Fungi and human health:
Targeting outdoor sources of allergens, volatiles from allergenic fungi, and indoor microbial communities with their relations to allergy

Fabian Christopher Weikl
a touch of microbes is good for your health

Sironi and Clerici (2010)
ABSTRACT

Fungi are omnipresent forms of life. They are essential for the functioning of soil, which is critical for supporting the vastness of life on our planet. However, some fungi associated with soil, or more generally speaking some environmental fungi, may cause various health impairments in humans. Naturally, such relations are mostly investigated from the direction of medical sciences. However, a comprehensive account on the topic has to consider the lifestyle of the involved organisms in context to their environment. In this thesis, three contemporary health concerns in connection with fungi were approached from a microbial and ecological perspective to extend beyond their known ecological context and improve our understanding on possible health impairments.

The first problem centred on fungal volatile organic compounds which are discussed in recent literature as cause for building related sicknesses. A narrow focus on the putatively well-known fungus *Alternaria alternata* revealed it to be a strong emitter of various sesquiterpenes, a class of highly versatile info-chemicals in inter- and intra-species communication and manipulation. This finding highlighted a general lack in ecological data foundations for volatile organic compounds, which renders a risk assessment of volatile organic compounds for human health questionable until further fundamental microbiological research has been accomplished.

The second concern was approached with a wider focus on the spatial and ecological scale, i.e. on the allergenic potential of outdoor fungi in farming environments. After pollen, fungi are the second most important causative of outdoor airborne allergies, yet their distribution and frequency are not well described. There is no established protocol to measure fungal allergenic potentials and direct measurements of fungal allergens are problematic. To improve this situation, a method for quantification of the most relevant outdoor allergenic fungi from
various environmental source-materials was developed and applied to cereal fields. Therein, plant materials at harvest-time were identified as main source. Individual field conditions had strong influences on the amounts of allergenic fungi. Despite those variations, the levels of the main allergy causatives, *A. alternata* and *Cladosporium cladosporioides*, remained substantial in all cases. This suggests that high numbers of allergenic fungi may be an inevitable side effect of farming in several crops.

The third subtopic concerned the relation between the microbial indoor environment and its effects on infant immune systems. This issue was studied from a very wide angle through a population based birth cohort. To that goal, fungal and bacterial molecular fingerprints of living-room dusts from homes with infants were analysed. Reaching epidemiologically relevant sample sizes, a link between fungal dust-communities during early childhood and later allergic symptomatic could be established. Additionally, factors influencing the composition of the home dust microbiome were identified. Among others, a strong association was observed between dust microbiome composition and the seasonal (fungi) or phenological (bacteria) course, with implications on the design of future studies on the indoor microbiome.

This thesis approached three major concerns of fungi mediated health effects with microbiology-centred views and showed that such perspectives are crucial for understanding human health issues and for elaborating actions towards their improvement.
ZUSAMMENFASSUNG


Als erstes Problem wurden volatile organische Verbindungen von Pilzen betrachtet, welche in aktueller Literatur als Ursache für gebäudebedingte Erkrankungen diskutiert werden. Ein enger Fokus auf den vermeintlich gut untersuchten Pilz *Alternaria alternata* zeigte, dass dieser ein starker Emittent verschiedener Sesquiterpene ist, einer Klasse vielseitiger Botenstoffe in der Kommunikation und Manipulation zwischen und innerhalb von Arten. Dieses Ergebnis unterstreicht einen generellen Mangel an grundlegenden ökologischen Daten zu volatile organischen Verbindungen, was Einschätzungen zum von diesen Substanzen ausgehenden Risiko für die menschliche Gesundheit ungewiss macht, bis weitere mikrobiologische Grundlagenforschung zu diesem Thema vorliegt.


Diese Arbeit begegnete drei bedeutenden Fragen zu pilzvermittelten Gesundheitseffekten aus mikrobiologischem Blickwinkel und zeigte, dass solche Perspektiven entscheidend für das Verständnis menschlicher Gesundheitsprobleme sind und um Anstrengungen zu ihrer Verbesserung unternehmen zu können.
LIST OF ORIGINAL ARTICLES

I  Weikl F*, Ghirardo A*, Schnitzler JP, Pritsch K. Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*: Effects of age, nutrient availability, and co-cultivation. Article submitted to *Scientific Reports* on November 11, 2015
*contributed equally to this work

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*contributed equally to this work

AUTHOR’S CONTRIBUTION

I  Fabian Weikl wrote the paper, except the part on GC-MS methodology. He performed all experimental work up to GC-MS analysis, which he did together with and under the guidance of Dr. Andrea Ghirardo. He planned the experiments together with co-authors and interpreted the results together with co-authors.

II  Fabian Weikl wrote the paper. He developed the idea, proofed its theoretical background and performed the experimental work. He planned the experiments together with co-authors and interpreted the results together with co-authors.

III  Fabian Weikl and Dr. Christina Tischer share first authorship. Fabian Weikl performed the experimental microbiological work and was responsible for the molecular-microbiological analysis of the results (community variation and diversity measures, multivariate tests), for which Dr. Alexander J. Probst gave support in statistics. Fabian Weikl wrote the microbiological-molecular part of the paper, assisted in writing the other parts together with co-authors, and interpreted the results together with co-authors.

IV  Fabian Weikl wrote the paper, contributed the idea to analyse temporal dynamics, and performed the microbiological experimental work. He planned the tRFLP experiments together with co-authors and interpreted the results together with co-authors.
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<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
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<tr>
<td>BVOC</td>
<td>biogenic volatile organic compound</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>DG18</td>
<td>dichloran-agar with 18% glycerol</td>
</tr>
<tr>
<td>DOI</td>
<td>digital objects identifier</td>
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<tr>
<td>EPA</td>
<td>Environmental Protection Agency (of the United States of America)</td>
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<tr>
<td>EPS</td>
<td>extracellular polysaccharides</td>
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<tr>
<td>GEE</td>
<td>general estimation equation</td>
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<td>HLA</td>
<td>human leucocyte antigen</td>
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<td>IFN</td>
<td>interferon</td>
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<td>IgE</td>
<td>immunoglobulin E</td>
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<td>IL</td>
<td>interleukin</td>
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<td>INSD</td>
<td>international nucleotide sequence database</td>
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<td>ITS</td>
<td>internal transcribed spacer</td>
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<tr>
<td>LISAplus</td>
<td>influence of life-style factors on the development of the immune system and allergies in East and West Germany plus the influence of traffic emissions and genetics</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
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<td>MRPP</td>
<td>multi-response permutation procedure</td>
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<td>MSQPCR</td>
<td>mould specific quantitative polymerase chain reaction</td>
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<td>MVOC</td>
<td>microbial volatile organic compound</td>
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<tr>
<td>NGS</td>
<td>next generation sequencing</td>
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<tr>
<td>OPLS</td>
<td>orthogonal partial least squares</td>
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<tr>
<td>PCA</td>
<td>principal component analysis</td>
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<td>PCo</td>
<td>principal coordinate</td>
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<tr>
<td>PCoA</td>
<td>principal coordinate analysis</td>
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<tr>
<td>qPCR</td>
<td>quantitative polymerase chain reaction</td>
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<tr>
<td>RAST</td>
<td>radioallergosorbent test</td>
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<tr>
<td>rDNA</td>
<td>ribosomal deoxyribonucleic acid</td>
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<tr>
<td>SBS</td>
<td>sick building syndrome</td>
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<tr>
<td>SEA</td>
<td>soil extract agar</td>
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<tr>
<td>T cell</td>
<td>lymphocyte expressing a T-cell-receptor</td>
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<tr>
<td>Th</td>
<td>T-helper lymphocytes</td>
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<tr>
<td>TNF</td>
<td>tumour necrosis factor</td>
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<tr>
<td>tRFLP</td>
<td>terminal restriction fragment polymorphism</td>
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<tr>
<td>VOC</td>
<td>volatile organic compound</td>
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<td>WHO</td>
<td>World Health Organization</td>
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1 INTRODUCTION

1.1 Fungi: ecological importance and relevant health impairments

Compared to other higher-level phylogenetic ranks of life such as the kingdoms Animalia and Plantae or the domain Bacteria, the importance of the kingdom Fungi is less broadly acknowledged (Rambold et al. 2013). However, the ecological importance of fungi for terrestrial environments is extraordinary. They likely assisted plants to adapt to life on land (Redecker et al. 2000), and are key drivers of soil functioning and the global material cycles (Rambold et al. 2013, Gadd 2006, Six et al. 2006).

Despite their fundamental ecological role, some fungi are also known for the health impairments they can cause. The list of health issues related to fungi ranges from often deadly infections, such as systemic aspergillosis and candidiasis, to skin diseases (e.g. athlete’s foot), intoxications, allergies, and to loosely defined health issues, e.g. sick building syndrome (Köhler et al. 2015, Fischer and Dott 2003). Most fungal pathogens are opportunists, with some of them being able to act as saprobes, plant- or human pathogens, depending on the circumstances (Köhler et al. 2015). The majority of all fungal health problems are caused by ubiquitous taxa like *Aspergillus fumigatus* for Aspergillosis, or *Alternaria, Cladosporium*, and the *Penicillium-Aspergillus* group for allergies. While the number of genuine fungal pathogens (i.e. those not limited to immune compromised persons) is small (Köhler et al. 2015), toxicity seems to be widespread among the kingdom (Spiteller, 2015). Despite this, the chemical ecology of fungi has not been investigated systematically until now (Spiteller, 2015). Apart from health problems that need direct contact to fungi, distance based health issues also exist. One of the latter is the potential health risk that originates from volatile chemical compounds emitted by fungi (Fischer and Dott 2003). Another class of distance based effects are hypersensitivity reactions such as immune globulin E mediated allergies (e.g. allergic rhinitis, allergic asthma, atopic dermatitis) or hypersensitivity pneumonitis, which
are both typically caused by airborne particles, e.g. fungal propagules (Simon-Nobbe et al. 2008).

In addition to the medical perspective, which is strong in describing agents and mechanisms of disease, a more microbial ecological perspective on fungal health issues is necessary for a better understanding of the relationship between fungal life and humans, specifically to bring ecology and human health into a perspective that eventually improves our quality of life. This thesis was planned as such an effort to extend the known ecological context of non-pathogenic fungi which are associated with allergies and other possible immune modulating effects.

1.2 Volatile organic compounds

1.2.1 Microbial volatile organic compounds

‘Volatile organic compounds’ (VOCs) are loosely defined as a group of carbon based compounds which are typically solid or liquid at 20 °C and normal pressure (Morath et al. 2012) and have at least medium vapour pressures (> 0.00001 kPa) (Korpi et al. 2009). Commonly used subcategories include ‘biogenic volatile organic compounds’ (BVOCs) for all atmospheric trace gases other than CO₂ and CO with probable biogenic origins (Kesselmaier and Staudt 1999) or ‘microbial volatile organic compounds’ (MVOCs) (Korpi et al. 2009). BVOCs are products of the primary or secondary metabolism and typically can be characterized either as alcohol (e.g. 2-pentanol), aldehyde (e.g. octanal), hydrocarbon (e.g. 1,3-octadiene), acid (e.g. octanoic acid), ether (e.g. 2,3,5-trimethylfuran), ester (e.g. ethyl-2-methyl propionate), ketone (e.g. 2-heptanone), lactone (e.g. γ-decalactone), terpenoid (e.g. β-elemene), sulphur compound (e.g. dimethyl disulphide), or nitrogen compound (e.g. 2-methoxy pyrazine) (Korpi et al. 2009). Biogenic emissions can also originate from uncontrolled loss of metabolites during microbial degradation, and they are not always easily
distinguished from emissions caused by physical–chemical degradation, e.g. in soil (Insam and Seewald 2010).

Though scents (i.e. VOCs) are omnipresent, microbial (i.e. fungal and bacterial) volatile emissions were often overlooked due to hampered absorption and detection methods (Lemfack et al. 2014, Schmidt et al. 2015). The development of capable techniques, especially headspace solid phase microextraction (Zhang et al. 1994) in recent years has improved the ability to investigate these compounds, and MVOC analysis has become an emerging branch of different research disciplines. This is reflected in an assortment of recent reviews for a variety of subtopics. Examples from the ecology perspective include VOC emissions from soil (Penuelas et al. 2014, Insam and Seewald 2010), belowground VOC mediated interactions (Effmert et al 2012, Wenke et al. 2010) and MVOC mediated microbial interactions (Schmidt et al. 2015) or MVOCs and plant health (Bitas et al. 2013, Junker and Tholl 2013, Farag et al. 2013). However, research on VOCs is highly interdisciplinary. Morath et al. (2012) listed a number of disciplines which contributed to knowledge on fungal volatiles: food flavouring and aroma, fungal development (quorum sensing, auto-induction), rhizosphere biology, mycofumigation, biocontrol, biofuels, indirect markers of fungal growth (food storage, indoor health), building related illnesses, separation science, and chemotaxonomy.

Irrespective of the potential merit of the research subject for different fields of research, a recently started comprehensive database on microbial volatiles only found reports in the literature of MVOC from 349 bacterial and 69 fungal species, with a total of 846 emitted compounds (Lemfack et al. 2014). Against the background of innumerable microbial phylotypes and the vast structural variety within volatile compound classes like sesquiterpenes (Kramer and Abraham 2012), it is evident that our knowledge on MVOC production and its ecology is limited. Just recently, Hung et al. (2015) and Bennett (2015)
strongly encouraged the intensification of crosstalk between the traditionally separated disciplines dealing with volatiles (with their different approaches, conceptions and fragmentary knowledge) to elucidate the ecological role of fungal volatiles, which has so far been neglected in research.

1.2.2 Fungal volatiles and human health effects

The most prominent health issue that is regularly associated with fungal VOC is the ‘sick building syndrome’ (SBS), a combination of various mucosal, skin, and general health symptoms of individuals in temporal relation to a building; its incidence, foundations and triggers are part of a debate that is by now continuing for more than 30 years (e.g. Burge 2004, Stolwijk 1991). Though fungi often seem to contribute to the syndrome (Zhang et al. 2012, Saijo 2011), the actual role of fungi in SBS remains vague. The clarification of risk factors for SBS needs studies with large numbers of samples and participants. Sahlberg et al. (2013) recently undertook such an effort. They aimed at revealing possible associations between airborne microbial exposure (VOCs and propagules), formaldehyde, and plasticizers in dwellings with the symptoms of SBS. The authors found positive associations between SBS and the levels of five MVOCs (2-pentanol, 2-hexanon, 1-octen-3-ol, 2-pentylfuran 3-methylfuran), however those compounds were not associated with the numbers of airborne viable bacteria or moulds. Because airborne microbes in general were taken as surrogate for microbial growth in the investigated homes, the authors could not uncover the actual source of the detected MVOCs.

Investigating health effects of fungal VOCs by focussing on single substances and their mode of action can serve as an approach to obtain evidence beyond the indeterminate associations found in the context of SBS, as was recently shown for the pan-fungal compound 1-octen-3-ol (‘mushroom alcohol’): Fungal C₈ volatiles including 1-octen-3-ol provoked a much higher
mortality (100 % after one day) in Drosophila melanogaster compared to any tested industrial chemical of the same concentration, e.g. toluene, benzene, and formaldehyde (max. 50 % mortality after 15 days) (Inamdar et al. 2012a). Correspondingly, 40- to 80-fold less 1-octen-3-ol than toluene was sufficient to eliminate 50 % of a given number of human embryonic stem cells (Inamdar et al. 2012b). 1-octen-3-ol was also retarding the development in the plant Arabidopsis thaliana (Hung et al. 2014). Surprisingly, 1-octen-3-ol additionally caused neurodegeneration by damaging the dopamine system in D. melanogaster, which the authors reasoned may be a missing piece towards explaining Parkinson’s disease, since epidemiological studies also connect this type of neuronal impairment with mould and mushrooms (Inamdar et al. 2013). Furthermore, the same group that reported the other results on 1-octen-3-ol given above showed that this substance also induced NO mediated inflammation pathways in D. melanogaster which are similarly found in humans (Inamdar and Bennett 2014). Intriguingly, 1-octen-3-ol is not an exceptionally rare compound; it can be detected in most mould-infested buildings (Korpi et al. 2009, Kuske et al. 2005). The prevalence of more complex emissions such as sesquiterpenes is still rarely described, and toxicity analyses of blends of compounds or studies on their mode of action are lacking (Cabral 2010, Kopri et al. 2009). However, it is known that non-toxic irritations from VOC can be the result of several mechanisms that are either inflammatory reactions or nerve stimulations (Kopri et al. 2009).

1.2.3 Fortifying the knowledge foundations: focusing on producers

VOCs are a multidisciplinary topic with potential in biomarker research for the fungi themselves (e.g. food spoilage), fungus related conditions (e.g. building contamination or ecotyping), and in ecology (info-chemicals). The knowledge regarding VOCs has also been tested for its use in potential routine analysis. While this seems to work reliably for certain
cases in the food industry (cf. Morath et al. 2012), contradictory results are obtained in indoor health research. For example, Moularat et al. (2011) were able to match the detection of defined sets of (small) fungal VOCs with visible and invisible mould contamination while Schleibinger et al. (2008) and Sahlberg et al. (2013) reported that they could not associate the mould status of buildings with MVOC.

The basic problem for a reliable utilisation of the knowledge on VOC, be it for applications on human health sustenance or hypotheses on their ecological role, is the patchiness of the underlying knowledge which became apparent with the advent of improved detection techniques (Lemfack et al 2014, Schmidt et al., 2015). For that reason, Article I of this thesis was defined as a study to install reliable foundations on the VOC production capabilities of the main human-allergenic, plant-pathogenic and food-spoilage fungus *A. alternata* that should include different growth conditions which aimed at simulating different environmental conditions.

1.3 Fungal allergies

1.3.1 The vague incidence of fungal allergy

Fungal airborne allergies are a long recognized problem. Thirty-five years ago, Gravesen (1979) gave estimations for threshold airborne concentrations of *Alternaria* (100 spores / m³) and *Cladosporium* (3000 spores / m³) for evoking allergic symptoms in sensitized individuals. However, the prevalence of sensitization to fungal material remained vague at that time and is still debated until today.

There are several reasons for this. One is the difficulty in obtaining standardized extracts for diagnosing fungal allergies. Gravesen (1979) wrote on the matter of prevalence:
“Precise statements are, however, impossible to give, until clinicians have been furnished with highly purified, standardized diagnostic extracts. The different diagnostic in vivo (skin test, nasal/bronchial provocation) and in vitro (RAST\(^1\), histamine-release) tests, and the environmental parameters have to be combined in the allergological clarification. Cases exclusively based on skin tests with commercially available fungal allergen extracts are often less reliable because the tests are restricted to a standard repertoire dependent upon successful preparation procedures from the different manufactures and are not always in accordance with the moulds to which the patient is really exposed.”

Surprisingly, progress on the standardization of allergen-extracts remains very modest until today, i.e. still reference standards are not available (Esch 2004, Kesphol et al. 2013). A recent example for the dilemma was given by Kesphol et al. (2013) who compared commercially available allergen extracts of four fungi from six manufacturers and concluded that the quantitative and qualitative heterogeneity of the extracts rendered their comparability questionable. The prognosis for establishing standardized fungal allergens extracts are pessimistic, due to several reasons including manufacturing differences, frequency of fungal somatic mutations and genetic instability during batch cultivation or even the variety of potentially allergenic substances (proteins, glycoproteins, polysaccharides and other substances) making up the extracts (Esch 2004).

Of course, diagnosis of allergy and detection of allergens would strongly profit from the use of recombinant allergens. However, only a handful of recombinant fungal allergens are available at the moment (five according to Crameri et al. 2014), which limits their usability.

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\(^1\) author’s note: RAST – radioallergosorbent test; an in vitro technique which detects specific IgE antibodies in samples of blood-sera by visualizing their binding to insolubilized suspected allergens (e.g. allergenic extracts of pollen or moulds); in modernized forms (e.g. immunoCAP™) still the second common diagnosis tool, aside from skin and prick tests (Smits et al. 2003)
For *A. alternata* and *Aspergillus fumigatus*, the probably best-studied allergenic fungi, 35 different allergenic proteins have been approved by the responsible institution (the Allergen Nomenclature Sub-Committee of WHO and International Union of Immunological Societies (www.allergen.org; accessed June 18, 2015). In contrast, most major allergens from other relevant sources such as pollen, mites, and food have already been cloned (Chapman et al. 2007). According to Crameri et al. (2014), this lack in recombinant fungal allergens is due to only few laboratories specialising in fungal allergenic sources and a pertained neglect of fungal allergens in molecular allergology, despite their importance in allergic asthma.

Diagnosis of fungal allergy is hampered further by cross-reactivity to other fungi. For example, the major allergen Alt a 1 (a protein of unknown function) from *A. alternata* shows cross-reactivity with about 50 similar proteins in other species of its phylum. To obscure the issue more, there are also cross-reactivities spanning phylogenetic kingdoms (Simon-Nobbe et al. 2008).

Finally, polysensitisations, i.e. allergenic reactivity of an individual against structurally unrelated allergens in different sources (e.g. moulds, pollen, and food), seem to be common with fungal allergens (Simon-Nobbe et al. 2008). This may be another reason for the uncertain prevalence of fungal allergies. For example, an allergy against *A. alternata* may easily be overlooked when combined with an allergy against grass pollen, mugwort (*Artemisia*) or ragweed (*Ambrosia*) as all those are abundant in the air at the same time (Twaroch et al. 2015). However, pollen monitoring is excellent which encourages false correlations of allergenic symptoms (Twaroch et al. 2015).

For these complex issues, diagnosis of fungal allergy is still “an unsolved medical need” (Cramer et al. 2014), although the prevalence can be roughly estimated by now. One of the latest comprehensive reviews on this topic (Simon-Nobbe et al. 2008) sums up results of several studies as follows:
“The incidence of mould allergy ranges from 6 to 24% in the general population\textsuperscript{2,3}, up to 44% among atopics\textsuperscript{4} and 80% among asthmatics\textsuperscript{5}. The incidence of mould allergy within asthmatic children is 45% whereas it is 70% in asthmatic adults\textsuperscript{6}.”

The high prevalence of fungal allergy among asthmatics has recently been recognized as serious health problem (Knutsen et al. 2012, Crameri et al. 2014) and led to the proposal of severe asthma with fungal sensitization (SAFS) as disease pattern (Denning et al. 2006). The most common genera that were associated to SAFS are \textit{Alternaria}, \textit{Aspergillus}, \textit{Cladosporium}, and \textit{Penicillium} (Denning et al. 2006).

1.3.2 Detecting fungal allergenic sources in outdoor environments

A link between allergic symptoms and visible growth of moulds in indoor environments is obvious to establish. As a result, indoor fungal growth has long been recognized as potential cause for human health impairment; Górny (2004) even refers to the bible (Leviticus 14: verses 34 to 45 give duties for dealing with mould on indoor walls). Consequently, there is profound interest in estimating the risk an indoor mould infestation poses for the inhabitants, especially as humans spend most of their time indoors.

Detecting allergens directly is impracticable and almost unfeasible, since the above mentioned issues with allergen diagnosis affect detection. While microbial culture techniques have routinely been used to evaluate risks on indoor allergies (Pasanen 2001), the identification of fungal cultures needs very specialized expertise and is prone to misidentification (Vesper 2011). Therefore, the use of molecular techniques is spreading rapidly at the moment. Mould-

\begin{small}
\begin{itemize}
\item\textsuperscript{2} Tariq et al. (1996)
\item\textsuperscript{3} Salvaggio and Aukrust (1981)
\item\textsuperscript{4} Corey et al. (1997)
\item\textsuperscript{5} Lopez and Salvaggio (1985)
\item\textsuperscript{6} Hsieh and Shen (1988)
\end{itemize}
\end{small}
specific quantitative PCR (MSQPCR, Haugland and Vesper 2002), a technique developed by the United States Environmental Protection Agency (EPA), is used as an unsanctioned reference standard for routine tests on indoor moulds (Beusse et al. 2013) and in research. As of June 19, 2015, the EPA listed more than 60 scientific publications using this technique (http://www.epa.gov/nerlcwww/mouldtech.htm).

As detailed in Article II of this work, corresponding methods for an allergenic fungal source tracking in outdoor environments are lacking. However, it is acknowledged that fungi are, together with pollen, the primary sources for outdoor allergenic particles (Burge and Rogers 2000). Consequently, the main aim in II was to provide such a method and give an outlook to its applicability for a selected agricultural environment. Details are given in II. An experiment that tried a source tracking with classical culture based microbiology is presented in chapter 2.2.2. (eII).

1.4 Indoor microbiomes

1.4.1 Developments in indoor microbial research

Fifteen years ago, progress for indoor microbiology was mostly made by organismal microbiology, for example through descriptions of new wall-growing mycobacteria (Vuorio et al. 1999). Most achievements in microbiological ecology of the built environment of that time were strongly linked to disciplines like toxicology (Andersson et al. 1997) or human epidemiology, rendering indoor microbiology a very interdisciplinary field. Although for epidemiology, microbes are one of many possible factors of influence (determinants) on human health. Nevertheless, the typically high sample-sizes in epidemiological studies and the application of different methods that were used through the course of time allowed substantial insights into typical indoor microbial communities. For example, cultivation/count methods applied in epidemiological studies revealed a seasonal quantitative oscillation of
viable fungi (Koch et al. 2000) and total fungal propagules (Heinrich et al. 2003) in living-
room dusts, or quantitative size-related seasonal variations in indoor airborne gram-positive 
bacteria (Moschandreas et al. 2003). In addition, indirect proxies such as β(1,3)-glucans, 
endotoxin, and fungal extracellular polysaccharides were introduced to relate microbial ‘taxa’ 
to health outcomes and are still in use today (e.g. Tischer et al. 2011).

In the last ten years, molecular microbial ecology techniques spread in the field of indoor 
microbiology. For indoor allergenic fungi, the development of MSQPCR found broad 
acceptance (1.3.2). The first studies that used rDNA clone-library sequencing to analyse 
fungal (Pitkäranta et al. 2008) and bacterial (Rintala et al. 2008) communities in indoor dusts 
made adequate interpretations of their result necessary, which were more complex than for 
those gathered with culture-studies. This resulted in a comprehensive interest on the 
mechanisms of indoor microbial ecology. The introduction of modern sequencing and 
microarray techniques recently led to a wide range of microbiome studies, from huge 
approaches dedicated solely to epidemiological outcomes (Lynch et al. 2014), to efforts 
fockussing on studying the built environment for the sake of explaining its ecology (Adams et 
al. 2013 and 2014). The latter notably suggested that the microbiological hypothesis of 
‘everything is everywhere’ (an explanation of the term can be found in O’Malley 2007) is 
invalid for indoor environments; instead the outdoor biogeography and the indoor habitat 
seem to shape indoor microbiomes.

Nowadays, the human built environment is recognized as “the modern ecological habitat of 
Homo sapiens sapiens” and it is acknowledged “that our understanding of indoor microbial 
diversity remains extremely sparse” compared to previous – culture-dependent – opinions 
(Kelley and Gilbert 2013). In fact, the recognition of the built environment as human habitat 
has led to a certain publicity (cf. the digest by Humphries 2012) and solid funding for a 
number of collaborative projects (MicroBE.net, hospitalmicrobiome.com; both accessed June
Such projects also include novel efforts on public outreach by using social media hubs such as facebook (https://www.facebook.com/MicroBEnet, accessed June 22, 2015).

1.4.2 Expectations on working with an epidemiological study

The elucidation of indoor microbial ecology recently gained momentum and now involves projects solely dedicated to ecological aspects (1.4.1). However, collaborative efforts that analyse samples from epidemiological studies are still rewarding for microbial ecology and epidemiology. In our case, the particular epidemiological study encompassed approximately 300 settled dust samples, each from the living room floor of a different family with three-month-old infants, and each sample was accompanied with a large set of explanatory data. The original study design of the birth cohort LISAplus (Heinrich et al. 2012) involved collecting dozens of descriptive parameters of home and children (Fahlbusch et al. 1999). The value of this meta-data was further increased through the results of evaluation studies thereon (e.g. Heinrich et al. 2002, Gehring et al. 2007, Tischer et al. 2011, Casas et al. 2013).

