children in the HESE-study. In conclusion, fungal DNA and pet allergens were common in day care centers and schools and respiratory symptoms in school children were common. The associations between VM concentration and fungal DNA levels in the schools and respiratory health effects in school children indicated a need for improvement of these environments. Moreover, risk constructions should be avoided and buildings should be maintained to avoid dampness and microbial growth. Health relevance of microbial exposure and biodiversity needs to be further studied using molecular methods.

Keywords: Day care centers, Quantitative PCR, Fungal DNA, Allergens, Indoor environment, Building dampness, Bacteria, Mycotoxins, Respiratory symptoms, Asthma, School environment, Viable moulds, School children

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ISSN 1651-6206
ISBN 978-91-554-8788-1
urn:nbn:se:uu:diva-209597 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-209597)

To my family

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Cai GH, Bröms K, Mälarstig B, Zhao Z-H, Kim JL, Svärdsudd K, Janson C, Norbäck D. (2009) **Quantitative PCR analysis of fungal DNA in Swedish day care centers and comparison with building characteristics and allergen levels.** *Indoor Air,* 19(5):392-400.

II Cai GH, Mälarstig B, Kumlin A, Johansson I, Janson C, Norbäck D. (2011) **Fungal DNA and pet allergen levels in Swedish day care centers and associations with building characteristics.** *J Environ Monit,* 13(7):2018-24.

III Cai GH, Hashim JH, Hashim Z, Ali F, Bloom E, Larsson L, Lampa E, Norbäck D. (2011) **Fungal DNA, allergens, mycotoxins and associations with asthmatic symptoms among pupils in schools from Johor Bahru, Malaysia.** *Pediatr Allergy Immunol,* 22(3):290-7.

IV Simoni M, Cai GH, Norback D, Annesi-Maesano I, Lavaud F, Sigsgaard T, Wieslander G, Nystad W, Canciani M, Viegi G, Sestini P. (2011) **Total viable moulds and fungal DNA in classrooms and association with respiratory health and pulmonary function of European schoolchildren.** *Pediatr Allergy Immunol,* 22(8): 843-52.

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Abbreviations

AADCs	Allergen-Avoidance Day care Centers
Asp/Pen	*Aspergillus* spp. and *Penicillium* spp.
A. versicolor	*Aspergillus versicolor*
Can f 1	Dog allergen
CE	Cell Equivalents
CI	Confidence Interval
Der f 1	House dust mite allergen
Der p 1	House dust mite allergen
ELISA	Enzyme-Linked ImmunoSorbent Assay
Equ c x	Horse allergen
ETS	Environmental Tobacco Smoking
Fel d 1	Cat allergen
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced Vitality Capacity
GC-MSMS	Gas Chromatography-tandem Mass Spectrometry
HESE	The Health Effects of the School Environment study
HPLC-MSMS	High Performance Liquid Chromatography-tandem Mass Spectrometry
MuA	Muramic Acid
MVOC	Microbial Volatile Organic Compounds
ODCs	Ordinary Day care Centers
OR	Odds Ratio
PVC	Polyvinyl Chloride
qPCR	Quantitative Polymerase Chain Reaction
RH	Relative Humidity
S. chartarum	*Stachybotrys chartrum*
T	Temperature
TLR-2	Toll-like Receptor 2
VM	Viable Moulds

Introduction

Asthma and allergy prevalence among children

Atopic diseases such as atopic dermatitis, asthma, and allergic rhinitis are among the most common chronic diseases in the developed world. Asthma alone has become one of the most common chronic diseases affecting about 300 million people worldwide [1]. This now poses a considerable disease burden on individuals and economic disease burden on healthcare systems and society [2, 3]. Wheezing has been suggested as the most important symptom in identifying asthma in population studies [4]. The prevalence of asthma and allergy increased markedly over the second half of the last century, especially in westernized societies, as documented by a large number of epidemiological studies [5-10]. There is large global variation of the prevalence of asthmatic and rhinitis symptoms between countries. The International Study of Asthma and Allergies in Childhood (ISAAC) Phase III study demonstrated that wheeze ranged from 4.1-32.1 % for the 6-7y age-group and 2.1-32.2 % for the 13-14y age-group. Allergic rhinoconjunctivitis ranged from 2.2-24.2 % for the 6-7y age-group and 4.5-23.2% for the 13-14y age-group [8]. Recent reports have claimed that asthma is decreasing or has plateaued in industrialized countries [8, 11-15]. However, a recent review article concluded that there are, at present, no overall signs of a declining trend in asthma prevalence; on the contrary, asthma prevalence is in many parts of the world still increasing. The reduction in emergency healthcare utilization for asthma being reported in some economically developed countries most probably reflect improvements in health care [16].

The majority of people, 60 % of total global population, live in Asia [17] and many countries in Asia have rapid economy development with new building constructions. Asia has different climate zones including temperate e.g. Japan, Korea and tropic climate e.g. Malaysia. The ISAAC Phase III study showed a large variation of the prevalence of asthmatic and rhinitis symptoms between countries in Asia. Moreover, Asia Pacific and India were the only regions where increases of all three disorders (asthma, allergic rhinoconjunctivitis, and eczema symptoms) occurred more often in both age-groups [8].

In Sweden, researchers have paid special attention to the schools and day care centers environments in relation to children's health. The prevalence of

asthma and allergic rhinitis increased from 1970s in Sweden [5]. However, the number of studies on asthma incidence in preschool children is limited [18-20], with reported incidence rates of 20/1,000/year among 0–2y old children, approximately 10/1,000/year in 4–7y old, and 11/1,000/year across all ages. Moreover, the incidence of physician-diagnosed asthma was 1 % per year among 7-13y old school children [21]. The ISAAC study, however, reported a slight decrease of allergic rhinoconjunctivitis from Phase I to III (from 8.0 % to 6.9 % for 6-7y age-group and from 11.1 % to 10.4 % for 13-14y age-group) in Sweden (but only one city in Sweden participated) [8]. In contrast, there is study showed that users of asthma medication increased significantly from 1996 to 2006 [22]. Moreover, there were a significantly greater proportion of children with asthma using inhaled corticosteroids (ICS) in 2006 than in 1996. This increase was parallel to a major decrease in severe asthma symptoms such as disturbed sleep because of wheeze (49 % vs. 38 %) and troublesome asthma (21 % vs. 11 %) [23]. Moreover, there has been a major increase in allergic sensitization from 1996 to 2006 in North Sweden measured by skin print test (SPT). This may lead to a further increase in clinical manifestations of allergic diseases in the pre-teenage and teenage years in the future [24].

The many faces of the hygiene hypothesis

The global variation of the prevalence of asthma and allergies between countries suggest that the factors causing these diseases vary between different locations and countries. The causative factors could be related to socio-economic status, lifestyle, dietary habits, microbial exposure, indoor or outdoor environment, climate conditions and awareness of disease and management of symptoms [8, 25]. During the last decades, there has been a focus on the role of early life microbial exposure. The theory has been called the “hygiene hypothesis” and was firstly coined by the researcher Strachan in 1989 suggesting that reduction of early childhood infections in the modern society could explain the increase of asthma and allergies [26].

A large scientific audience has discussed and studied this idea over the last two decades, and new angles and aspects of the hygiene hypothesis have been proposed [27]. At least four different aspects of the hypothesis have been launched. One is that a decrease in exposure to infections such as viruses and bacteria in early childhood may alter the maturation of the immune system [28]**.** Another hypothesis is that microbial products such as endotoxin could affect the development of children’s immune systems early in life and the development of tolerance to allergens ubiquitous in natural surroundings [29, 30]. However, the effect can be depending on exposure timing, dosage, environmental cofactors and genetics [31]. Some studies have reported a lower prevalence of allergic sensitization and

physician-diagnosed asthma in children exposed to higher levels of endotoxin at home [32-35]. Radon has summarized the effects of endotoxin with respect to different phenotypes of asthma [36]:

> ‘‘The risk of atopic asthma, mainly dominated by eosinophilic response, is decreased in those exposed to endotoxins. In contrast, the risk of nonatopic asthma, characterized by neutrophilic response, is enhanced in subjects with higher endotoxin exposure’’.

A third hypothesis is that different genetic patterns in the promoter region for CD14 may modify response to microbial exposure [37]. A fourth hypothesis is that the composition of the intestinal flora in early life may influence the development of an allergic phenotype [38-40] and influence the immune response to infections [41]. Moreover, a recent study reported that higher maternal total aerobic bacteria and enterococci bacteria in the intestine were related to increased risk of infant wheeze which implied that maternal intestinal flora may be an important environmental exposure in early immune system development [42].

Indoor environment for children

People in the industrialized world spend about 60 % of their time in the dwelling and about 90 % could be spent indoors [43]. There are various types of airborne pollutants that may play a substantial role in the development and morbidity of asthmatic respiratory illness and allergies. The major indoor pollutants include both chemicals (nitrogen dioxide, ozone, sulfur dioxide, particulate matter, and volatile organic compounds) and biological parameters (dust mites, pet allergens, and mould) [44-46]. Children may have greater susceptibility to these pollutants than adults, because they breathe higher volumes of air relative to their body weights and their tissues and organs are growing [47, 48]. Home, day care centers and schools are the three most important indoor environments for children. Published data suggest that schools can be important sites of exposure to cat and dog allergens, particularly for susceptible individuals (e.g. sensitized children who do not have pets at home), and sometimes the school represents a location of greater exposure than the home [49-54]. School absenteeism is more frequent among asthmatic children than healthy children, and the absenteeism increase with severity of the disease [49, 55].

There is a trend that more and more pre-school children stay in day care centers. In Singapore, more than 90 % of the children attend day care centers [56]. In Sweden, 83 % of all children attended day care centers in 2010 [57]. The national campaigns for allergy prevention and better indoor environments has resulted in the creation of special day care centers in

Sweden, called ‘allergen avoidance day care centers’, or ‘allergy-adapted day care centers’ (AADCs). The first AADC was opened in 1979 in northern Sweden. These special day care centers exist in all areas of Sweden, and are financed within existing municipal budgets. In AADCs, neither children nor staff are allowed to have pets at home, and staff members are not allowed to be smokers or use perfumes or cosmetics that smell. General cleaning is enhanced and there is a reduction in the amount of textiles, carpets, open shelves and pot plants in the rooms [58].

Indoor allergen exposure and asthma and allergy

Indoor allergen exposure may be important in childhood atopic disease development [44, 59, 60] and influence morbidity [61]. Common indoor allergens are produced by house dust mites, cockroaches, animals (cats, dogs, and rodents), and moulds [62]. Numerous studies have shown that animal allergens can be present in environments in which no animals reside and are transferred from other environments by clothing or human hair [63-66]. Asthma severity in children can be related to the level of exposure to common indoor allergens such as dust mite and cat allergens [67]. However, it is unclear if high exposure to indoor allergens causes more asthma and allergies. A review article concluded that allergen exposure may cause asthma, be protective, or have no effect, depending on the type of allergen, age of exposure, route of exposure, dose of exposure and underlying genetic susceptibility [62]. On the other hand, there is strong evidence that indoor allergens play a key role in triggering and exacerbating allergy and asthma symptoms in sensitized subjects [68, 69].

The conception of building dampness

The conception of ‘‘dampness’’ includes both high relative humidity in indoor air and moisture in the construction and have been associated with health problems [70, 71]. Different parts of the world may have different kinds of ‘‘dampness’’ problems. In Scandinavia visible mould and condensation on walls is rare while hidden dampness in the construction is more frequent. In more humid climate visible mould and condensation on walls are more common. Water damage in buildings can be due to construction flaws, leakages, flooding, and moisture accumulation caused by energy-effective ways of construction, insufficient airing, and insufficient maintenance [72, 73]. High relative humidity is an indicator of poor ventilation, which may result in increased levels of a wide range of other potentially harmful indoor pollutants. Dampness may increase dust mites and moulds, or promote wood-rotting bacteria, yeasts and survival of viruses [71]. However, this has received little attention in the literature.

Furthermore, dampness can damage building materials, leading to off-gassing of chemicals (e.g. formaldehyde) and release of particles [45, 74]. There is study showed that dampness in the floor can cause chemical degradation of plasticizers in polyvinyl chloride (PVC) floor coatings and glues, with the emission of ammonia and 2-ethyl-1-hexanol [74]. Moreover, (1–3)-β-D-glucan, endotoxin and mycotoxins may be dispersed into the air in damp buildings [71, 75].

Chemical microbial markers

(1–3)-β-D-glucan is a biologically active polyglucose molecule composing as much as 60 % of the mould cell wall, and is also found in some soil bacteria and plants [76]. Endotoxins are part of the outer membrane of Gram negative (G-) bacteria, ubiquitous, and can be also found in normal indoor environments in house dust [36]. Muramic acid (MuA), as a peptidoglycan, is present in both G- and G+ bacteria. Since the cell wall of G+ bacteria is thicker, MuA is mainly a marker for G+ bacteria [77]. Recently it has been shown that these fungal components may also be carried by smaller ultrafine or nanosize fragments [78-80]. Because of their small size, fungal fragments can stay in the air longer than larger spores with the potential to penetrate deep into the alveolar region when inhaled [79].

Health effects of building dampness

Moisture damage and indoor mould contamination have been commonly reported in homes, schools, offices, and hospitals. The conception of "dampness" varies in different part of the world, however, the reported risks for health effects are in the same range. Recent reviews and meta-analyses have concluded that sufficient epidemiological evidence is available from over 100 studies, conducted in different countries and under different climatic conditions, to show that the occupants of dampness or mouldy buildings are at increased risk of respiratory symptoms, respiratory infection, and asthma [71, 75, 81-83] and headache, fatigue, eye symptoms or sick building symptoms (SBS) [84-86]. Even if the mechanisms are unknown, there is sufficient evidence to take preventive measures against dampness in buildings, and the practical advice is to avoid dampness in buildings [70, 71] . Other studies have shown that remediating the water-damage and mould in asthmatics' homes resulted in improvements in the asthmatics' health [87-89].