For this study, it was hypothesized that the exploration of the samples with tools of microbial ecology (molecular fingerprinting of marker genes) should allow assignment of (a) environmental influential factors on the examined indoor microbial communities and (b) effects of this microbial environment on the long-term health development of the children exposed to it.

An important example for the impact of environmental factors on indoor microbial communities is the time of sampling during the year. Though its relevance has been partly acknowledged for some time (Koch et al. 2000, Rintala et al. 2008), its assessments lacked temporal resolution and different locations for an exhaustive survey. Details on environmental influence factors with focus on the sampling time are presented in Article IV.
The assessment of influences from the microbial environment on health development of children was planned as a contribution to the current dispute that tries to explain how the microbial environment modulates maturing immune systems. This produced Article III and the extended project eIII (2.2.3). The greater question addressed was whether indications for the validity of the hygiene hypothesis (Strachan 1989) can be found for children growing up in an urban environment. In brief, the hygiene hypothesis currently proposes that too much cleanliness (i.e. reduced exposure to microbes) prevents the development of a well-balanced immune response and can result in chronic inflammatory diseases, e.g. allergies (Okada et al. 2010, Sironi and Clerici 2010). Until now, epidemiological evidence for this hypothesis has been gathered from more than 30 studies on rural and mostly farm environments (von Mutius and Vercelli 2010). For example, an extraordinarily wide range of microbial exposures for children living on farms was protective for asthma and atopy (Ege et al. 2011). Evidence whether the microbiome in urban indoor environments also affects the emergence of allergic diseases in children is scarce as detailed in Article III.
1.5 Aims of the thesis

Fungi are able to impair human health through non-pathogenic mechanisms. This work aims at contributing to the predominantly medical topics of allergies and related conditions from the perspective of microbial ecology and at showing the importance of multidisciplinary approaches to identify health risks associated with non-pathogenic fungi. Three potential fungal health effects were investigated at different scales: volatile emission, outdoor sources of allergens, and indoor fungal communities.

The specific aims in this thesis are:

1. To explore the dimensions VOC production in the major allergy causative *Alternaria alternata* and the co-occurring *Fusarium oxysporum*. (I)

2. To establish a high spatial resolution method for qualitative and quantitative assessments of outdoor allergenic fungi and to apply it under different farming regimes for a first statement on sources of fungal allergens in agriculture. (II)

3. To enlighten long-term effects of the microbiome in household dust on infants and to identify major influence factors on this microbiome as well as directions for future studies on the indoor microbiome. (III and IV)

Although all parts of the thesis relate to microbial ecology, their overlap with other disciplines such as epidemiology and immunology is distinct (Fig. 1).
Figure 1 The author’s opinion on locating projects I, II, III, and IV in the space of established research disciplines. Left: Microbial Ecology and two disciplines with common intersections to the projects. Right: The degree of overlap for subtopics of the thesis.
2 MATERIALS AND METHODS

2.1 Overview of the analyses and methods used in the articles (I, II, III, IV)

Detailed descriptions of the procedures and references are given in the respective articles (I, II, III, and IV). A short summary of the approaches is presented here. Important project related work exceeding the focus of these articles is documented concisely in chapter 2.2 as extended projects eII, and eIII.

I: The study compared VOC emissions of *A. alternata* and *F. oxysporum* in five different cultivation setups: sterile control, solitary (i.e. pure cultures of) *A. alternata*, solitary *F. oxysporum*, split-plate (hemispheres of a petri dish separated by a vincible physical barrier) cultivation and direct confrontation of both fungi on one plate. The experiment was conducted with five replicates each for malt extract gelrite and synthetically nutrient poor gelrite, to compare nutrient rich and poor conditions. Volatiles were sampled at different dates after inoculation to capture different stages of culture maturation. VOCs were collected using headspace sorptive extraction with Gerstel Twisters (Gerstel, Mühlheim an der Ruhr, Germany), i.e. a stir bar sorptive extraction method. After thermo-desorption, VOC samples were analysed with a gas chromatograph-mass spectrometer (GC-MS). Chromatograms were analysed with appropriate software (MSD ChemStation E.02.01.1177, Agilent Technologies, Santa Clara, USA) in a stepwise calculation approach. Result interpretation was supported by multivariate statistics (correlation matrices, principal component analysis (PCA), and orthogonal partial least squares analysis (OPLS)).

II: The study established a method to estimate the amount of allergenic fungi found in outdoor environments with special focus on agriculture. Field samples from the Scheyern research station (Schröder et al., 2008) were used to adapt the MSQPCR procedure to
agricultural sample types and to exert a limited exemplary monitoring. The study was divided into three parts. The first part involved setting up the method. This encompassed finding a suitable DNA-extraction procedure for agricultural samples (supplementary material of II), testing the accuracy of different calculation methods ($E^{-\Delta CT}$ and $E^{-\Delta CT}$ approach, cf. Haugland and Vesper 2002), and testing the reproducibility of the whole setup. The second part used the method for a time-based monitoring of allergenic fungi on all fields with wheat in the crop rotation during the period between harvest and the next sowing (August – March). The third part gave an outlook on the levels of allergenic fungi of several common crops at harvest-time. The dimensions of each experiment are compiled in Table 1 of study II.

III and IV: These studies used a molecular fingerprinting technique (tRFLP) to assess general community parameters for fungi and bacteria (community dissimilarity and diversity measures) in dust samples obtained from the cohort-study LISAplus. The dust samples had been collected by LISAplus investigators via vacuuming living room floors of homes in which new-borns (two to three months old) had lived. Most of the homes were part of the area around Munich (cf. Figure 1 (III) and S1 Supporting Information (IV)). For each dust-sample, a set of descriptive variables (meta-data) existed. These included questionnaires completed by the parents (on environmental parameters and on the children’s health), measurements of the children’s health status up to 10 years (e.g. total and specific serum immune globulin E, doctor diagnosed asthma), and data from environmental monitoring (greenness of the neighbourhood, urbanization and exhaust measurements).

III and IV vary in the meta-data associated with the dust-samples analysed therein. For the samples of III, data on IgE and the children’s health development had been available, while IV encompassed all samples of III and additionally included samples in its analyses that lacked health related data. IV was an analysis of a broad variety of environmental parameters.
that potentially influence the indoor microbial community and was particularly focused on the major environmental determinant sampling time (i.e. the course of seasons). Its set of fingerprints (286 for fungi, 283 for bacteria) was examined from a molecular ecology perspective; main steps included normalization of restriction fragment data with rarefying, construction of Bray-Curtis community dissimilarity matrices and calculation of diversity measures (Shannon diversity, Simpson diversity, Pielou Evenness, Richness). Multivariate statistical testing on community dissimilarities was conducted with Adonis, MRPP and partial Mantel tests. Kruskal—Wallis tests were used for non-parametric univariate testing.

In III, only data of microbial diversity (Simpson Index) was used, however the study included a statistical analysis from an epidemiological viewpoint. To that goal, adjusted odds ratios (adjusted for sex, maternal education and season of sampling) were calculated for the association between tertiles of Simpson diversity and health outcomes after logistic regression and general estimation equation (GEE) models.

2.2 Extended projects: additional work with relation to the articles (II and III)

2.2.2 Allergenic fungi in agriculture, a cultivation approach (eII)

Overview

The postharvest time series of field samplings for qPCR quantification described in study II was accompanied by cultivation attempts for the dates of 19/09/11, 25/10/11, and 27/03/12. This had two aims. First: to find out whether basic knowledge about actively growing allergenic fungi in decaying plant matter of agricultural environments can be gained by a technically unpretentious cultivation approach. Second: to analyse the VOC emission patterns of acquired field-isolates of allergenic fungal strains and determine the intra-taxon variability, which would be an extension to study I.
Field samplings and processing of samples

Field samplings for cultivation were completed in parallel and in the same manner as described in II for molecular biology samples of the postharvest time series, except that only decaying shoots and roots of wheat were sampled, and that the samples were stored at 0 °C – 4 °C until processing.

Preparation of samples and inoculation was conducted no longer than 48 hours after sampling. Before preparation, subsamples of each field were mixed to one field-sample for roots and one for shoots of each field. Each of these field samples was prepared separately. First, soil was removed from all samples under running tap water. Afterwards, approximately 20 g of material per sample were put into a 50 ml tube filled with tap water and shaken for 5 min with a frequency of 4 rounds s⁻¹. The washing water was discarded and the procedure was repeated twice with fresh tap water. The tap water was exchanged with sterile deionized water and the procedure was repeated three further times. Afterwards, the sample material was cut into pieces < 1 cm² in a sterile environment. Prepared samples were stored no longer than two hours before inoculation. The whole procedure was developed upon recommendations given by Frankland et al. (1990).

Inoculation

Two media were prepared for inoculation: (1) dichloran-agar with 18% glycerol (DG18) after Samson et al. (2010), and (2) soil extract agar (SEA) after the recommendations by Rosenbrock (1997). Before autoclaving, both media were supplemented with 4 g L⁻¹ of sieved (mesh: 0.9 mm) pieces of wheat straw. Final concentrations of antibiotics in inoculation-media were 100 µg L⁻¹ chloramphenicol and streptomycin to inhibit bacterial growth. For inoculation, 10 pieces < 1 cm² each of straw and root material were randomly selected per field, and from these pieces, one piece < 1 mm² was cut and placed one a culture plate.
containing DG18 or SEA, respectively. This resulted in 20 inoculated media per field for each of the three sampling dates: 10 inoculations with straw material and 10 inoculations with root material, half of each inoculated on DG18 and half on SEA; i.e. in total 480 isolation attempts.

**Further cultivation**

Growth was inspected regularly. Up to three separation attempts were undertaken for plates showing growth of mixed cultures. For long-term storage, purified cultures and inseparable mixed-cultures were deposited as airtight slant-agar cultures at 4°C.

**Taxonomic identity of the cultures**

Identification to family-level, and for potentially allergenic fungi genus-level, was targeted. To that goal, phylogenetic marker DNA was used as described below. The results for each isolate were validated through light microscopy. Isolates for which DNA-based identification did not match diagnostically important growth and micro-morphological features according to the relevant descriptions (Domsch et al. 1993, Guarro et al. 2012, Samson et al. 2010) were sorted into an ‘unknowns’ category. Mixed cultures (defined according to morphology or dubious results in DNA marker sequencing) were sorted into a ‘mixed-cultures’ category.

**DNA based identification of isolates**

DNA was extracted from 10 – 50 mg of surface culture of each isolate using a DNeasy 96 plant kit (Qiagen, Hilden, Germany) following the manufacturer’s recommendations for fresh tissue and with tungsten-carbide beads. DNA of fungal internal transcribed spacer (ITS) rDNA, i.e. the fungal barcode region (Schoch et al. 2012), was PCR-amplified using primers ITS1F (Gardes and Bruns 1993) and ITS4 (White et al. 1990). In order to get sequence
information of the barcode-region, purified PCR-products were reused for linear PCR (separate reaction for forward and reverse primers, ‘sequencing reaction’) with and according to the BigDye Terminator Kit v.3.1 (Applied Biosystems, Foster City, USA), with the reaction volume downscaled to 5 µl. The products were cleaned via ethanol precipitation, suspended in molecular-biology-grade water and their sequence was read with an Applied Biosystems 3730 48-capillary sequencer.

DNA sequences were checked manually for quality (ambiguous nucleotides, putative chimeric sequences, correct placement of conserved regions) using SeaView (Gouy et al. 2010) for editing and FinchTV (v.1.4.0) to display electropherograms. Contigs were made from each isolate’s forward and reverse fragments.

UNITE’s massBLASTERer tool was used to align each sequence against UNITE database and International Nucleotide Sequence Database (INSD) (Abarenkov et al. 2010, Kõljalg et al. 2005) (database releases of June, 2012). Only conclusive phylogenetic relationships were used to classify the isolates. For cases showing ambiguous INSD relationships, erroneous entries in INSD were assumed (Nilsson et al., 2006) and only alignments from the manually curated UNITE database were used for classification. If no conclusive matches to UNITE were available, the respective isolate was categorised as ‘unknown’.

Statistical analyses

The Adonis (permutational multivariate analysis of variance using distance matrices) and MRPP (multi-response permutation procedure) functions from the vegan package (Oksanen et al, 2014) implemented in R (R Core Team, 2013) were used with $10^5$ iterations to assess the significance (Adonis $P$, MRPP $\delta$) of covariates (sampling date, farming, field, sampled material) and to estimate their relevance as proportion of explained community dissimilarities (Adonis $R^2$, MRPP $A$ ($A$: chance corrected within group agreement)).
**2.2.3 Indoor microbiomes and health: prospects from further analyses (eIII)**

*Extended analysis on the data presented in study III*

Study III’s set of microbial fingerprints with its complementary health data was used to show associations between the microbial community variation (Bray—Curtis community dissimilarity) in indoor dusts and the later health outcome in children that were exposed to these dust communities. The route of analysis was identical to the presentation given in IV for environmental parameters, except that only the Adonis test was used for evaluation of significances. In addition to III, data on the total IgE levels in the children’s blood at six and ten years of age, asthma at six and ten years of age, and data on skin eczema at one year were analysed.

*Analysis of additional data on white blood cell measures*

The LISAplus database provided information on children’s white blood cell (leucocyte) subpopulations for a random subset. This subset overlapped with the subset of study III in 22 dust samples. For these 22 samples, 29 variables (table 1, chapter 3.2.2) had been measured at the respective cohort children’s two-year check-up and these data were not incorporated in III. The measurements and their preparations had been conducted by LISAplus investigators and are described in detail by Tischer et al. (2015). Briefly, whole blood samples of peripheral blood had been chemically stimulated to produce cytokines, fixated, tagged with fluorescence-labelled monoclonal antibodies against lymphocyte surface markers (cluster of differentiation 3 (CD3), CD4, CD8, CD16, CD19, CD25, CD45RA, and CD45RO) as well as human cytokines (interferon γ (IFNγ), tumour necrosis factor α (TNFα), interleukin 2 (IL2), and interleukin 4 (IL4)) and analysed by flow cytometry. Results were obtained from LISAplus as percentages of cytokine-producing cells of the respective cell population.
The first steps to analyse relationships between leucocyte measures in the children’s blood at two years of age and the microbial dust communities in their homes after birth were performed as reported in study IV for environmental parameters. Briefly, Bray—Curtis dissimilarity matrices and diversity indices were each calculated from $10^3$ times randomly rarefied tRFLP data and averaged. Significances of associations between each leucocyte measure and community dissimilarities for fungi and bacteria were tested using Adonis.

With regard to the sample-size of 22 and the continuous nature of the variables, the further analysis steps differed from studies IV. To allow visual interpretations on the nature of significant (Adonis $P < 0.05$) relationships found between community dissimilarities and leucocyte measures, principal coordinate analyses (PCoA, implemented in vegan) were done for the fungal and bacterial dissimilarity matrices in a first step. Then, the significant leucocyte measures were plotted against the $1^{st}$ principal coordinate (PCo1) (representing the major part of dissimilarity between the samples) of the PCoAs. In a last step, linear regressions and respective Pearson correlations were calculated for each plot using the function cor.test implemented in R; non-normality of the data in Shapiro—Wilk tests was disregarded in accordance with McDonald (2014). Monotonous correlations between diversity indices (Simpson, Shannon, and Pielou evenness) of the samples and children’s leucocyte measures were analysed with Spearman correlation tests using the cor.test function.
3 RESULTS

3.1 Overview of the results presented in the articles

3.1.1 Volatile organic compounds in *Alternaria alternata* and *Fusarium oxysporum* (I)

The tested fungi differed greatly in quantity and quality of their VOC emissions. *A. alternata*'s sesquiterpene production was much higher under nutrient rich compared to nutrient poor conditions. Nutrient poor culture conditions resulted in reduced but similar emission profiles compared to nutrient rich conditions for both fungi. Emissions did not stop during the time-course of four weeks (five weeks for nutrient poor conditions) but declined strongly (calculated to mycelia surface areas) from the first measurement on (i.e. three days for nutrient rich conditions and 14 days for nutrient poor conditions). The different co-cultivation setups did not provoke strongly shifted emission profiles.

At nutrient rich conditions, *A. alternata* and *F. oxysporum* showed equal growth behaviour as solitary cultures and in co-cultivation, while *F. oxysporum* grew faster than *A. alternata* at nutrient poor conditions. On co-cultivation setups, *A. alternata* produced less VOC per mycelium surface area than as solitary culture, which suggested VOC mediated interactions between *A. alternata* and *F. oxysporum*.

PCA and OPLS analyses could differentiate between solitary cultures of both fungi and the emission of co-cultivations. Taking all high nutrient setups and sampling time-points into account, emissions of most compounds were correlated to each other (Spearman correlation matrices).

3.1.2 Targeting allergenic fungi in agricultural systems (II)

The MSQPCR procedure could be adapted for agricultural samples. Accuracy tests showed matching results for the $E^{-\Delta CT}$ and $E^{-\Delta\Delta CT}$ calculation methods.

An extensive post-harvest time series indicated plant materials as major sources for the
allergenic fungi *A. alternata* and *C. cladosporioides* in agricultural environments during that time, i.e. from autumn to spring, but it also indicated that the bulk soil still often contained more than $10^3$ conidial equivalents (CE) of *C. cladosporioides* per gram of dry material in that period. *Trichoderma viride/atroviride/koningii* was detected in a patchy pattern, mostly in low amounts. Amounts of *F. culmorum* increased in the order bulk soil < root-soil congeries < decaying roots < decaying shoots.

The plot based pre-harvest experiment found very high amounts (> $10^6$ CE g$^{-1}$) of *A. alternata* and *C. cladosporioides* on wheat and rye ripe for harvest, and additionally significantly (ANOVA $P < 0.001$) higher levels (approximately 10-fold) of *A. alternata* and *C. cladosporioides* on organic plots of wheat compared with conventional plots. An increase in fungal numbers from lower stem parts of the plant to the seed heads was also found for *A. alternata* and *C. cladosporioides*. The *Trichoderma* group and *F. culmorum* were found in low to medium numbers (maxima in the order of $10^4$ CE g$^{-1}$), with a patchy pattern and preferentially at the lower parts of the plants.

A final experiment using spot-samples of different mature crops (wheat, rye, barley, oat, field bean and pea) at harvest found similar or higher numbers of the tested fungi than in the plot-based experiment.

### 3.1.3 Microbial communities in house dust: Impact on infants’ health development (III)

A higher exposure to fungal diversity (Simpson index) at birth was associated with a significantly reduced risk for sensitization to inhalant allergens at 6 years, but not at 10 years and not in the longitudinal view (GEE models) until the age of 10 years. A higher fungal diversity was also inversely associated with ever wheezing until the age of 10 years. No significant association was found between exposure to bacterial diversity and any of the health outcomes tested.
3.1.4 Determinants of indoor microbial communities (IV)

In comparison to bacterial community variation, more environmental determinants were significantly (Adonis $P$ and MRPP $\delta \leq 0.05$) associated with the fungal community variation. These included: mould at home, tightness of the windows, central heating, type of living room floor, age of the building, vegetation level in 100 m around the home, degree of urbanisation, particulate matter concentrations ($< 2.5 \mu m$ and coarse particulates) and sampling times during the year. Only the ventilation behaviour (wintertime) was significantly associated with bacterial community variation.

The diversity of uncommon fungal groups was significantly associated with the ventilation behaviour (Shannon index, Kruskal—Wallis $P = 0.04$), while the diversity of common fungal groups was significantly associated with the vegetation level in a 100 m range around the home (Simpson Index, Kruskal—Wallis $P = 0.03$). The bacterial diversity was associated with the type of living room floor and the position of the home (level above ground) (Shannon and Simpson Index, Kruskal—Wallis $P \leq 0.04$).

It was possible to obtain chronological trends from an epidemiological sampling scheme, i.e. spatially and temporally distributed samples. The variation between the dust communities of different homes was not spatially correlated.

An estimation of the ecological relevance of the sampling time on community variation showed best results for a categorisation based on phenological periods. Conservative estimates (MRPP chance-corrected within group agreement: $A$) suggest a tenth of the fungal community variation and a fifth of the bacterial community variation between the dust samples to be caused by the phenological change in the outdoor environment.

In the overview of all dust samples, fungal community variation changed from a winter to a summer state and vice versa, while fungal diversity indices only slightly lowered in late
summer, compared to the rest of the year. Bacterial community variation experienced a thorough change in the phenological periods of early and full spring and gradually returned to its winter state during the year. This trend was mirrored in bacterial diversity in contrast to observations for fungal diversity.

3.2 Results of the extended projects

3.2.1 Allergenic fungi in agriculture approached from the culture-dish (eII)

425 inoculations (89% of all attempts) showed fungal growth. From these, 334 pure cultures were obtained and a further 142 cultures could not be purified. This share of mixed cultures was roughly similar between the sampling time-points (23% – 32%), while the portion of unsuccessful isolation attempts varied more between the time-points (19/09/11: 10%, 25/09/11: 24%, 27/03/12: 0%).

For the pure cultures, combined identification based on microscopy and marker-genes allowed classification of the isolates into distinct groups at different taxonomic levels (taxa in Fig. 2). The majority of all pure cultures belonged to Ascomycota (95%) while Basidiomycota (3%) and Zygomycota (2%) were represented in low numbers. Most isolates within the Ascomycota belonged to the orders Hypocreales (43%) and Pleosporales (34%). The overall share of the highly allergenic genera *Alternaria* (a subgroup of Pleosporales) and *Cladosporium* on all pure cultures was only 4% each. 34% of all purified isolates were *Acremonium, Trichoderma* or Nectriaceae (incl. *Fusarium*), which have been described in the literature as occasional causatives of allergies (cf. Simon Nobbe et al. 2008). No pure culture belonged to the indoor allergenic group encompassing *Aspergillus, Penicillium* and *Paecilomyces*, though their unequivocal identification was possible through both, microscopy and ITS marker genes (cf. Samson et al. 2010).

The frequency of identified taxonomic groups is shown in Fig. 2 for different main
parameters of the sampling design (a, b, c) and the isolation media (d). Multivariate analyses on differences between communities (frequency of each identified taxonomic group, mixed cultures and failed isolation attempts) using the Adonis function revealed no significant difference between the communities isolated from shoots and roots (P = 0.189). The best-fitted Adonis model showed significant influences for the sampling date ($R^2 = 0.033, P < 0.0001$), the sampled field ($R^2 = 0.033, P < 0.0001$) and the interactions date × field ($R^2 = 0.048, P < 0.0001$), field × material ($R^2 = 0.025, P = 0.0006$), date × field × material ($R^2 = 0.036, P = 0.016$). Another fitted model in which the spatial factor ‘individual field’ was exchanged by the spatial factor ‘general field type’ (i.e. conventional vs. organic farm management) showed a significant influence of the farm management ($P = 0.005$). However, the ecological relevance ($R^2 = 0.008$) was estimated much lower compared with an analysis based on individual fields, and no significant interactions with the sample material were found when using ‘farming management’ as influence factor in the Adonis model. All significant differences in communities were reconfirmed by results of the MRPP procedure (sampling date: $A = 0.029, \delta < 0.0001$; field: $A = 0.02, \delta < 0.0001$; farm management: $A = 0.006, \delta < 0.0001$; material: $A = 0.0007, \delta = 0.186$). As expected, the two isolation-media, DG18 and SEA, were found to differ significantly in their isolation spectrum of fungi (Adonis: $R^2 = 0.009, P < 0.0001$; MRPP: $A = 0.007, \delta < 0.0001$). However, most of the differences were caused by a bigger number of pure cultures obtained for many taxonomical groups on DG18, compared to SEA (Fig. 2d).
Figure 2 Numbers of identified isolates from project eII shown with different separation parameters (a, b, c, d). Taxa written in black: Ascomycota, taxa in grey: Basidiomycota, taxa in light grey: Zygomycota. Taxonomic representation is simplified with composite groups: ‘Basidiomycota other’ = Cantharellales (1x), Tremellales [Cryptococcus] (1x), not determined (2x); ‘Ascomycota other = incertae sedis [Minimidoichium] (1x), Eurotiales (1x), Magnaporthales [Gaeumannomyces] (1x), Microascales [Trichurus] (1x), undetermined (1x); ‘Xylariales’ = Monographella; n.d. = not determined.
3.2.2 Indoor microbiomes and health: prospects from further analyses (eIII)

Extended analysis on the data presented in study III

Current asthma at six years of age and skin eczema at one year were associated with a significant change in fungal community variation in the respective dusts at the children’s early infancy (Adonis $P = 0.032$ and $P = 0.004$, respectively) but no relationship between fungal community variation and wheezing or IgE-levels of the children later in childhood were observed (Table 1). Differences in the bacterial community variation among the dust samples at early infancy were significantly associated with total IgE levels when the respective children were six years old (Adonis $P = 0.017$), but not with any of the other health outcomes tested (Table 1).

Table 1 Significance of associations between microbiome community dissimilarity and health outcomes (Adonis test). Bold values: Adonis $P < 0.05$.

<table>
<thead>
<tr>
<th>atopic health outcomes</th>
<th>Fungi</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P$</td>
<td>$R^2$</td>
</tr>
<tr>
<td>Current asthma at 6 years</td>
<td>0.032</td>
<td>0.020</td>
</tr>
<tr>
<td>Current asthma at 10 years</td>
<td>0.778</td>
<td>0.002</td>
</tr>
<tr>
<td>Ever wheezing until 10 years</td>
<td>0.291</td>
<td>0.007</td>
</tr>
<tr>
<td>Eczema at 1 year</td>
<td>0.004</td>
<td>0.035</td>
</tr>
<tr>
<td>IgE at 6 years: total levels</td>
<td>0.373</td>
<td>0.018</td>
</tr>
<tr>
<td>IgE at 6 years: inhalant allergens</td>
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<td>0.008</td>
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<td>IgE at 10 years: total levels</td>
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<tr>
<td>IgE at 10 years: inhalant allergens</td>
<td>0.901</td>
<td>0.002</td>
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</tbody>
</table>

Analysis of additional data on white blood cell measures

Significant (Adonis $P < 0.05$) associations between community dissimilarity of household dusts and exposed (i.e. living in the respective houses) children’s white blood cell measures at the age of two years were found for six different measures with fungal communities and for two different measures with bacterial communities (Table 2). Significant linear correlation coefficients (Pearson $P < 0.05$) were detected for fungi between a major part of community dissimilarity (PCo 1) and leucocyte measures that also showed significant Adonis results (Fig. 3) but not for bacteria (data not shown). Significant monotonous correlations (Spearman $P <
between diversity and the selected leucocyte measures were only detected for fungi (Table 3).

Table 2 Significance of associations between microbial community dissimilarity and measures of leucocytes (Adonis test). CD19+: expressing cluster of differentiation 19; HLA-DR+ (human leucocyte antigen DR): expressing MHC (major histocompatibility complex) class II cell surface receptor (human leucocyte antigen DR); IL4+, IL2+, IFNγ+, TNFa+: expressing receptors for interleukin 4, interleukin 2, interferon γ, or tumor necrosis factor α; CD3-: not expressing CD3; bold values: P (Adonis) < 0.05.