Health effects of selected exposure in damp buildings

Fungal allergens, (1–3)-β-D-glucan, Microbial Volatile Organic Compounds (MVOC), and mycotoxins are among the proposed components that may contribute to some of these adverse health effects [45], however, inconsistent associations have been reported. Some studies have shown that increased concentration of fungi (total level or specific species) in the indoor environment is associated with increased risk of respiratory health outcomes [90-95], yet, other studies found no association [96, 97]. One study found a positive association between increased concentrations of (1–3)-β-D-glucan and prevalence of atopy [98], while the other found protective effects on atopic wheeze in school children [99]. Some studies reported positive associations between certain MVOC and nocturnal breathlessness and doctor-diagnosed asthma [100] and allergic rhinitis [101]. Moreover, chemical compounds caused by chemical degradation of certain building materials have been shown to influence respiratory health. An association between 2-ethyl-1-hexanol in the air and the secretion of lysozyme from the nasal mucosa and the occurrence of ocular and nasal symptoms has been reported [74].

Some studies found negative associations between endotoxin and the asthmatic symptoms and atopy [30, 102], and another study reported negative association with asthma for home endotoxin but positive association with non-atopic asthma for school endotoxin levels [103]. Other studies have reported positive associations between levels of endotoxin in house dust and respiratory illness [104] and wheeze [105]. In addition, MuA levels in dust, has been found inversely associated with wheezing and asthma [77] and with wheeze and daytime attacks of breathlessness [106].

Hypothesis on mechanisms for effects of microbial exposure on asthma

Mechanisms behind observed effects of microbial exposure are not well characterized. There may be differences in the health effects of microbes growing in their natural environment as compared to those growing in mouldy houses [107]. Moulds can produce distinct immune responses e.g. elevated different IgE titers and Th2 adjuvant activity [108-110]. Moreover, spores of the gram-positive bacteria *Streptomyces* spp. are able to cause cytotoxicity [107, 111], inflammation in lungs and systemic immunotoxicity [112], production of inflammatory mediators, such as cytokines, nitric oxide (NO), and reactive oxygen species (ROS) in immunological cells [113, 114]. NO, ROS, and cytokines are essential mediators in host defense, but if produced in excess they may cause inflammatory diseases including asthma [115-117]. It has also been suggested that fungal exposure might promote

adjuvant effects on allergic immune responses [118, 119]. The bacteria component endotoxin has strong immune-stimulatory properties [120-122]. Other bacteria components, such as MuA, can also act as immuno-modulators. MuA can be recognized by Toll-like receptors TLR-2 receptor, and this receptor also reacts to compounds in intestine parasite's cell walls [123, 124]. Some other studies reported that multiple microbial exposures (endotoxin and bacteria) in the home [125] and a wider range of microbes in farms [126] may protect against asthma or allergy in childhood which suggested that exposure to many different microbes is beneficial.

Indoor exposure in day care centers

Studies about the indoor environments of day care centers have been conducted mostly in the North America and Scandinavian countries [49]. Two studies from the USA and Canada measured CO_2 in daycare centers, and concluded that the ventilation is often inadequate, with CO_2-levels exceeding 1000 ppm [84, 127]. Most day care centers studies have assessed allergen levels, among which, cat (Fel d 1), dog (Can f 1), dust mite (Der f 1 and Der p 1), cockroach (Bla g 1 and Bla g 2), and mouse (Mus m 1 and mouse urinary protein [MUP]) allergens are most frequently studied [49, 128-134]. In addition, exposure to lead [135, 136], organic pesticides [137] and other persistent organic pollutants e.g. polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), phthalates have been measured in day care centers [138, 139]. Approximately 45 % of the 87 day care centers in Bergen, Norway, contained Pb and PAHs levels in dust above recommended action levels [138]. Finally, some studies have measured pollutants from the outdoor environment [NO, NO_2; TSP (total suspended particulates) and PM10] inside and outside day care centers [140-143].

Dampness and mould growth in day care centers

Dampness problems and indoor mould growth seems to be common in day care centers. In a nationwide survey of Swedish day care centers study, more than a third of the buildings had a history of mould growth or building dampness [58]. In a Finnish day care centers study, 70 % of the day care centers had water damage and 17 % had mould odour [144]. Two other studies from Taiwan [85] and Turkey [145] reported increased mould levels in some day care centers, but in general reports of airborne moulds in day care centers were uncommon. There were studies reported quantifiable levels of allergens from the mould species *Alternaria alternata* in settled dust in day care centers [49, 131, 132]. Chemical microbial compounds, such as (1–3)-β-D-glucan, bacteria [146] and endotoxin [130, 133, 146], have

been measured in daycare centers. There are large differences in the measured endotoxin levels between different studies. Endotoxin levels in dust samples in day-care units in Norway (0.005–0.050 ng/mg) [133] were approximately 5-60 times lower than in Finland (0.2–0.3 ng/mg) [146] and in Brazil (1–3 ng/mg) [130]. Differences in the analytical methods could have contributed to these differences.

Asthma and allergies among children and environmental factors in day care centers

There are limited data available to evaluate to what extent environmental exposure in day care centers contribute to allergic sensitization and exacerbation of allergic symptoms. We have not found any studies on health effects of measured indoor exposure on children at daycare centers. However, one study reported that specific environmental factors (e.g. pets, rugs, carpets) within day care centers may increase the risk of recurrent ear infections in the first year of life among children with familial history of atopy [147]. Another study reported lower prevalence of asthma and allergy, and respiratory symptoms in children attending natural ventilated day care centers [56] while the other study did not find any association between type of ventilation or dampness problems and the studied symptoms and diseases [148]. Some studies have been made on respiratory effects or sick building syndrome (SBS) in daycare center staff, in relation to moulds and dampness [85, 144].

Asthma and allergies and day care attendance

Studies investigating associations between asthma and allergy and day care attendance have produced conflicting results. There is evidence for an increase in respiratory infections among children attending day care centers [147, 149-152] and day care attendance may increase the risk of allergies and even asthma [152, 153]. In contrast, some studies have demonstrated protective effect of early attendance at day care on the risk of atopy [154-156] and asthma [157] later in childhood. The effect of day care on sensitization and atopic wheezing may differ among children with different variants of the TLR-2 receptor [158-161]. These genetic variations are thought to be responsible for variations in the individual susceptibility to effects of endotoxins [159, 162]. An alternative potential explanation for the protective effect of day care attendance is that children raised in this environment may be that they are exposed to lower levels of indoor allergens [163]. However, there is study concluded that the protective effect of day

care attendance (on atopy) cannot be explained by the reduced exposure to indoor allergen (house dust mite and cat) at day care centers [156]. Thus, further work is needed to determine the exposure that is responsible for the respiratory health effects of day care attendance.

Indoor exposure in schools and respiratory health effects

Indoor problems in schools include poor ventilation [164-167], high room temperature [168] and poor cleaning [21]. In an intervention study, new ventilation systems with increase ventilation flow improved indoor air quality and reduced asthma symptoms among students in intervened schools [166]. Moreover, the school environment may contain indoor pollutants such as moulds, bacteria, airborne dust, volatile organic compounds (VOC), MVOC and formaldehyde [167-170]. Exposure to allergens from furry pets in the school is common in the western world, especially cat and dog allergens [21, 49, 52, 53, 168, 171]. Some studies also reported presence of allergens from house dust mites (Der f 1/p 1), cockroach (Bla g 1), mouse (Mus m 1) [170, 172-174] and horse (Equ c x) [171] in schools [49]. However, remarkably few studies to date have evaluated associations between asthma and allergy and indoor allergen exposures in schools. Some Swedish studies have suggested that indirect exposure to cat and dog allergens in schools might influence asthma morbidity, asthmatic symptoms, or the incidence of asthma diagnosis [21, 53, 54, 171, 175].

Dampness and moulds exposure in schools and respiratory health effects among children

Some studies have reported positive associations between respiratory morbidity (e.g. asthma) among children and exposure to moulds in schools [176-180]. It has been reported that exposure to spores, toxins, and other metabolites of moulds may act as a nonspecific triggers for allergic sensitization, leading to the development of atopy [180]. Another study found an association between moisture and mould problems in a school building and the occurrence of respiratory infections and wheezing in school children [181]. Studies from China reported that observed indoor moulds were associated with asthma attacks among pupils [182] and microbial exposure indicated by certain chemical markers (e.g. MuA) could be protective for asthmatic symptoms, but the effect of lipopolysaccharide (LPS) (endotoxin) varied by different lengths of fatty acids of LPS [106]. One longitudinal study found that children without a history of atopy at

baseline had more new asthma diagnosis at higher concentration of total moulds in the classroom air [21]. Moreover, endotoxin exposure at schools, which were higher levels than at homes, was positively associated with non-atopic asthma in pupils [103].

Traditional methods for mould detection

Traditionally, mould quantification is performed by culturing moulds from the sample on various media or by counting cells under a microscope. Although culture-based analysis is one of the most economical ways to identifying moulds at species level, it requires different media for different species to grow and needs to be performed by qualified personnel. Moreover, non-viable and non-cultural mould are not detected by this method, and non-infectious health effects of microorganism are not related to viability [183]. Counting-based methods have limited measurement range and the counting can be influenced by the skill of the person doing the counting and can be disturbed by other particle [183, 184]. These traditional methods are not likely to measure the relevant microbial exposures accurately. Because of these limitations, it has been suggested that there is a need for molecular methods of mould analysis [75].

Quantitative PCR methods for mould specific analysis

By using different primers and probes, quantitative Polymerase Chain Reaction (qPCR or sometimes called real time PCR) [185] is a fast method for specific identification and quantification of viable and non-viable fungal agents, and is being used more frequently because its low detection limit and high accuracy [183]. EPA scientists has designed and tested probes and primers for about 130 moulds (http://www.epa.gov/microbes/mouldtech.htm) and designated the method as mould specific qPCR [184]. This method can detect general sequences of fungi DNA (e.g. *Aspergillus/Penicillium*) [186], as well as specific sequences (e.g. *Stachybotrys chartarum*) [187]. The method has been used in many studies in hospitals [183, 188, 189], in homes [183, 190] and in shopping centers [191]. Other sequences have been developed and used in agricultural environments [192, 193] and in hotel rooms [194]. A national dust sampling and analysis campaign using mould specific qPCR in US homes produced a scale for comparing the mould burden in homes, called the Environmental Relative Mouldiness Index (ERMI) [195], which was useful for the characterization of homes of severely asthmatic children [196]. The ERMI scale can be used to rank homes in terms of relative water-damage and mould growth and may be useful in finding hidden mould problems [195,

197-199]. However, it is expensive and time consuming since it needs to analyze 36 ERMI species.

Mycotoxins

Mycotoxins are low molecular weight (generally <1 kDa) natural products, produced as secondary metabolites by moulds. The term mycotoxin is restricted to those secondary metabolites that pose a potential health risk to animals or humans. However, most toxicological data for mycotoxins are from *in vitro* cell, bioassays and human or animal toxicity data is limited [200-202]. Many moulds that thrive in damp indoor environments are potent mycotoxin producers. Important mycotoxins includes sterigmatocystin, a carcinogenic mycotoxin produced mainly by *Aspergillus versicolor* (*A. versicolor*), and citrinin, gliotoxin and patulin, produced *by Aspergillus spp. and Penicillium spp*. Other examples are verrucarol and trichodermol, hydrolysis products of macrocyclic trichothecenes (including satratoxins), and trichodermin, predominately produced by *Stachybotrys chartarum* (*S. chartarum*) [203, 204]. Aflatoxins are mainly produced by *Aspergillus* spp., including *A. versicolor* and *A. flavus* [73]. However, there are few epidemiological studies measuring mycotoxins as indicators of mould exposure.

Mycotoxins can be analyzed by different methods. Mass spectrometry (MS)-based methods, especially tandem MS (MS/MS), are nowadays commonly used because of the high analytical specificity and sensitivity. Vishwanath and co-authors published a method for the simultaneous determination of 186 fungal and bacterial secondary metabolites in indoor matrices using HPLC MS/MS [205]. A Swedish researcher has developed a HPLC MS/MS method to detect the following mycotoxins: aflatoxin B1, gliotoxin, satratoxin G and H, and sterigmatocystin. Moreover, a gas chromatography MS/MS method was developed to detect trichodermol and verrucarol mycotoxins [73, 204]. Competitive enzyme-linked immunosorbent assay (ELISA) tests and array biosensors have also been used to analyze mycotoxins [206, 207].

Dust sampling methods

Before analyzing indoor microbial exposure, dust or particles must be collected by a dust sampling method. A variety of such methods exist and some are widely used [183, 208-211]. For air sampling, one widely used device is the Andersen N6 single-stage impactor (Thermo-Electron, Atlanta, GA, USA [212, 213]. It has long been accepted as the “gold–standard” method for the evaluation of fungal aerosols. However, this method can only

sample air particles for short time (a few minuts) and is combined with cultivating methods. For a relative longer time (hours), the airborne micro-organisms can be collected on Nuclepore filters, and analyzed by the CAMNEA method measuring both total and viable moulds and bacteria by adding cultivation methods [214]. Surface sampling can determine whether a spot on a wall is from fungal growth or has some other cause. Surface sampling can also assess the effectiveness of remediation and clean-up of indoor environments [183]. Cotton swab sampling has been used in school buildings to collect settled dust on the surfaces and mouldy spots [215] and in cases and matched control dwellings [216]. Swab sampling enables the sampling of dust that has accumulated over a longer period of time (several months), but during an unknown period. Moreover, the area of the contaminated surfaces should be measured to assess the potential risk linked to spore contamination [216].

In larger population studies, dust sampling from floors or mattresses and upper horizontal surfaces with a vacuum cleaner is the most common method since it is easily applied and is inexpensive. The main advantage of this method is that the collected dust can be analyzed by different techniques and it is possible to measure a variety of relevant components in these samples, like mite and pet allergens, endotoxins, and (1-3)-β-D-glucans [183, 210, 217-219]. However, part of the collected dust fraction consists of large particles that may never become airborne. Moreover, the dust composition of the samples might depend on the size of the area sampled, the sampling time, the power of the vacuum cleaner [220] and the sampling device trapping the dust (e.g. ALK filters, ALK Allergologisk Laboratorium A/S, Denmark [211, 221] or nylon-sock samplers, Allied Screen Fabrics, Hornsby, Australia [210, 211, 222] or Dustream collector, indoor biotechnologies, Charlottesvill [223]. Although health associations have been shown for components measured in vacuumed dust, it may be argued that methods sampling dust that has been airborne may be more representative of inhaled particle exposures.

Different methods to sample airborne dust has been used, such as active airborne dust sampling with an ion charge device [224, 225] or dust fall collector [226, 227]. However, these methods either have high equipment costs or have been applied only for short-term measurement. A new electrostatic dust fall collector (EDC) was designed by combining several of their features to measure endotoxin [228]. The EDC consists of a custom-fabricated polypropylene sampler that has electrostatic cloths attached to it to provide a sampling surface. Airborne dust settles on this surface and is captured by the electrostatic properties of the cloth (2-8 weeks). EDC may thus be a low-cost means of assessing long-term fungal exposure with a defined sampling time and sampling area [208, 228-232]. In addition, Petri dish sampling method has been used to measure allergens in schools environment [100, 233, 234]. This method can collect settling

airborne particles for a relative longer period (1-4 weeks) as compared to conventional pumped sampling [234-236]. The lack of standardized dust sampling methodology is problematic when comparing results from different studies.

Background to this thesis

Beside homes, day care centers and schools are important indoor environments for children. Previous studies have shown that allergens, moulds and dampness are quite common in these environments. However, there has been no previous study on associations between levels of indoor mould measured by molecular methods and building characteristics in these environments. Exposure to moulds may result in a variety of respiratory illnesses, but very few epidemiological studies exist from day care centers and schools, and very few using molecular methods. Mycotoxins are among the potential agents that could contribute to adverse health effects and occupants in damp buildings, but few epidemiological studies exist on health effects of indoor exposure to mycotoxins. Moreover, since most available studies in day care centers and schools are from developed countries in temperate climate zones, there is a need for more studies in different climate zones.

Aims of present investigations

The overall aims was to measure levels of selected mould indicators and furry pet allergens in day care centers and schools and study their associations with respiratory health in school children. The specific aim of the thesis was:

1. To measure levels of five selected fungal DNA sequences (including one gram-positive bacteria), airborne viable moulds (VM), selected mycotoxins, furry pet allergens and indoor climate in Swedish day care centers and schools in Europe and Malaysia.
2. To study associations between levels of fungal DNA, VM and furry pet allergens in Swedish day care centers and schools and selected building or room characteristics.
3. To study associations between levels of fungal DNA, VM, mycotoxins and furry pet allergens in schools and asthma, rhinitis, respiratory symptoms, airway infections and self-reported allergy in school children.
4. To study associations between levels of fungal DNA and VM in schools in Europe and lung function in school children.
5. To study differences in levels of fungal DNA and furry pet allergens between two types of Swedish day care centers (AADCs and ODCs).

Summary of Study Design

Table 1. Summary of study design for the four studies

Paper	I	II	III	IV (HESE)
Environment	Day care centers	Day care centers	Schools	Schools
Country	Sweden	Sweden	Malaysia	Europe (N=5)*
Numbers of				
Citys/Areas	6	1 (Österåker)	1 (Johor Bahru)	6
Selected buildings	22	26	8	21
Selected rooms	70	103	32	46
Measurements				
Indoor climate	Yes	No	Yes	Yes
Inspection	Yes	Yes	Yes	Yes
Dust sampling	Swab/Petri-dish/ vacuumed	Swab/Petri-dish	Swab/Petri-dish	Vacuumed/pump
Analysis				
Fungal DNA	Yes	Yes	Yes	Yes
Allergens	Yes	Yes	Yes	No
Mycotoxins	No	No	Yes	No
Viable moulds	No	No	No	Yes
Health study	No	No	Yes	Yes

*Five European countries: Italy, France, Norway, Sweden, and Denmark

Materials and methods

Study design and population

The first study (paper I)

In a previous survey, AADCs in Sweden were identified nationwide from technical and environmental sections of all 288 municipalities in Sweden, as well as all outpatient and inpatient paediatric clinics. Seventy-two AADCs and the closest situated ODCs were identified [58]. In this study, we selected a subset of matched pairs of day care centers (AADCs and closest ODCs) within near travelling distance of two major cities in Sweden (Malmö and Göteborg). Three to five rooms in each selected day care center (depending on the size) were investigated for fungal DNA and pet allergens. The rooms were arbitrary selected. For vacuum cleaning and climate measurement, we could only do three rooms per day care center. In total, 11 AADCs with 33 rooms and 11 ODCs with 37 rooms, in southern and western parts of Sweden, were included. Sampling was performed during the winter season (Jan-Feb) 2007.

The second study (paper II)

One mid-Swedish municipality (Österåker) was selected because there had been a general survey of the building conditions of all day care centers (N=24) performed by a major building inspection company. Three of the day care centers were excluded in this study since they were located in school buildings, and could be influenced by the school environment. The remaining 21 day care centers (26 separate buildings) were included. Measurements and room inspections were performed in 3-5 randomly selected rooms (depending on the size of the buildings) within each building. Totally 103 rooms were investigated on March-April 2007.

The third study (paper III)

Eight schools were randomly selected from the junior high schools in Johor Bahru, Malaysia. For each selected school, four classrooms were randomly selected. Finally, 15 students in each class were randomly selected. A total

of 462 pupils (224 male and 238 female) participated (participation rate 96%). The study was approved by an ethical committee in Malaysia.

The fourth study (paper IV)

Details of the Health Effects of the School Environment (HESE) study have been previously reported [164]. Briefly, the study involved six cities in five European countries (Italy, France, Norway, Sweden, and Denmark). Twenty-one schools (46 classrooms) with heterogeneous characteristics, new and old buildings, near and not near heavy traffic roads, were selected. The study was approved by the ethical committees of each institution. It was carried out in the heating season of 2004–2005 using the same standardized procedure, during a full week in each location.

Indoor climate measurement and building inspection

In paper I, III, and IV, Temperature (T, ℃), Relative Humidity (RH, %) and concentration of CO_2 (ppm) were measured during normal activities within 1-2h with Q-Trak^{TM} IAQ monitor (TSI Incorporated, St. Paul, Minnesota, USA), by logging average values over one minute. The instruments were regularly calibrated by Comfort Control, the Swedish service laboratory for TSI equipment. Indoor climate was not measured in paper II since the data from paper I showed that the Swedish day care centers are well-ventilated with CO_2 levels below the current standard of 1000 ppm [237].

The day care centers and school buildings and rooms were inspected and details on construction, building materials and age, type of ventilation and heating system, amount of open shelves, textiles and number of pot plants were noted. In paper I, II and III, the room volume (m^3), shelf (m/m^3), textile (m^2/m^3) [238] and pot plant (number of potplants/m^3) factors were calculated for each room.

The first study (Paper I)

In a previous study, a questionnaire was sent to the local directors of all day care centers in 2000 [58]. It included three questions on water leakage or flooding, signs of floor dampness, and visible mould growth in any part of the building the last 12 months using previously published questions [239]. Moreover, there was an additional question asking if there had been water leakage or mould growth at any time in the building (irrespectively of recall period). There were two yes/no questions on odours in the building, one question asking about mouldy odour and another asking about other types of odour [239]. A room inspection for signs of water leakage, flooding, damp spots, floor dampness (bubbles under the PVC-floor), visible mould growth,

and mouldy odour and window condensation was performed in the selected rooms in 2007 at the same time as sampling dust. In order to cover the time period from 2000-2007, additional data on reported dampness and mould growth was collected by a structured telephone interview with the local directors, using the same set of questions as in the previous questionnaire survey.

The second study (paper II)

Previously, the inspection company had classified the buildings into three groups by a two step procedure. Firstly, according to the types of construction, the building was classified as non-risk (level 0) or risk construction. Moreover, the risk construction was classified into two levels depending on absence (level 1) or presence (level 2) of visible water damage/moulds (Table 2).

Table 2. Principles for classification of risk construction buildings

Classifications principles	Non-risk constructions	Risk constructions	
Concrete slab on the ground	with underlying insulation	with overlying insulation	
Basement walls	with insulation outside	with insulation inside	
Outdoor ventilated crawl space	NO	YES	
Risk construction levels	Non-risk level 0	Risk level 1	Risk level 2
Visible water damage/moulds	NO	NO	YES

The fourth study (paper IV)

Information on classrooms characteristics was collected through a standardized questionnaire filled in by the teachers. Moreover, detailed inspections were made by the investigators, and data on school buildings/classrooms (including construction materials, type of ventilation system, and the presence of visible moulds/dampness) were recorded.

Dust sampling methods

Swabbing dust samples (paper I, II and III)

Sample collection by swabbing was performed with a sterile cotton swab initially designed for medical use (Copan Innovation, Brescia, Italy; www.copanitania.com). The dust samples in Paper I and paper II were collected by swabbing a 60 cm^2 surface (1×60 cm) of half of the upper part of the doorframe on the main entrance door to each room in the selected AADCs and ODCs. If the main entrance door had a supply or exhaust ventilation duct above the doorframe, another doorframe without any ventilation duct was selected. Two samples were collected by dividing the doorframe into a left and a right side, whereby the left-side one was sent for fungal DNA analysis. In paper III, settled dust were collected by swabbing 60 cm^2 of surface (1×60 cm per swab) from the top frame of the blackboard in each classroom. The blackboard top frame was divided into a left and right part, with the left side dust samples used for fungal DNA analysis and the right side samples for mycotoxin analysis.

Vacuumed dust samples (paper I and IV)

Vacuumed dust was collected in the same rooms as the other measurements. Two samples of settled dust were collected in each room by dividing the room into roughly a half entrance side and a half window side [168, 171, 233], using a vacuum cleaner equipped with a special dust collector (ALK Abello, Copenhagen, Denmark) fitted with a Millipore filter (pore size 6 μm). We sieved dust samples through a 0.3 mm mesh screen to obtain the fine dust [85], weighed the amount of the collected fine dust and then stored it at -20℃ until extraction. The vacuumed dust in paper I was used for allergen analysis, and in paper IV for fungal DNA analysis.

Airborne dust samples (Petri dish and pump)

In paper I, II and III, airborne dust was collected on two Petri dishes in each room, placed on the top of open book shelves or similar areas (at about 1.5-2.0 m height) [234]. Moreover, dust samples (for VM analysis) in paper IV were pumped on 25-mm nucleopore filters (pore size of 0.4 μm) with a sampling rate of 1.5 l/min for 4 h [214].

Analytical methods

Fungal DNA analysis (by qPCR)

The dust samples for fungal DNA analysis by qPCR were sent to a professional lab anoZona AB, Uppsala, Sweden which got license from Environmental Protection Agency (EPA). For DNA extraction, the cotton swabs were cut into 2 ml tubes, diluted with 400 µl of AP1 buffer (DNeasy Plant Mini Kit, Qiagen, Hilden Germany) and vortexed briefly. After removal of the swab, the cellular material and cell debris in the sample pellet was disrupted and homogenized by the addition of a tungsten carbide bead (Qiagen, Hilden Germany) and the subsequent beating and grinding effect of the bead on the sample material when shaken in the micro-centrifuge tube on a TissueLyser (Qiagen, Hilden Germany) at 30 Hz for 10 min. In contrast, 10 ml of double distilled water was added to the Petri dish, transferred to a 10 ml tube and then centrifuged at 12 000 rpm (8 000 g) for 5 minutes. After removal of the supernatant completely, added 250 µl AP1 buffer and transferred to a 2 ml tube. After this preparation, the respective target DNA extracts and total genomic DNA from the homogenates were extracted with the DNeasy Plant Mini Kit (Qiagen, Hilden Germany) according to the manufacture's instructions. The DNA extracts were kept at -70 ºC until amplification. Moreover, fungal DNA in vacuumed dust samples was extracted from 100 mg dust using the YeaStar^{TM} Genomic DNA Kit (Zymo Research, Orange, CA, USA). The quantity of the unknown samples was calculated based on the calibration curve of standardized DNA solutions versus the corresponding cycle threshold (Ct) value. If internal control was not detected or the Ct was over a certain value, the samples were further diluted. In respect of the sampling method in this study, the mould level was calculated by fungi cell concentrations expressed as cell equivalents (CE) for each target mould or mould group assuming one DNA copy per cell [194]. The final result was presented as CE/m^2 for cotton swab, CE/g dust for vacuumed dust and CE/m^2/day for Petri dish dust samples.

The first and second study (paper I and paper II)

Amplification and detection of the DNA extracts for *Aspergillus* or *Penicillium* genera (*Asp/Pen*), *Stachybotrys chartarum* (*S. chartarum*) and total fungal DNA were performed on Mx3000P/MXpro real-time PCR machine (Stratagene, La Jolla, CA, USA) in the TaqMan Master Mix (Applied Biosystems, Carlsbad, CA USA) according to the manufacture's protocols. A partial fungal DNA sequence common for a large number of moulds (Universal Fungal assay 1) was analysed, here described as total fungal DNA. The species list is available online at http://www.freepatentsonline.com/6387652.html. The standard qPCR cycling was performed using the following protocol: 50 °C for 2 min, 95 °C

for 10 min, 95 °C for 0.25 min, and 60 °C for 1 min, 45 cycles. Standard curves were created for respective analysis, from total genomic DNA extracts by pure cultures. The DNA extracts from the pure cultures were quantified using limiting dilution analysis [240].