<table>
<thead>
<tr>
<th>Leucocyte measure</th>
<th>Fungi</th>
<th>Bacteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>leucocytes / µl [blood]</td>
<td>0.284</td>
<td>0.056</td>
</tr>
<tr>
<td>lymphocytes / µl [blood]</td>
<td>0.006</td>
<td>0.189</td>
</tr>
<tr>
<td>share of sub-populations on leucocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>granulocytes</td>
<td>0.048</td>
<td>0.121</td>
</tr>
<tr>
<td>monocytes</td>
<td>0.033</td>
<td>0.127</td>
</tr>
<tr>
<td>lymphocytes</td>
<td>0.018</td>
<td>0.153</td>
</tr>
<tr>
<td>share of sub-populations on lymphocytes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B lymphocytes (CD19+)</td>
<td>0.151</td>
<td>0.079</td>
</tr>
<tr>
<td>natural killer cells (CD16+ CD56+)</td>
<td>0.159</td>
<td>0.077</td>
</tr>
<tr>
<td>T lymphocyte (CD3+ HLA-DR+)</td>
<td>0.105</td>
<td>0.091</td>
</tr>
<tr>
<td>cytotoxic T cell (CD3+ CD8+)</td>
<td>0.629</td>
<td>0.030</td>
</tr>
<tr>
<td>T lymphocytes (CD3+)</td>
<td>0.656</td>
<td>0.028</td>
</tr>
<tr>
<td>T helper cell (CD3+ CD4+)</td>
<td>0.738</td>
<td>0.024</td>
</tr>
<tr>
<td>activated T lymphocyte (CD25+ CD3+)</td>
<td>0.132</td>
<td>0.082</td>
</tr>
<tr>
<td>share of sub-populations on T lymphocytes (CD3+)</td>
<td>0.953</td>
<td>0.015</td>
</tr>
<tr>
<td>IL4+</td>
<td>0.547</td>
<td>0.034</td>
</tr>
<tr>
<td>IL2+</td>
<td>0.788</td>
<td>0.022</td>
</tr>
<tr>
<td>IFNγ+</td>
<td>0.802</td>
<td>0.021</td>
</tr>
<tr>
<td>TNFa+</td>
<td>0.938</td>
<td>0.014</td>
</tr>
<tr>
<td>share of sub-populations on cytotoxic T cells (CD3+ CD8+)</td>
<td>0.022</td>
<td>0.145</td>
</tr>
<tr>
<td>IL4+</td>
<td>0.649</td>
<td>0.029</td>
</tr>
<tr>
<td>IL2+</td>
<td>0.942</td>
<td>0.015</td>
</tr>
<tr>
<td>IFNγ+</td>
<td>0.600</td>
<td>0.031</td>
</tr>
<tr>
<td>TNFa+</td>
<td>0.224</td>
<td>0.065</td>
</tr>
<tr>
<td>share of sub-populations on T helper cells (CD3+ CD4+)</td>
<td>0.446</td>
<td>0.042</td>
</tr>
<tr>
<td>IL4+</td>
<td>0.487</td>
<td>0.038</td>
</tr>
<tr>
<td>IL2+</td>
<td>0.253</td>
<td>0.060</td>
</tr>
<tr>
<td>IFNγ+</td>
<td>0.063</td>
<td>0.108</td>
</tr>
<tr>
<td>TNFa+</td>
<td>0.169</td>
<td>0.075</td>
</tr>
<tr>
<td>share of CD25+ lymphocytes on CD3- lymphocytes</td>
<td>0.169</td>
<td>0.075</td>
</tr>
<tr>
<td>share of TNFa+ cells on non T cells</td>
<td>0.169</td>
<td>0.075</td>
</tr>
<tr>
<td>share of IFNγ+ cells on non T cells</td>
<td>0.032</td>
<td>0.134</td>
</tr>
<tr>
<td>CD3+ CD4+ / CD3+ CD8+ cells</td>
<td>0.945</td>
<td>0.014</td>
</tr>
<tr>
<td>CD3+ CD45RA+ (T naive) / CD3+ CD45RO+ (T memory) cells</td>
<td>0.135</td>
<td>0.082</td>
</tr>
</tbody>
</table>
Figure 3 The fungal community in dust in correlation to infants’ leucocyte measures. Different measures of leucocytes in the blood of two-year-old infants are plotted against the main part of community dissimilarity between the fungal communities in dust of the children’s homes three months after birth. Leucocytes measures used in this analysis were selected for their significance in Adonis tests ($P < 0.05$) (Table 2). PCo1: $1^{st}$ principal coordinate of a PCoA done on a averaged Bray—Curtis community dissimilarity matrix built from tRFLP results, $r^2$: squared value of Pearson correlation coefficient $r$, $P$: significance level for the true correlation being not equal to 0, line: linear regression with 95 % confidence interval.

Table 3 Monotonous correlations between indices of fungal diversity and selected measures of white blood cells. $\rho$: Spearman correlation coefficient, $P$: significance level for the true correlation being not equal to 0, bold values: $P$ (Spearman) $< 0.05$.

<table>
<thead>
<tr>
<th>white blood cell measures</th>
<th>Simpson index (1-D) $\rho$</th>
<th>$P$</th>
<th>Shannon index ($H'$) $\rho$</th>
<th>$P$</th>
<th>Pielou evenness (E) $\rho$</th>
<th>$P$</th>
<th>$P$</th>
</tr>
</thead>
<tbody>
<tr>
<td>leucocytes / µl [blood]</td>
<td>0.306</td>
<td>-0.23</td>
<td>0.228</td>
<td>-0.27</td>
<td>0.204</td>
<td>-0.28</td>
<td></td>
</tr>
<tr>
<td>lymphocytes / µl [blood]</td>
<td>0.007</td>
<td>-0.57</td>
<td>0.004</td>
<td>-0.59</td>
<td>0.003</td>
<td>-0.61</td>
<td></td>
</tr>
<tr>
<td>share of sub-populations on leucocytes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>granulocytes</td>
<td>0.002</td>
<td>0.63</td>
<td>0.003</td>
<td>0.61</td>
<td>$&lt;0.001$</td>
<td>0.66</td>
<td></td>
</tr>
<tr>
<td>monocytes</td>
<td>0.018</td>
<td>0.50</td>
<td>0.011</td>
<td>0.53</td>
<td>0.035</td>
<td>0.45</td>
<td></td>
</tr>
<tr>
<td>lymphocytes</td>
<td>$&lt;0.001$</td>
<td>-0.69</td>
<td>$&lt;0.001$</td>
<td>-0.69</td>
<td>$&lt;0.001$</td>
<td>-0.72</td>
<td></td>
</tr>
<tr>
<td>share of IL4+ cells on cytotoxic T cells</td>
<td>$&lt;0.001$</td>
<td>0.76</td>
<td>$&lt;0.001$</td>
<td>0.69</td>
<td>$&lt;0.001$</td>
<td>0.73</td>
<td></td>
</tr>
<tr>
<td>share of IFNy+ cells on non T cells</td>
<td>0.008</td>
<td>-0.56</td>
<td>0.017</td>
<td>-0.51</td>
<td>0.037</td>
<td>-0.45</td>
<td></td>
</tr>
</tbody>
</table>
4 DISCUSSION

This thesis approached three health concerns mediated by fungi. In the first part of the discussion, a review is provided on insights from the extended projects and their potential merit for the design of future studies (4.1). The general discussion on the three health concerns, based on the articles I–IV, is given in the following chapters (4.2–4.6).

4.1 Extended projects beyond the articles (eII and eIII)

Extended project eII: outdoor allergenic fungi in agriculture

Project eII demonstrates the limitations of a cultivation based quantification approach for a very basic source monitoring of allergenic fungi. It suffered the same limitations (e.g. identification issues, cultivation bias, only living organisms detectable, long incubation times) as indoor cultivation based mould determinations (Vesper 2011).

Allergenic fungi were only a minor fraction of all isolates. Such results were conceivable during preparation of the study since fungal diversity studies in litter (Poll et al. 2010, Sadaka and Ponge 2003) demonstrated that only minor shares of fungal isolates belonged to highly allergenic genera (such as Aspergillus, Penicillium, Cladosporium, Alternaria, Epicoccum; cf. Simon-Nobbe et al. 2008). Consequently, the experiment included a long-term storage of the isolates to allow later usage of its strain collection. In future studies, these isolates could be used, e.g. for a VOC experiment on differences in sesquiterpene emissions from strains of Alternaria, Cladosporium, and other fungal strains isolated from the same environments, as such comparative data-sets are still missing in the literature.

The results of eII suggest that a cultivation technique as solitary tool for a reliable source monitoring of allergenic fungi or for revealing relevant source materials is unrealistic due to lack of accuracy (cultivation bias and small share of allergenic fungi on total isolates) and cost of time and personal resources (isolation, identification, and culture maintenance).
Nevertheless, eII revealed unexpected insights. First, DG18 can be used as replacement for soil extract medium, which requires filtered and sterilized soil extracts and is therefore laborious to make. While soil extract medium is used to provide organisms with conditions that are at least vaguely similar to those found in the soil they originate from, DG18 is a standard medium to isolate fungi from indoor environments (Samson et al. 2010). DG18 features a lowered water activity \( (a_w = 0.95, \text{ a result of 18\% glycerol}) \) compared to most other media \( (a_w \approx 0.999 \text{ to } 0.997) \) and dichloran (2,6-dichlor-4-nitroaniline), which constrains hyphal extension of many Zygomycota to prevent overgrowth of plates (Hocking and Pitt, 1980). However, the isolation medium was the least important among the variables tested for their influence on differences in cultivated isolates (based on Adonis \( R^2 \) and MRPP \( A \)). The strongest differences between the isolation profiles of DG18 compared to SEA were the absence or near-absence of Acremonium, Cladosporium and Mucorales on the latter medium. This suggests that DG18 is a suitable alternative to SEA for the isolation of many typical soil fungi such as Nectriaceae, Trichoderma, or Pleosporales with the benefit of an easier handling than soil extract medium. Additionally, DG18 seems to allow a better isolation ratio of fungi like Acremonium or Cladosporium from soil environments than SEA.

A second important insight was that a multivariate approach done with qPCR based monitoring (Supplementary Material of eII) produced outcomes alike the multivariate statistical analysis on the dissimilarity between different conditions of all isolates (sampling time-point, farming management, type of plant material, individual field). Both, qPCR for allergenic fungi and cultivation results indicate that the influence of sampling time-point and individual field conditions on the difference in occurrence between groups were higher than the influence of farming management. However, the conditions tested for the cultivation experiments accounted for less of the observed dissimilarity between groups (approximately 10 times lower \( R^2 \) values in the Adonis test) compared to the qPCR tests on allergenic fungi,
in which sampling time-point and individual field conditions together explained more than half of the observed dissimilarities in allergenic fungi between samples (Supplementary Material of II).

*Extended project eIII: prospects from further analyses on indoor microbiomes and health*

Study III presents its message in a very concise way that did not permit including tests on the relationship between community variation and health outcomes. Community variation has been calculated from pairwise differences in the communities of the samples (cf. IV). Its statistical analysis could therefore detect changes that are not resulting in changes of diversity indices. Nevertheless, results of significance tests on community variation are difficult to interpret when they are not supported by taxonomic information (changes cannot be addressed with an unambiguous direction as opposed to the “up” and “down” of diversity indices) and were therefore not analysed in study III.

In the extended analysis, the multivariate tests for community variation of the 189 samples from study III showed significant associations between the fungal microbiome and health outcomes for children asthma development in children at six years of age and eczema development in infants, as well as between the bacterial microbiome and elevated total IgE at six years. Although no microbial taxa were determined due to the fingerprinting method used here, its results pose excellent selectors for further studies. For example, sequencing projects could focus on revealing the identities of microbes in just that subset of samples that were responsible for the significant association between fungal community variation and asthma development. A simple pre-test on the tRFLP data (ANOVA testing per OTU, data not shown) already indicated that only a few (< 10) OTUs were responsible for this association. This suggests that a future sequencing project may be able to identify marker-taxa for asthma development as has been shown recently by Arrieta et al. (2015) and others (cf. chapter 4.5).
The small number of samples (22) that could be analysed regarding a relationship between leucocyte subpopulations of two-year-old children and their surrounding dust microbiomes after birth requires a modest interpretation of the results. The linear regression with Pearson correlation in Fig. 3 served the visualisation of significant Adonis results, i.e. it was used as an auxiliary method to illustrate the shift in community dissimilarity, which a significant Adonis test result (Table 2) represents. It is likely that an in depth analysis of a larger number of samples would find that different modes of correlation describe the relation between some leucocyte measures and the dust microbiome better than linear correlation, e.g. for the share of monocytes on leucocytes. Still, the Adonis results themselves were calculated from a Bray—Curtis dissimilarity matrix meaning that the change in OTU abundances of each sample to each other sample were included in the test. This makes it highly probable that a similar relationship between changes in community variation and leucocyte measures will be observed when a higher number of samples is analysed in the same way, provided the randomly selected 22 dust samples that were used here were not made up mostly of outliers. For that in turn, no indication was found because the samples were dispersed evenly in a principal coordinate analyses (PCo 1 shown in Fig. 3).

Although the analysis of a small sample size indicated an association between levels of leucocytes in young children and their microbial surroundings, a larger sample size is required to confirm this observation. A bigger study on the topic should additionally provide information about individual microbial taxa, as many conditions can result in increases or decreases of blood cell populations (Denning et al. 2014). A more detailed microbial data set could allow a better elucidation of the highly complex interaction between immunological parameters and microbiomes (Okada et al. 2010). Therefore, this analysis on 22 dust samples and their meta-data can be seen as outlook on an epidemiological study setup that could
provide valuable information on potential immunological mechanisms behind the hygiene hypothesis (cf. 4.5).

**4.2 New results accentuate a knowledge gap in VOC research (I)**

The results communicated in study I clearly show why and where more fundamental and descriptive research about VOC is needed. Recent studies found unknown sesquiterpene emissions from well investigated fungi that had not been previously detected. This is probably due to earlier studies having used less efficient methodological approaches (I, Hung et al. 2015), indicating that basic assumptions might be overturned just by sampling with more sensitive techniques. For example, it is currently supposed that bacteria emit terpenes more frequently than fungi (Penuelas et al. 2014). This postulation has been built on the fact that just 2% of the compounds described to be emitted by fungi were terpenes while for bacteria it was 17% (data extracted from mVOC database by Penuelas et al. 2014). In absolute values, this data was gathered from 349 bacterial and only 69 fungal species that had until then been investigated (Lemfack et al., 2014). However, it should be taken into account that early investigations might have overlooked whole compound classes like sesquiterpenes and that there are uncounted species of fungi (Blackwell 2011) (and also bacteria, e.g. Pedrós-Alió 2012) which could vary in VOC capabilities from strain to strain (Demyttenaere et al. 2004). It is therefore evident that what is known about fungal VOC production so far is just some pieces of the whole puzzle. Nevertheless, without having solid information about ‘what is out there’, it is hard to answer advanced questions such as ‘why is it there’ and ‘how does it interact’ (based on the definition of ecology in Townsend et al. 2002). Naturally, the same applies to the properties of sesquiterpene emissions that have been found up to now, as can be seen on the early onset of sesquiterpene emissions for the fungi tested in study I. This result could not be expected from earlier studies, as sesquiterpene production was thought to be a
feature of aging cultures and secondary metabolism (Kramer and Abraham 2012).

Generally speaking, the fungal ‘volatome’ (cf. Heddergott et al. 2014) needs to be described better before the ecological role of VOCs can be explored in full depth and environmental health applications of VOCs can show their full potential. This does not imply that every fungus needs a measurement-based characterisation of its volatome. Concerning the various sesquiterpenes, future reports on the emission properties of cloned sesquiterpene synthases (SQSs) can help to complete the picture, as has been shown for putative SQSs of the basidiomycete *Coprinus cinereus* (Agger et al., 2009). However, this auxiliary approach is still hampered at the moment, because only few fungal SQS have been functionally characterised until now (Quin et al. 2014) and the enzymatic mechanisms leading to individual sesquiterpenes are only partly understood (Miller and Allemann 2012).

Nonetheless, a lack of basic descriptive data does not infer that ecological and applied studies should be postponed until the dimensions of the fungal volatome are known comprehensively. Recent works on the info-chemical potential of some compound classes like sesquiterpenes (cf. Kramer and Abraham, 2012, Schmidt et al., 2015) demonstrate the reasons why the fungal volatome needs to be analysed thoroughly. Descriptive and explanatory research needs to go hand in hand, but at the moment the knowledge base in VOC research seems too overstrained as to support all interpretations that have been made. As further example, sets of VOCs have already been suggested as indicators of indoor fungal growth (e.g. specifically C8 compounds by Ryan and Beucham (2013), or different compounds in earlier works, cf. Kuske et al. (2005)). The value of such efforts will strongly increase when the range of possible emitters for a compound is better known. This likewise applies to results of study I. While the sesquiterpene profiles from *A. alternata* and *F. oxysporum* are technically very good biomarkers, not much is known about the phylogenetic level (e.g. pathotype, species, genus) on which it is sensible to use them, since comparable studies are lacking.
4.3 Managing fungal outdoor allergies (II)

Fungal allergy is a widespread condition with a sometimes demanding diagnosis (1.3). Its prevalence illustrates the need for a management of fungal allergies by treatment or with allergen avoidance strategies.

Apart from short term suppression of allergic symptoms (pharmacotherapy), for example with steroids and antihistamines, treatments of fungal allergy concern immunotherapy with allergens or peptide-structures derived from them (Twaroch et al. 2015). In immunotherapy, allergen extracts are administered to a patient in gradually increasing doses to ameliorate symptoms with later exposures to that allergen (Bousquet et al. 1998). Allergen extracts used to that goal are often referred to as therapeutic vaccines with ‘vaccines’ defined as immune modifiers that influence varying immunologic mechanisms for different allergens (Bousquet et al. 1998). Twaroch et al. 2015 analysed results of studies that tested immunotherapy approaches for *Alternaria* and *Cladosporium* allergies and concluded that immunotherapy can currently not be recommended for patients with mould allergies. The main reasons for their evaluation were that relevant recombinant allergens were not available and that natural allergen extracts caused side-effects and were additionally of poor quality (which mirrors the issues with diagnosis detailed in 1.3.1). Similar conclusions were drawn repeatedly in the last years (Cramer et al. 2014, Simon-Nobbe et al. 2008).

As with all forms of allergy, avoiding exposure is still the best treatment (Cramer et al. 2014). Additionally, with immunotherapy being out of reach at the moment, avoidance of exposure to fungal allergens is the only symptom prevention strategy that can be used on a day to day basis. This approach is used for indoor mould allergy by eliminating sources (e.g. infected walls) and is also effective when only visible mould is eliminated (Burr et al. 2007). However, exposure to outdoor fungi cannot be completely avoided as they are a ubiquitous part of our outdoor airborne environment (Cramer et al. 2014). Nevertheless, the life quality
of patients could be improved considerably, if they were able to avoid fungal outdoor allergen exposition with minimal constraints in outdoor activities. To that goal, a day by day fungal monitoring in a form similar to modern pollen monitoring is desirable. However, that is too costly to be done reliably in routine analysis for several reasons. Spores of many species are morphologically nearly indistinguishable (Vesper 2011) and fragments of fungal matter (e.g. hyphae) are completely non-relatable by morphology but may be of much higher importance than spores themselves (Reponen et al. 2007, Green et al. 2005, Górny 2002). In addition, spore release can occur event based on various spatiotemporal scales (thunderstorms, appropriate weather conditions, farming actions, cf. II). As described and exemplified in study II, source monitorings maintained for certain periods of time and on different locations can help to gather data that describe the potential of an environment to release fungal allergens. With enough data, this may serve as useful substitute of a direct airborne monitoring, in the same way a person with hazel pollen allergy does not need to know the latest forecast on hazel pollen as long as he or she knows when hazels flower and where to keep mind of hazel trees. II mainly provides a technique and gives an outlook to actually presenting fungal allergenic potentials. Yet, some results are strong enough to give cautious predictions for allergic persons. For example, a mere stopover next to a cereal field ready for harvest may provoke symptoms in patients allergic to C. cladosporioides as only a gentle breeze of air from the field could transport enough allergenic fragments to exceed typical thresholds, because general atmospheric levels are often already around the threshold level during summer (Twaroch et al. 2015) (more than 10⁷ conidia equivalents g⁻¹ cereal material were found at harvest (II) and approximately 1000–3000 spores m⁻³ air are needed to provoke symptoms for C. cladosporioides (Gravesen 1979, Twaroch et al. 2015)).

However, the results presented in II should not be interpreted in a too general way. To avoid incorrect (public) interpretations on the differences found in allergenic fungi between organic
and conventional management, II explained that the farming management is only one factor out of a group of factors (tillage system, fertilizer, etc.) that also can include adverse approaches within one farming management depending on individual field conditions. For example, a farmer might handle soil erosion on a field with steep slope with strip tillage systems (e.g. Nowatzki et al. 2011) while on a field with a less steep slope he might decide to counter erosion with contour ploughing (Van Oost et al. 2006). For any generalized statement on differences in the presence of allergenic fungi between farming managements, statistically relevant numbers of agricultural enterprises (i.e. individual ways of practices) have to be monitored. The possibility that the actual importance of the farming management for development of allergenic fungi could be of lesser importance is given in the supplementary material of II. Therein, a multivariate analysis (Adonis) on the measured differences in allergenic fungi could explain a much bigger share of all differences when the explanatory spatial variable ‘individual field’ was used instead of the spatial variable ‘farming management’ ($R^2 = 0.39$ compared to $R^2 = 0.05$). Other factors could be comparatively more important than farming management for future studies on allergenic sources and for estimating the risk of allergens coming in contact with the human immune system. II showed that high levels of *A. alternata* and *C. cladosporioides* could be a common side effect of agriculture with cereals and other crops like legumes, which would also help explaining the high number of these fungi found in the atmosphere during summer (Fernández-Rodriguez et al. 2014, Twaroch et al. 2015), if affirmed by other studies. While agricultural impact related to water pollution by fertilisers and pesticides or to atmospheric pollution by greenhouse gas emissions have received relatively much attention, solutions could also be found to reduce the amount of allergenic material released into the air. Such strategies of allergen avoidance could possibly be simple, for example equipping harvesters near settlements in a way that releases less of the relevant fragments and propagules into the air to flatten the highest annual peaks in
airborne fungal allergens, or using farming techniques that could slow down growth of
relevant (epiphytic) fungi (*Alternaria*, *Cladosporium*) like ploughing of cover-crops in late
autumn instead of spring (as long as they were planted only to improve nutrient quality). But
such solutions remain speculative until the completion of more research on fungal outdoor
allergies. The interest of the public should be given with the worldwide rising incidence of
allergy (Pawankar et al. 2011).

4.4 Indoor dust communities represent neighbouring microbiomes (III and IV)

The results on determinants of indoor microbiomes given in IV showed that the composition
of indoor microbial communities was strongly influenced by the outdoor surroundings and
building characteristics, with these having a more fundamental effect on the fungal
composition than the bacterial. Another potential major determinant, briefly touched in the
discussion of IV, are the inhabitants themselves, for instance as carriers of their skin
microbiomes (e.g. Lax et al. 2014). Such contributions from different sources (IV) also stress
that indoor dust communities are to a large degree a passive assemblage, which has
constrained microbial growth due to the low water activity (Nevalainen et al. 2015, Samson et
al. 2010). Indoor microbiomes including those of dusts are a major research focus at the
moment (1.4). The reason for this attention is that humans nowadays spend most of their life
indoors and indoor (dust) microbiomes are one of the major sources for microbes we are
exposed to. In fact, indoor biomes already cover a substantial part of earth’s terrestrial area as
documented by the NESCent working group on the evolutionary biology of the built
environment (NESCent et al. 2015). Additionally, the passiveness of indoor dust communities
makes them a time-integrated alternative for airborne sampling (Nevalainen et al. 2015).
Passiveness here implies that dust microbial communities are metabolically not very active.
Nevertheless, they are still constantly changing in their composition and, for instance, show a
seasonal course (e.g. IV). Overall, indoor dust communities reflect the state of other biomes. Knowing which factors influence indoor dusts will in turn allow future studies to infer information on these factors from only having data on dusts. For example, a number of bacterial strains from house dusts have been associated with dog ownership (Kettleson et al. 2015), which could make it possible in future to infer dog ownership from dust samples alone. To allow such conclusions, information on particular microbial species is necessary, which was not obtained by the molecular technique used in studies III and IV. When those studies were planned, the decision was made to use the tRFLP method as it was a reproducible technique (Prakash et al. 2015, Pilloni et al. 2012) with a good technical and economical ratio between analysis effort for 300 samples and achieved result detail (Prakash et al. 2015, Camarinha-Silva et al. 2012). However, in recent years, next generation sequencing (NGS) techniques became more simple and affordable (van Dijk et al. 2014). New studies on the matter of indoor microbial communities should therefore at least partly rely on NGS. The samples obtained from LISAplus (i.e. the extracted DNA) can be reused in future studies. Using given epidemiological samples in III and IV was a compromise as the sampling scheme could not be influenced (i.e. no replicates per location, random spatial and temporal scattering of samples). Nevertheless, the data associated with the samples made them precious objects of study. It contained a decades-spanning involvement of participating households and investigators to organize different kinds of samplings and gather medical data as well as legal permissions (Heinrich et al. 2012). This associated meta-data make a future exploration of the samples from III and IV valuable and practical.

4.5 Human health is affected by microbial communities (III)

The hygiene-hypothesis (Strachan 1989) originally proposed that childhood infections transmitted by unhygienic contacts (e.g. infant to infant) might confer protection against hay
fever. With time, it developed into the assumption that early life contact to a broad range of microorganisms stimulates developing immune systems in a way that inhibits later development of allergies and related inflammatory diseases (1.4.2). Furthermore, it is increasingly acknowledged that microbial communities in human environments (affected by hygiene) and inside humans are not independent, especially with regard to their impact on immune regulation (Kinross et al. 2011, Okada et al. 2010). However, the term ‘hygiene’ is not fitting when discussing internal exposure to microbes, so recent studies often use the ideas originally promoted by the hygiene-hypothesis but they avoid to use the term (cf. use of language in Legatzki et al. 2014).

The first mechanism that had been proposed to explain observations underpinning the hygiene-hypothesis was that exposure to microbes is needed to promote a shift in T-helper lymphocytes (Th) from Th2 type (producing pro-inflammatory cytokines like IL4) typically found in immature immune systems of newborns and young children to Th1 type (producing anti-inflammatory cytokines like IFNγ) (Martinez and Holt 1999, Sironi and Clerici 2010). The mechanisms are now thought to be much more complex (Okada et al. 2010). For example, microbial exposure during early life may also persistently influence other immune functions such as natural killer cells (Olszak et al. 2012). Concerning Th cells, it has for instance been known that persistent helminth infections can provoke a strong shift towards Th2 type immune response without increasing the prevalence of allergy, which suggests mechanisms of counter-regulation (Yazdanbakhsh et al. 2002). All in all, the mechanistic background for the hygiene hypothesis seems to be heterogeneous which makes practical implications difficult (Schaub et al. 2006).

Study III was not mechanistic but gathered epidemiological evidence for associations between microbial communities and the development of allergies in urban surroundings. It indicated that a higher fungal diversity shortly after birth is associated with a significant lower
risk for developing allergic symptoms. As written in III, a similar association was found for farm environments (Ege et al. 2011), while for urban surroundings prior to III only (1,3)-β-D-glucan had been used as proxy for fungi in epidemiological studies on that matter and without conclusive results (Tischer et al. 2015). When reduced diversity in microbes is seen as an effect of ‘hygiene’, then the results of III are a first hint that the principle of the hygiene hypothesis can be applied also to fungal microbial communities in urban indoor environments.

Recent discoveries suggested extending the hygiene hypothesis to most chronic inflammatory diseases including type 1 diabetes, multiple sclerosis and inflammatory bowel diseases (Okada et al. 2010). Furthermore, it is increasingly evident that not only higher frequency infections or a rich microbial diversity are preventative for chronic inflammatory diseases, but also individual microbial taxa may be involved and play a role. For instance, Lynch et al. (2014) found that reduced exposure to Firmicutes and Bacteriodetes (especially Prevotellaceae, Lachnospiraceae, and Ruminococcaceae) in urban house dust during the first year of life was associated with developing atopy and atopic wheeze. Similar results were obtained by Valkonen et al. (2015) for mattress dust exposure to Mycobacterium, Bifidobacteriaceae and Clostridium in a rural area. Another example was recently given by Arrieta et al. (2015), who associated a reduction of the gut bacteria Lachnospira, Veillonella, Faecalibacterium plus Rothia in infants with a significantly increased risk of asthma and were able to confirm their findings in a mouse model.

As with overall diversity, there seems to be a lack of studies dealing with the health effect of specific environmental fungal taxa as compared to studies on bacterial taxa. This is a good point to advocate for using the existing DNA and meta-data of study III for a NGS project that gathers epidemiological data on specific fungal taxa. Existing data on community variation could guide an analyses on subsets of samples that goes beyond ITS (barcode)
sequencing (Schoch et al. 2012), to allow an improved assignment of fungal taxa and an analysis of their association to health outcomes.