The third and fourth study (paper III and IV)

Five multiplex reactions were performed in five separate tubes targeting the DNA of the following species: total fungi, *Asp/Pen*, *A. versicolor*, *S. chartarum* and *Streptomyces spp.* (Table 3) [194]. The primers and probes used for amplification and detection were designed using the design software Primer Express 2.0 (Applied Biosystems, Foster City, CA USA), by a well known company working with molecular diagnostics (Dynamic Code AB, Linköping, Sweden). Primers and probes for total fungal DNA, *A. versicolor* and *S. chartarum* DNA are in the region of internal transcribed spacer 1, 5.8 S rRNA and internal transcribed spacer 2. Primers and probes for *Asp/Pen* DNA are in the gene for 28S rRNA and for *Streptomyces* DNA in the gene for 16S rRNA. The reaction targeting *A. versicolor* simultaneously amplified an internal positive control that was used to detect PCR inhibition. Amplification and detection were performed on a 7300 Real-time PCR Instrument (Applied Biosystems, Foster City, CA, USA) using the Taqman® Universal Master Mix (Applied Biosystems, Foster City, CA, USA). Standard curves were created for respective analysis using total genomic DNA extracts from pure cultures and were quantified using limiting dilution analysis [241]. We have used the term ‘‘total fungal DNA’’ for this sequence since it covers a wide range of indoor fungi, mainly *Ascomycetes*, but it does not cover all indoor fungi.

In addition, pumped dust samples for viable mould analysis were cultivated on two different media [214]. The detection limit was of 30 colony-forming units (cfu) per m^3 of air.

Table 3. The detected species for the fungal DNA sequences by qPCR method

Fungal DNA sequences	Detected species (number)
Total fungal DNA	*Acremonium* (7), *Alternaria* (61, *including A. alternata*), *Aspergillus (*including *A. fumigatus)* (86), *Aureobasidium mansonii, Aureobasidium pullulans, Cerebella and ropogonis, Cladosporium* (38, including *S. herbarium*), *Curvularia* (14) , *Cylindrocarpon lichenicola, Davidiella* (3), *Epicoccumnigrum, Eupenicillium* (27), *Eurotium* (6), *Fusarium* (8), *Hemicarpenteles paradoxus, Mycosphaerella macrospora, Mycosphaerella tassiana, Nectria haematococca, Neosartorya* (17), *Paecillomyces* (15), *Penicillium* (157), *Petromyces* (3), *Ramichloridium mackenziei, Rhinocladiella* (9), *Sclerocleistaornata, Stachybotrys* (12), *Thermoascus* (3) and *Trichoderma* (48)
Asp/Pen DNA	*Aspergillus* (37, including *A. fumigatus*), *Davidiellatassiana, Eupenicillium* (14), *Eurotium* (15), *Hemicarpentelesparadoxus, Neosartorya* (7), *Paecilomyces variotii* and *Paracoccidioides cerebriformis, Penicillium* (62) *and Thermoascus*

	aurantiacus
A. versicolor DNA	*A. versicolor* (13 *GenBank accessions*)
S. chartarum DNA	*S. chartarum* (14 *accessions*) and *S.chlorohalonata* (6 *accessions*)
Streptomyces DNA	*Streptomyces (*187) and *Micromonospora megalomicea*

Allergens analysis

Two-site sandwich ELISA (Enzyme-Linked Immunosorbent Assay) was applied to determine the allergen levels of cat (Fel d 1), dog (Can f 1) and dust-mite (Der p 1 and Der f 1) (only in vacuumed dust samples) (Indoor Biotechnologies Ltd, Manchester, UK), and horse (Equ c x) (Mabtech, Stockholm, Sweden) [242], as previously described [100] for both vacuumed and airborne dust samples by using monoclonal antibodies. The allergen levels in vacuumed dust were expressed as ng/g dust, except for horse allergen concentration which was expressed as U/g dust, where 1 Unit equalled 1 ng protein of horse hair and dander extract used as standard (Allergon, Valinge, Sweden and NIBSC, Hertfordshire, UK).

Amplified ELISA was used for cat allergen analysis in Petri dish airborne dust samples, for cases when the cat allergen levels were lower than 1.0 ng/ml by the conventional ELISA. It was completed with a commercial signal amplification kit, basically by following the manufacturer's protocol [234]. The allergen levels in airborne dust samples were expressed as $ng/m^2/day$, by dividing the amount of allergen on the Petri dish by the sampling time, and the total surface area of Petri dish (0.0124 m^2 for sum of both lids) [100]. Total allergen level (ng/g dust) was calculated by adding cat and dog allergens (ng/g dust) and horse allergens (U/g dust).

Mycotoxin analysis

The swabs dust samples were extracted and analysed for aflatoxin B_1, gliotoxin, satratoxin G, satratoxin H and sterigmatocystin by using high pressure liquid chromatography (HPLC)-MSMS. Derivatives of trichodermol and verrucarol were analysed by GC-MSMS. Details on extraction, hydrolysis, derivatization and analytical conditions are provided elsewhere [204, 243]. Satratoxin G and H could not be quantified due to lack of pure reference compounds. The amount of each mycotoxin in the swab samples were expressed as pg/m^2 of swabbed surface area.

Assessment of health data

The third study (paper III)

A self-administered questionnaire, which had previously been used in school studies in Sweden, Korea and China, was used to assess the health data [100, 233]. It contained questions obtained from the large international ISAAC

study [8] and the European Community Respiratory Health Survey (ECRHS) [244], with additional questions on doctor-diagnosed asthma, current asthma medication, asthma attacks and allergies during the last 12 months [100]. It contained no question on lifetime wheeze but a question on lifetime asthma. In addition, there was a set of questions about airway symptoms related to asthma during the last 12 months, without using the term 'asthma'. These symptoms included wheezing or whistling in the chest, daytime attacks of breathlessness during rest and after exercise and nocturnal attacks of breathlessness in the last 12 months [233]. Moreover, the questionnaire contained questions on current smoking, allergy to cat, dog and pollen, parental asthma/allergy, and the number of respiratory infections during the last three months. The questions on cat, dog and pollen allergy contained three response options: yes, no, don't know [233]. The questionnaire was first distributed to the selected pupils during the same week as the technical measurements and was answered with the help of parents at home. Then a school nurse went through the questionnaires during a face-to-face interview with the students, to clarify any uncertainly in the questions.

The forth study (paper IV)

Data on children were collected through two standardized questionnaires filled in by the pupils and their parents, respectively. For the present study, we considered the following outcomes: 1) past year wheeze, 2) past year dry cough at night, 3) past year rhinitis, and 4) any current persistent cough (for 4 or more days per week, outside of common colds). Among children with both self and parental report (68 % of total), the outcomes were present when reported by either children or parents, or absent, when unreported by both children and parents. Information on children with only self-report (16.4 %) or only parental report (15.6 %) was also included in the data. Overall, health status was derived for 654 children. Moreover, there was a short questionnaire about the environment in the classroom answered by the teachers before the measurements were done by us. When answering the questionnaire, the students and teachers had no information on the measurement data from the classrooms.

Non-invasive clinical tests were performed on 5 randomized selected pupils in each class. For the present analyses we focused on forced expiratory volume in one second (FEV_1) and forced vitality capacity (FVC), as measured in 224 children. Pulmonary function was measured by experienced medical staff using a portable pocket spirometer (Spirobank™, IntraMedic Inc, Sweden). Percent predicted FEV_1 and FVC were computed by using reference equations for European children and adolescents, which take into account gender, age and height [245].

Statistical analysis

In crude analysis, Mann-Whitney U-test was used to compare differences between two groups, and Kruskal–Wallis test was used to compare more than two groups. Associations between categorical variables were tested by the chi-square test in contingency tables. Associations between continuous variables were tested by Kendal Tau-β rank correlation test. In paper II, to adjust for the hierarchic structure of data and for mutual adjustment, linear mixed models were used to analyze associations between fungal DNA, allergens levels and building factors. Within- and between-buildings variability was evaluated in paper II and III using linear mixed models with a random intercept. Data on total fungal DNA and allergens was log-transformed to get approximately normally distributed variables. In addition, the variance ratios also called 'fold-ranges' within- and between-buildings ($_wR_{0.95}$ and $_bR_{0.95}$) were calculated from the variance components of the 97.5th and 2.5th percentiles of the log-normally distributed exposure [246]. As an example: A $_wR_{0.95}$ of 3 means that 95 % of the mean value for each building can vary with a factor 3 between rooms. A $_bR_{0.95}$ of 3 indicates that the 95 % of the mean values for the buildings are with a range of factor 3.

Different types of multiple regression analysis were used to study health associations in paper III and IV. In paper III, associations between environmental allergens, fungal DNA, mycotoxin exposure and respiratory health effects were examined by hierarchical logistic regression, controlling for environmental variables of sex, race, smoking and heredity. Data on total fungal DNA, *Asp/Pen* DNA and allergens were log-transformed to obtain approximately normally distributed residuals. The clustered nature of the sample was controlled for using random intercepts on the school and classroom levels. Since there were relatively strong correlations between total DNA and *Asp/Pen* DNA, these two exposure variables could not be kept in the same model, and thus two models were applied. One was created to include only total fungal DNA and personal factors and another was created to include personal factors and the four specific fungal DNA and cat allergen. Odds ratio (OR) with 95 % confidence intervals (95 % CI) were calculated.

In paper IV, associations of health outcomes with mould exposure (VM or Fungal DNA) were assessed by logistic regression models with each symptom/disease as the dependent variable (0 = absent, 1 = present) and log-transformed exposure data as continuous independent variables. VM were entered in the models either as binary exposure variable (elevated vs. low) or as continuous variable. Data of VM and fungal DNA levels were log-transformed to obtain approximately normally distributed residuals. Besides conventional logistic regression models, we fitted random intercept two-level models for binary dependent variables using the STATA gllamm

(generalized linear latent and mixed models) command with logit link function, which estimates the maximum likelihood. Two hierarchical levels were considered: first level, the child, and second level, the classroom. Both crude and adjusted (for gender, age, passive smoking at home, and lifetime asthma) OR and 95 % CI were reported. The associations between lung function tests and mould exposure were assessed by linear regression analyses with FEV_1 and FVC as continuous dependent variables and log-transformed mould data as independent continuous variables. Both crude and adjusted (for gender, age, height, passive smoking at home, and any of lifetime asthma, dry cough at night, or cough) partial regression coefficients (B) and 95 % CI were reported. Besides linear regression models, we fitted the generalized least-squares random-effects model (GLS-RE) with fixed effect of the classroom using the STATA xtreg command.

All statistical tests were two-tailed, and a p-value below 0.05 was used to indicate statistical significance. Statistics were performed with the Statistical Package for the Social Sciences (SPSS). Besides, STATA was used in study III and study IV.

Results

Paper I

In total, 11 AADCs and 11 ODCs were studied (70 rooms). All had a mechanical ventilation system and most were heated by district heating and waterborne radiators. The mean construction year was 1983 (SD=13) (Table 4). Data from the local directors showed that some day care centers had a history of dampness, mould growth and current odour, but none of the directors classified the odour as mouldy odour. Buildings with reported odour had more vacuumed fine dust (Mean 752 mg; SD=301) as compared to buildings without (Mean 372 mg; SD=491) (p<0.001). In addition, 56 % of the buildings with reported odour had reported dampness, as compared to 22 % reported dampness in buildings without reported odour (p=0.009). Moreover, 33 % of the stone/brick buildings and only 13 % of wooden facade buildings had odour (p=0.05). Finally, 50 % of the stone/brick building and only 10 % of the wooden facade buildings had reported dampness (p<0.001).

Table 4. Characteristics of investigated rooms (N=70) and indoor climate parameters in eleven allergen avoidance day care centers (AADCs) and eleven ordinary day care centers (ODCs)

Building characteristics/Parameters		AADCs (n=33) n (%)	ODCs (n=37) n (%)	All day care center rooms (n=70) n (%)	P-value
Floor heating[a]		3 (9 %)	3 (8 %)	6 (9 %)	0.56
Wooden facade buildings[a]		15 (50 %)	16 (43 %)	31 (44 %)	0.45
Basement[a]		6 (18 %)	7 (19 %)	13 (19 %)	0.96
Dining room[b]		16 (31 %)	33 (57 %)	49 (46 %)	0.008**
Observed dampness[b]		0 (0 %)	9 (24 %)	9 (13 %)	0.11
Reported window condensation[c]		0 (0 %)	15 (41 %)	15 (21 %)	<0.001***
Reported dampness[c]		9 (27 %)	15 (41 %)	21 (34 %)	0.24
Any reported odour[c]		0 (0 %)	16 (43 %)	16 (23 %)	<0.001***
Type of floor[b]	linoleum	16 (48 %)	16 (43 %)	32 (46 %)	
	PVC	14 (42 %)	19 (52 %)	33 (47 %)	
	Wood	3 (10 %)	2 (5 %)	5 (7 %)	0.73
Year of construction (Mean±SD)		1985±11	1981±14	1983±13	0.09
Indoor climate (Mean±SD)	CO_2 (ppm)	680±92	630±87	655±92	0.02*
	T (ºC)	21.7±2.0	21.8±1.1	21.8±1.6	0.56
	RH (%)	26±7	27±7	26±7	0.82

*: P-value <0.05 **: P-value <0.01 ***: P-value <0.001

[a] Characteristics of the building where the investigated rooms were situated

[b] Observation in the investigated rooms

[c] In any part of the building where the inspected rooms are situated

The GM levels of cat allergen was 6 times lower, dog allergen was 2 times lower and horse allergen was 2 times lower in AADCs, as compared to ODCs. The total allergen levels (sum of cat, dog and horse allergens) were about 3 times lower in AADCs. Similar results were observed for airborne dust by the Petri dish method (Table 5).

Table 5. Allergen levels in allergen avoidance day care centers (AADCs) and ordinary day care centers ODCs

	Allergens	AADCs (GM±GSD)	ODCs (GM±GSD)	*P*-value
In vacummed dust samples (ng/g)	Cat allergen (Fel d 1)	79.4±2.8	625.7±2.8	<0.001
	Dog allergen (Can f 1)	84.7±2.8	831.6±3.3	<0.001
	Horse allergen (Equ c x)	90.2±2.5	263.4±4.6	0.003
	Total (cat+dog+horse)	326.2±2.1	2104.7±2.7	<0.001
In Petri dish samples ($ng/m^2/day$)	Cat allergen (Fel d 1)	0.4±3.4	2.4±3.6	<0.001
	Dog allergen (Can f 1)	2.1±1.2	4.1±2.5	<0.001
	Horse allergen (Equ c x)	1.1±1.1	2.0±3.3	0.003
	Total (cat+dog+horse)	3.8±1.3	10.8±2.3	<0.001

GM: Geometric Mean. GSD: Geometric Standard Deviation.

Total fungal DNA in swab samples was detected in 91 %, *Asp/Pen* DNA in 34 % and *S. chartarum* DNA in 6 % of the rooms. Total fungal DNA levels were positively associated with reported dampness, any reported odour, stone/brick façade buildings, and linoleum floor material (Table 6). Moreover, total fungal DNA levels were positively associated with carpet factor (p=0.03), total allergen levels in vacuumed dust samples (p=0.009) and in Petri dish samples (p=0.003), but negatively associated with RH (p=0.03).

Table 6. Mean levels of total fungal DNA in swab samples and in relation to building characteristics and room characteristics

Building characteristics		Total fungal DNA (N=70)			
		N	GM ($\times 10^7$ CE/m^2)	GSD	*P*-value
Reported dampness	no	46	1.02	3.39	0.02
	yes	24	1.78	4.07	
Observed dampness	no	61	1.23	3.50	0.69
	yes	9	1.29	5.18	
Window condensation	no	55	1.18	3.85	0.81
	yes	15	1.46	3.04	
Any reported odour	no	54	0.93	3.71	<0.001
	yes	16	3.22	1.85	
Wooden building[1]	no	36	2.08	2.70	0.003
	yes	31	0.81	3.17	
Linoleum[2]	no	33	0.83	3.63	0.02
	yes	32	1.60	3.45	

Dining room	no	42	1.20	4.16	0.84
	yes	28	1.29	2.99	
Floor heating	no	64	1.23	3.79	0.85
	yes	6	1.30	2.47	
Basement	no	57	1.31	3.51	0.46
	yes	13	0.96	4.42	
Region of Sweden	south	42	1.21	4.05	0.90
	west	28	1.27	3.16	
Type of day care	AADCs	33	0.97	4.14	0.18
centers	ODCs	37	1.53	3.17	

1: Three rooms with other facade material were omitted. 2: Five rooms with wooden floors were omitted.
GM: Geometic Mean; GSD: Geometic Standard Deviation.
CE/m^2=cell equivalents of DNA per square meter

Paper II

All buildings were wooden buildings (wooden construction and wooden façade) with mainly one floor level. The mean of the building construction year was 1975 (SD=20). Details of the building characteristics are shown in Table 7. Data of the fungal DNA and allergen levels are shown in Table 8. There was less variation between-buildings than within-building, particularly for total fungal DNA in Petri dish samples (Table 9).

Table 7. Building characteristics in 21 Swedish day care centers (26 buildings, 103 rooms)

Building characteristics		N (buildings)	N(rooms)	*Percent (%)
Number of floors	1	21	86	81
	2	2	8	8
	3	3	9	11
Risk construction	0	4	13	15
classification	1	8	31	31
	2	14	59	54
Type of floor	Linoleum	-	93	90
	PVC	-	10	10
Heating system	radiator	-	95	92
	Floor heating	-	7	8
Rotating heat	Yes	16	66	62
exchanger	No	10	37	38
Type of roof	Leaning roof	23	94	88
	Flat roof	3	9	12
Type of ventilation	Supply exhaust	25	100	96
	mixing			
	supply	1	3	4
Dining room	Yes	-	62	60
	No	-	41	40
Wooden outside	Yes	26	103	100
	No	0	0	0
Barrack	Yes	3	9	12
	No	23	94	88

* The percent data was calculated at building levels except those factors which were on room levels (type of floor, heating system and dinner room)

Table 8. Total fungal DNA and allergen levels from swab and Petri dish dust samples in 21 Swedish day care centers (N=103 room)

Dust samples	Fungal DNA/Allergens	[1]Percent (%)	[2]GM	[3]GSD	range
Cotton swab (N=103) (CE/m^2)	Total fungal DNA	99	$4.2*10^6$	2.1	($<1.0*10^4$, $3.2*10^7$)
	Asp/Pen DNA	53	$8.4*10^3$	4.5	($<3.0*10^3$, $3.9*10^5$)
Petri dish (N=101) (CE/m^2/day)	Total fungal DNA	100	$2.9*10^5$	1.8	($6.0*10^4$, $9.8*10^5$)
	Asp/Pen DNA	68	385	4.4	(<100, $1.6*10^4$)
Petri dish (N=97) (ng/m^2/day) (for horse allergen: $Unit/m^2$/day)	Cat allergens	100	9.4	2.6	(0.9, 78.6)
	Dog allergens	81	7.2	2.9	(1.2, 72.5)
	Horse allergens	63	5.0	5.8	(0.6, 208.7)
	[4]Total allergens	100	29.0	2.7	(2.9, 272.3)

1: positive detected values which were above of the detection limits.
2: GM: Geometric Mean. 3: GSD: Geometric Standard Deviation.
4: Total allergens = Cat allergen + Dog allergen + Horse allergen

Table 9. Within and between buildings variation of total fungal DNA, allergens and fold ranges for variation in 26 Swedish day care centers buildings

Fungal DNA/Allergens[1]		Variations within-building (%)	Variations between-buildings (%)	${}_wR_{0.95}$*	${}_bR_{0.95}$*
Total fungal DNA (swab)		57	43	2.7	2.3
Total fungal DNA (Petri dish)		90	10	2.6	1.4
Allergens	Cat (Fel d 1)	79	21	4.3	2.1
	Dog (Can f 1)	75	25	4.8	2.5
	Horse (Equ c x)	60	40	10.6	6.9
	[2]Total allergens	61	39	3.8	2.9

[1]Total fungal DNA analyzed both in swab and Petri dish dust, allergens only in Petri dish dust samples. [2]Total allergens = Cat allergen + Dog allergen + Horse allergen.
* ${}_wR_{0.95}$ and ${}_bR_{0.95}$: the variance ratios ('fold-ranges') within- and between buildings, calculated from the variance components of the 97.5^{th} and 2.5^{th} percentiles of the log-normally distributed exposure

There were associations (by linear mixed models) between total fungal DNA in swab samples (e logarithm values) and risk construction classification (β=0.105; 95 % CI 0.003-0.201), and rotating heat exchanger (β=0.184; 95 % CI 0.037-0.332). After mutual adjustment, both risk construction classification (β=0.106; 95 % CI 0.002-0.194), rotating heat exchanger (β=0.235; 95 % CI 0.074-0.357), and linoleum floor material (β=0.273; 95 % CI 0.100-0.487) remained significant variables (the β value values and 95 % CI were for e logarithm values). In the Petri dish samples, there were associations between total fungal DNA levels and levels of cat (p=0.02), dog (p<0.001), horse (p=0.001) allergens, and total allergens (p<0.001). Moreover, there were associations between *Asp/Pen* DNA levels in Petri dish samples and levels of cat (p=0.007), dog (p=0.02) allergens, and total allergen (p=0.004). There was an

association between the two sampling methods (cotton swab and Petri dish) for *Asp/Pen* DNA (p=0.02), but not for the other measured fungal DNA.

Data on total fungal DNA levels in swab and Petri dish dust samples, stratified for building characteristics are shown in Table 10.

Table 10. Geometric mean values for total fungal DNA in swab and Petri dish dust samples in 21 Swedish day care centers (N=103 rooms), stratified for building characteristics

Building characteristics		Total fungal DNA in Swab samples			Total fungal DNA in Petri dish samples		
		N/room	GM[a]	GSD	N/room	GM[b]	GSD
Risk construction classification	0	13	3.0	1.9	13	3.8	1.5
	1	31	3.7	2.5	30	2.8	1.7
	2	59	4.8	1.9	58	2.7	1.9
Type of roof	Flat	9	6.1	2.4	8	2.7	2.1
	Leaning	94	4.0	2.1	93	2.9	1.8
Type of floor	Linoleum	93	4.5	2.0	91	2.8	1.8
	PVC	10	2.1	2.2	10	3.5	1.6
Rotating heat exchanger	Yes	66	4.8	1.9	66	2.9	1.9
	No	37	3.2	2.3	35	2.8	1.7
Barrack	Yes	9	5.5	2.5	8	3.1	1.9
	No	94	4.1	2.1	93	2.8	1.8

GM: Geometric Mean; GSD: Geometric Standard Deviation.
a: $\times 10^6$ CE/m^2 b: $\times 10^5$ CE/m^2/day

Paper III

The participation rate in the questionnaire study was 96 % (462 of 480 invited pupils). Among the participants, 43 % were Malay, 42 % Chinese and 15 % Indian. Their mean age was 14 yr (range 14-16). There were more female pupils reporting breathlessness at rest and after exercise, any daytime breathlessness, respiratory infections in the last 3 months, pet allergy, pollen allergy and pollen or pet allergy (Table 11).

Table 11. Prevalence of respiratory symptoms and infections, asthma, allergy and smoking among male and female pupils (N=462) in eight secondary schools in Johor Bahru, Malaysia

Health variables	Male N (%)	Female N (%)	p-value *	Doctor-diagnosed asthma N (%)		p-value*	Overall N (%)
				Yes	No		
Wheeze	21 (10)	26 (11)	0.58	21 (35)	25 (6)	<0.001	47 (10)
Breathlessness during wheeze	11 (5)	20 (9)	0.33	15 (25)	15 (4)	<0.001	31 (7)
Breathlessness at rest	13 (6)	32 (14)	0.005	12 (21)	33 (8)	0.003	45 (10)
Breathlessness after exercise	65 (29)	101 (43)	0.003	37 (64)	128 (32)	<0.001	166 (36)
Daytime breathlessness (exercise or rest)	72 (32)	114 (48)	0.001	39 (67)	146 (37)	<0.001	186 (41)

Night-time breathlessness	12 (5)	20 (9)	0.19	14 (24)	17 (4)	<0.001	32 (7)
Respiratory infections in last 3 months	27 (12)	60 (25)	<0.001	19 (32)	67 (17)	0.006	87 (19)
Ever had asthma	35 (16)	25 (11)	0.11	-	-	-	60 (13)
Doctor-diagnosed asthma	35 (16)	25 (11)	0.11	-	-	-	60 (13)
Asthma attacks in last 12 months	6 (3)	7(3)	0.85	13 (22)	0 (0)	<0.001	13 (3)
Medication for asthma	9 (4)	7 (3)	0.53	14 (24)	2 (1)	<0.001	16 (4)
Parental asthma/allergy	40 (19)	53 (25)	0.18	22 (38)	71 (20)	0.002	93 (22)
Pet allergy	19 (9)	43 (18)	0.003	15 (25)	47 (12)	0.005	62 (13)
Pollen allergy	15 (7)	42 (18)	<0.001	12 (20)	45 (11)	0.054	57 (13)
Pet or pollen allergy	30 (14)	67 (28)	<0.001	20 (34)	77 (19)	0.011	97 (21)
Smoking	19 (9)	3 (1)	<0.001	7 (12)	15 (4)	0.007	22 (5)

*Differences between male and female, asthmatic and non-asthmatic, pupils were tested using the Chi-square test

None of the schools had a mechanical ventilation system, but all classrooms had openable windows with venetian blinds on both sides that were kept open during lectures. None of the classrooms had a carpet, but curtains were common. The mean building age was 16 yr (range 3–40). Five classrooms had signs of water leakage, but none had visible indoor mould growth. Eight classrooms were situated in two older school buildings, where black mould growth on the outside walls was noted. The mean value of both indoor and outdoor temperature was the same, namely 29 ℃ (range 27–31). The mean value of indoor relative humidity (RH) was 70 % (range 60–78) and the CO_2 level was 492 ppm (range 376–689). The mean value of outdoor RH was 73 % (range 64–87) and the mean outdoor CO_2 level was 408 ppm (range 384–518).

Total fungal DNA, *Asp/Pen* DNA and cat allergen levels (geometric mean levels) are shown in Table 12. *Asp/Pen* DNA was detected in all classrooms both for Petri dish and swab samples. *A. versicolor* DNA was detected in 70 %, *S. chartarum* in 13 % and *Streptomyces* DNA in 87 % of Petri dish samples. In contrast, *A. versicolor* DNA was detected in 56 %, *S. chartarum* DNA in 3 % and *Streptomyces* DNA in 28 % of swab samples. For total fungal DNA, there were less variations between-buildings than within-building for both sampling methods. However, for *Asp/Pen* DNA and cat allergen, there were more variations between-buildings than within-building (Table 12).

Table 12. Fungal DNA and cat allergen levels in swab and Petri dish dust samples in eight secondary schools in Johor Bahru, Malaysia

Dust samples	Fungal DNA/Allergen	GM±GSD[1]	Variations within-building (%) ($_wR_{0.95}$*)	Variations between-buildings (%) ($_bR_{0.95}$*)
Cotton swab (CE/m^2)	Total fungal DNA	5.7×10^8±2.1	61 (9.2)	39 (5.9)
	Asp/Pen DNA	0.5×10^8±3.3	28 (12.4)	72 (59.2)
Petri dish (CE/m^2/day)	Total fungal DNA	9.2×10^6±1.9	56 (6.6)	44 (5.3)
	Asp/Pen DNA	1.6×10^6±3.0	44 (17.2)	56 (24.6)
Petri dish (ng/m^2/day)	Cat allergen	5.9±3.1	35 (14.8)	65 (38.3)

[1]: GM: geometric mean, GSD: geometric standard deviation
* $_wR_{0.95}$ and $_bR_{0.95}$: the ranges in which 95% of the individual schools' mean values lie

Cat (Fel d 1) allergen was detected in all Petri dish dust samples, but dog (Can f 1) or horse (Equ c x) allergen was not detected in any sample. Totally, 6 % of pupils had a cat and 3 % had a dog at home. Aflatoxin B_1 was detected in 3 % (one classroom) of the swab dust samples, while sterigmatocystin was detected in 6 % and verrucarol in 12 %. The arithmetic mean of the amount (in pg/m^2) of aflatoxin B_1 was 67, while it was 2 547 (max. 50 500) for sterigmatocystin and 17 (max. 467) for verrucarol.