Some of the results mentioned in this chapter may be startling, but setting them into a context that allows drawing comprehensive conclusions, e.g. for clinical applications, will take a great deal of work. In the words of Legatzki et al. (2014): “We are at the very beginning of unraveling the complex network between the human body and the indigenous and environmental microbiome in health and disease.”

4.6 Health impairments caused by fungi: side effects of fungal life or human lifestyle?

The non-infectious fungal health impairments this work is concerned with are generally thought not to be caused by fungi that manipulate or evade human immune systems the way pathogens would do (Köhler et al. 2015). It is supposed that diseases such as allergy and asthma can be referred to as imbalanced responses of the immune system (Sironi and Clerici 2010), which renders fungal allergies into an arbitrary side effect of sharing our environment with fungi. Arguable, the case is less easy to decide for fungal volatiles. The role of VOCs in human pathogenic fungi seems to be unknown, despite efforts to use them for infection diagnosis via patient-exhaled breath (Bazemore et al. 2012). Additionally, fungal VOC emissions may just occasionally be a by-product of a degrading metabolism (Insam and Seewald 2010) as specialized enzymes for their production exist. It has become more and more evident that fungal VOCs are means of communication and manipulation (1.2). The roles of single compounds can be multitrophic as it has been reported for caryophyllene, a sesquiterpene that causes interactions of fungi, bacteria, plants and animals (Kramer and Abraham 2012), or for 1-octen-3-ol as detailed in the introduction (1.2.2). While fungal volatiles may not specifically target humans, they are sensed in minute amounts by our noses (Hung et al. 2015) and deliberate reactions to exposition are obvious (e.g. leaving VOC rich
mould-infested buildings). Uncontrolled (e.g. wheezing, dizziness, headaches, intoxication symptoms) reactions of exposed humans are described (cf. Kopri et al. 2009). The typical response of humans exposed to strong bouquets of fungal VOC is avoidance. This effect of leaving the vicinity of fungi is – at least for some volatiles – caused by an actual meddling of a volatile with the concerned person’s nervous system (Kopri et al. 2009). Keeping a broad range of animals in distance through VOCs can result in holding them back from obstructing an emitting fungus. Hence, humans may as well be one of a broad range of targets for fungal volatiles.

Concerning allergies and related diseases, recent findings gathered evidence that these diseases are not just an aberrant reaction of the immune system caused by chance, but a likely consequence of suboptimal training of early life immune systems by interaction with a reduced diversity of microbiota (4,5). From that perspective, allergies, are not random unswayable side-effects of arbitrary encounters with our environment but most likely expressions of a poorly balanced immune development that could have been guided better with deliberate actions (e.g. ensuring a diverse microbial environment for young children).

It may be reasoned in future works that all non-infectious health impairments caused by fungi are based on a clear rationale and can be remedied (stop of VOC exposition, allergy immunotherapy) or prevented before they develop (training of developing immune systems). Our current knowledge strongly points in that direction.

As a last and more comprehensive point, “no species is an island, entire of itself, not even Homo Sapiens” (Heal 2000). A lot of evidence has been collected showing that we need biological diversity, from the grand scale for maintaining an inhabitable world (e.g. MacDougall et al. 2013, Hooper et al. 2005, Naeem and Li 1997) down to the microscopic scale for maintaining our health (e.g. Legatzki et al. 2014, Martín et al. 2014, Pflughoeft and Versalovic 2012). The present thesis tried to elucidate a minute part of this scheme. It
emphasized that more effort should be invested to provide foundations of ecological knowledge (I and IV), to find relationships between environment and health (III), and to help managing health outcomes (II).

5 CONCLUSION AND OUTLOOK

An approach from the angle of environmental microbiology on non-infectious health concerns mediated by fungi illustrated requirements for a holistic description of the interactions between humans and fungi and possible routes of action to improve human health.

A focus on the volatile production of two allergenic fungi highlighted the importance of providing solid data foundations to avoid findings in ecological and applied health research on the subject that will prove ephemeral. A thorough reinvestigation of sesquiterpene class volatiles produced by the two putatively well examined fungi Alternaria alternata and Fusarium oxysporum revealed extensive profiles and high quantities of emitted volatile compounds, thereby highlighting that the fungal volatome needs a systematic description as similarly done for microbiomes. This is also a prerequisite to evaluate the connection of VOC to other non-infectious health impairments, i.e. chronic inflammatory diseases such as allergy and asthma.

Developing a quantification routine for the assessment of outdoor allergenic fungi was an endeavour on a wider spatial and ecological scale. The project did not only describe conditions that may pose a problem for science or health, despite some of the results suggesting that the growth of allergenic fungi in agriculture could be unavoidable to a certain extent. Instead, it provided a procedure for outdoor monitoring on sources of allergenic fungi
that could contribute to reduce the burden outdoor fungal allergies pose, by developing advanced strategies for allergen avoidance or mitigation of allergen release.

Finally, the wide angle of a birth cohort study was used to gather evidence for the influence of environmental determinants on indoor dust microbial communities and to investigate health development of children in relation to the dust collected from their homes. The first part showed different dependencies of fungal and bacterial dust communities that need consideration in future microbiome studies, especially through the choice of sampling times. The second part indicated that the fungal and bacterial communities in indoor dusts of urban homes are associated with the health development in children exposed to them. This highlights the universal requirement of a balanced exposure to microbes and gives further evidence for mechanistic studies working on a healthy immune development and the clinical application of that knowledge.
6 REFERENCES


Bible, Leviticus, 14th chapter, verses 34-45.


Effects on patients with asthma of eradicating visible indoor mould: a randomised controlled trial. Thorax 62, 767-772.


Green, B.J., Sercombe, J.K., Tovey, E.R., 2005. Fungal fragments and undocumented conidia function as new aeroallergen sources. Journal of Allergy and Clinical Immunology 115, 1043-1048.


German Birth Cohorts: GINIplus and LISAplus]. Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz 55, 864-874.


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Koch, A., Heilemann, K.-J., Bischof, W., 2000. Indoor viable mold spores ± a comparison between two cities, Erfurt (eastern Germany) and Hamburg (western Germany). Allergy 55, 176-180.


Nowatski, J., Endres, G., DeJong-Hughes, J., Aakre, D., 2011. Strip till for field crop production, report AE-1370 (Revised), North Dakota State University Extension Service, Fargo, USA


Microbiology 5, 647-651.


Rambold, G., Stadler, M., Begerow, D., 2013. Mycology should be recognized as a field in biology at eye level with other major disciplines – a memorandum. Mycological Progress 12, 455-463.


syndrome (SBS) and biomarkers of inflammation in a 10 year follow-up study. Science of the Total Environment 430, 75-81.

I Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*: Effects of age, nutrient availability, and co-cultivation

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Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*:

Effects of age, nutrient availability, and co-cultivation

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*Alternaria alternata* is one of the most studied fungi to date because of its impact on human life—from plant pathogenicity to allergenicity. However, its sesquiterpene emissions have not been systematically explored. *Alternaria* regularly co-occurs with *Fusarium* fungi, which are common plant pathogens, on withering plants.

We analyzed the diversity and determined the absolute quantities of volatile organic compounds (VOCs) in the headspace above mycelial cultures of *A. alternata* and *Fusarium oxysporum* under different conditions (nutrient rich and poor, single cultures and co-cultivation) and at different mycelial ages.

Using stir bar sorptive extraction and gas chromatography–mass spectrometry, we observed *A. alternata* to strongly emit sesquiterpenes, particularly during the early growth stages, while emissions from *F. oxysporum* consistently remained comparatively low. The emission profile characterizing *A. alternata* comprised over 20 sesquiterpenes with few effects from nutrient quality and age on the overall emission profile. Co-cultivation with *F. oxysporum* resulted in reduced amounts of VOCs emitted from *A. alternata* although its profile remained similar.

Both fungi showed distinct emission profiles, rendering them suitable biomarkers for growth-detection of their phylotype in ambient air.

The study highlights the importance of thorough and quantitative evaluations of fungal emissions of volatile infochemicals such as sesquiterpenes.
Introduction

Sesquiterpenes are a class of highly reactive volatile terpenoids (C_{15}H_{20}). They function as infochemicals\textsuperscript{1,2} and play crucial roles in plant-to-plant, plant-to-microbe/animal and microbe-to-microbe interactions\textsuperscript{3}. Industrially, sesquiterpenes can act as precursors of advanced biofuels with properties similar to petroleum-based fuels\textsuperscript{4,7}. Sesquiterpenes can affect atmospheric chemistry and impact climate in a similar manner to other volatile terpenoids\textsuperscript{8,9}, although – due to analytical difficulties – a large uncertainty exists about sesquiterpene emissions\textsuperscript{10}. Microorganisms and especially fungi have recently been recognized as potentially important sources of volatile organic compounds (VOCs)\textsuperscript{3}. Thus, studying microbial volatile terpenoids is both industrially valuable and essential to understand biotic and biosphere-atmosphere interactions.

The genus \textit{Alternaria} comprises saprotrophic and plant-pathogenic fungi and includes some ubiquitous species, such as \textit{A. alternata}\textsuperscript{11,12}. Recently, the genomes of 25 \textit{Alternaria} spp. were sequenced, paving the way for the molecular exploration of their different lifestyles and their underlying metabolomics networks\textsuperscript{13}. Studies on metabolites of the genus \textit{Alternaria} mainly focused on agricultural spoilage via mycotoxins\textsuperscript{14} or toxin-mediated plant pathogenicity\textsuperscript{15}, while fewer studies targeted VOCs. A recent compilation of original work on microbial VOCs\textsuperscript{16} mostly includes substances for \textit{Alternaria} that are commonly found throughout the fungal kingdom, such as 1-octen-3-ol or 3-octanone. Accounts of sesquiterpene biosynthesis in \textit{Alternaria} are scarce, and of 268 metabolites reported for the genus\textsuperscript{17}, just two were sesquiterpene-derived compounds, commonly called 'oxygenated sesquiterpenes' (oSQTs).

In addition to its role as a plant pathogen\textsuperscript{11} and producer of mycotoxins\textsuperscript{14}, \textit{A. alternata} is a major fungal allergen source\textsuperscript{18}, which has led to it becoming one of the most thoroughly studied fungi. Still, the microbial VOC database\textsuperscript{19} lists only one compound (6-methoxyheptanol) for \textit{A. alternata}, which is the only representative of the genus in the
database (accessed 20/09/15). However, sesquiterpenes have been reported for *A. alternata* and two compounds (\(+\)-\(\beta\)-cedrene and (-)-thujopsene) have been identified.

Knowledge about sesquiterpene emissions is greater for some species of *Fusarium* compared to *Alternaria*. Eighteen different sesquiterpenes were identified in two strains of *F. sambucinum* and one strain of *F. sporotrichioides*, as well as no or six sesquiterpenes in two different strains of *F. graminearum*\(^{21}\), respectively.

As previously mentioned, sesquiterpenes can act as biological infochemicals, and several examples of volatile-mediated interactions have been described for *Fusarium*. Volatiles (including sesquiterpenes) from a non-pathogenic *Fusarium oxysporum* strain suppressed the growth and gene expression of plant pathogenic strains of this species\(^{22}\). Other strains of *F. oxysporum* with different VOC profiles have been shown to inhibit the growth of nematodes\(^{23}\) or fungal pathogens\(^{24}\).

Most of these findings have been recently made because the analytical techniques for the detection of a broad spectrum of VOCs have constantly improved over the last two decades\(^{25}\). Headspace sampling and stir bar sorptive extraction (SBSE) coupled to gas chromatography–mass spectrometry (GC-MS) have recently been applied for *in vitro* ecotyping of fungi based on their volatile profiles\(^{26}\). The same method was used to reveal reprogramming of root architecture through sesquiterpene signaling, thus highlighting the role of sesquiterpenes in plant-microbe interactions\(^{1}\). However, absolute quantification of sesquiterpene emissions that would allow comparisons between studies is rare in fungal VOCs research.

In this study, we aimed to comprehensively identify and quantify sesquiterpene production from *A. alternata* and *F. oxysporum* as a function of the growth stage, nutrient conditions, and fungus-fungus interactions. With investigating the variability of fungal sesquiterpene emissions, we provide fundamental data for exploring the different ecological functions of
fungal VOCs i.e., sesquiterpenes related to the different lifestyles and for applied approaches such as the use of sesquiterpene biomarkers for fungi.

Results

Growth characteristics

Under nutrient-rich conditions, the mycelia of both fungi grew similarly with growth ending between day 14 (co-cultivation) and day 21 (solitary cultivation). Neither fungus was able to overgrow the other and cultures were aged after day 28, as indicated by partly collapsed and hyaline hyphae.

Under nutrient poor conditions, *F. oxysporum* grew faster than *A. alternata*; however, the growth of *F. oxysporum* under co-cultivation slowed between day 14 and day 21. Between days 21 and 28, *F. oxysporum* started to overgrow peripheral parts of *A. alternata* mycelia. At day 35, both fungi were still growing when cultivated alone. (Fig. 1). Fungal morphology is shown in Supplementary Figs. S2 and S3 online.

Overview of VOC emission diversity and strength

The chemical compounds in VOC emissions were quantitatively and qualitatively diverse, and the quantity was higher for *A. alternata* compared to *F. oxysporum*. Altogether, we detected 27 different volatile compounds of unambiguous fungal origin, 25 sesquiterpenes and two alcohols (Table 1). From *A. alternata*, 26 compounds were emitted – in total up to $183.5 \pm 82.3$ pmol cm$^{-2}$ h$^{-1}$ (day 3, nutrient rich), while *F. oxysporum* emitted 10 compounds – in total up to $6.7 \pm 4.7$ pmol cm$^{-2}$ h$^{-1}$ (day 3, nutrient rich). All VOCs from *F. oxysporum* were also detected in *A. alternata*, except for δ-elemene (Table 2).

All 27 substances were detected when both fungi grew in direct contact (direct confrontation plates) under nutrient rich conditions. Fewer different VOC were detected when the fungi grew alone, were physically separated (split-plates), or were placed under nutrient poor
conditions (Table 2). Solitary cultures of *A. alternata*, split-plate cultures and direct
confrontation cultures of the same age differed only in the compounds that were present in the
lowest amounts. The emission profiles were highly similar between *A. alternata* grown alone
and co-cultivated with *F. oxysporum*, except for the appearance of δ-elemene in the co-
cultivation (Figs. 2a, 3a).

Under nutrient rich conditions, the emission rate per plate was strongest at day 7, while the
emission rate per mycelium area were strongest at day 3 (Fig. 2). Under nutrient poor
conditions, the emission rate was highest at day 14 (Fig. 3), the first time-point measured.

Under nutrient rich conditions, the average emission rate per mycelium area of *A. alternata*
grown alone was more than an order of magnitude higher than those of *F. oxysporum* (e.g., 27
times more at day 3, 58 times more at day 7, and 117 times more at day 14) Similar
differences were obtained under nutrient poor conditions (e.g., 19 times more at day 14 and
26 times more at day 21). The total emission intensity of *A. alternata* decreased exponentially
with culture age (Fig. 2c).

The emission rate per mycelium area from nutrient rich split-plates at day 14 of co-cultivation
were significantly lower than those of the same age of *A. alternata* grown alone (t-test \( P =
0.04 \)) (Fig. 2). Over all cultivation conditions and all time-points, the emission rate (pmol cm\(^{-2}\)
h\(^{-1}\)) decreased in the order: *A. alternata* alone > direct confrontation > split-plate > solitary *F.
oxysporum*.

The emission rate (day 14) in solitary cultures of *A. alternata* and co-cultivations with *F.
oxysporum* were much higher under nutrient rich than nutrient poor conditions, when
calculated per plate (pmol h\(^{-1}\)) (Fig. 3a). However, those differences became insignificant (t-
tests \( P > 0.05 \)) when calculated based on the mycelium area of *A. alternata* (Fig. 3b).

In contrast, the emission rate of solitary *F. oxysporum* under nutrient rich and poor conditions
were roughly similar when calculated per plate (Fig. 3b) as well as when based on the
mycelium area of *F. oxysporum* (Supplementary Table S1 online).
Emission of specific compounds

Under all nutrient conditions and at all time-points, the sesquiterpenes thujsene and β-cedrene were the most abundant VOCs of _A. alternata_. Together, they constituted more than two-thirds of the total emissions (Fig. 2). The next highest emissions generally were an unknown SQT (sesquiterpene) #1, a mixture of thujsane-2β-ol plus unknown SQT #4, α-himachalene, and β-acoradiene (Table 1, Supplementary Fig. S4 online). While the general emission patterns of _A. alternata_ did not differ notably with culture age, the contributions of thujsene and β-cedrene varied by up to 25% between sampling time-points (Fig. 2).

The emission pattern of _F. oxysporum_ was more variable (detection was approximately at the limit of quantification for some substances) and the overall emission rate was more than 100-fold lower at most time-points than for _A. alternata_ (Fig. 2, Supplementary Fig. S5 online). For example, at day 3 under nutrient rich conditions, nine compounds were detected for _F. oxysporum_ (highest: δ-elemene, 2-methyl-1-butanol, and α-himachalene), while three weeks later (day 28), only two compounds (δ-elemene and α-himachalene) remained detectable. Three substances (γ-curcumene, germacrene D, and an unknown SQT #2) were only detected in trace amounts (<0.003 pmol cm$^{-2}$ h$^{-1}$) on one or two sampling dates.

Common to all cultures was a much more rapid decline in the emission rate of alcohols compared to sesquiterpenes. By day 14, only traces of alcohols were detected (e.g., 0.04 ± 0.008 pmol cm$^{-2}$ h$^{-1}$ 2-methyl-1-butanol emitted from solitary cultures of _A. alternata_).

Amounts of compounds under nutrient poor cultivation resembled those of the nutrient rich cultivations for _A. alternata_ (except for the absent compounds, Table 2). For _F. oxysporum_, emissions were very low (day 14: 0.24 ± 0.15 pmol cm$^{-2}$ h$^{-1}$, day 21: 0.03 ± 0.01 pmol cm$^{-2}$ h$^{-1}$, day 35: 0.003 ± 0.001 pmol cm$^{-2}$ h$^{-1}$), therefore no temporal trends for the amounts of individual compounds from _F. oxysporum_ were inferred for nutrient poor conditions.
Almost all individual compounds from *A. alternata* were found in lower amounts on split-plates compared to solitary cultivation (Fig. 4). When grown together with *F. oxysporum*, some sesquiterpenes of *A. alternata* were significantly less abundant, e.g., β-cedrene (days 14 and 21; t-tests $P = 0.001$) and thujaopsene (days 7 and 14; t-tests $P = 0.005$).

**Multivariate analyses and correlation matrices**

VOC profiles strongly differed in multivariate analyses (PCA and OPLS) – in quality and quantity – between *A. alternata* and *F. oxysporum* cultures growing alone, as shown by the large distance and separation between *A. alternata* and *F. oxysporum* samples in the first significant principal component in a PCA, which explained 94% of the total VOC variance (Fig. 5a).

Furthermore, VOC emissions from co-cultivated fungi could be separated from those emitted from cultures containing only one fungus, as demonstrated by the separation of the second significant principal component. PCA indicated differences in emissions between fungi growing in direct contact or on split plates, but these were rather marginal.

Overall, high emission rates of sesquiterpenes were negatively correlated in solitary cultures of *F. oxysporum* (PCA: Fig. 5b, OPLS: Supplementary Fig. S6 online). Only the sesquiterpene δ-clemene, unique to *F. oxysporum*, was highly and negatively correlated to solitary cultures of *A. alternata* with a scaled and centered regression coefficient of $-0.82$ in the OPLS model (Supplementary Fig. S6 online).

The OPLS model described changes of VOC emissions from the different fungal cultures very well; the cumulative $R^2(X)$ and $Q^2$(cum) were 98.6% and 70.1% respectively, using 3 predictive components. The analysis of variance testing the cross-validated predictive residuals (CV-ANOVA) indicated that the OPLS model discriminated the cultures of a single fungus in a highly reliable manner ($P$-values: *A. alternata* $< 2 \times 10^{-7}$, *F. oxysporum* $< 4 \times 10^{-4}$, direct confrontation $= 0.0499$). Regression lines for observed versus predicted $Y$-values.
were $R^2 = 0.955$ (A. alternata) and $R^2 = 0.996$ (F. oxysporum). Classification exercises indicated that an OPLS model based on VOC emission profiles collected at day 7 can correctly predict the presence of a solitary culture of A. alternata and F. oxysporum at day 3, 7, 14 (threshold set to 0.5 predicted Y values) (Supplementary Fig. S6 online).

Spearman correlation matrices that included all time-points of solitary cultivations showed positive correlations (Spearman $P < 0.05$, Supplementary Fig. S7 online) for emission rates of most compounds for each fungus. Nearly half of the sesquiterpenes produced by A. alternata were almost totally correlated (Spearman correlation coefficient $\rho > 0.9$), as were $\delta$-elemene and $\alpha$-himachalene for F. oxysporum ($\rho = 0.97$).

4. Discussion

Our sampling and detection system allowed the detailed assessment of sesquiterpene formation in the two fungi. The quantitative differences in VOC emissions between them were very distinct; less abundant compounds from A. alternata were still emitted at the same magnitude as the most abundant VOC released from F. oxysporum.

As observed in this study, A. alternata had the highest sesquiterpene emissions relative to other fungi in an earlier study$^{26}$. However, direct comparison with our data is impeded by the lack of absolute quantification in most of the earlier studies. A recent study$^{26}$ applied the same detection system and a synthetic medium comparable to the nutrient poor medium that we used in this study. That study analyzed emission profiles and source strengths from eight fungi of different ecotypes (ectomycorrhizae, pathogens, and saprophytes) and observed the highest sesquiterpene emissions for Trichoderma viride ($\sim 0.7$ pmol cm$^{-2}$ h$^{-1}$ at an age of 3 days). At the first sampling date in our study (day 14), the emission rate for A. alternata was still approximately six-fold higher than for T. viride in the earlier study, while those for F. oxysporum were three times lower. However, the emission rate in nutrient rich medium in this
study at day 3 was more than 250 times higher for A. alternata and ten times higher for F. oxysporum, compared to the earlier data on Trichoderma viride.

In plants – a global major source of terpenes$^{10}$ – lower sesquiterpene emission rates were often reported$^{27}$ than the emission rate from A. alternata found here. A. alternata is geographically widespread and abundant in nature$^{32}$. Therefore, A. alternata might potentially contribute significantly to the emission of sesquiterpenes into the atmosphere, as generally hypothesized for fungi$^3$.

This study expanded the sesquiterpenes of A. alternata described from two major compounds$^{30}$ to a complex variety of substances. We used a passive sampling system specifically suitable to trap non-polar compounds such as sesquiterpenes but with a low affinity for relatively polar compounds such as alcohols, ketones, and aldehydes. Therefore, their contribution to the total VOC emitted from A. alternata in the present analysis cannot be completely assessed. Physicochemical differences in the VOC collection system and trapping material can explain why previous studies found different chemical compounds for Alternaria$^{16}$.

We detected oSQT as minor constituents in the A. alternata emission profile. Few oSQT have been described for fungi so far$^{28}$. To the best of our knowledge, this is the first report on the production of widdrol, thujopsane-2β-ol and cedren-13-ol, 8 in fungi, while isomers of 10-epi-γ-eudesmol have been described for freshwater fungi (Beltrania rhombica)$^{29}$ and mushrooms (Inonotus obliquus)$^{30}$. Our most predominant VOC, β-cedrene, has only been reported in fungal odor profiles from A. alternata$^{30}$ and Penicillium decumbens$^{31}$. The structurally related di-epi-α-cedrene and α-cedrene have been found in some other ascomycetes$^{28}$. The second most abundant substance in our study, thujopsene, has been reported in various fungi including Penicillium decumbens$^{31}$. This fungus emitted thujopsene in addition to β-cedrene and other compounds, which included substances that were also
detected in this study for *A. alternata*, e.g., (E)-β-farnesene, β-acoradiene, β-chamigrene, and α-chamigrene. Thujaopsene was found to have an auto-regulatory function for the growth of *P. decumbens* and inhibitory effects on several ascomycetes. Compared to earlier studies on fungal VOCs, the number of sesquiterpenes from *A. alternata* was relatively high. This confirmed results from recent measurements showing that many fungi emit a suite of many different sesquiterpenes. Our attempt to relate sesquiterpene synthases (SQTS) to this multitude of sesquiterpenes by using emission correlation matrices, as has been shown recently, gave ambiguous results and suggested that either the same enzyme was responsible for the formation of all sesquiterpenes or that different SQTS were biochemically active in concert. The formation of multiple products by single sesquiterpene synthases has been documented in fungi, but multiple SQTS homologs were also found in single fungi of different groups. The high number of different sesquiterpenes is generally mirrored in the *Alternaria* genome: A search of the *A. alternata* genome library (*Alternaria alternata*, SRC1HrK2f v1.0) revealed 30 putative terpene synthase genes ([http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Altal1, accessed 04/11/2015](http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Altal1, accessed 04/11/2015)). VOC emissions from our *F. oxysporum* strain differed – except for acoradiene – from other strains described so far, indicating that VOC profiles are strain-dependent in *F. oxysporum*. The lower number of compounds for *F. oxysporum* resulted in a much simpler correlation matrix compared to *A. alternata*. On the basis of this correlation matrix, we speculate that the same SQTS synthesized δ-elemene as the primary product along with several additional minor compounds. In accord with the lower number of detectable sesquiterpenes, only five putative terpene synthase genes were annotated in the *Fusarium oxysporum* genome ([http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Fuso1, accessed 04/11/2015](http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Fuso1, accessed 04/11/2015)).
To translate the observed sesquiterpene emission pattern of both fungi to a molecular basis, future work will focus on the heterologous expression of the putative terpene synthase genes, genetic differences between strains and their functional biochemical characterization. Our results clearly demonstrated highest sesquiterpene emissions in very young (3 days old) mycelia in both fungi. Contradictory to our findings, sesquiterpene emissions have been attributed to a later growth phase. However, according to our literature survey, *Aspergillus versicolor* was the only truly indicative example for strongly increased sesquiterpene emissions with age, showing a 10³-fold increase between day 3 and 14 of incubation. The early peak of emission in our study suggests that sesquiterpene formation is associated with rapid growth in *A. alternata* and *F. oxysporum*, at least on nutrient rich medium. For cultures on nutrient poor medium, we started VOC sampling when *A. alternata* cultures reached a size of 3 cm², which occurred only after 14 days of incubation. Therefore, no information on emissions at early growth stages under nutrient poor cultivation conditions is available. Nonetheless, growth on nutrient poor media before day 14 was slower than during the following two weeks. If emissions are related to rapid growth as found under nutrient rich conditions, the growth pattern suggests no intensely increased emissions under nutrient poor conditions at an earlier time. However, this still needs to be studied further. The general VOC pattern for *A. alternata* was stable during aging, except for an overly rapid decline in the alcohol emissions. This temporal emission pattern for small hydrocarbons is in accordance with other works. For example, in *Penicillium expansum*, C₈ compounds were emitted mainly between an age of 3 and 9 days. In contrast to the stable pattern found here, the VOC diversity of *Trichoderma atroviride* increased by 24 substances (three sesquiterpenes) in a comparison between 5- and 14-day-old cultures. However, the effect of aging mycelia on sesquiterpene emissions has rarely been intentionally investigated and knowledge of the prevalence of sesquiterpene emissions in aging fungi remains fragmentary.
Emission quantities for *F. oxysporum* did not differ considerably between nutrient poor and rich media. Emissions from *A. alternata* from cultures of the same age strongly differed under rich and poor nutrient conditions, yet only when calculated for the entire plate but not with respect to the mycelium area. However, the physiological conditions between cultures of the same age under different nutrient conditions probably diverged strongly, which may explain the insignificant differences based on mycelial area. For example, *A. alternata* cultures on nutrient rich media had almost completed growing at day 14 and showed their final coloration, while *A. alternata* cultures on nutrient poor media at the same time covered only a tenth of the surface compared to their nutrient rich equivalents and were unpigmented.

As mentioned above, cultures of *A. alternata* on nutrient poor medium likely never emitted VOCs in similar amounts as the cultures on nutrient rich medium. This suggests that the nutrient supply plays an essential role in sesquiterpene biosynthesis in *A. alternata*, although the mechanism remains unclear. A recent study on *Aspergillus fumigatus* showed that transcript levels of mevalonate kinase, one of the key enzymes in terpenoid biosynthesis, were decreased upon iron starvation, which resulted in a strong decline of sesquiterpene emissions. The nutrient poor medium in our study did not contain iron, which may have been a reason for limited sesquiterpene biosynthesis.

Apart from the least abundant compounds, the volatile profile of *A. alternata* was the same under both nutrient conditions. Corresponding results were reported from *Penicillium polonicum, Aspergillus ustus* and *Periconia britannica* strains with similar sesquiterpene profiles when grown on malt extract, wallpaper, or plaster board, or when several *Fusarium* spp. were cultivated on different substrates.

Co-cultivation with shared headspace and separate growing space (split-plate) limited interactions between the fungi to their headspaces until the glass barrier was overgrown around day 10 after inoculation under nutrient rich conditions. However, the observed drop in
total emissions from split-plates compared with the solitary cultivation of *A. alternata* was only significant at day 14, when possible effects of headspace interactions (e.g., change in emission profiles or amounts, adsorption of VOC by *F. oxysporum*) could not be separated from effects triggered by physical contact or exudates. However, during co-cultivation on split-plates, the headspace concentration of nearly all compounds was already lower very early at day 3 and 7 compared to the concentration found in solitary cultures of *A. alternata*. Because border effects were similar for both setups during this early stage, an active/passive adsorption of compounds in *F. oxysporum* mycelia is probable, especially because strains of *F. oxysporum* can transform terpenes\(^{39,40}\) and various fungi are known to grow with terpenes or similar compounds as a sole carbon source\(^{41}\). Another possibility is the reduced production of sesquiterpenes by *A. alternata* after recognition of *F. oxysporum* volatiles (i.e., δ-elemene). Although the exact mechanism must be elucidated in future studies, our experiment suggests a VOC-mediated interaction between the two fungal species.

The multivariate analysis clearly separated the sesquiterpene profiles of both fungi, suggesting that the profile might serve as a very good biomarker to discriminate between the tested strains of *A. alternata* and *F. oxysporum*. Such specific sesquiterpene profiles could also be used in ambient air to detect fungal growth. An earlier study\(^{42}\) concluded that sesquiterpene biomarkers may be the best approach to detect indoor fungal growth, after *Aspergillus*, *Chaetomium*, and *Epideicum* were identified from water-damaged buildings via their sesquiterpene profiles. Examples of the use of different classes of VOCs as fungal biomarkers include screening for fungal causes of lung diseases\(^{43}\) and ecotyping\(^{26}\). The peculiar sesquiterpene profile of *A. alternata* is an ideal target for detecting those allergenic fungi in indoor environments.

The available data on VOC emissions of different *F. oxysporum* strains\(^{21-24}\) suggests that sesquiterpenes are useful biomarkers on the sub-species level for this species, as has been
proposed earlier\textsuperscript{21}. The stability of sesquiterpene emissions by \textit{A. alternata} under various
conditions suggests that such emissions are especially good biomarkers for the growth of the
species. However, intraspecific variability in \textit{A. alternata} has not been assessed; therefore
more \textit{A. alternata} strains must be chemotyped. Because strains of \textit{Alternaria} are known to
produce different terpenoids (bi- and tricycloalcanenes) and other classes of toxins\textsuperscript{14}, it may
be speculated that a pathotype relation with sesquiterpene profiles could be established in a
similar manner as has been demonstrated for \textit{F. oxysporum}\textsuperscript{21}.

This study showed the early, diverse and long-lasting emission of sesquiterpenes at a high rate
and with a stable profile for the tested strain of \textit{A. alternata}. If this observation holds true for
different isolates of \textit{A. alternata}, these properties make its emission profile useful for future
chemotyping approaches in different fields, such as mycotoxin prevention or indoor health
prediction. While this is also principally true for the investigated strain of \textit{F. oxysporum}, low
quantities of emissions might hamper such use.

Our novel findings concerning two frequently studied fungi highlight the importance of a
thorough quantitative and qualitative (re-)evaluation of fungal VOC emissions of complex
volatiles such as sesquiterpenes. Such fundamental analyses are necessary to provide a
comprehensive knowledge base for mechanistic, ecological and applied research.

\textbf{Methods}

\textit{Culture media}

A synthetic nutrient ‘poor’ and a complex nutrient ‘rich’ medium were prepared. The nutrient
poor medium was based on synthetic nutrient poor agar and the nutrient rich medium on malt
extract agar\textsuperscript{44}. To minimize the volatile background, 15 g L\textsuperscript{-1} gelrite (SERVA electrophoresis,
Heidelberg, Germany) were used for solidification instead of agar. The poor medium
contained (L\textsuperscript{-1}): KHPO\textsubscript{4} 1 g, KNO\textsubscript{3} 1 g, MgSO\textsubscript{4} \times 7 H\textsubscript{2}O 0.5 g, KCl 0.5 g, glucose (D\textsuperscript{+}) 0.2 g.
sucrose 0.2 g, ZnSO₄ × 7 H₂O 0.01 g, CuSO₄ × 5 H₂O 0.005 g; the rich medium contained (L\(^{\dagger}\)); malt extract 20 g, ZnSO₄ × 7 H₂O 0.01 g, CuSO₄ × 5 H₂O 0.005 g. The media were poured (20 ml plate\(^{\dagger}\)) into sterile degreased glass petri-dishes without division (direct confrontation) or with a glass-barrier of 7 mm height dividing the plate into two equal halves (split-plate). The split-plates ensured that only VOC-mediated interactions were possible during early growth, while on direct confrontations exudates into the medium and early exploratory hyphae were additional routes for interactions. After solidification, each dish of nutrient poor medium was provided with four cellulose filters (30 mm, No. 1001-329, Whatman GE, Dassel, Germany) that served as a polymer carbon source and a supporting matrix for mycelia to approximate the situation in a withering plant. Sterile culture plates were stored at least one week at 4 °C in odorless roasting tubes (Toppits Cofresco, Minden, Germany) before use.

Strains and cultivation

The experimental plates were inoculated with 3 × 50 conidial spores from spore-solutions made from *Alternaria alternata* (Fr.) Keissler (DSMZ 62006) or *Fusarium oxysporum* f. *aequimeae* (Fr.) Schltldl. (DSMZ 62297) cultures grown as recommended\(^{14}\). For the rich medium, the spores were applied in three droplets onto one half of the plate and approximately 1 cm from the rim with equal spacing between the droplets. For the poor medium, two droplets were applied on half of the cellulose-filter next to the rim of the dish and one droplet was placed in between the filters. The plates were sealed with Parafilm M (Bemis, Oshkosh, WI, USA) and incubated at 20 °C in the dark.

On both media, five growth setups were tested in five replicate plates each: (1) plates with sterile media for sampling the VOC-background, (2) *A. alternata* on one half of a split-plate with the second half containing sterile medium (‘solitary *A. alternata*’), (3) *F. oxysporum* on a half of a split-plate with the second half containing sterile medium (‘solitary *F. oxysporum*’), (4) both fungi on different halves of a shared split-plate, (5) both fungi on opposite sides of a
barrier-less plate (‘direct confrontation’). For each growth condition, three extra replicates were incubated under exactly the same conditions except for absence of VOC sampling, to monitor possible changes in the growth dynamics compared to the sampled plates because sampling is a potential growth disturbance (e.g., short exposure to light and lifting of the lid).

Collection of VOCs and the sampling scheme

VOCs were collected from the glass Petri dishes by head-space sorptive extraction using the SBSE method based on non-polar, polydimethylsiloxane coated stir bars (Twister, film thickness 0.5 mm, Gerstel, Mülheim an der Ruhr, Germany) as recently described, with the following modification - magnetic discs were fixed to the dishes in the center of each lid’s outer surface. This guaranteed a central positioning of the magnetic sorptive stir bars in the air-space of the plates and rapid, contamination-free handling (Supplementary Fig. S1 online). The collection time was 48 hours for cultures on poor and 20 hours for cultures on rich medium.

Sampling began when the slower growing fungus covered approximately 1 to 3 cm² of the plate surface. Four time-points were sampled from nutrient poor and five from nutrient rich medium. Time-points for sampling from nutrient poor plates were days 14, 21, 28, and day 35 after inoculation, and the time-points for nutrient rich plates were days 3, 7, 14, 21, and day 28 (each date was the beginning of a VOC collection, e.g., sampling for day 3 started 72 hours after inoculation).

Analyses of VOCs

In our approach, as in recent work, VOC samples were analyzed using a thermo-desorption unit (TDU, Gerstel) coupled to a GC-MS (GC type: 7890A, MS type: 5975C inert XL MSD with a triple axis detector, both from Agilent Technologies (Palo Alto, CA, USA) using a 5% phenyl 95% dimethyl arylene siloxane capillary column (60 m × 250 μm × 0.25 μm DB-5MS
Calibration was achieved by injecting pure standard mixtures dissolved in hexane at seven different concentrations ranging from ~20 to 800 pmol μL⁻¹. Each mixture was made independently in triplicate for each concentration and measured in duplicate. The resulting MS signal responses were found to be linear with an increasing standard concentration with $R^2 > 0.99$. Non-isothermal Kovats retention indices were calculated according to generally accepted standards, based on chromatography retention times of a saturated alkane mixture ($C_7 - C_{40}$, Sigma-Aldrich, Taufkirchen, Germany) and other alkanes ($< C_7$) occurring in the chromatogram background.

Limits of detection (LOD) were set to twice $\sigma$, and the limit of quantification (LOQ) to 10-folder of LOD. Emission rates were calculated on a fungal mycelium area (pmol cm⁻² h⁻¹) or plate (pmol h⁻¹) bases.

Statistical analysis

Relationships between VOC emissions from *A. alternata* and *F. oxysporum* growing solitary or in co-cultures in nutrient rich medium were analyzed using principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS) (SIMCA-P v13, Umetrics, Umeå, Sweden). For this, day 7 was chosen because all VOCs were detected and the highest plate-based emission rates were measured at this date. Using plate-based emission rates allowed the inclusion of data for the co-cultivation setups. Established procedures to analyses and evaluate MS data were followed in a similar manner as previously described. Prior to analysis, all x-variables were logarithmically (log10) transformed, centered, and each type of data was scaled block-wise with 1 sd⁻¹. Calculated significant principal components were validated using ‘full cross-validation’, with 95% confidence level on parameters and 7 as number of cross-validation groups. The prediction ability of the OPLS
model to discriminate *A. alternata* and *F. oxysporum* was evaluated using VOC data collected at day 3, 7, 14.

Spearman correlation-matrices for the emissions of the volatile substances to each other were calculated in the R programming environment using algorithms of the Hmisc package (v3.10-1). Hierarchical clustering (single linkage) of the results was performed with the corplot package (v0.73).

References


36. Ma, L.-I. et al. Comparative genomics reveals mobile pathogenicity chromosomes in
93

527  Matsylik, S., Herbarth, O. & Mueller, A. Determination of volatile metabolites originating from
528  mould growth on wall paper and synthetic media. J Microbiol Methods 75, 182-187,
531  compounds emitted by Trichoderma atrovirens on plant growth. Arch Microbiol 197, 723-727,
533  Molina, G., Butron, M. L., Bicas, J. L., Dolder, M. A. & Pastore, G. M. Comparative study of the
534  bioconversion process using R-(+)- and S-(−)-limonene as substrates for Fusarium oxysporum
536  Morstica, M. R. & Pastore, G. M. Production of r-(+)-α-terpineol by the biotransformation
537  of limonene from orange essential oil, using cassava waste water as medium. Food Chem
539  Prenafeta-Boldu, F. X., Summerbell, R. & Sybren de Hoog, G. Fungi growing on aromatic
540  hydrocarbons: biotechnology’s unexpected encounter with biohazard? FEMS Microbiol Rev
542  Poliizli, V. et al. Identification of volatile markers for indoor fungal growth and
543  chemotaxonomic classification of Aspergillus species. Fungal Biol 116, 941-953,
545  Bazemore, R. A., Feng, J., Cseke, L. & Podilla, G. K. Biomedically important pathogenic fungi
546  detection with volatile biomarkers. J Breath Res 6, 016002, doi:10.1088/1752-
547  7155/6/016002 (2012).
549  KNAW Fungal Biodiversity Centre, 2010).
550  Ghrirado, A., Heller, W., Fladung, M., Schnitzler, J. P. & Schroeder, H. Function of defensive
551  volatiles in pedunculate oak (Quercus robur) is tricked by the moth Tortrix viridana. Plant Cell
553  Kreuzwieser, J. et al. The Venus flytrap attracts insects by the release of volatile organic
555  van den Dool, H. & Kratz, P. D. A Generalization of the retention index system including linear
557  (1962).
558  Veilkova, V. et al. Knocking down of isoprene emission modifies the lipid matrix of thylakoid
559  membranes and influences the chloroplast ultrastructure in poplar. Plant Physiol 168, 859-
561  Kaling, M. et al. UV-B mediated metabolic rearrangements in poplar revealed by non-
564  compounds in isoprene emitting and non-emitting poplar. Plant Cell Environ,
566  Vanzo, E. et al. S-nitroso-proteome in poplar leaves in response to acute ozone stress. Plos One 9,
568  Way, D. A. et al. Increasing atmospheric CO2 reduces metabolic and physiological differences
569  between isoprene- and non-isoprene-emitting poplars. New Phytol 200, 534-546,
571  Ghrirado, A., Sorensen, H. A., Petersen, M., Jacobsen, S. & Sondergaard, I. Early prediction of
572  wheat quality: analysis during grain development using mass spectrometry and multivariate
574  Team, R. C. R: A language and environment for statistical computing, (R Foundation for
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Author Contributions Statement

F.W., A.G., J.S., and K.P. designed the study. F.W. and A.G. performed the experiments together and analysed the data together. F.W. coordinated cultivation and VOC sampling and A.G. GC-MS identification and quantification. F.W. wrote the first draft of the manuscript and prepared the figures. All authors contributed to interpretation of the findings and edited and approved the manuscript.

Additional Information

Competing financial interests

The authors declare no competing financial interests.

Supplementary Information

Supplementary Materials File

Figure Legends

Figure 1 Mycelial expansion during VOC sampling. Solid line: Alternaria alternata, dashed line: Fusarium oxysporum; points and error bars depict means ± s.d. (n = 5) of mycelium areas at VOC sampling time-points. Grey strips: approximate size of mycelia when hyphae started to grow across the glass borders separating the gelrite hemispheres of the plates.

Figure 2 Time-course of VOC emission rates from fungi growing under nutrient rich conditions. (a) Total VOC emission rates under different cultivation conditions; F: Fusarium oxysporum alone, A: Alternaria alternata alone, FA: split-plate with both fungi, FA: direct confrontation of both fungi; pie chart areas are proportional to the emission rate per culture
plate, grey numbers: means (n = 5) of total VOC emission rate (pmol plate⁻¹ h⁻¹). (b) Total VOC emission rate normalized to the projected mycelium area (pmol cm⁻² h⁻¹) of *A. alternata* on each plate (F|A and FA setups: ignoring the minute share of *F. oxysporum* on total emissions); means ± s.e. (n = 5); *P* = 0.04, t-test. (c) Total VOC emissions as function of the culture age.

Figure 3 Emission rate after 14 days of cultivation on nutrient rich and poor media. (a) Total emission rate of VOC under different cultivation conditions; F: *Fusarium oxysporum* alone, A: *Alternaria alternata* alone, F|A: split-plate with both fungi, FA: direct confrontation of both fungi; pie chart areas are proportional to the emitted volatiles, grey numbers: means (n = 5) of total VOC emission rates (pmol plate⁻¹ h⁻¹). Colors are coded as in Fig. 2a. (b) Total emission rate normalized to the projected surface area (pmol cm⁻² h⁻¹) of *A. alternata* on each plate (F|A and FA culturing: ignoring the minute share of *F. oxysporum* on the total emissions); means ± s.e. (n = 5).

Figure 4 Emission rate per mycelium surface area of *Alternaria alternata* compared between solitary cultures of *A. alternata* and split-plates with the headspace shared by *A. alternata* and *Fusarium oxysporum*. Points with standard errors (n = 5, each): emissions of single compounds, red: day 3 VOC sampling, blue: day 7 VOC sampling; the diagonal line indicates equal results for both setups. The insert shows the results for the strongest emissions on a larger scale. Full names of abbreviated compounds are given in Table 1.

Figure 5 Principal component analysis (PCA) of VOC emission rates of the five biological replicates of all different fungal setups (7-day-old cultures, nutrient-rich media). PC1, PC2: principal first and second components with total explained variance given as percentage. (a) Score plot: solitary fungal culture of *Fusarium oxysporum* (green) and *Alternaria alternata*
(blue) are depicted with circles; direct confrontations are indicated by beige triangles and split plates by grey triangles; the ellipse indicates the tolerance based on Hotelling’s \( T^2 \) with a significance level of \( \alpha = 0.05 \). (b) Correlation scaled loading plot: white squares: sesquiterpenes, black dots: alcohols; the outer and inner ellipses indicate 100% and 75% explained variance, respectively.

Table 1 VOC analysis using stir bar sorptive extraction (SBSE) and GC-MS. Means of VOC emission rates ± s.e. (n = 5) from 7 days old *Alternaria alternata* and *Fusarium oxysporum* co-cultures (confrontation plates) growing on nutrient rich medium (malt extract gelrite).

<table>
<thead>
<tr>
<th>Compounds</th>
<th>RT (min)</th>
<th>Kovats' RI</th>
<th>CAS</th>
<th>LOD (pmol plate(^{-1}) h(^{-1}))</th>
<th>emission (pmol plate(^{-1}) h(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td><strong>alcohols</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Isobutanol</td>
<td>6.827</td>
<td>619</td>
<td>78-83-1</td>
<td>&lt;0.01</td>
<td>4.88 ± 0.97</td>
</tr>
<tr>
<td>2-Methyl-1-butanol</td>
<td>8.895</td>
<td>724</td>
<td>137-32-6</td>
<td>&lt;0.01</td>
<td>8.44 ± 1.92</td>
</tr>
<tr>
<td><strong>sesquiterpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \delta )-Elemene</td>
<td>32.864</td>
<td>1335</td>
<td>20307-84-0</td>
<td>&lt;0.01</td>
<td>13.39 ± 1.03</td>
</tr>
<tr>
<td>( \beta )-Elemene</td>
<td>35.967</td>
<td>1391</td>
<td>515-13-9</td>
<td>0.05</td>
<td>4.98 ± 0.34</td>
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<tr>
<td>Di-epi-( \alpha )-Cedrene</td>
<td>35.822</td>
<td>1410</td>
<td>50894-66-1</td>
<td>&lt;0.01</td>
<td>11.67 ± 0.62</td>
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<td>( \alpha )-Cedrene</td>
<td>36.422</td>
<td>1427</td>
<td>11028-42-5</td>
<td>&lt;0.01</td>
<td>14.3 ± 0.7</td>
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<tr>
<td>( \beta )-Cedrene</td>
<td>36.861</td>
<td>1439</td>
<td>546-28-1</td>
<td>&lt;0.01</td>
<td>421.31 ± 20.92</td>
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<tr>
<td>Thujopsene</td>
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<td>1449</td>
<td>470-40-6</td>
<td>&lt;0.01</td>
<td>206.11 ± 18.37</td>
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<tr>
<td>( \psi )-( \psi )-Farnesene(^2)</td>
<td>37.355</td>
<td>1452</td>
<td>18794-84-8</td>
<td>0.02</td>
<td>6.18 ± 0.34</td>
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<tr>
<td>( \beta )-Acoradiene</td>
<td>38.083</td>
<td>1472</td>
<td>24048-44-0</td>
<td>&lt;0.01</td>
<td>24.36 ± 1.29</td>
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<tr>
<td>unknown SQT(^2) #1</td>
<td>38.304</td>
<td>1478</td>
<td>n.a.</td>
<td>0.06</td>
<td>92.5 ± 5.72</td>
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<td>( \alpha )-Himachalene</td>
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<td>18431-82-8</td>
<td>&lt;0.01</td>
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<td>allo-Aromadendrene</td>
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<td>1501</td>
<td>25246-27-9</td>
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<td>14.65 ± 0.94</td>
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<td>( y )-Curcumene</td>
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<td>1508</td>
<td>28976-68-3</td>
<td>&lt;0.01</td>
<td>15.08 ± 2.06</td>
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<tr>
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<td>1517</td>
<td>23986-74-5</td>
<td>&lt;0.01</td>
<td>20.66 ± 1.22</td>
</tr>
<tr>
<td>Eremophilene</td>
<td>39.86</td>
<td>1529</td>
<td>10219-75-7</td>
<td>&lt;0.01</td>
<td>6.35 ± 0.43</td>
</tr>
<tr>
<td>( \alpha )-Chamigrene</td>
<td>40.257</td>
<td>1545</td>
<td>19912-83-5</td>
<td>&lt;0.01</td>
<td>6.78 ± 0.41</td>
</tr>
<tr>
<td>( y )-Cadinene</td>
<td>40.323</td>
<td>1548</td>
<td>1460-97-5</td>
<td>&lt;0.01</td>
<td>10.76 ± 0.58</td>
</tr>
<tr>
<td>unknown SQT #3</td>
<td>41.478</td>
<td>1553</td>
<td>n.a.</td>
<td>0.03</td>
<td>2.76 ± 0.22</td>
</tr>
<tr>
<td><strong>oxygenated sesquiterpenes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thujopsene-( \beta )-ol</td>
<td>41.761</td>
<td>1606</td>
<td>150737-93-2</td>
<td>&lt;0.01</td>
<td>33.07 ± 3.95</td>
</tr>
<tr>
<td>= unknown SQT #4</td>
<td></td>
<td></td>
<td>n.a.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cedren-13-ol, 8-</td>
<td>42.399</td>
<td>1641</td>
<td>18319-35-2</td>
<td>&lt;0.01</td>
<td>1.35 ± 0.14</td>
</tr>
<tr>
<td>Widdrol</td>
<td>42</td>
<td>1619</td>
<td>6892-80-4</td>
<td>&lt;0.01</td>
<td>3.47 ± 0.31</td>
</tr>
<tr>
<td>10-epi-( y )-Eudesmol</td>
<td>42.247</td>
<td>1632</td>
<td>15051-81-7</td>
<td>&lt;0.01</td>
<td>3.14 ± 0.26</td>
</tr>
</tbody>
</table>

\(^2\)verified by authentic standards, otherwise tentatively identified; \(^2\)SQT: sesquiterpene; \(^\ast\)co-eluted peaks
Table 2 Fungal origin of volatile organic compounds (VOC) (above the limit of quantification) collected from solitary cultures of *Alternaria alternata* and *Fusarium oxysporum* grown on nutrient rich medium (malt extract gelrite).

<table>
<thead>
<tr>
<th>Venn plot</th>
<th>VOC unique to <em>Alternaria alternata</em> (17)</th>
<th>VOC detected from both species (9)</th>
<th>VOC unique to <em>Fusarium oxysporum</em> (1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Di-epi-α-Cedrene</td>
<td>Isobutanol&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-Cedrene</td>
<td>1-Butanol,2-methyl&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thujopsene</td>
<td>β-Elemene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-β-Farnesene</td>
<td>β-Cedrene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unknown Sqt #1</td>
<td>α-Chamigrene&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>β-Chamigrene&lt;sup&gt;a&lt;/sup&gt;</td>
<td>β-Accordiene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>allo-Aromadendrene</td>
<td>δ-Elemene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Eremophilene&lt;sup&gt;b&lt;/sup&gt;</td>
<td>γ-Cadinene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>α-Chamigrene</td>
<td>unknown Sqt #2&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>γ-Cadinene</td>
<td>unknown Sqt #3&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unknown Sqt #2</td>
<td>γ-Curcumene</td>
<td></td>
</tr>
<tr>
<td></td>
<td>unknown Sqt #3</td>
<td>Thujopsane-2β-ol</td>
<td>Germacrone D&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>(+ unknown Sqt #4)</td>
<td>Cedren-13-ol, 8'-&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Widdrol</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>10-epi-γ-Eudesmol</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>absent on nutrient poor medium, <sup>b</sup>on nutrient poor medium only detected for *A. alternata*
<Figure 1>

(mycelium surface area [cm²] over days after inoculation for different conditions)

- F. oxysporum solitary
- A. alternata solitary
- F|A (split plate)
- F (direct confrontation)
Supplementary Materials

Sesquiterpene emissions from *Alternaria alternata* and *Fusarium oxysporum*:

Effects of age, nutrient availability, and co-cultivation

Fabian Weikl, Andrea Ghirardo, Jörg-Peter Schnitzler, Karin Pritsch

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Figure S1 Example of a PDMS twister (polydimethyldisiloxane coated stir bar) attached to the inside of a glass culture-dish’s lid and held in place by a magnet on the outside of the dish (pre-test with a Cladosporium isolate).

Figure S2 Time series (dates of VOC samplings) on the morphology of a shared split-plate with nutrient rich conditions. Left halves: Fusarium oxysporum, right halves: Alternaria alternata. d: days after inoculation.
Figure S3. Culture morphology under nutrient poor conditions. Upper panel: at the first VOC sampling (day 14 after inoculation); lower panel: at the last VOC-sampling (day 35). From left to right: Alternaria alternata solitary, Fusarium oxysporum solitary, shared split plate (left half F. oxysporum, right half A. alternata), direct confrontation (left half F. oxysporum, right half A. alternata).

Table S1. Emissions under nutrient rich vs. nutrient poor conditions. Comparisons of emission rates between cultures of approximately similar size and different age (day 3 nutrient rich vs. day 14 day nutrient poor) or different size and the same age (day 14).