In Petri dish samples, levels of *A. versicolor* DNA were positively associated with wheeze (*P*=0.002) and daytime attacks of breathlessness (*P*=0.026), while *Streptomyces* DNA was positively associated with doctor-diagnosed asthma (*P*=0.049). However, levels of *S. chartarum* DNA were negatively associated with daytime breathlessness (*P*=0.019). In swab samples, there were no associations between fungal DNA and respiratory health effects. There was negative association between verrucarol and daytime breathlessness (*P*=0.033) (Table 13). Moreover, there was an association between sterigmatocystin and *A. versicolor* DNA (*P*=0.01) and positive association between *S. chartarum* DNA and CO_2 levels (*P*=0.007).

Table 13. Associations (OR with 95 % CI) between fungal DNA and cat allergen in Petri dish dust samples, mycotoxins in swab dust samples, and respiratory health effects among pupils (N=462) in eight secondary schools in Johor Bahru, Malaysia

Fungal DNA/ Allergen	Wheeze	Daytime breathlessness	Night-time breathlessness	History of atopy	Respiratory infections in last 3 months	Doctor-diagnosed asthma
Total fungal DNA[1]	0.71 (0.32-1.59)	1.02 (0.66-1.58)	0.58 (0.27-1.26)	1.02 (0.71-1.45)	1.16 (0.55-2.46)	1.32 (0.89-1.95)
Asp/Pen DNA[2]	0.84 (0.21-3.35)	0.91 (0.40-2.08)	0.44 (0.05-3.97)	1.36 (0.63-2.92)	2.35 (0.52-10.63)	0.99 (0.40-2.44)
A. versicolor DNA[3]	1.19 (1.07-1.32)**	1.14 (1.02-1.28)*	0.74 (0.49-1.10)	1.03 (0.94-1.13)	0.91 (0.74-1.12)	1.04 (0.93-1.15)
S. chartarum	1.03	0.88	0.95	0.99	1.03	1.01

DNA[4]	(0.90-1.18)	(0.80-0.98)*	(0.82-1.09)	(0.91-1.09)	(0.88-1.23)	(0.92-1.11)
Streptomyces	0.95	1.05	0.73	0.93	1.15	1.18
DNA[5]	(0.73-1.24)	(0.89-1.24)	(0.47-1.13)	(0.79-1.10)	(0.88-1.50)	(1.00-1.40)*
Cat allergen	0.97	1.00	1.04	0.99	0.99	1.01
	(0.91-1.04)	(0.96-1.05)	(0.97-1.11)	(0.95-1.03)	(0.91-1.07)	(0.96-1.06)
Verrucarol	0.87	0.96	1.02	0.98	1.01	1.01
	(0.71-1.07)	(0.93-1.0)*	(0.96-1.08)	(0.95-1.02)	(0.96-1.07)	(0.97-1.04)

[1]OR calculated for 10^7 CE/m^2/day increase in total fungal DNA
[2]OR calculated for 10^7 CE/m^2/day increase in *Asp/Pen* DNA
[3]OR calculated for 10^3 CE/m^2/day increase in *A. versicolor* DNA
[4]OR calculated for 100 CE/m^2/day increase in *S. chartarum* DNA
[5]OR calculated for 10^3 CE/m^2/day increase in *Streptomyces* DNA
**p*-value<0.05 ***p*-value<0.01

Paper IV

The participation rate in the questionnaire study was 84 % (654 pupils were invited). Mean age of the children was 9.9 ± 0.8 yr, 53 % were female. The prevalence of respiratory symptoms was 34 % for dry cough at night, 32 % for rhinitis, 26 % for cough and 13 % for wheeze. About 34 % of the children were exposed to environmental tobacco smoke (ETS) at home. The prevalence of lifetime asthma (ever asthma) was 16 %.

Mechanical ventilation was used in all Swedish, half of Norwegian and 13 % of Italian classrooms, while all classrooms in France and Denmark had natural ventilation only. Totally, 30 % of all classrooms had mechanical ventilation. Poor ventilation rate (<8L/s/p) was more prevalent in classrooms with natural than with mechanical ventilation (97 % vs. 13 %, $p < 0.001$). Both viable moulds (VM) and total fungal DNA were detectable in all monitored classrooms. Airborne viable mould species included *Cladosporium* spp. (detectable in 44.3 % of classrooms), *Penicillium* spp. (34.8 %), *Aspergillus* spp. (13 %), *Alternaria* spp. (11 %), *A. versicolor* and *Streptomyces* (6.5 %). *S. chartarum* was never detected in air samples.

VM exceeded the standard suggested by ASHRAE [247] for good indoor air (300 cfu/m^3) in 33 % of the classrooms, in which Reims (France) had the highest VM concentration. In contrast, Uppsala and Oslo centers had the lowest concentration of VM. *Asp/Pen* and *Streptomyces* DNA in vacuumed dust were detected in all classrooms and *A. versicolor* and *S. chartarum* DNA in 89 % and 24 % of classrooms, respectively. The concentrations of VM (Fig. 1) and total fungal DNA (Fig. 2) varied between the classrooms within the same center.

Figure 1. Concentrations of total viable moulds in monitored classrooms. The horizontal line indicates the maximum standard value proposed by the American Society of Heating. Refrigerating and Air-Conditioning Engineers (ASHRAE) for good indoor air (300 cfu/m^3)

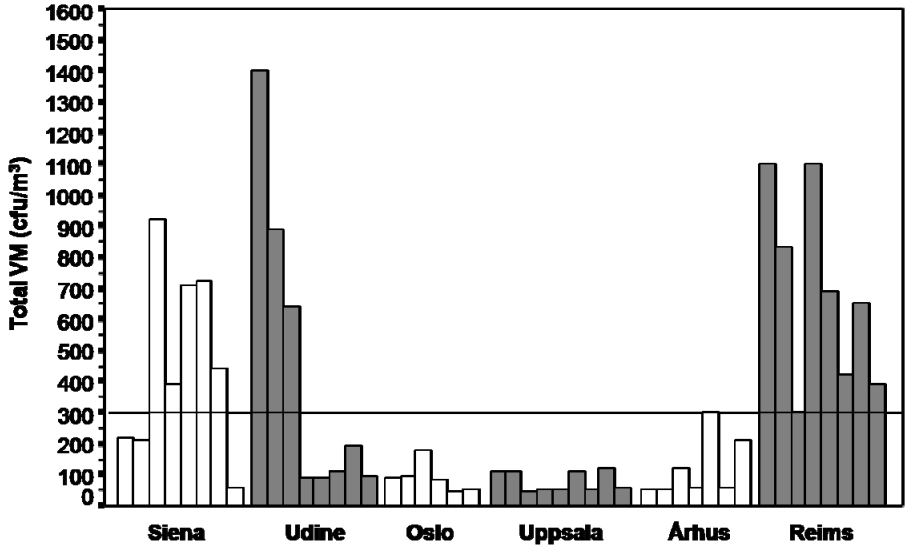
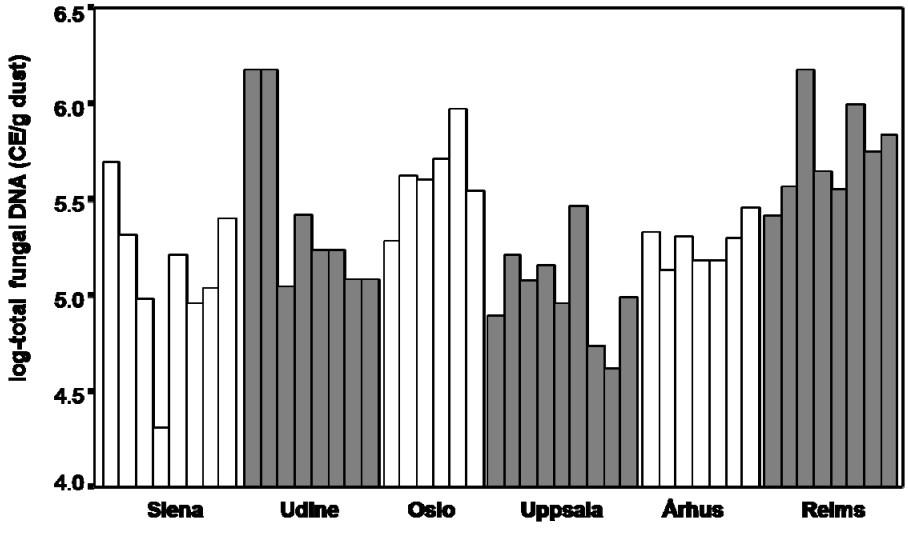


Figure 2. Concentrations of total fungal DNA in monitored classrooms.



The teachers reported visible moulds in 30 % and moulds odour in 33 % of the schools respectively. There were significant correlations between total fungal DNA levels in vacuumed dust and reported signs of dampness/moulds, reported

mould odour, and mechanical ventilation, and between *A. versicolor*, *Streptomyces* DNA levels and mechanical ventilation. Classrooms with mechanical ventilation had lower levels of fungal DNA (Table 14).

Table 14. Geometic mean levels of fungal DNA in total (n=654, total in 8 centers) and in relation to building characteristics

[a]Fungal DNA GM(CE/g)	Reported dampness or mould growth			Reported mould odour			Mechanical ventilation		
	Yes	No	*P*-value	Yes	No	*P*-value	Yes	No	*P*-value
[b]Total fungal DNA($\times 10^5$)	2.50	1.59	0.03	3.00	1.51	0.05	2.65	1.58	0.06
Asp/Pen($\times 10^4$)	0.25	0.22	0.22	0.32	0.20	0.41	0.28	0.23	0.43
A.versicolor ($\times 10^2$)	0.37	0.23	0.24	0.35	0.21	0.31	0.41	0.12	0.05
Streptomyces ($\times 10^2$)	0.33	0.20	0.61	0.31	0.16	0.24	0.33	0.10	0.06

a: GM, geometric mean; CE = cell equivalent; b: total fungal DNA=*Asp/Pen+A.versicolor+S.chartarum+Streptomyces*;
P-value: statistical difference between school characteristics by Mann-Whitney non parametric test.

After accounting for gender, age, ETS at home, and lifetime asthma, both adjusted conventional and hierarchical regression models indicated that schoolchildren exposed to elevated levels of VM (>300 cfu/m^3), compared with those exposed to low levels, had more dry cough at night, rhinitis and cough. Similar results were also found when continuous values of VM were included in the statistical models. Total fungal DNA levels were positively associated with rhinitis, but only in conventional regression models. The effects of specific fungal DNA differed, depending on the type of outcome and fungal DNA (Table 15).

Table 15. Associations of exposures to total viable moulds (VM, cfu/m^3) and fungal DNA (CE/g dust) with respiratory disorders in schoolchildren. Odds ratios (OR) and 95% Confidence Interval (CI).

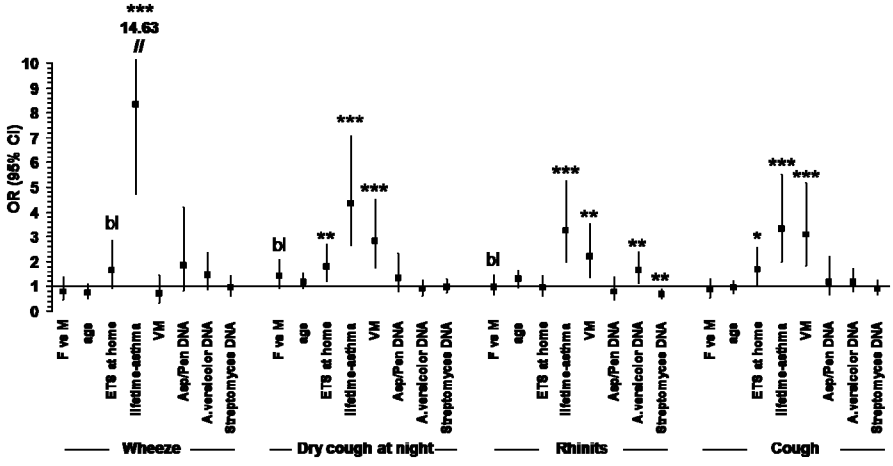
	Conventional logistic regression adjusted[b]	**2-level[a] hierarchical regression adjusted[b]**
VM *elevated* level[c]		
Wheeze	1.41 (0.80-2.47)	1.41 (0.80-2.48)
Dry cough at night	2.92 (1.95-4.36)***	3.10 (1.61-5.98)***
Rhinitis	2.84 (1.91-4.23)***	2.86 (1.65-4.95)***
Cough	3.70 (2.41-5.69)***	3.79 (2.40-5.60)***
Lg-VM		
Wheeze	1.11 (0.62-1.99)	1.12 (0.62-1.99)
Dry cough at night	2.68 (1.77-4.05)***	2.88 (1.41-5.88)**
Rhinitis	2.67 (1.77-4.03)***	2.59 (1.45-4.62)***
Cough	3.48 (2.28-5.46)***	3.60 (2.17-6.00)***

[1]Lg-total fungal DNA		
Wheeze	1.72 (0.88-3.38)	1.71 (0.87-3.35)
Dry cough at night	1.03 (0.64-1.63)	1.10 (0.51-2.39)
Rhinitis	1.73 (1.09-2.77)*	1.66 (0.80-3.45)
Cough	1.57 (0.96-2.58)[bl]	1.43 (0.69-2.97)
[2]Lg-*Asp/Pen* DNA		
Wheeze	2.17 (1.06-4.42)*	2.14 (1.05-4.36)*
Dry cough at night	1.63 (1.01-2.64)*	1.67 (0.66-4.23)
Rhinitis	1.46 (0.91-2.33)	1.28 (0.60-2.71)
Cough	1.91 (1.14-3.22)*	1.80 (0.87-3.74)
[3]Lg-*A. versicolor* DNA		
Wheeze	1.48 (0.99-2.22)[bl]	1.48 (0.99-2.21)[bl]
Dry cough at night	1.32 (1.00-1.73)*	1.24 (0.75-2.04)
Rhinitis	1.83 (1.38-2.43)***	1.75 (1.18-2.60)**
Cough	1.76 (1.29-2.39)***	1.75 (1.17-2.62)**
[3]Lg-*Streptomyces* DNA		
Wheeze	1.01 (0.68-1.49)	1.01 (0.68-1.49)
Dry cough at night	1.03 (0.78-1.35)	0.95 (0.56-1.59)
Rhinitis	0.80 (0.61-1.04)[bl]	0.71 (0.45-1.12)
Cough	1.02 (0.76-1.36)	1.00 (0.64-1.55)

Moulds exposure variables are separately included in the statistical models.
Asp/Pen = Aspergillus/Penicillium; *A. versicolor = Aspergillus versicolor.*
[a] 1st level: child, 2nd level: classroom; [b] analysis accounted for passive exposure to tobacco smoking at home, gender, age, lifetime-asthma; [c] > 300 cfu/m^3; [1] OR calculated for 10^5 CE/g dust increase; [2] OR calculated for 10^4 CE/g dust increase; [3] OR calculated for 100 CE/g dust increase.
Statistical significance = * $p<0.05$, ** $p<0.01$, *** $p<0.001$, [bl] $0.05 < p < 0.1$.