<table>
<thead>
<tr>
<th>Nutrient Poor Setup</th>
<th>Nutrient Poor Emissions (pmol cm⁻² h⁻¹) ± s.e. [n = 5]</th>
<th>Nutrient Rich Setup</th>
<th>Nutrient Rich Emissions (pmol cm⁻² h⁻¹) ± s.e. [n = 5]</th>
<th>P-values (t-test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14 d solitary A. alternata</td>
<td>4.41 ± 1.97</td>
<td>3 d solitary A. alternata</td>
<td>183.48 ± 82.29</td>
<td>0.061</td>
</tr>
<tr>
<td>14 d solitary A. alternata</td>
<td>4.41 ± 1.97</td>
<td>14 d solitary A. alternata</td>
<td>11.23 ± 2.78</td>
<td>0.080</td>
</tr>
<tr>
<td>14 d split-plate</td>
<td>4.12 ± 1.55</td>
<td>3 d split-plate</td>
<td>130.08 ± 34.04</td>
<td>0.006</td>
</tr>
<tr>
<td>14 d split-plate</td>
<td>4.12 ± 1.55</td>
<td>14 d split-plate</td>
<td>3.17 ± 1.8</td>
<td>0.699</td>
</tr>
<tr>
<td>14 d confrontation</td>
<td>22.05 ± 8.33</td>
<td>3 d confrontation</td>
<td>148.15 ± 35</td>
<td>0.008</td>
</tr>
<tr>
<td>14 d confrontation</td>
<td>22.05 ± 8.33</td>
<td>14 d confrontation</td>
<td>9.36 ± 2.58</td>
<td>0.183</td>
</tr>
<tr>
<td>14 d solitary F. oxysporum</td>
<td>0.24 ± 0.15</td>
<td>3 d solitary F. oxysporum</td>
<td>6.74 ± 4.7</td>
<td>0.205</td>
</tr>
<tr>
<td>14 d solitary F. oxysporum</td>
<td>0.24 ± 0.15</td>
<td>14 d solitary F. oxysporum</td>
<td>0.1 ± 0.03</td>
<td>0.367</td>
</tr>
</tbody>
</table>
Figure S4 Comparisons between solitarily grown Alternaria alternata (solitary Fusarium oxysporum in the case of δ-elemene) and split-plates with A. alternata and F. oxysporum on nutrient rich medium. Emission rates of each compound calculated based on A. alternata mycelium area, except for δ-elemene, for which emission rates were calculated based on F. oxysporum mycelium area; error bars: s.e. (n = 5).
Figure S5 Emission rates of solitary grown *Fusarium oxysporum* on nutrient rich medium. Emission rates of each compound calculated based on *F. oxysporum* mycelium area; error bars: s.e. (n = 5). The substances ϑ-curcumene, germacrene-D, and unknown SQT #2 were only detected in low traces (< 0.003 pmol cm⁻² h⁻¹) and are not displayed.
Figure S6: Observed versus predicted Y-values of orthogonal partial least square (OPLS) analysis referred to *Alternaria alternata* (a) and *Fusarium oxysporum* (b) modeled using VOC profile data from 7 days old culture and growing with nutrient rich media, and corresponding correlation coefficient plots (c, d); prediction plots of VOC profiles from day 3, 7, 14 (nutrient rich) (e, f); predicted Y-values > 0.5 are classified as *A. alternata* in (e) and *F. oxysporum* in (f). Colored dots: blue: solitarily grown *A. alternata*, green: solitarily grown *F. oxysporum*, grey: split-plates with both fungi, beige: direct confrontations. Error bars: confidence intervals based on jack-knifing uncertainty method. Regression lines: goodness of fit. OPLS model fitness: $R^2(X) = 98.6\%$, $R^2(Y) = 100\%$, $R = 76.8\%$, $Q^2$(cum) = 70.1% using 3 predictive components. RMSEE: *A. alternata* = 0.102; *F. oxysporum* = 0.029; RMSECV: *A. alternata* = 0.101; *F. oxysporum* = 0.031. P-values of cross-validated ANOVA: *A. alternata* (solitary), $P < 2 \times 10^{-5}$; *F. oxysporum* $P < 4 \times 10^{-14}$. Partly overplotted text (e, f): t1 (day 3 VOC sampling), t7 (day 7), t14 (day 14)
Figure S7 Correlation matrices for emissions from solitary cultures on nutrient rich medium. (a,b): Lower triangle correlation matrices including data from all time-points and replicates. Compounds ordered by hierarchical clustering (single linkage). Numbers: Spearman correlation coefficient ($\rho$) expressed as percentage; X: correlation insignificant ($P > 0.05$). (a) *Alternaria alternata*, (b) *Fusarium oxysporum*. (c) *A. alternata*: correlation matrices including data of all replicates of two consecutive time-points each. Matrices were prepared as for (a), order of compounds differs since clustering was used. Colored frames around the matrices correspond to the respective times covered in the graphs to the left (emission rates and mycelium surface areas).
II  Targeting allergenic fungi in agricultural environments aids the identification of major sources and potential risks for human health

Weikl F, Radl V, Munch JC, Pritsch K.

Targeting allergenic fungi in agricultural environments aids the identification of major sources and potential risks for human health

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HIGHLIGHTS

- Detection system for outdoor allergenic fungi enabling source monitoring
- Allergenic fungi are ubiquitous in agricultural soils and plant materials.
- Plant residues in fields after harvest are major sources of allergenic fungi.
- Crop residues at harvest, particularly cover crops, show high allergenic fungal loads.

ABSTRACT

Fungi are, after pollen, the second most important producers of outdoor airborne allergens. To identify sources of airborne fungal allergens, a workflow for qPCR quantification from environmental samples was developed, thoroughly tested, and finally applied. We concentrated on determining the levels of allergenic fungi belonging to Alternaria, Cladosporium, Fusarium, and Trichoderma in plant and soil samples from agricultural fields in which cereals were grown. Our aim was to identify the major sources of allergenic fungi and factors potentially influencing their occurrence. Plant materials were the main source of the tested fungi at and after harvest. Amounts of A. alternata and C. cladosporioides varied significantly in fields under different management conditions, but absolute levels were very high in all cases. This finding suggests that high numbers of allergenic fungi may be an inevitable side effect of farming in several crops. Applied in large-scale studies, the concept described here may help to explain the high number of sensitization to airborne fungal allergens.

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1. Introduction

Respiratory allergies and associated diseases show a rapidly growing incidence worldwide, with allergic rhinitis alone affecting between 10% and 30% of the population (Pawankar et al., 2011). In recent years, the extensive contribution of fungi to allergies and asthma has received increased attention (e.g., Denning et al., 2006; Burge and Rogers, 2000). However, some features of fungal allergy still remain elusive such as the complex relations of allergy and allergens in fungi; for example, the sheer number of allergens, cross-reactivities, poly-sensitizations, or the hampered diagnosis of allergy (Simon-Nobbe et al., 2008). Up to 80% of asthmatics are sensitized to fungal allergens (Lopez and Salvaggio, 1985) and a disease pattern of severe asthma with fungal sensitization (SAFS) has been recently proposed (Denning et al., 2006).

So far, most in-depth environmental studies have focused on the more obvious issues with indoor houses such as children’s homes (Reponen et al., 2010), office buildings (Pitkäranta et al., 2008), or moisture-damaged building materials (Andersson et al., 1997). Recently, assessments of potentially allergenic fungi in indoor environments have been greatly enhanced because of the use of molecular techniques, such as molecular recognition PCR (MSPCR, Haukland and Vesper, 2002), which can be performed on a collected integrated sample of fungal load in indoor air. So far, no corresponding methods for outdoor environments have been available, despite growing evidence that fungi detected in most indoor environments may be...
dominated by the local outdoor airborne community (Amend et al., 2010; Adams et al., 2013).

For monitoring allergic fungi in the outdoor air, spore traps and other impact samplers, which deliver data over large time scales, have been used (Levetin, 2004). For example, results from spore monitoring indicate that allergic fungi, such as Alternaria, which are naturally associated with plants, follow a seasonal course with a strong peak in summer or early fall (Corden and Millington, 2001). However, allergenic fungi, such as Cladosporium spp., are present in high levels during most of the year (Sautour et al., 2009), suggesting more diverse environmental sources. Propagation of fungi is localized and often triggered by certain preconditions such as suitable temperature and humidity (Katala et al., 1997), thunderstorms (Naser and Pulmood, 2009), farming operations (Friisen et al., 2001), or crop cultivation in combination with warm and dry weather (Corden et al., 2003). For these reasons, time-integrating methods with poor spatial resolution may not provide insight into cause-effect relations. Therefore, it is highly desirable to have methods, which deliver a more differentiated picture of the presence of potentially allergic fungi in the environment. These methods would enable studying conditions that favor their development, which are prerequisites to the production and release of allergens. Consequently, the origins of allergenic fungal matter need to be considered. With that aim, we established and rigorously tested a workflow that was compatible with existing probe-based qPCR tests and can be used with varying source materials such as soil or plant residues. For developing the procedure, we focused on samples from an agricultural environment and on four fungal groups: the two most relevant outdoor allergen producers Alternaria alternata and Cladosporium cladosporioides, with approximately 20% of patients with respiratory allergy alone reacting to Alternaria in skin and prick tests (Barta et al., 2009), and Fusarium culmorum as well as the Trichoderma viride/Atroviride/Konigii group. The latter two belong to genera frequently known to cause hypersensitivity (e.g., Kurup et al., 2000), although with uncertain prevalence, and they were included because they were frequently found in agricultural environments, including the present study site (Hagn et al., 2003).

After developing a workflow adapted to the relevant sample types in an agricultural context (soil and plant materials), our next aim was to identify the main factors influencing the amounts of allergic fungi in these environmental samples. Therefore, we analyzed agricultural field samples with the aim of detecting 1) major sources of allergic fungi; 2) critical time points in the annual cycle from harvest to the next growing season; 3) the influence of spatial heterogeneity within fields; 4) the role of agricultural management; and 5) fungal colonization of different crops at maturity just before harvest. With a thoroughly tested procedure and the first set of applications, the overall aim of the study was to develop a strategy enabling environmental monitoring efforts for elucidating the paths of fungal allergens from the environment to sensitized persons. 2. Material and methods 2.1. Fungal cultures and conidial supplements

Fungal cultures from A. alternata (Fr.) Keissler (DSMZ 62006), C. cladosporioides (Fr. ex. G.A. de Vries) (DSMZ 62112), T. viride Pers. Ex. Gray (DSMZ 63065), F. culmorum (W.G. Smith) Sacc. (DSMZ 62191), and Penicillium roqueforti Thom (DSMZ 1080) were grown, as recommended by Samson et al. (2010). Accordingly, the media used for sporeulation were 'potato carrot agar' for A. alternata, 'synthesicher nährstoffärmer agar' (supplemented with cellulose filters (4.5 cm Whatman 1001-329, GE Healthcare, Little Chalfont, UK) as support for mycelia) for F. culmorum and 20% malt-extract agar' for the other fungi. Conidia (aerial spore) stocks were prepared by rinsing spore cultures on plates with 0.01% Tween 80, as described by Haugland et al. (2004). Conidia from the reference fungus (P. roqueforti) were used to spike all samples prior to DNA extraction as an internal standard, allowing normalization of possible extraction differences during calculations and screening for PCR inhibition. 'Calibrator' samples, which contained only conidia from the reference fungus P. roqueforti and one or more target fungi, were prepared and extracted with each batch of environmental samples. These calibrators provided the basis for the comparative quantification according to the MSQPCR principle and served as positive controls for all qPCR runs. The amounts of conidia in calibrators were set to 4 × 10^4 for P. roqueforti, 2 × 10^5 for C. cladosporioides and T. viride, and 1 × 10^4 for A. alternata and F. culmorum.

To determine an alternative reference fungus, in silico comparisons for amplification of P. roqueforti and a Geotrichum candidum strain used in the literature (Haugland and Vesper, 2002) have been made beforehand. The results showed a comparable and equally unlikely amplification of both fungi from our test environments i.e., plant residues and soil. A pre-experiment with different soil-plant congeries from our environment had revealed only minimal amplification of P. roqueforti. Its very low occurrence in the target samples and its easy handling (nonpathogenic, well sporulating) lead to the choice of P. roqueforti as reference fungus. Details in Suppl. 2.

2.2. Field sampling of soil and plant residues

Samples were collected at the Scheyern Research Station, 40 km north of Munich (48.50°N, 11.45°E, 450–490 m asl) with a mean annual precipitation of 803 mm and mean annual temperature 8.4 °C. Two farm management systems (organic and conventional) have been used on the farm since 1992 with different crop rotations and use of synthetic fertilizers and pesticides, respectively (Schröder et al., 2008). Field samples were used for the following two major targets: 1) establishing a standardized workflow for outdoor environmental samples and 2) identifying major sources of target fungi and environmental factors associated with allergenic fungal growth. The major focus of the study was on wheat (Triticum aestivum L.) as the major crop cultivated under organic and conventional farming at the research station. In addition to wheat, other crops were taken into account for the study of fungal loads at the time of harvest (see below).

For a postharvest time series, eight wheat fields (four organic, four conventional) were sampled after wheat harvest in 2011. Samples were collected at least 2 m from the margins of each field. Soils on the farm are cambisols and luvisols. Four different sample types were collected, each composed of 10 subsamples per field: 1) decaying stems and (2) roots from 1 to 10 cm soil depth with the adhering soil shaken off manually but without further cleaning, (3) soil directly attached to and shaken off of decaying roots; (root soil), and (4) bulk soil from 1 to 4 cm depth. Ten subsamples were collected on each field at least 10 m distant from one another in the six larger fields, and at least 5 m distant in the two smaller fields. For each subsample, four blocks within 1 m² were separated with a spade. From each block one source material was collected. Equal weights but at least 10 g of each source material were gathered per subsample and the subsamples of a source material were merged to one sample per field. Time points were one week after harvest, after tillage, in late autumn, and before the next sowing to cover a time series of crop residue decay after harvest (Table 1). For the development of the procedure and for sensitivity tests, plant residues and soil samples from the late-autumn date were used. Within-field heterogeneity was studied in one of the fields, which was chosen for its heterogeneous soil conditions.

The field was divided into four sections of 0.7 ha, gradually descending from a hillside of 480 m asl (section 1) to a depression of 470 m asl (section 4) with soil conditions changing from sandy-loamy partially stagnic cambisol with high-stone content (section 1) to cambisol with colluvic material (section 4). In each section, three independent replicate samples were used for reproducibility testing. Each replicate sample consisted of 10 merged subsamples each of bulk soil and decaying
Table 1

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Sampling dates</th>
<th>Plot sizes (plot n.)</th>
<th>Plot type</th>
<th>Source materials</th>
<th>Subsamples per source and plot</th>
</tr>
</thead>
<tbody>
<tr>
<td>Accuracy of different calculation</td>
<td>10/25/11</td>
<td>Spot samples (4)</td>
<td>Harvested wheat fields</td>
<td>Bulk soil [spiked with conidia]</td>
<td>12 [conidia dilution series of each fungus]</td>
</tr>
<tr>
<td>approaches</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Reproducibility of whole procedure</td>
<td>10/25/11</td>
<td>Spot sample (1)</td>
<td>Harvested wheat fields</td>
<td>Bulk soil, decaying roots, decaying shoots</td>
<td>6 [3 spiked with conidia of target fungus]</td>
</tr>
<tr>
<td>Postharvest time series</td>
<td>08/17/11</td>
<td>0.29 ha - 0.6 ha (8)</td>
<td>Harvested wheat fields*</td>
<td>Bulk soil, root/soil, decaying roots, decaying shoots</td>
<td>10 [merged to 'field sample']</td>
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<td></td>
<td>09/09/11</td>
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<td>10/25/11</td>
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<td>03/27/12</td>
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<tr>
<td>Within-field heterogeneity</td>
<td>10/25/11</td>
<td>0.7 ha (4)</td>
<td>Sections of 1 field*</td>
<td>Bulk soil, decaying shoots</td>
<td>10 [merged to 'field-section sample']</td>
</tr>
<tr>
<td>Preharvest test of individual plants</td>
<td>07/23/14</td>
<td>0.01 ha (1)*</td>
<td>Fields with mature crops*</td>
<td>Lower part of plants, upper part of plants, seed heads</td>
<td>4 plants [individuals not merged]</td>
</tr>
<tr>
<td>Preharvest field trial</td>
<td>07/23/14</td>
<td>0.014 ha (39)</td>
<td>Field trial plot: mature crops*</td>
<td>Lower part of plants, seed heads</td>
<td>25 [merged to a 'plot-sample']</td>
</tr>
</tbody>
</table>

* B. organic, 4 conventional.
* Divided into 3 replicates per subsample before merging to test reproducibility.
* Different fields for each species: wheat, rye, barley, oat, pea, field bean.
* Plots for organic wheat (n=10), organic rye (n=15), conventional wheat (n=9).

Shoot samples, respectively. Samples were collected anew for the heterogeneity test and in the same manner for the postharvest time series.

After plant materials were identified as a major source of the tested allergenic fungi, a series of second sampling (preharvest) was performed immediately before harvest, a time point representing farming operations critical for release of potentially high amounts of fungal colonized material. Wheat and rye (Secale cereale L.) plants were collected from 15 organic plots each, omitting a 1 m border strip. Because rye had not been a part of the crop rotation, only wheat was collected from 9 conventional plots of the field trial. From each plot, 25 randomly sampled plants were divided into two parts (stems 0-30 cm above soil and seed heads), which were separately analyzed.

Additional plant samples were collected at the same date at a 5-km distance from the research station, i.e., field bean (Vicia faba L.), oat (Avena sativa L.), pea (Pisum sativum L.), winter barley (Hordeum vulgare L.), winter rye, and winter wheat (in four replicates (10 m distant from each other) per plant); these samples were divided into three sections (0-30 cm above the soil, 30-100 cm above the soil, and seed heads), which were separately analyzed.

All samples were frozen on-site dry ice and stored at -20 °C until further processing. An overview over all samplings is given in Table 1.

2.3. Extraction of DNA

The optimal DNA extraction method was determined in pre-experiments and showed that the PowerSoil-hp ™ 96 Well Soil DNA Isolation Kit (Mo-Bio Laboratories Inc, Carlsbad, USA) was most suitable in comparison with the other extraction protocols with respect to DNA quality, PCR functionality, handling, and cost (Suppl. 1). Soil samples were treated according to the manufacturer's recommendations for spores in soils, except for sample processing after centrifugation following the mechanical basis step. Here, for each extraction, the lowest volume of a sample was determined as the volume to be transferred in the next step for all extracts on the plate. To avoid high plate to plate variability, only calibrators samples from the same extraction plate as the respective samples were used later for calculation.

Plant residues and plant-soil mixtures were homogenized as follows: a stainless steel grinder (UM 620, Kärcher AG, Birkenfeld, Germany) was precooled with dry ice, loaded with 4-6 g of frozen samples and approximately 10 g of pelleted dry ice, and ground to a particle diameter of <2 mm. The resulting composites could be handled like soil samples after evaporation of dry ice. Input weights for the extractions were approximately 0.25 g after the manufacturer’s recommendations for the kit, except for very light homogenized materials, which only allowed input of >0.15 g. The exact input of fresh-weight was noted for later calculations and dry-weights were determined separately.

2.4. qPCR assays

Prior to qPCR, in silico tests were conducted to choose a suitable GenBank™ assay for the calibrator (Suppl. 2). The following assays (original names) were used: Pq (P. raoultii), Ccld1 (C. cladosporioides), Aasr (A. alternata), Svir (T. viride/fuscum/irinophilum) from Haugland and Vesper (2002), and the assay of F. culmorum from Cullen et al. (2005). Oligonucleotides were synthesized by MWG-Euromics GmbH (Ebersberg, Germany). Reactions were carried out in optical-grade 96-well reaction plates from Applied Biosystems (Foster City, CA, USA) with 5 µl undiluted DNA-templates, reaction mix, and PCR conditions according to Haugland and Vesper (2002). PCR was performed on an Applied Biosystems 7300 system (Foster City, CA, USA).

2.5. Calculation of conidial equivalents

The amount of fungal DNA in unknown samples was estimated on the basis of the amount of DNA extracted from known numbers of conidia (determined by microscopic counting) and expressed as conidial equivalents (CE). To quantify the target fungus in samples, two variants of the comparative CT (cycle threshold) method exist (Haugland and Vesper, 2002). Both rely on calibrator-samples consisting of known amounts of extracted conidia instead of standard curves. The first, directly associates the CT of a test sample with the CT of a corresponding calibrator sample, using an assay of only the target fungus (Eq. (i)) and the second, includes normalization via reference fungus (here: P. raoultii) (Eq. (ii)):

\[
N = N_0 \times 2^{-\Delta CT} \quad (i)
\]

\[
N = N_0 \times 2^{-\Delta CT} \quad (ii)
\]

where \(N_0\) = the number of target fungus conidia in the calibrator; \(\Delta CT = CT_{\text{sample-target fungus}} - CT_{\text{calibrator-target fungus}}\); \(\Delta CT = CT_{\text{sample}} - CT_{\text{calibrator}}\); \(\Delta CT = CT_{\text{sample-reference fungus}} - CT_{\text{calibrator-reference fungus}}\); \(\Delta CT\) = efficiency of the target assay. The calculated \(N\) therefore is the number of target fungus conidial equivalents (CE) in the PCR-reaction of the respective sample.

Results are shown as CE/g (dry-weight of sample). The E-\(\Delta CT\) approach was used for all experiments after comparisons of both.
Reaction efficiencies (E) were calculated using CTs from dilution series (4-fold steps) of the calibrators, via the slopes of log-transformed dilution vs. CT plots. The final calculation results were means from comparative calculations with CTs from the first three dilutions of the calibrator, as similarly described by Pitkäranta et al. (2008). The dilution series were therefore only used to get mean values from different PCR-reactions, thus increasing the accuracy of calculation. For that and the determination of E, only dilutions in the linear range (similar ACT between dilution steps) were used. With our dilution steps, the linear range usually ended around 0.000088x (e.g., resembling the equivalent of 5 conidia of A. alternata in the PCR reaction of a diluted calibrator). The theoretical detection limit is not coupled to a standard-curve for MSQPCR and similar approaches. However, if no CTsample|target fungus is available, Eq. (i) or (ii) is incomplete. With our qPCR setup, we reached that calculated theoretical limit at 10 CE/g for the assay on C. cladosporioides and at <1 CE/g for the other assays. For routine analyses, a lower detection limit of 5 x 10^4 CE/g is recommended (see Discussion).

2.6. Tests for accuracy and sensitivity

The accuracy of different calculation approaches in field samples were compared using soil samples from four different fields. These soils were previously identified to having low-levels of test fungus DNA (not shown). Subsamples of each soil were spiked with different amounts of conidia from C. cladosporioides (2 x 10^3–2 x 10^6) and T. viride (2 x 10^3–2 x 10^6) as well as fixed amounts of P. roqueforti (4 x 10^5) and subsequently extracted.

Accuracies of different calculation approaches near the detection limit were assessed with six extractions from an artificial conidial mixture, containing 1 x 10^5 conidia of A. alternata and F. culmorum, 2 x 10^3 of C. cladosporioides and T. viride, and 4 x 10^3 conidia of P. roqueforti per extraction.

Possible amplification bias in the PCR reactions between calibrators (low-total DNA) and field samples (high-total DNA) was accounted for by supplementing three of the six replicates with 2 μg of salmon sperm DNA (Carl Roth GmbH, Karlsruhe, Germany) as nontemplate DNA (NTDNA) prior to extraction.

Reproducibility of the whole procedure, including DNA extraction, was tested by dividing one sample each of bulk soil, wheat stems, and wheat roots into six subsamples. For each material, three of the subsamples were spiked with conidia from all target fungi. DNA from the subsamples was then individually extracted and analyzed by qPCR. Replicate extractions from the within-field heterogeneity test were used to assess reproducibility in native field conditions.

2.7. Statistical analysis

The following factors potentially contributing to allergenic fungal presence in the environment were taken into account: a) major reservoirs (soils and decaying plant residues), b) time after harvest until spring, c) agricultural management (organic vs. conventional), and d) occurrence in crop species at harvest. The postharvest experiment was analyzed using one fitted ANOVA model (with type III sum of squares) for the results of each fungus. In addition, results for each fungal group and sampling material were treated as effects and analyzed using the Adonis function (PERMANOVA) in the vegan package (version 2.0-10) for R (R Core Team, 2013) with 10,000 permutations and Bray-Curtis dissimilarities. This analysis enabled evaluation of the overall effects of sampling time and management or individual field characteristics for the whole population of tested allergenic fungi. For fitting ANOVA with preharvest results, those results were grouped into the following three sets of comparable data: (1) all conventional plots, (2) all organic plots, and (3) all wheat plots. Input data were augmented by 2 and transformed to base-10 logarithms for all ANOVA. For Adonis, original values were used. The R programming environment was used for all computation.

3. Results

3.1. Outdoor fungal detection system

All qPCR assays were successfully applied to the following sample types: soil, soil-plant mixtures, and different plant constituents (Suppl. 3). Accuracies of different calculation approaches were first compared using spiked soil samples (Fig. 1A) and secondly using diluted conidial suspensions (Fig. 1B) to evaluate performance at low-conidia levels. Overall, both calculation approaches well represented the numbers of added conidia. The simplified calculation setup (E^ACT) exhibited a tendency to underestimate conidial counts for large supplements (max.: 0.33-fold for 2 x 10^6 C. cladosporioides conidia from microscopical counts). The E^-ΔCT setup exhibited a tendency to underestimate conidial counts for small supplements (max.: 1.99-fold for 2 x 10^3 counted conidia of C. cladosporioides). The ratios of calculated to counted conidia for individual extracts were between 0.28-1.16 (E^-ΔCT, median: 0.62, mean 0.63) and 0.82-2.5 (E^-ACT, median: 1.26, mean 1.36). If the samples spiked with 2 x 10^6 conidia are ignored because of the chance of interference with small amounts of conidia native to the soils, the ratios were between 0.28-0.83 (E^-ΔCT) and 0.82-1.96 (E^-ACT). Using diluted conidia, both approaches appeared equally precise at low-conidia levels (Fig. 1B). In the same experiment, three of six low-conidia samples were supplemented with an excess of nontemplate DNA prior to extraction. Results for supplemented and nonsupplemented extracts were similarly heterogeneous. However, variability was significantly reduced in samples complemented with nDNA (p < 0.05, t tests with E^-ACT results of all fungal test assays).

The reproducibility of the entire detection system was high within all fungal assays and sample types, which were first tested using replicated DNA extractions of spiked environmental samples (standard deviations (s.d.): 4.5%-24.9% for bulk soil, 2.6%-11.5% for root material, 7.3%-14.4% for shoots) and secondly tested using replicated sampling (n = 3) plus DNA extraction from four subsamples of one wheat field (s.d. <3% for results >10^4 CE; Fig. 2).

3.2. Quantity of allergenic fungi in environmental samples

In the postharvest period, the major factor determining quantity of each fungus was sample type (ANOVA p < 0.001, except Trichoderma; p = 0.003). Decaying shoots were the major source of the tested fungi, with the exception of the Trichoderma group. For C. cladosporioides, high-numbers were also detected in decaying roots (Fig. 3). Fungal abundances decreased in the order of shoot > root > immediate root vicinity > bulk soil for all allergenic fungi, except for the Trichoderma group, which was in most cases found at low-levels and without a clear pattern.

Time point was a significant factor for C. cladosporioides and Trichoderma (ANOVA p < 0.001) with highest numbers of these fungi found in shoots at the late-autumn sampling date. Interaction effects of sample type with sampling time were found for C. cladosporioides, A. alternata, and F. culmorum (ANOVA p < 0.001). Amounts of C. cladosporioides and F. culmorum in decaying shoots remained substantial until the following spring (Fig. 3).

Management had a significant influence for all fungi (ANOVA p = 0.001 (C. cladosporioides, Trichoderma), p = 0.002 (F. culmorum), p = 0.016 (A. alternata)). The multivariate approach estimated the effect of individual fields as significant (Adonis-PERMANOVA p < 0.001) on the whole population of tested allergenic fungi and in the same magnitude as the coupled effect of management (details in Suppl. 4).

Within-field variations (Fig. 2) were more pronounced for bulk soil than for decaying shoots, less substantial for C. cladosporioides (soil: 13-fold, shoots: 5-fold) and A. alternata (soil: 2-fold, shoots: 4-fold)
compared to F. culmorum (soil: no detection in two sections of the field, shoots: 38-fold) and the Trichoderma group (soil: 200-fold, shoots: 8-fold) and in the same range as between-field variations. In most of the 150 preharvest samples of different crops and plant parts, C. cladosporioides and A. alternata were detected in substantial amounts (95% of samples > 10^5 CFU/g for C. cladosporioides and 95% of samples > 5 x 10^5 CFU/g for A. alternata). Quantities of C. cladosporioides were commonly 100-1000 times higher than those of A. alternata (Figs. 4 and 5). F. culmorum and the Trichoderma group were less frequently found (48% and 31% of samples if no detection limit was applied, or 30% and 27% of samples if a lower detection limit of 5 x 10^5 CFU/g was set) and typically in lower quantities than the other two fungi (Fig. 5).

Out of all the crops collected at harvest at separate locations, samples of field bean and pea had very high amounts of C. cladosporioides (> 10^9 CFU/g) and A. alternata (> 10^7 CFU/g) over all plant compartments. Levels for cereals (wheat, rye, oat, and barley) were lower in most cases (Fig. 4), yet at substantial levels (> 10^5 CFU/g or > 10^6 CFU/g, respectively). Differences between plant compartments were significant for C. cladosporioides (ANOVA p < 0.001) and A. alternata (ANOVA p = 0.002). Differences between levels for legumes and cereals were significant (ANOVA p < 0.001) for C. cladosporioides, A. alternata and F. culmorum.

Detailed sampling of 15 organic and 9 conventional plots revealed nearly identical fungal loads for rye and wheat on organic plots (Fig. 5), which were significantly higher than those for wheat on conventional plots (ANOVA p = 0.001 for C. cladosporioides and A. alternata). A significant influence (ANOVA p = 0.015 for C. cladosporioides and A. alternata) of the sampled plant section was found with higher fungal loads on seed heads than on lower stems. Other factors (tillage, fertilizer, and interactions) had only minimal influence on one of the three compared plot types (conventional, organic, or wheat plots; details in Suppl. 4).

4. Discussion

4.1. Evaluation of the outdoor fungal detection system

Accuracies of the assays under the protocols adapted in this study matched that of qMSQPCR, with the ratios of calculated to counted conidia falling in a small range when compared, for example, with the ratio from 0.1 to 2.25 (median: 0.70) in Haugland et al. (2004) for similar assays and was consistent with the range of both calculation approaches of this study. Integration of further assays for the fungi of interest may be carried out without extensive re-evaluations.

E−ΔΔCt calculations tended to underestimate large amounts of conidia and E−ΔΔCt calculations tended to overestimate small amounts, but in general, both methods were in good agreement and always approximated the magnitude of conidia counts with the hematocytometer. By referring to Haugland and Vegter (2002), this resemblance should be verified before the E−ΔΔCt approach may be discontinued in favor of the E−ΔΔCt method. Consequently, we recommend utilizing the cheaper and faster E−ΔΔCt method, provided that similar sample types and analogous qPCR assays are used. Nonetheless, addition of reference fungus conidia is advisable when PCR inhibition cannot be excluded, as mentioned by Roe et al. (2001). This addition may also serve as an internal positive control for DNA extraction, such as for very heterogeneously structured samples. P. roqueforti proved useful as a reference fungus, as it will not amplify from most terrestrial environmental sources as readily as Geotrichum candidum, which was used as reference by MSQPCR. Examining potential reference fungi may be done through checking primer-sensitivity and possible PCR products against nucleotide-databases (in silico PCR), if environmental sequences are available.

The study disproved a bias because of sharply differing DNA amounts between samples and calibrators. However, supplying calibrators with nDNA prior to extraction significantly reduced disparity between replicate extractions and can therefore be recommended in future experiments. For all assays, a detection threshold was defined
and was recommended as a convenient routine for future applications, for several reasons. Meaningful results near the theoretical detection limit demand appropriate replicates to overcome stochastic effects (Pecoud and Jacob, 1996). Yet, replication conflicts with the need for high-throughput along with low-cost, which is necessary for large-scale surveillance. Additionally, limited false positive signals were provoked in a strongly artificial setup with huge overloads of DNA from a single-related fungus. A similar behavior cannot be ruled out for other environmental samples. However, limited detection suggests low-abundance, which renders an allergenic response less likely in most sensitized individuals, considering the dose dependence of allergenic immune reactions (Arts et al., 2006). Yet, thresholds for respiratory allergens are still not conclusively defined (Ward et al., 2010). Until the immunological relations are better understood, a conservative detection limit of $5 \times 10^4$ CE/g is a straightforward strategy to overcome such issues. It could be used in analogy to the threshold estimated for air samples with $1 \times 10^5$ spores of Alternaria per m$^3$ air and $3 \times 10^4$ spores of Cladosporium per m$^3$ air (Graeven, 1975).

An important finding for large-scale applications was the high reproducibility of results in artificial setups as well as in all environmental samples. This finding implies that our mode of sample preparation is generally appropriate without the need of extraction replicates. This situation can facilitate the processing of large sample numbers and enables a rapid indication of the allergenic potential of a given outdoor environment. Similarly, technical replicates of the qPCRs can be reduced to a minimum, as suggested by Kitchin et al. (2010) and Bengtsson et al. (2006), among others.

4.2. Quantifying allergenic fungi in environmental samples

The results from the postharvest period clearly demonstrate that decomposing plant debris is a larger reservoir of the allergenic fungi Alternaria, Cladosporium, and Fusarium than the soils we studied. This result suggests a focus on sampling decomposing plant constituents during this period. Bulk soil in the winter half-year is of negligible importance for fungal allergenicity, with the possible exception of Cladosporium. In contrast, fields with exposed plant residues, such as those, which are often seen with cover crops or green manure, may be of high-concern as a reservoir for local dispersal of fungal allergens or allergen producers and require focused studies. The species-specific decline in levels of test fungi from decaying shoots to bulk soil reflects the quality of the nutrient source available. The strong decline of Alternaria on nearing the winter season in comparison with its high-prevalence at harvest can be explained by its mesophilic physiology and growth as an endophyte in a wide range of plants. Analogous results of a decline during winter and a peak around harvest time were obtained by airborne Alternaria spore monitoring (Fernandez-Rodriguez et al., 2014), demonstrating the link between the two steps (along the route taken by outdoor fungal allergens to arrive in the human body) of growth of allergenic fungi and dispersal in the air. However,
C. cladosporioides showed at most a marginal decline in the environmental sample materials with time. Thus, all tested sample types may serve as a reservoir for the species and lead to a high-abundance found in airborne spore monitoring (Fernandez-Rodriguez et al., 2014), and, in the present study, in plant samples at harvest. Furthermore, results from the postharvest sampling suggest that during the winter period, effects of different management were strongly superimposed by individual characteristics of the fields. Similarly, Schneider et al. (2010) and Hagn et al. (2003) found only minor influences of conventional and organic farming systems on soil fungal communities and identified the sampling time as the most influential factor of community structure. The high-levels of C. cladosporioides and A. alternata in all plant materials before harvest indicate that their establishment is inevitable. This confirms Burge’s (2002) proposal that plant materials are the primary source of airborne fungi. Interestingly, an absolute difference between C. cladosporioides and A. alternata of a similar magnitude, as in our study, has also been found for airborne spores (Fernandez-Rodriguez et al., 2014). This finding suggests that sampling source materials of allergens could serve as an alternative to air sampling for measuring environmental fungal allergen potential. Such sampling would obviate the installation and maintenance of impact-sampling devices, a measure strongly limiting temporal and spatial resolution owing to its cost.

The pre-harvest results showing differences between fungal loads of wheat under organic and conventional farming suggest that agricultural practices affects the amounts of allergenic fungi growing on fields. In our case, management differences comprised several factors, such as different tillage systems, fertilizers, pesticides, and crop rotation, precluding general conclusions about fungal allergens in organic or conventional systems. The influence of specific management practices on allergenic fungal growth and dispersal awaits more detailed study. The high numbers of A. alternata and C. cladosporioides in air samples and their broad host range are reflected in our results. We found identical fungal colonization of rye and wheat under identical farming practices. Along with the results from other cereals (oat and barley) at a different location, this result suggests that spotting, for example, on grids or gradients at a defined spatial scale, would be a sufficient strategy for larger surveys. Strongly elevated counts of fungi in pea and field beans highlight the importance of a focused observation of cover crops, as many of them are legumes and might increase the number of fungal airborne allergens during winter.

Our pilot tests provide a means of planning monitoring programs. Two of the assays used here (A. alternata and C. cladosporioides), represent a core set of outdoor allergenic fungi (e.g., Simon-Nobbe et al., 2008) and should be complemented with additional assays, according to the environment sampled. In our example, the additions were F. culmorum and the T. viride group, known as minor allergenic fungi often reported from the sampled environment.

The present study may serve as a template for using or developing comparably built assays for individual needs as well as for extensive surveillance. Together with the MSQPCR application for indoor dust and air-sampling applications (e.g., Medkina et al., 2007), this study contributes to a comprehensive quantitative picture of the route leading from allergen-producing sources to dispersal and human contact.

5. Conclusion

The standardized protocol and suggested line of action allow rapid indication of the actual allergenic fungal potentials in any plant or soil sample. Our pilot experiments provide strong evidence for the ubiquity and quantity of major fungal allergen producers on a range of agricultural plants. The results also suggest a focus on decaying plant debris for sampling allergenic fungi during winter and the inclusion of cover crops along with a range of other crops to evaluate the abundance of outdoor allergenic fungi in monitoring programs during the growing season.

6. Outlook

Quantifying allergenic fungi is a first step toward actually defining outdoor fungal allergenic potentials. The next step forward is to collect data from different environments and to connect the presence of fungi with probabilities of their release into the air. In due course, this measure will enable developing the estimates for the risk of contact of fungal environmental allergens with human immune systems. It will thereby support recommendations for personal avoidance strategies as well as approaches to source control (Burge and Rogers, 2000) or mitigation of release by farming operations.

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Appendix A Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.scitotenv.2015.05.056.

References


Supplementary Information

Contents:
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       1. Univariate analyses of the post-harvest data
       2. Multivariate analyses of the post-harvest data
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       1. Overview of pre-harvest ANOVA analyses
       2. Outputs of fitted ANOVA models
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SL.6 Supplementary References
SL.1 Choice of DNA-extraction method

Four techniques for soil DNA extraction were evaluated with bulk soil from twelve different plots of the Scheyern research site. Bulk soil was presumed to have the highest concentration of PCR-inhibitors (e.g. humic acids) and lowest amount of fungal DNA. Methods PCS (Töwe et al., 2011) and SV (Sagova-Marecka et al., 2008) are phenol-chloroform based extractions optimized for soil-samples, whereas method PS and MV root on the PowerSoil DNA Isolation Kit (Mo-Bio Laboratories Inc., Carlsbad CA, USA), PS according to the manufacturer’s guidelines and MV with a pretreatment of CaCO₃ to the soil (Sagova-Marecka et al., 2008). Based on the data shown in suppl. table 1, method PS was chosen for all further analyses.

<table>
<thead>
<tr>
<th>Method</th>
<th>DNA / µg [0.5 g dry soil]</th>
<th>Absorbance 260 nm / 280 nm</th>
<th>Absorbance 260 nm / 230 nm</th>
<th>Fragmentation DNA</th>
<th>PCR* from undiluted DNA</th>
<th>Handling</th>
<th>Costs</th>
<th>Total score</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCS</td>
<td>37.2 (59) 3</td>
<td>1.39 (8) 2</td>
<td>1.94 (7) 1</td>
<td>6 - 3 kbp 2</td>
<td>weak 1</td>
<td>2</td>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>SV</td>
<td>158 (43) 5</td>
<td>1.74 (4) 4</td>
<td>2.07 (24) 1</td>
<td>8 - 3 kbp 3</td>
<td>strong 1</td>
<td>2</td>
<td>4</td>
<td>22</td>
</tr>
<tr>
<td>PS</td>
<td>83.2 (42) 4</td>
<td>1.74 (3) 4</td>
<td>-1.00 (57) 4</td>
<td>8 - 6 kbp 4</td>
<td>overload 5</td>
<td>4</td>
<td>2</td>
<td>27</td>
</tr>
<tr>
<td>MV</td>
<td>38.1 (15) 3</td>
<td>1.60 (5) 3</td>
<td>-0.18 (18) 4</td>
<td>&gt; 10 kbp 5</td>
<td>overload 5</td>
<td>2</td>
<td>2</td>
<td>24</td>
</tr>
</tbody>
</table>

Suppl. table 1: Evaluation of soil-DNA extraction methods. n [soil-DNA-extractions] = 12; brackets: % standard deviation; italics: score (5 = excellent, 0 = fail); *ITS1/ITS4 (White et al., 1990)

SL.2 Review and choice of qPCR assays

Primer-probe combinations for fungal groups relevant for the study were found in existing literature (Suppl. table 2). To find the necessary reference fungus for outdoor agricultural environments, oligo-sequences from candidate assays were aligned with ModellInspector (release 5.6.8.3, Genomatix Software GmbH, Munich) against GenBank database (release 184 for invertebrates and plants). Numbers of templates showing in silico amplification for the tested assays were 21 for “Geo” an assay on Geotrichum candidum strain UAMH7863, which is the reference fungus for MSQPCR (Haugland and Vesper, 2002), 21 for “Gcand2” on another assay on G. candidum (Haugland and Vesper, 2002) and 35 for “Proq” an assay on P. roqueforti (Haugland and Vesper, 2002). The scarce environmental information on the hits in the searches lead us to assume an approximately equally low chance of native growth in our sampling materials soil and plant debris. For its easy handling, we selected P. roqueforti as reference fungus for the study.

2
Suitability of *P. roqueforti* as reference fungus was further tested by applying the corresponding assay on non-spiked samples (soil-root and soil-stem congeries) from 8 different wheat-plots. In agreement with the in silico results, *P. roqueforti* was not detected to considerable extents: 7 of 16 independent samples showed minimal amplifications of 180 ± 57 CE / g, being without consequences for regular conidial supplements of 4*10^5 conidia / sample.

<table>
<thead>
<tr>
<th>Target organism</th>
<th>original name of assay</th>
<th>Oligo-sequences 5'-3': F (forward), R (reverse), P (probe)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alternaria alternata</em></td>
<td>Aatr*</td>
<td>F GGCGGGCTGGAAACCTC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R GCAATTACAAAAAGGTTTATGTGTCGTA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P TTACAGCCCTTGCTGATTTATGCCCTTGCTT</td>
</tr>
<tr>
<td><em>Cladosporium cladosporioides</em></td>
<td>Cclad1*</td>
<td>F CATTACAAATGGACCCTGGCTAAC</td>
</tr>
<tr>
<td>svar.1</td>
<td></td>
<td>R CCCGGAGGGAACAGAG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P CCCGGAGATGTTGTAACCTACCTTGCTTGGC</td>
</tr>
<tr>
<td><em>Trichoderma viride/atrovenide</em></td>
<td>Tviri*</td>
<td>F CCAAAATCCTGGAACCA</td>
</tr>
<tr>
<td><em>kaningi</em></td>
<td></td>
<td>R TCGCAGAGGCAGACGGTACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P CCAAACGGATGCTGCTCCGGC</td>
</tr>
<tr>
<td><em>Fusarium culmorum</em></td>
<td>FcU*</td>
<td>F TTGGCTGATGGGGGACTGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CTATGGAATCGCTGACGGTACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P CCTGCATGCTGCCCTCCCAATGTC</td>
</tr>
<tr>
<td><em>Penicillium roqueforti</em></td>
<td>Proqu*</td>
<td>F CGGGCCGGCCGGTAAAC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R TAAATTAATTTATATTTTGTTCAGACTGCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P CGGGCCGGCCGGAGACA</td>
</tr>
<tr>
<td>Fungi</td>
<td>5.8*</td>
<td>F AACCGCCAAACGAGATCTCGTGG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R CCATGCAAGAAGCTGATGTTACC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>P CATGCTGAAGAACGCCAGCGCAGAATGAC</td>
</tr>
</tbody>
</table>

Suppl. table 2: qPCR assays. Probes were labeled according to respective reference (5-FAM, 3-TAMRA); *Haugland and Vesper (2002), **Cullen et al. (2005), assay renamed

**SL3 performance of the selected assays**

All qPCR assays were reproducibly deployed on the various sample types as shown in suppl. table 3.

False positives were detected in non-template controls and highly concentrated non-target DNA samples in late cycles (suppl. Table 3).

<table>
<thead>
<tr>
<th>Assay</th>
<th>E (R² ≥ 0.998)</th>
<th>Independent runs of assay</th>
<th>Highest provoked false positives [CE / extract]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aatr</td>
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<tr>
<td>Cclad1</td>
<td>1.92 ± 0.02</td>
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<td>17</td>
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<td>Tviri</td>
<td>1.84 ± 0.01</td>
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<tr>
<td>FcU</td>
<td>1.87 ± 0.00</td>
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<td>Proqu</td>
<td>1.69 ± 0.03</td>
<td>27</td>
<td>29 (unprovoked: non-template control)</td>
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<tr>
<td>Uni</td>
<td>1.93 ± 0.02</td>
<td>7</td>
<td>not applicable</td>
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Suppl. Table 3: general properties of the used assays during validation of the qPCR assays
SL.4 Statistical analyses

SL.4 A: Post-harvest time series

1. Univariate analyses of the post-harvest data

Outputs of fitted ANOVA models calculated with R (R Core Team, 2013) in summarized form; Input data were augmented by 2 and transformed to base-10 logarithms.

### C. cladosporioides

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<tr>
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### A. alternata

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<td>4.381</td>
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### F. culmorum

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### Trichoderma viride/atroviride/koningii

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<td>0.614</td>
<td>2.746</td>
<td>0.0165 *</td>
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<tr>
<td>Residuals</td>
<td>98</td>
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<td>0.224</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
2. Multivariate analyses of the post-harvest data

QPCR results were ordered in a way that allowed the fungal group and sampling material to be treated as effect-variables and analyzed with the Adonis function (Permutational Multivariate Analysis of Variance Using Distance Matrices) using Bray-Curtis dissimilarities and 10000 permutations both implemented in the vegan-package (Oksanen et al., 2013) for R.

Adonis results (below) and visualizations via dimension-reductions (suppl. figure 1) highlight the importance of the sampling time in relation to the likewise significant factors individual field, respectively agricultural management.

Summarized Adonis outputs:

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<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
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<td>timepoint</td>
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<td>4.3775</td>
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<td>manag.</td>
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<td>0.1837</td>
<td>0.18367</td>
<td>1.5765</td>
<td>0.05197</td>
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<td>2.3301</td>
<td>0.11651</td>
<td>0.65938</td>
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<tr>
<td>Total</td>
<td>23</td>
<td>3.5338</td>
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</table>

Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

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<tr>
<th>Df</th>
<th>SumsOfSqs</th>
<th>MeanSqs</th>
<th>F.Model</th>
<th>R2</th>
<th>Pr(&gt;F)</th>
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<tbody>
<tr>
<td>field</td>
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<td>1.3621</td>
<td>0.19459</td>
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<td>Residuals</td>
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<td>Total</td>
<td>23</td>
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Signif. codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
Suppl. figure 1:
Two visualizations of the combined variance in abundance of the tested fungi between the fields at the sampling dates: Principal Component Analysis (PCA) and Yue Clayton similarity (YC) after Kruskal’s Non-metric Multidimensional Scaling. PCA using R function procomp (stats-package 3.15.3) with square-root transformation and YC calculated as in (http://www.mothur.org/wiki/Theetayc, April 2014) with non-metric multidimensional scaling using isoMDS (MASS package 7.3-13).

1. Overview of pre-harvest ANOVA analyses

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<tr>
<th>assay</th>
<th>factor</th>
<th>n / category (n with N = 0)</th>
<th>Df</th>
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<th>F value</th>
<th>P</th>
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<td>plant-section</td>
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<td>1</td>
<td>3.781</td>
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<td>&lt; 0.001</td>
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<tr>
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<td>plant-section</td>
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<td>1.152</td>
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<tr>
<td></td>
<td>tillage</td>
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<td>0.563</td>
<td>3.788</td>
<td>0.048</td>
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<tr>
<td>Trichoderma group</td>
<td>plant-section</td>
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<td>13.035</td>
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</tr>
<tr>
<td>F. culmorum</td>
<td>plant-section</td>
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<td>17.739</td>
<td>17.56</td>
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</tr>
<tr>
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<tr>
<td>C. cladosporioides</td>
<td>plant-section</td>
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<td>1</td>
<td>17.995</td>
<td>230.270</td>
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<tr>
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<td>1</td>
<td>29.059</td>
<td>280.932</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>fertilizer</td>
<td>&gt; 24</td>
<td>1</td>
<td>0.502</td>
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<tr>
<td></td>
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<tr>
<td>C. cladosporioides</td>
<td>management</td>
<td>&gt; 18</td>
<td>1</td>
<td>11.046</td>
<td>152.99</td>
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<td>9.521</td>
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Suppl. table 4:
Overview of pre-harvest ANOVA analyses. Significant (p < 0.05) factors as seen in fitted ANOVA models (below) on log-transformed values. Df: degrees of freedom, SSq: sums of squares, IA: interaction component.
2. Outputs of fitted ANOVA models

Calculated with R, presented in summarized form; input data were augmented by 2 and transformed to base-10 logarithms. As organic and conventional treatments have different plot designs, the plots were grouped into three sets for separate ANOVA, resembling comparable treatments: (1) all conventional plots, (2) all organic plots and (3) all wheat plots.

<table>
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<td>Mean Sq</td>
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<td>Mean Sq</td>
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<td>Mean Sq</td>
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<td>Mean Sq</td>
<td>F value</td>
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SI.5 Supplementary table 4: qPCR measurements used for Figure 3

Values in conidial equivalents (CE) per gram (dry material).

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SI.6 Supplementary References


Urban dust microbiome: Impact on later atopy and wheezing.


*contributed equally to this work

Article submitted to *Environmental Health Perspectives* on November 20, 2015
Urban dust microbiome: Impact on later atopy and wheezing.

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Joachim Heinrich*a, PhD, and Karin Pritsch*t, PhD

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1 Funding:

2 The first two years of the LiSAplus study were mainly supported by grants from the Federal
3 Ministry for Education, Science, Research and Technology, Helmholtz Center Munich (former
4 GSF). The four, six, and ten year follow-up examinations of the Munich LiSAplus study were
5 covered by Helmholtz Center Munich (former GSF). The European Study of Cohorts for Air
6 Pollution Effects has received funding from the European Community’s Seventh Framework
7 Program (FP7-2007-2011) under grant agreement number: 211250.

8

9 Key words:

10 Dust microbiome, microbial diversity, atopy, wheezing, and children

11

12 Short summary:

13 Exposure to higher fungal diversity shortly after birth is associated with a significant lower risk
14 for developing allergic sensitization to aero-allergens at 6 years, but not in later childhood.
ABSTRACT

Background:
Investigations in urban areas have just begun to explore how the indoor dust microbiome may affect the pathogenesis of asthma and allergic diseases. We aimed to investigate the early fungal and bacterial microbiome in house dust with allergic sensitization and wheezing later in childhood.

Methods:
Individual dust samples from 189 homes of the LISAplus birth cohort study were collected shortly after birth from living room floors and profiled for fungal and bacterial microbiome. Fungal and bacterial diversity was defined by the Simpson diversity index. Information on wheezing outcomes and co-variates until the age of 10 years was obtained by parental questionnaires. Information on specific allergic sensitization was available at 6 and 10 years. Logistic regression and General Estimation Equation (GEE) models were used to examine the relationship between microbial diversity and health outcomes.

Results:
Logistic regression analyses revealed a significantly reduced risk of developing sensitization to aero-allergens at 6 years and ever wheezing until the age of 10 years for exposure to higher fungal diversity (adjusted Odds Ratio aOR (95%CI): 0.26 (0.10-0.70), and 0.42 (0.18-0.95), respectively), in adjusted analyses. The associations were attenuated for the longitudinal analyses (GEE) until the age of 10 years. There was no association between higher exposure to bacterial diversity and the tested health outcomes.

Conclusion:
Higher early exposure to fungal diversity might help to prevent from developing sensitization to aero-allergens in early childhood, but the reasons for attenuated effects in later childhood require further prospective studies.

Words: 242
INTRODUCTION

Exposure to higher microbial loads and higher microbial diversity in rural settings has been observed to confer protection for asthma and allergic diseases in a number of European studies (Karvonen et al. 2014; von Mutius and Vercelli 2010). In this context, it has been suggested that microbial profiles in house dust around birth, the ‘dust microbiome’, might be in particular decisive to confer protection by affecting the children’s developing immune system towards a non-allergic immune response (Karvonen et al. 2014; Valkonen et al. 2015). Evidence regarding the microbiome composition in dust from urban environments and its influence on the occurrence and development of allergic diseases is still scarce and comprehensive understanding is lacking. The microbial profile in urban environments might differ considerably from those in rural areas in levels, composition and diversity (Pakarinen et al. 2008) and therefore, might also have different effects on atopic outcomes. Until now, the assessment of the urban microbiome in dust has been only considered in a few studies with small sample sizes (not exceeding 100 subjects) or studies that were mainly focused on exposure assessment rather than on health outcomes (Adams et al. 2013a, 2014, 2013b; Barberán et al. 2015; Dannemiller et al. 2015, 2014; Kembel et al. 2012; Lynch et al. 2014).

Moreover, to conclude on the impact of early exposure to the urban dust microbiome in relation to health outcomes in later childhood, cohort studies with a prospective study design and appropriate analyses methods are warranted.

In the present study, we investigated the diversity of the fungal and bacterial microbiome in dust from a population based birth cohort from the city of Munich, Germany. We aimed to study whether early life exposure to fungal and bacterial diversity is related to the development of allergic sensitization and wheezing later in childhood. These outcomes are major risk factors for asthma and allergic diseases.
MATERIALS AND METHODS

1. Study overview and participants

LISAplus (The influence of life-style factors on the development of the immune system and allergies in East and West Germany PLUS the influence of traffic emissions and genetics study) is an ongoing birth cohort study with four research centers in Germany (Munich, Leipzig, Bad Honnef and Wesel). Screening, recruitment and exclusion criteria have been described in detail elsewhere (Heinrich et al. 2002; Zutavern et al. 2006). The current analysis is based on a subgroup of children from the Munich study center with an available dust sample from the living room floor obtained at three months of age and follow-up information on outcomes until ten years of age (N = 189).

2. Assessment of health outcomes

The subjects were tested for specific sensitization at six and ten years. Specific allergic sensitization was defined as a positive response (> 0.35 kU/l) to the ‘sx1 aero-allergen mixture’ (timothy, rye, mugwort, mite [Dermatophagoides pteronyssinus], cat, dog and mould [Cladosporium herbarum] allergens). Wheezing in the past 12 months was obtained at age of 6, 12, 18, 24 months and at 4, 6, and 10 years of age.

3. Dust sampling and assessment of the fungal and bacterial microbiome

Settled dust samples from living room floors in the area of Munich (radius: 37.5 km, Figure 1) were obtained by trained inspectors when the children were 2-3 months old by using vacuum cleaners (Phillips, Hamburg, Germany) equipped with ALK filter holders (ALK, Hørsholm, Denmark) containing a paper filter. The sampling was done by vacuuming 1 m² for 2 minutes for textile surfaces or 4 m² for 4 minutes for smooth floors. The sampling period lasted 301 days. A detailed description of the dust sampling and analysis procedures has been published previously (Casas et al. 2013; Heinrich et al. 2002).
Frozen filter boxes with vacuumed dust were equilibrated to ambient conditions in a clean PCR chamber with deactivated airflow for 60 minutes. Dust was released from the filter boxes, freed from obvious extraction obstacles (stones, etc.) and 100 mg were used to extract DNA with a PowerSoil-htp96 Soil DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, USA). For tRFLP DNA-fingerprinting, DNA was PCR-amplified using a TopTaq DNA polymerase kit (Qiagen, Hilden, Germany) with primers ITS1F (5’-CTTGGTCATTTAGAGGAAT-3’) (Gardes and Bruns 1993) and ITS4 (5’-TCCTCGGGTATGGATATGC-3’) (White et al. 1990) for fungal ITS (internal transcribed spacer) DNA, or Bac27f (5’-AGAGTTTGATCMGGCTCAG-3’) (Jiang et al. 2006) and 907r (5’-CCGTCATTTGAGTTTT-3’) (Mühling et al. 2008) for bacterial 16S rRNA genes.

Forward primers were labelled with 6-FAM and reverse primers with 6-HEX fluorescent dyes, respectively. PCR profiles were [4 min 94 °C; 32 cycles of 60 s 94 °C, 60 s 50 °C, 90 s 72 °C; 5 min 72 °C] (fungi) and [5 min 94 °C; 30 cycles of 45 s 94 °C, 45 s 59 °C, 45 s 72 °C; 5 min 72 °C] (bacteria). Products from two PCR reactions were pooled, purified and digested with restriction enzyme HpyCH4IV (fungi) or MspI (bacteria). HpyCH4IV was selected after in silico enzyme digestions using REPK v1.3 (Collins and Rocap 2007) against an artificial set of fungal sequences commonly found in dust. Cleaned fragments were transferred to HiDi Formamide (Applied Biosystems, Foster City, USA) containing MapMarker 1000-ROX (1:400; Bioventures, Murfreesboro, USA) and separated with an ABI 3730 capillary sequencer (Applied Biosystems). Raw fragment tables were built with peak-scanner 2.0 (Applied Biosystems). T-REX v1.14 (Culman et al. 2009) was used for noise filtering (peak height, multiplier 1) and binning of fragments (threshold 1 bp).

4) Fungal and bacterial diversity in dust

In order to assess possible relationships between the fungal and the bacterial microbiome in dust with later health outcomes, we determined the diversity of the fungal and bacterial
microbiome with the Simpson index (Simpson 1949) (shown as 1-D, i.e. range from 0 (no
diversity, all individuals belong to the same species) to 1 (maximum diversity)). Thus, we
accounted for both richness and proportion of each species within a sample. For doing so, OTU
(operational taxonomic unit) abundances were rarefied to the lowest amount of signal present
in the samples and the Simpson index calculated for each sample (vegan (Oksanen et al. 2013)
and GUniFrac package (Chen 2012) in R (R Team 2012)). This step was repeated 10^3 times and
averaged. Results were calculated separately for forward and reverse terminal restriction
fragments (including labelled forward or reverse primers) and averaged.