Based on the tertile distribution of fungal DNA, dose–response effects were found for all considered outcomes, and were particularly for *A. versicolor* DNA (Fig. 3).

Figure 3. Mutiple regression conventional models. Risk (Odds ratio, OR) and 95 % Confidence Interval (CI) for reporting respiratory disorders (dependent binary variables, yes/no). Total viable moulds (VM, cuf/m^3) and specific fungal DNA (CE/g dust) are log-transformed.



When VM and all specific fungal DNA were included in the same model (mutual adjustment), VM were significantly related to higher risk for dry cough at night (OR: 2.80, 95 % CI: 1.74–4.50), rhinitis (OR: 2.18, 95 % CI: 1.36–3.50), and cough (OR: 3.08, 95 % CI: 1.84–5.17), whereas *A. versicolor* DNA was related to higher risk for rhinitis (OR: 1.65, 95 % CI: 1.15–2.37). In contrast, there was an inverse association between *Streptomyces* DNA and rhinitis (OR 0.66, 95 % CI: 0.49–0.88) (Table 16).

Table 16. Associations of pulmonary function with total viable moulds (VM, cfu/m^3) and fungal DNA (CE/g dust). Partial regression coefficients (B) and 95 % Confidence Interval (CI).

	Linear regression models		**GLS-RE models**
	Crude	**adjusted[a]**	**adjusted[b]**
Log-*A. versicolor* DNA			
FEV_1	-0.08 (-0.16;-0.01)*	-0.04 (-0.14 ;0.06)	-0.10 (-0.27;0.07)
FVC	-0.16 (-0.25;-0.06)**	-0.10 (-0.21;0.01)[bl]	-0.17 (-0.35;0.003)*
Log-*Streptomyces* DNA			
FEV_1	-0.13 (-0.21;-0.04)**	-0.14 (-0.23 ;-0.05)*	-0.16 (-0.34;0.02)[bl]
FVC	-0.15 (-0.26;-0.05)**	-0.14 (-0.23 ;-0.04)**	-0.18 (-0.36;0.01)[bl]

GLM-RE = generalized least squares random-effects model; [a] for gender, age, height, passive smoking at home, and any of lifetime-asthma, dry cough at night, cough; [b] as [a] plus fixed effect of the classroom; [c] > 300 cfu/m^3; *Asp/Pen = Aspergillus/Penicillium*; *A. versicolor* = *Aspergillus versicolor*; FEV_1 = forced expiratory volume in 1 second; FVC = forced vital capacity.
Statistical significance = * $p<0.05$, ** $p<0.01$, [bl] $0.05 < p < 0.1$.

The associations were significant or borderline significant (depending on the model of analysis) for *A. versicolor* DNA with FVC and *Streptomyces* DNA with both FEV1 and FVC**.** Multiple linear regressions with VM and all specific fungal DNA included in the same model (mutual adjustment), confirmed the significant inverse associations of *Streptomyces* DNA with FEV1 ($p = 0.003$) and FVC ($p = 0.005$) and of *A. versicolor* DNA with FVC ($p = 0.05$).

General Discussion

This thesis showed that risk construction buildings, dampness/moulds, fungal DNA, and pet allergens were common in Swedish day care centers or schools from Malaysia and Europe. Moreover, there was a high prevalence of respiratory symptoms among pupils. The epidemiological studies provide evidence of a positive association between total fungal DNA levels and building inspection data (risk construction buildings), reported dampness/moulds, reported odour), certain building factors (type of wall construction, rotating heat exchangers), certain room factors (linoleum floor, textile carpets) and allergens (cat, dog and horse) levels in dust.

It was found that airborne viable mould (VM) and fungal DNA levels were positively associated with respiratory health. Children exposed to VM levels ≥300 cfu/m^3 in the classrooms showed higher risk for rhinitis, dry cough at night and persistent cough. There were associations between Asp/Pen DNA and wheeze, between *A. versicolor* DNA and wheeze, rhinitis, cough, and daytime breathlessness and between *Streptomyces* DNA and doctor-diagnosed asthma. However, the associations were inverse between *S. chartarum* DNA, as well as the verrucarol mycotoxin and daytime breathlessness. Moreover, there were inverse associations for *A. versicolor* DNA in vacuumed dust and forced vitality capacity (FVC) and for *Streptomyces* DNA and both FVC and forced expiratory volume in one second (FEV_1) which indicates lower lung function at higher exposure levels to these two species.

Comments on internal validity

In the two Swedish day care center studies, the first one was a part of a larger day care center survey in Sweden [58]. For practical reasons, we included day care centers that were located within travelling distance from western and southern Sweden. As we studied the two types of day care centers (AADCs and ODCs) located close to each other, it is unlikely that there were systematic difference in outdoor fungal levels that would have biased the comparison between AADCs and ODCs. The second study included all day care centers situated in separate buildings within one municipality, and different types of rooms were arbitrary selected within each building. In the two schools studies, schools, classrooms and students

were randomly or arbitrary selected and the questionnaire surveys were completed with high response rate (96 % in paper III and 84 % in paper IV). Thus, selection bias is less likely.

Information bias could affect an epidemiological study, but the students and teachers in the schools had no information about the results of the analysis of environmental samples or measurements. Environmental sampling was conducted within the same week as the questionnaire study. All samples in each study were analysed after questionnaire data collection was completed and in the same batch in the laboratories. Thus, it is unlikely that the study was seriously influenced by information bias.

Comments on external validity

For the Swedish day care centers studies, the first study was not randomly selected among Swedish day care centers. However, there was no selection of buildings with health complaints, dampness or microbial growth or a particular building construction. Moreover, we found no difference in fungal DNA levels when comparing all day care centers between western and southern Sweden. In the second study, the municipality (Österåker) was chosen because there was a previous survey of all day care centers on the dampness conditions, irrespectively of complaints. It is a small municipality with rural surrounding and coast area, so it can be representative for mid-Sweden. We consider our day care centers studies to have reasonable external validity.

Johor Bahru, in Malaysia, has a tropical climate with a similar outdoor climate all year round, so the fact that the first school study was performed in November is less likely to influence the outcome, as the ventilation characteristics and both indoor and outdoor exposure parameters would be expected to be relatively constant over the year. In the second school study (HESE), the school buildings were arbitrary selected from new and old buildings, and near and far from traffic. The limitation in this study is that limited numbers of centers were chosen from a few countries, yet they were from different climate zones and the results could be possibly generalized to Western Europe. Moreover, the study for the first time provides data on both viable mould (VM) and fungal DNA in European schools, and studied associations with children health, in a wide range of locations from different countries, using the same standardized procedure.

Comments on fungal DNA levels and reported or observed dampness and odour

There was an association between total fungal DNA levels in swab samples and reported history of dampness/mould growth in Swedish day care centers. In contrast, the prevalence of observed dampness was low and not associated with fungal DNA levels. One reason for the discrepancy could be that the inspection only covered the selected rooms, while the reported data covered all parts of the building. In addition, inspection only covered current situation, while the reported dampness/mould covered the history of dampness and mould growth for several years. A hotel study, using the same swab sampling method, reported that hotel rooms with observed signs of dampness or mould growth had higher levels of total fungal DNA and *Asp/Pen* DNA [194]. Hidden microbial growth in the construction may result in fungal emissions to the indoor environment, including odourous and irritant substances and/or allergens [70] and hidden microbial growth is difficult to discover at a visual inspection. Some studies have reported associations between visible mould damage and the concentration of airborne viable mould and bacteria [91, 248, 249] and the concentrations of several microbial species in house dust determined by PCR [250]. Other studies, however, have found no association between visible mould growth and airborne or dust borne viable mould levels [92, 93, 251, 252]. This illustrate that some of the mould contamination may be missed by mere inspection and that many traditional methods of measuring moulds and other microorganisms are insufficient to detect mould contamination [71].

There was a strong association between total fungal DNA levels in swab samples and odour reported by the local directors in the Swedish day care centers, but none of the directors classified the odour as “mouldy odour”. Even though mouldy odour is common in water-damaged buildings only a few studies have reported association with mouldy odour. The hotel study, using the same swab sampling method, found an association between odour and levels of *S. chartarum* DNA, but not levels of total fungal DNA [194]. Other studies reported that dampness odour was associated with increased concentration of airborne *Aspergillus, Penicillium*, and *Cladosporium* fungi [253] and microbial contaminants at homes (endotoxin, dust beta-glucan and ERMI) [82, 254]. Finally, mould odour has been reported be associated with an increased risk of developing asthma [83] and was associated with asthma, rhinitis and eczema symptoms in children [255]. Thus, odour, both mouldy odour and other types of odour, appears to be an important indicator for indoor air quality and should be included in the exposure and health assessment.

Comments on fungal DNA levels associations with building characteristics

In Swedish day care centers, there were higher levels of total fungal DNA in swab samples in risk construction buildings, especially those with visible water damages/mould growth (risk level 2). This association maintained significant even after mutual adjustment for other building factors. To our knowledge, this association has not been previously demonstrated. The prior risk classification survey from the day care centers (Österåker) showed that most of the buildings are risk construction buildings. One typical risk construction, for example, is concrete slab on the ground with overlaying thermal insulation, which was commonly used for smaller buildings such as day care centers during the 70'ies and 80'ies. Another type of risk construction is basement walls with insulation inside. The reason that these constructions are often damaged by moisture is that the concrete get similar high relative humidity (close to 100%) as the underlying/surrounding soil. Another typical Swedish risk construction is the outdoor ventilated crawl space. In this case, the crawl space has a lower temperature than the outdoor air during summer. In summer, the crawl space is ventilated by warm and humid air and the relative humidity reach levels high enough for condensation and mould growth.

Moreover, there were higher levels of total fungal DNA in the swab samples in buildings with rotating heat exchanger than those with plate heat exchanger or no heat recovery system. To our knowledge, this is a new finding, with respect to fungal contamination. It has been previously shown that rotating heat exchangers can transfer water soluble chemicals from the exhaust air to the supply air [132, 256]. Moreover, microbes and the subsequent biofilms can grow within heat exchangers, however, fungi concentration was higher in aluminum (378 CFU/cm^2) than in the copper (1 CFU/cm^2) heat exchangers which maybe because of the antimicrobial properties of copper [257]. Further studies on rotating heat exchanger and microbial contamination are needed.

Finally, total fungal DNA levels in swab samples were higher in rooms with linoleum floor covering as compared with those with PVC floors. Linoleum floors are commonly used in Sweden. If there is a dampness problem in the floor construction, fungal growth could arise in the jute material on the back of the linoleum material. In addition, we found higher levels of total fungal DNA in rooms with more carpet area per m^3. Associations between carpeting and airborne levels of viable fungi [258] and ergosterol [259] have previously been reported.

Comments on allergens levels and associations with fungal DNA

Cat and dog allergen contamination was common in the Swedish day care centers, which is in agreement with other studies [49, 133]. Moreover, cat allergen was common in the Malaysian schools. In addition, horse allergen was common in the Swedish day care centers. Horse allergen contamination has been demonstrated in Swedish schools [171], but we did not find any other study on horse allergens in day care centers. We found associations both between cat, dog, horse allergens and total fungal DNA. To our knowledge, the association between fungal DNA and allergens has not been reported previously and it illustrates the need to sample both allergens and micro-organisms in epidemiological studies on health effects of indoor bio-aerosols. The observed association between fungal and allergen contamination in the Swedish day care centers indicated a 'hygiene factor' related to different types of biological contaminants.

Comments on airborne viable mould (VM) levels

Airborne indoor VM were commonly found in the monitored European schools, and levels were higher in building without mechanical ventilation and when the teachers reported indoor mould growth in the schools. Our study was performed in the cold season and 70 % of the classrooms had higher indoor VM levels than outdoors. The VM concentrations (GM=180 cfu/m^3) in our study is similar to levels in a previous Swedish school environment study (GM=210 cfu/m^3) [21]. Another Swedish schools study performed in the warm season (May-June) reported higher levels of indoor VM (mean=360 cfu/m^3) even though there were no visible moulds or signs of dampness in the classrooms, and the outdoor VM level (mean=980 cfu/m^3) was much higher than indoors. This higher level of indoor VM compared to our study maybe because the study was performed in the warm season [260]. There was study reported that outdoor concentrations, for most genera, are the main source of influence on indoor spore levels in the warm season [261].

Comments on within- and between-buildings variations

Pet allergens and fungal DNA were very common in our studies. There were higher variations within-buildings than between-buildings for total fungal DNA and allergens (cat, dog and horse allergens) levels in Petri dish samples in the Swedish day care centers (Paper II). Similar results were found for total fungal DNA in both Petri dish and swab samples in the Malaysian

school study (Paper III). However, there were less variations within-buildings than between-buildings for *Asp/Pen* DNA and cat allergens levels in both Petri dish and swab samples. We found no other studies on within- and between-buildings variability for fungal DNA or allergens levels, but one variability study on pollutant levels in Michigan schools showed that the variability of CO_2, VOC, and bioaerosol concentrations within schools exceeded the variability between schools [262]. In addition, a multicenter study reported that specific allergen levels (dust mite, cat, and cockroach) varied depending on the geographic location, season, and type of room in schools [263]. These imply that sufficient number of rooms and multiple locations must be investigated in indoor exposure studies in order to get a reliable estimate of average levels in a building. Moreover, analysis of health associations on a classroom level in schools is preferable if possible.

Comments on prevalence of respiratory health among school children

The prevalence of daytime attacks of breathlessness (41 %), doctor-diagnosed asthma (13 %) and respiratory symptoms were high among junior high school pupils in Malaysia. Girls reported more daytime breathlessness (exercise or rest) or airway infections in the last 3 months compared to boys. Moreover, there was a higher rate of prevalence of pollen and/or pets allergy among girls. Similar gender differences have been reported among pupils in other studies in Asia [106, 182]. A high prevalence of daytime attacks of asthma (23-41 %) has been reported from studies in other Asian countries using the same questionnaires [182, 233, 260]. One-fifth reported a history of atopic sensitization to pollen or furry pets, around 10 % of the pupils had wheeze during the last 12 months and 13 % reported doctor-diagnosed asthma. Previous studies from 1990-2001 of schoolchildren in Malaysia reported a lower prevalence of wheeze (5-8 %) and asthma (3-10 %) [264-266]. One explanation for the higher prevalence of wheeze and asthma in our study could be that it was performed many years later, as there are indications that asthma is on the rise in Asia [8]. In our European school study (mean age 10 yrs), the prevalence of wheeze in the past 12 months was 13 %, and cough (26 %), dry cough at night (34 %) and rhinitis (32 %) in the past 12 months were common. The ISAAC study reported that the prevalence of wheeze in the last 12 months was 9.7 % for 13-14y age-group in Sweden [8].

Comments on health associations with fungal DNA/viable mould/mycotoxins

There was a positive association between total fungal DNA levels in vacuumed dust samples and rhinitis in school children in HESE study, but only in conventional regression models. In addition, increased levels of Asp/Pen DNA was positively related to wheeze, cough, and dry cough at night, and the risk for wheeze was confirmed by the hierarchical analysis. Viable airborne moulds were measured only in the HESE study. School children exposed to elevated levels of VM (>300 cfu/m^3), compared with those exposed to low levels, had more cough, dry cough at night and rhinitis. A recent review study reported that exposure to mould was significantly associated with a higher risk of allergic respiratory disorders including asthma, wheezing and allergic rhinitis in children [267]. These health associations were only found in the HESE school study, not in the Malaysia school study. The reasons for this could be because the species composition is different in different climate zone. In the temperate climate zones the variability of fungal species is reported to be larger than in the tropics [268] and it has been demonstrated that a larger proportion of the total fungal DNA in a warmer climate consist of *Asp/Pen* DNA (50 % in tropical climate versus 20 % in temperate climate zone) [194]. There has been reported that mould exposure is associated with several effects on health, depending on the species involved [92]. In addition, differences in sampling methods between the HESE study and the Malaysia study could be another reason. The Petri dish sampling capture mostly smaller particles while vacuuming capture both small and large particles.

To date, we haven't found any other health study on fungal DNA in day care centers or school environments. However, associations between VM levels in schools and asthmatic symptoms [168] and new asthma [21] has been reported from Sweden. A Finnish pilot study reported an association between total fungal DNA in home floor dust (summer data) and lower lung function FVC and FEV1 [269]. Moreover, children in homes with higher ERMI values are more likely to have asthma [196, 270-272]. These results bolster the growing evidence linking dampness and mould exposures to asthma [71]. However, mould species are not equally relevant to respiratory illness [92]. Identifying specific moulds that are potentially associated with asthma could narrow down our search for the most health relevant moulds.

In both the HESE and Malaysian school studies, we found that increased exposure to *A. versicolor* DNA was associated with higher risk for the considered outcomes (wheeze, rhinitis, cough, and dry cough at night). Moreover, *A. versicolor* DNA levels were positively associated with daytime attacks of breathlessness in Malaysia school study. In the HESE school study, dose–response effects were found for all considered outcomes, particularly evident for *A. versicolor* DNA. Moreover, there were inverse

associations between *A. versicolor* DNA and forced vitality capacity (FVC) which indicating an effect on the lung functions. *A. versicolor* is a sporulating fungus often detected in moisture damaged materials or in the air of moisture-damaged buildings [179, 273, 274]. *Aspergillus* spores are small in size (aerodynamic size 2.0–3.6 μm) [275] and can be inhaled deeply into the lungs [276]. Moreover, the spores are possible carriers of nonvolatile mycotoxins [277]. Thus the penetration of these fungal spores into airways may induce inflammatory and other harmful reactions. Finnish studies showed that the spores of *A. versicolor* grown on plasterboards increased the production of different inflammatory mediators, such as tumor necrosis factor (TNF) and interleukin (IL)-6, in mouse lungs [278, 279].

We found associations between *Streptomyces* DNA and doctor-diagnosed asthma in the Malaysia study and lung function (FEV1 and FVC) in the HESE study. *Streptomyces*, gram-positive bacteria, are believed to thrive in moisture-rich environments, and a number of *Streptomyces* species, e.g., *Streptomyces* griseus and *Streptomyces coelicolor*, have been isolated from moisture-damaged building materials [280-282]. Several *Streptomyces* strains have been shown to be potent inducers of inflammatory reactions *in vitro* [283, 284] and *in vivo* [112]. The health effects of exposure to specific bacterial species have been less studied than fungal species.

In contrast, levels of *S. chartarum* DNA and verrucarol mycotoxin in the Malaysian school study were negatively associated with daytime breathlessness. Verrucarol is a hydrolysis product of macrocyclic trichothecenes, produced by *S. chartarum* (and other fungi not found indoors, such as the plant pathogen *Myrothecium verrucaria*) [280]. *Stachybotrys* spp. can produce highly toxic secondary metabolites [285, 286] and have profound toxic effects in mouse macrophages [287]. However, since *S. chartarum* DNA was only detected in a few samples and the levels were low, the negative associations for daytime breathlessness found in our study are less likely to have any protective effect on health. Endotoxin, a gram-negative bacteria surface structure, is known to have strong immune-stimulatory properties [120, 122] and some types of endotoxin have been demonstrated to have negative associations with asthmatic symptoms in school children in China (Zhao et al., 2008). Since both *stachybotrys* and gram-negative bacteria need high humid conditions to grow, it can be speculated that confounding by endotoxin could explain our findings regarding *S. chartarum* and verrucarol. Indoor epidemiology on microbial exposure may need to cover both bacterial and fungal components to get a better understanding of the associations for the complex mixture of moulds and bacteria indoors.

Conclusions and implications

Around the world, asthma and allergy in children will still be an important topic in the future both in industrial and developing countries. The prevalence of asthma and allergy has been high for long time in industrialised countries and has increased over the last decades especially in pacific Asia. Day care centers and schools are important indoor environments for children, and globally more and more children are attending day care centers. Contamination of allergens is common in day care centers and schools and can be a health problem, especially for the sensitized children. The creation of allergen avoidance day care centers (AADCs) is a good idea, even not completely free of allergens, but the allergens levels are much lower in this type of day care centers.

There is rising evidence of health effects due to building dampness and indoor mould, but the issue of building dampness is complex, and there is need for more studies using objective exposure measurements, not just reported or observed dampness and moulds. The question if it is the total indoor fungal load or exposure to specific species that causes the health effects are needed to be further investigated. Two important specific species (*A. versicolor* and *Streptomyces*) was identified as being health relevant fungi in this thesis, but studies on health effects for more species are needed. Both moulds and bacteria are needed to be monitored. Moreover, the health relevance of the total fungal biomass in buildings in different climate zones, as well as the health relevance of microbial biodiversity, needs to be further studied.

Measurements of airborne viable moulds have been used for long time, but have clear limitations. Compared to the traditional culture-based or counting-based methods, quantitative Polymerase Chain Reaction (qPCR or sometimes called real time PCR) can be a promising method to detect mould or bacteria since this method can detect and quantify both viable and non-viable fungi.

Most indoor studies consider the building as a relevant exposure unit. However, the higher variation for allergens and fungal DNA within-buildings than between-buildings implied that sufficient number of rooms and multiple locations must be investigated in exposure studies in order to get a reliable estimate of average levels in a building.

There is a wide variation of sampling methods available and each sampling method has its applications and limitations. Since different particle

sizes with different health effects may be captured by different sampling methods, evaluation of different sampling methods versus health is needed.

There are insufficient numbers of epidemiological studies on exposure and health effects in day care centers and school environments. Moreover, most of these studies have been performed in industrialised countries. More multi-center studies and studies in different climate zones are needed to be covered to get generalized knowledge on health effects of microbial and allergen exposure. Further studies in Asian countries are needed since Asia has more than half of the global population.

This thesis implicates that there is a clear need to improve indoor environments in day care centers and schools. However, there is limited information on how to choose and implement the most cost-effective intervention approach and the extent to which reductions in allergen exposures in these environments influence allergy- and asthma-related morbidity [49]. Multifaceted approaches might be needed. Efficient cleaning, pets keeping control, and use of special school clothing might help to decrease exposure to indoor allergens microbial compounds in day care environments and schools. Since day care centers and schools are crowded environments, increased ventilation flow using mechanical ventilation system is needed in most countries. Risk construction should be avoided and building should be maintained to avoid dampness and microbial growth.

Acknowledgement

A lot of people have contributed to this thesis, without their helps, this thesis will not be accomplished in an effective way. Hereby, I would like to express my sincere gratitude in particular to:

Assoc. Prof. Dan Norbäck: the best and kindest supervisor. In Chinese, I would refer to you as my "Gui-ren", meaning that you are the one who made it all good happen. You gave me the possibility and chance to become a PhD-student in Sweden, to enter the interesting and exciting world of epidemiology, the world of combining medicine, occupation and environment. Thanks for your positive attitude, your supporting without any hesitation and trust without any doubt, both in scientific work and private life.

Prof. Christer Janson, my Co-supervisor, for accepting me as his PhD-student, for his fully support during my PhD-period, for his constructive review of manuscripts and helpful suggestions and discussions to my half-time control, the thesis and future scientific work.

Prof. Eva Vingård, head of our department, for encouraging me to believe in myself and giving me the freedom to accomplish the work in the way that I wish, for supporting me behind and the kindness to me all the time.

Assoc. Prof. Robert Wålinder, head of the Clinic of Occupational and Environmental Medicine of UAS, for being very kind to me and giving me the great chance to apply my environmental knowledge into patient work. Thanks for the inspiration and suggestions for the future work.

Assoc. Prof. Lena Elfman, for her great help and introducing me to the very practical lab work of analyzing allergens, for the creation of an easy atmosphere in the lab, for the enjoyable time that we spent together at the work place.

Assoc. Prof. Gunilla Wieslander, for being so nice and kind to me all the time and listening to me and helping me whenever I had any trouble, whenever I needed.

Dr. Zhuohui Zhao, former colleague in Uppsala currently is working at Public Health Department of Fu-dan University, China, for introducing me the scientific lab work both in Shanxi, China and in Sweden and for the true friendship.

Dr. Jeong-Lim Kim, former colleague in Uppsala currently is working at Occupational and Environmental Medicine, Sahlgrenska University Hospital, Gothenburg, for her great help of collecting data from the Swedish day care centers and the revision of the manuscript, and not to forget **Mona Norbäc**k, for her help with collecting data from the Swedish day care centers.

All other persons at our department (***Anna, Corina, Helena, Jennie, Johanna, Juan, Lenita, Magnus, Margareta-T, Martin, Miles, Monica-L, Monica-R, Mostafa, Peter-P, Pia, Roma, Susanne, Tobias, Tomas, Xi, Åsa,***) and the persons who previously worked at the department (***Bitte, Bo, Calle, Greta, Ingvar, Karin, Malin, Peter-W, Torsten***) for their useful advices and discussions, for the harmony atmosphere and friendly working time, for the laugh and relaxing talk during coffee and lunch break, specially to **Erik Lampa**, for his useful help with the statistics, valuable suggestions and review of the manuscript.

Prof. Jamal Hisham Hashim, professor at United Nations Universiti International Institute for Global Health, Kuala Lumpur, Malaysia, and **Prof. Zailina Hashim**, from Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, Kuala Lumpur, Malaysia, for their efforts of making the possibility to do the school study in Malaysia and the great help and support, for the careful review of the manuscript and valuable suggestions.

Prof. Lennart Larsson, from Laboratory Medicine Department of Lund University and **Dr. Erica Bloom,** IVL Swedish Environmental Research Institute Ltd., Stockholm, for their help with analyzing the mycotoxins levels in dust samples, and for their critical revision and suggestions on the manuscript of the Malaysia school study.

Dr. Marzia Simoni, from Pulmonary Environmental Epidemiology Unit, CNR Institute of Clinical Physiology, Pisa, Italy, for her work with the European school study.

Prof. Zhuan-hua Wang, former master degree supervisor from Department of Institute of Biotechnology, Shanxi University, for her great help of introducing me to the Biochemistry and Molecular Biology scientific field, for building the bridge to connect the research and cooperation between China and Sweden, and for her care and kindness to me throughout these years.

Last but not least, my beloved family:
My parents, sisters, brothers, and all other relatives and friends in China, for their endless support and care for me, especially to my cousin ***Xiaohong Cai*** for helping taking care of our daughter Noomi at home so that I could focus on writing my thesis.

Johan Svensson, for your true love, sweetest surprises, continues patience, and care for the whole family so that I could balance between work and my free-time, so that I could be active and always fulfilled with energy. For your fully support without any complains and the big efforts at making us a sweet home, and helping me to improve my Swedish.

Noomi Cai, the lovely and sweetest little girl, for you being so kind and capable, for you managing so well both at home and in the day care center so that I could focus on my work without any concern or worries.

The whole ***Svensson family*** and specially to ***Hasse Svensson*** and ***Helena Svensson***, for active interest in my work process and giving me advice and support both in my work and private life, for the good relationship and friendship, and for the great time that we spent together.

I would also like to acknowledge the financial support from the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS), CFUG Gävleborg County, and the Swedish Asthma and Allergy Association's Research Foundation, from Swedish International Development Cooperation Agency (SIDA) and from the European Commission – Directorate General for Health and Consumer Affairs (DG SANCO) – Contract SPC.2002391.

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