5) **Statistical analysis**

In order to investigate possible relationships between exposure to fungal and bacterial
diversity (Simpson index) with later allergic sensitization and wheezing outcomes, logistic
regression and general estimation equation (GEE) models were used with the exposure (fungal
and bacterial diversity) expressed in tertiles. The logistic regression and GEE models have been
adjusted for sex, maternal education and season of dust sampling. All results are presented as
adjusted odds ratio (aOR) with corresponding 95% confidence interval (95% CI). Statistical
analyses were performed using the R programming environment (R Team 2012).

**RESULTS**

The study population characteristics are depicted in **Table 1**. About two-third of the mothers
(68%) held a high educational level compared to mothers with low or medium education
(32%). During the months in summer and autumn, more dust samples have been obtained
compared to winter and spring. Based on the Simpson index, **Table 1** displays that the average
diversity was higher for the fungal microbiome compared to the bacterial microbiome. There
was very weak correlation between fungal and bacterial diversity (Spearman's Rho: -0.05). At
the six year follow-up, 27% of the children were sensitized to aero-allergens with 40% at ten
years. Ever wheezing at the age of 10 years was reported for 43% of the children.

Regression analyses

All relationships between exposure to higher microbial diversity around birth and health
outcomes followed an inverse association (Table 2) which was significant for higher exposure
to fungal diversity and sensitization to aero-allergens at 6 years (3rd tertile versus 1st tertile:
0.26 (0.10-0.70)), adjusted for covariates. High fungal diversity in dust conferred also
protection for ever wheezing until the age of 10 years (3rd tertile versus 1st tertile: 0.42 (0.18-
0.95)). However, in the longitudinal view (GEE models), considering the impact of several
follow-ups and their correlation with each other, the inverse effects attenuated. The
association between exposure to higher fungal diversity and sensitization to aero-allergens as
well as wheezing did not attain statistical significance (0.63 (0.25-1.64), and 0.57 (0.26-1.24),
respectively). There was no significant association between exposure to bacterial diversity with
any of the outcomes tested.

DISCUSSION

To the best of our knowledge, the present prospective study is the first that specifically
considered the fungal and the bacterial microbiome in more than 100 households in an urban
environment. We observed that a higher exposure to fungal diversity in house dust around
birth was significantly inversely related to aero-allergen sensitization status at 6 years as well
as ever wheezing until the age of 10 years. However, considering several follow-up time points
during the study period, the magnitude of the effects attenuated and the association did not
attain statistical significance in the longitudinal view.
For farm and rural environments, studies have shown that early microbial exposure seems to be crucial for non-allergic immune response later in childhood and adulthood. Ege et al. (2011) observed in a cross-sectional analysis that children growing up on farms were exposed to a greater diversity of fungal and bacterial species, resulting in a lower prevalence of childhood asthma and atopy. As against farm studies, investigations in urban areas have just begun to explore how the indoor dust microbiome may affect the pathogenesis of asthma and allergic diseases. The CHAMACOS birth cohort study in California, U.S., used next-generation DNA sequencing of fungal ITS regions describing the fungal microbiome in settled house dust collected at 12 months of age. In this small case-control study (13 asthma cases and 28 controls), it has been observed that the asthma risk at 7 years of age was significantly increased for lower fungal diversity in dust within the first year of life (Dannemiller et al. 2014).

One birth cohort study (URECA) across 104 children residing in an exclusively urban environment investigated the association of combined early life exposure to allergens and bacteria on wheezing and atopic outcomes (Lynch et al. 2014). Lynch and colleagues observed that both, exposure to high levels of allergen and a certain subset of bacteria taxa decreased the risk of allergic sensitization and wheezing outcomes at the age of 3 years. Our present study partly confirms what has been found recently, however, a unique feature of our study is the long follow-up period until later childhood. Although we also observed inverse associations of higher microbial exposure in relation to allergic sensitization and wheezing, the effects were only significant for higher fungal diversity and only for early childhood (5 years) but not at later age (10 years). As of today, no study in an urban setting could confirm protective effects of higher microbial exposure in relation to atopic outcomes until later childhood or young adulthood. We suggest that a possible reason might be that with increasing age, the school environment and activities conducted in different places might start to become more important and the daily individual microbial exposure may change in composition and relevance (Tischer et al. 2015).
The present study has important strengths, such as a prospective study design, a larger sample size compared to previous studies and a longer follow-up period until the age of 10 years. However, our study faced some limitations, which should be noted. Although we had nearly double the sample size as compared to the available studies in the U.S. on the subject (Dannemiller et al. 2014; Lynch et al. 2014), caution is warranted when interpreting the findings, due to the reduced statistical power in the adjusted regression models. Furthermore, our analysis did not account for the phylogenetic relationship between bacteria and fungi within the samples as no sequence information was available. Moreover, it is not yet entirely clear how storage of dust samples over a period of several years might affect microbial DNA. Therefore, an unknown, not quantifiable storage effect might have biased the results of our study.

Conclusion

Our study is an important contribution to the field of the urban dust microbiome in relation to atopic and respiratory health. We observed a significant reduced risk for developing specific allergic sensitization to aero-allergens in early childhood after exposure to higher fungal diversity around birth but with attenuated effects until later childhood.
REFERENCES


Adams RI, Mileto M, Taylor JW, Bruns TD. 2013a. Dispersal in microbes: fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. ISME J 7:1460; doi:10.1038/ismej.2013.84.


### Table 1: Characteristics of the study population

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Study population</strong></td>
<td>189</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>87/189 (46%)</td>
<td></td>
</tr>
<tr>
<td><strong>Maternal education</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low &amp; Medium</td>
<td>60 / 187 (32%)</td>
<td></td>
</tr>
<tr>
<td>High</td>
<td>127 / 187 (68%)</td>
<td></td>
</tr>
<tr>
<td><strong>Season of dust sampling</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Winter</td>
<td>41 / 189 (22%)</td>
<td></td>
</tr>
<tr>
<td>Spring</td>
<td>29 / 189 (15%)</td>
<td></td>
</tr>
<tr>
<td>Summer</td>
<td>57 / 189 (30%)</td>
<td></td>
</tr>
<tr>
<td>Autumn</td>
<td>62 / 189 (33%)</td>
<td></td>
</tr>
<tr>
<td><strong>Fungal diversity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; tertile</td>
<td>[0.403 - 0.863]</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; tertile</td>
<td>[0.863 - 0.931]</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; tertile</td>
<td>[0.931 - 0.977]</td>
<td></td>
</tr>
<tr>
<td><strong>Bacterial diversity</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; tertile</td>
<td>[0.179 - 0.693]</td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; tertile</td>
<td>[0.693 - 0.835]</td>
<td></td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; tertile</td>
<td>[0.835 - 0.941]</td>
<td></td>
</tr>
<tr>
<td>IgE aero-allergens (≥ 0.35 kU/l) – 6 years</td>
<td>43/159 (27%)</td>
<td></td>
</tr>
<tr>
<td>IgE aero-allergens (≥ 0.35 kU/l) – 10 years</td>
<td>56/141 (40%)</td>
<td></td>
</tr>
<tr>
<td>Wheezing ever – 10 years</td>
<td>73 / 170 (43%)</td>
<td></td>
</tr>
</tbody>
</table>
Table 2: Adjusted* odds ratios (aORs 95% CI) for the association between fungal and bacterial diversity (Simpson index, tertiles) and health outcomes

<table>
<thead>
<tr>
<th>Logistic regression models</th>
<th>Fungal diversity</th>
<th>Bacterial diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitization to aero-allergens (6y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd tertile vs 1st tertile</td>
<td>0.67 (0.28, 1.59)</td>
<td>0.56 (0.23, 1.35)</td>
</tr>
<tr>
<td>3rd tertile vs 1st tertile</td>
<td><strong>0.26 (0.10, 0.70)</strong></td>
<td>0.46 (0.19, 1.14)</td>
</tr>
<tr>
<td>Sensitization to aero-allergens (10y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd tertile vs 1st tertile</td>
<td>1.14 (0.48, 2.73)</td>
<td>0.57 (0.25, 1.34)</td>
</tr>
<tr>
<td>3rd tertile vs 1st tertile</td>
<td>1.04 (0.42, 2.59)</td>
<td>0.43 (0.17, 1.07)</td>
</tr>
<tr>
<td>Wheezing ever (10y)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd tertile vs 1st tertile</td>
<td>0.59 (0.27, 1.28)</td>
<td>0.60 (0.28, 1.32)</td>
</tr>
<tr>
<td>3rd tertile vs 1st tertile</td>
<td><strong>0.42 (0.18, 0.95)</strong></td>
<td>1.00 (0.47, 2.16)</td>
</tr>
</tbody>
</table>

GEE models (longitudinal analysis)

| Sensitization to aero-allergens until 10y  |                  |                     |
| 2nd tertile vs 1st tertile                | 0.93 (0.36, 2.38) | 0.77 (0.31, 1.88) |
| 3rd tertile vs 1st tertile                | 0.63 (0.25, 1.64) | 0.56 (0.22, 1.44) |

| Wheezing until 10y                        |                  |                     |
| 2nd tertile vs 1st tertile                | 0.78 (0.40, 1.52) | 0.74 (0.36, 1.53) |
| 3rd tertile vs 1st tertile                | 0.57 (0.26, 1.24) | 0.99 (0.52, 1.86) |

*Adjusted for sex, maternal education and season of dust sampling.
Figure 1: Map of the Munich urban area

The map covers the Larger Urban Zone of Munich. It includes the position of 189 sampled households located within a radius of 37.5 km from the center of Munich.
IV  Fungal and bacterial communities in indoor dust follow different environmental determinants

Weikl F, Tischer C, Probst AJ, Heinrich J, Markevych I, Jochner S, Pritsch K.

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Fungal and Bacterial Communities in Indoor Dust

Follow Different Environmental Determinants

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Abstract

1 People spend most of their time inside buildings and the indoor microbiome is a major part
2 of our everyday environment. It affects humans' wellbeing and therefore its composition is
3 important for use in inferring human health impacts. It is still not well understood how
4 environmental conditions affect indoor microbial communities. Existing studies have mostly
5 focussed on the local (e.g., building units) or continental scale and rarely on the regional
6 scale, e.g., a specific metropolitan area.
7 Therefore, we wanted to identify key environmental determinants for the house dust
8 microbiome from an existing collection of spatially (area of Munich, Germany) and
9 temporally (301 days) distributed samples and to determine changes in the community as a
10 function of time.
11 To that end, settled dust samples that had been collected from the living room floors of 286
12 individual households, were profiled for fungal and bacterial community variation and
13 diversity using microbial fingerprinting techniques. The profiles were tested for their
14 association with occupant behaviour, building characteristics, outdoor pollution, vegetation,
15 and urbanization.
16 Our results showed that more environmental and particularly outdoor factors (vegetation,
17 urbanization, airborne particulate matter) affected the community composition of indoor
18 fungi than of bacteria. The passage of time affected fungi and, surprisingly, also strongly
19 affected bacteria. We inferred that fungal communities in indoor dust changed semi-
20 annually, whereas bacterial communities paralleled phenological periods. These differences
21 in temporal dynamics cannot be fully explained and should be further investigated in future
22 studies on indoor microbiomes.
Introduction

In industrialized countries, people spend a majority of their time indoors, and residential
floor space can surpass the land area in a city [1]. Therefore, the building environment can
be seen as the “modern ecological habitat of Homo sapiens sapiens” with all implications
that may have on human well-being [2].

Indoor microbial communities are a ubiquitous part of the building environment. The
season has been determined to be significant for fungal composition [3-5], but of minor or
no importance for bacteria [6-9]. The outdoor environment has been shown to have a major
impact on indoor the fungal community [4, 7, 10], while occupants [7, 11-14] and their
behaviour (ventilation) [11, 15] have been found to affect bacteria. However, DNA-based
studies with high numbers (> 100) of spatially distributed indoor dust samples mainly
focussed on epidemiology (i.e., the human health effects of microbial communities) [16, 17].

Only recently, studies on a continental and global scale revealed that the indoor microbial
community depended on the environmental parameters in an individual geographic region,
to a minor degree for bacteria, and to a larger degree for fungi [7, 10]. Scales in between
continental overviews and local investigations of individual building units have rarely been
considered in molecular analyses of indoor dust. Influential factors on a continental scale
(e.g., temperature or precipitation [7, 10]) are rather uniform on a regional scale, e.g. in a
specific metropolitan area, and factors that affect an individual building may not be
significant for the surrounding area. To carve out the reasons for the variation in the indoor
fungal and bacterial communities, studies on a regional scale with comprehensive
environmental data are required.
The potential effects of the time of the year on the composition of an indoor microbial community have been analysed by sampling at different times or over short periods of time [4, 5, 8]. Continuous observations (i.e., high frequency temporal sampling of indoor dust over several months) have used spore counts or cultivation [3, 18, 19]. In the adjacent outdoor air environment, seasonal changes have been well described in fungi [3, 20-22] and have recently been observed in bacteria during 14 months of monitoring [23], confirming studies that had shorter time-frames [24-26]. In addition, outdoor airborne bacteria can change within few days [27, 28]. Although large knowledge gaps exist, temporal changes in microbial communities seems to be common in a number of other environments as well [29]. For an actual assessment on how microbial communities in indoor dust are affected by the passage of time, comparing different short windows of time is not sufficient. Instead, it is necessary to perform analyses over a period of several months or years with frequent sampling, but such DNA-based studies are rare.

In this study, we determined the variation and diversity of the fungal and bacterial microbiome in dust samples from 286 households. The samples were distributed spatially (i.e., over an area of Munich, Germany) and temporally (i.e., over 10 months). We used fungal and bacterial fingerprints (terminal restriction fragment length polymorphism – tRFLP [30]) along with associated data sets on environmental parameters.

Our first objective was to identify key indoor and outdoor environmental factors that affected the microbial community. Secondly, we assessed and compared the temporal dynamics of the fungal and dust bacterial communities. Regarding that, we explored whether the existing sample design of spatially unrelated single samples collected in a defined time window could be used to infer the temporal dynamics for fungi and bacteria. In
addition, we showed that the seasonal effects on indoor communities can be different for fungi and bacteria.

Materials and Methods

Study design and sampling

We analysed microbial fingerprints derived from DNA extracts of settled dust from the living room floors of 286 homes. Each home was sampled once within 301 days from April 1998 to February 1999. All dust samples were collected in an urban area in Munich (radius: 37.5 km, S1 Supporting information) as part of the LISApuls study (i.e., The influence of life-style factors on the development of the immune system and allergies in East and West Germany PLUS the influence of traffic emissions and genetics study). LISApuls is an ongoing birth cohort study that has screening, recruitment and exclusion criteria that have been described elsewhere [31, 32]. LISApuls has been approved by local ethics committees (Ethikkommission der Bayrischen Landeärztekammer, Ethikkommission an der Medizinischen Fakultät der Universität Leipzig, Ärztekammer Nordrhein) and written informed consent was obtained from all participating families.

Information on indoor and most outdoor environmental characteristics for the sampled homes was obtained by self-completed questionnaires. Based on the residential addresses, we acquired further information on air pollution from traffic, the surrounding greenness based on satellite-data (i.e., vegetation density, the Normalized Difference Vegetation Index (NDVI)) and the urban index (i.e., the proportion of the built-up area). A detailed description of the environmental characteristics is given in S2 Supporting Information.
Samples were collected by trained inspectors using vacuum cleaners (Phillips, Hamburg, Germany) equipped with ALK filter holders (ALK, Hørsholm, Denmark) containing a paper filter when infants of participating families were two to three months old. A detailed description of the dust sampling and processing has been previously published [31, 33]. The filter boxes were stored below -20 °C.

Microbial fingerprinting

Frozen filter boxes with vacuumed dust were equilibrated to ambient conditions in a clean PCR chamber with the airflow deactivated for 60 minutes. Dust was released from the filter boxes, freed from obvious extraction obstacles (e.g., stones, etc.) and 100 mg were used for DNA extraction with a PowerSoil-htp96 Soil DNA Isolation Kit (Mo-Bio Laboratories, Carlsbad, CA, USA). For tRFLP DNA-fingerprinting, the DNA was PCR-amplified using a TopTaq DNA polymerase kit (Qiagen, Hilden, Germany) with the primers ITS1F (5'-CTTGGTCAATTAGAGGAAATGA-3') [34] and ITS4 (5'-TCCTCCGCTTATGATATGC-3') [35] for fungal ITS (internal transcribed spacer) DNA, or Bac27f (5'-AGAGTTTGATCMTGGCTCAG-3') [36] and 907r (5'-CGGCTACATATTGGGAGT-3') [37] for bacterial 16S rRNA genes. Forward primers were labelled with 6-FAM and reverse primers with 6-HEX fluorescent dyes, respectively. The PCR profiles were 4 min 94 °C; 32 cycles of 60 s 94 °C, 60 s 50 °C, 90 s 72 °C; 5 min 72 °C (fungi) and [4 min 94 °C; 30 cycles of 45 s 94 °C, 45 s 59 °C, 45 s 72 °C; 5 min 72 °C] (bacteria). Products from two PCR reactions were pooled, purified and digested with the restriction enzyme HpyCH4IV (fungi) or Mspl (bacteria). HpyCH4IV was selected after in silico enzyme digestions using REPK v1.3 [38] against an artificial set of fungal sequences commonly found in dust. Cleaned fragments were transferred to HiDi Formamide (Applied
Biosystems, Foster City, CA, USA) containing MapMarker 1000-ROX (1:400; Bioventures, Murfreesboro, TN, USA) and separated on an ABI 3730 capillary sequencer (Applied Biosystems). Raw fragment tables were built with peak-scanner 2.0 (Applied Biosystems). T-REX v1.14 [39] was used for noise filtering (peak height, multiplier 1) and binning of fragments (threshold 1 bp).

**Statistical analyses**

The R programming environment [40] was used for all statistical analyses. Bray—Curtis dissimilarities [41] between the samples (community variation) and biodiversity indices were calculated from $10^3$ times randomly rarefied (to the lowest amount of signal present in one sample) OTU (operational taxonomic unit) abundances using algorithms from the vegan [42] and GUInvFrac [43] packages. The results from forward and reverse terminal restriction fragments (including labelled forward or reverse primers) were averaged. Multivariate testing for the effect of environmental characteristics on the community was conducted using average Bray—Curtis dissimilarity matrices with the Adonis (Permutational Multivariate Analysis of Variance Using Distance Matrices) and MRPP (Multi-Response Permutation Procedure) functions, each with $10^5$ permutations available in the vegan package. Partial Mantel tests ($10^4$ permutations, implemented in the vegan package) were used to test for possible geographic correlations of community dissimilarities.

Microbial diversity was assessed with the Simpson (1—D) and Shannon ($H'$) indices, and seasonal influences were additionally evaluated with Pilou’s Evenness (J) and Richness (S). Details on these calculations are available in the vegan package [42]. Relationships between biodiversity indices and environmental characteristics were assessed using a Wilcoxon...
signed rank test for dichotomous variables and a Kruskall—Wallis test for variables with three or more categories due to the non-normal distribution of fungal and bacterial diversity (Shapiro—Wilk test \( P < 0.01 \)).

Results

Community variation

The variation in the fungal community was more sensitive to the tested environmental determinants than the variation in the bacterial community (Table 1). For indoor factors, variation in the fungal community in the living room floor dust was affected in particular by signs of mould inside the home, the tightness of the windows, heating inside the home, and the type of living room floor (i.e., Adonis \( P < 0.05 \), MRPP \( \delta < 0.05 \); Table 1). In contrast, the bacterial community variation was only significantly affected by ventilation (winter half year, Adonis \( P = 0.05 \), MRPP \( \delta = 0.05 \)), and in tendency (i.e., Adonis \( P = 0.08 \), MRPP \( \delta = 0.02 \)) as well as the position of the home (Adonis \( P = 0.1 \), MRPP \( \delta = 0.05 \)).

For outdoor factors (Table 1), a significant effect on the variation in the fungal community was due to the building age, the surrounding greenery within a 100 m buffer, the urbanization grade (urban index), and particulate matter (< 2.5 \( \mu \)m and coarse particulates) (Adonis \( P \leq 0.05 \), MRPP \( \delta < 0.05 \)). The surrounding greenery within a 30 m buffer (Adonis \( P = 0.06 \), MRPP \( \delta = 0.03 \)) and nitrogen oxides (NO\(_x\)) (Adonis \( P = 0.06 \), MRPP \( \delta = 0.01 \)) were also in tendency associated with the variation in the fungal community. The only outdoor characteristic that may have affected variation in the bacterial community was the position of the home (i.e., the level above ground) (Adonis \( P = 0.1 \), MRPP \( \delta = 0.05 \)). No spatial
correlation with the community variation was observed (simulated \( P > 0.25 \) with \( 10^5 \) replicates for fungi and bacteria in partial Mantel tests conditioned by sampling date).

The fungal and bacterial communities showed a significant change with the time of the year (Table 2); its ecological relevance (MRPP A, chance-corrected within group agreement) was greater than that of all other tested variables (Table 2 and S1 Table). Of all seasonal categorizations, the outdoor phenological period best described the association between the sampling date and the microbial community (fungi: Adonis \( R^2 = 0.19 \), MRPP A = 0.11; bacteria: \( R^2 = 0.34 \), A = 0.21) (Table 2).

Chronological presentations of the first Principal Coordinates (PCo) in a PCo analysis (PCoA) showed different time courses of the community variation for fungi and bacteria (considering all samples, Fig 1). The PCoA indicated that fungal community variation changed semi-annually; it gradually changed from winter to summer and vice versa (for the 1st PCo in Fig 1b: approximately 5% of the total variation between samples occurred during the first 5 months). In contrast, the bacterial community variation changed rapidly and extensively during the phenological periods of early and full spring (for the 1st PCo in Fig 1d: approximately 35% of the total variation between samples occurred during the first 2 months) and gradually returned to a winter state until the beginning of phenological winter.

<table>
<thead>
<tr>
<th>Environmental characteristics</th>
<th>Fungi ( p )</th>
<th>Bacteria ( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indoor characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N° of rooms within the flat</td>
<td>0.75</td>
<td>0.71</td>
</tr>
<tr>
<td>Dampness</td>
<td>0.69</td>
<td>0.16</td>
</tr>
<tr>
<td>Mould at home</td>
<td>0.04</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td>0.03</td>
<td>0.13</td>
</tr>
</tbody>
</table>
Results from Adonis (P) and MRPP (δ), bold: P or δ ≤ 0.05; all R² (Adonis) A (MRPP chance corrected between groups agreement) values are given in S1 Fig. a R² < 0.06, b A < 0.03, c R² < 0.02, d A < 0.01.

Table 2. Significance of associations between sampling time and microbial parameters.

<table>
<thead>
<tr>
<th>representation of sampling times</th>
<th>Community sampling indices</th>
<th>Diversity indices</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Adonis</td>
<td>MRPP</td>
</tr>
<tr>
<td>fungi four astronomical seasons a</td>
<td>&lt;10⁻⁵</td>
<td>0.131</td>
</tr>
<tr>
<td>bacteria</td>
<td>&lt;10⁻³</td>
<td>0.245</td>
</tr>
<tr>
<td>fungi four meteorological seasons b</td>
<td>&lt;10⁻⁵</td>
<td>0.145</td>
</tr>
<tr>
<td>bacteria</td>
<td>&lt;10⁻³</td>
<td>0.248</td>
</tr>
<tr>
<td>fungi nine phenological periods c</td>
<td>&lt;10⁻⁵</td>
<td>0.187</td>
</tr>
<tr>
<td>bacteria</td>
<td>&lt;10⁻³</td>
<td>0.241</td>
</tr>
<tr>
<td>fungi continuous (metric)</td>
<td>0.0267</td>
<td>0.013</td>
</tr>
<tr>
<td>bacteria</td>
<td>&lt;10⁻⁵</td>
<td>0.228</td>
</tr>
</tbody>
</table>

Categorization of the sampling time and its significance for differences in community variation (based on Bray—Curtis dissimilarities) and diversity changes for fungi and bacteria.
(Shannon Index (H'), Simpson Index (1-D), Pielou's Evenness (J), Richness (S)). * e.g., spring starting at March equinox, ending at June solstice; * e.g., spring starting March 1, ending May 31; * for the area of Munich in 1998–1999, displayed and supported in Fig 1d.

**Fig 1. Microbial community variation and temporal dynamics.** Points: dust samples from different homes. (a, c) first coordinates of a principal coordinate analysis (PCoA) on Bray-Curtis dissimilarities of the fungal (a) and bacterial (c) communities; in brackets: explained variance, colours: meteorological seasons. (b, d) the principal coordinate that explains most of the variation for fungi (b) or bacteria (d) sorted by the sampling date; regression (b, d): a locally weighted polynomial fit with 95% confidence interval; colour strip (b): meteorological seasons: spring, summer, autumn, and winter; colour strip (d): phenological periods of the geographic area during the time-frame of sampling: early spring, full spring, early summer, midsummer, late summer, early autumn, full autumn, late autumn, and winter (dates obtained from: http://www.dwd.de/, http://www.phenocal.chira.de; accessed 18 Jul 2014).

**Diversity**

The determinants of significantly different community variations in fungi and bacteria were not associated per se with significantly different diversities (Simpson and Shannon indices, Table 3: significant variables, S2 Table: all variables).

For indoor environmental factors, infrequent ventilation of a flat via the windows in the summer half year was significantly associated with a higher diversity of less abundant fungi (Shannon index P = 0.04, Kruskal–Wallis test). Homes with carpets had a lower bacterial diversity than homes with smooth floors, but the highest bacterial diversity was sampled
from mixed floors (Shannon index $P = 0.01$ and Simpson index $P = 0.04$, Kruskal–Wallis tests).

Regarding outdoor factors, low and high (1st and 3rd tertile) surrounding greenery within a 100 m buffer significantly increased the fungal diversity in the dust compared to the third of samples from homes with a medium level of surrounding greenery (2nd tertile) (Simpson index $P = 0.03$, Kruskal–Wallis test). The bacterial diversity was significantly lower for homes above the 1st floor (Shannon index $P = 0.04$ and Simpson index $P = 0.03$, Kruskal–Wallis tests).

Fungal diversity was only lower in late summer compared to the rest of the year (Fig 2a). However, the effect of the season on fungal diversity indices was strongly significant when the sampling times were categorized according to outdoor plant phenology or the meteorological seasons ($P < 0.001$, Kruskal–Wallis tests on Simpson and Shannon indices, Evenness, and Richness; Table 2). In contrast, the bacterial diversity precisely mirrored the shift in the bacterial community variation during spring (Fig 2b). Differences between the maximum and minimum diversity (determined from the localized regression shown in Fig 2) were also much less for fungi (approximate difference: Shannon index 20%, Simpson index 11%, evenness 11%) than for bacteria (approximate difference: Shannon index 61%, Simpson index 52%, evenness 42%).
Table 3. Significant associations between environmental determinants and microbial diversity.

<table>
<thead>
<tr>
<th>Environmental characteristics</th>
<th>Fungi 1-D median (p25-p75)</th>
<th>Fungi H' median (p25-p75)</th>
<th>Fungi 1-D median (p25-p75)</th>
<th>Fungi H' median (p25-p75)</th>
<th>Bacteria 1-D median (p25-p75)</th>
<th>Bacteria H' median (p25-p75)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vent. living room: summer</td>
<td>0.08</td>
<td>3.16</td>
<td>0.71</td>
<td>2.00</td>
<td>0.62</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>seldom/never/via another room</td>
<td>(0.85-0.93)</td>
<td>(2.79-3.47)</td>
<td>(0.53-0.86)</td>
<td>(1.47-2.57)</td>
<td>(0.53-0.86)</td>
<td>(1.47-2.57)</td>
<td></td>
</tr>
<tr>
<td>once/several times a day (short)</td>
<td>0.93</td>
<td>3.54</td>
<td>0.77</td>
<td>2.21</td>
<td>0.77</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>once/several times a day (long)</td>
<td>(0.88-0.94)</td>
<td>(3.04-3.72)</td>
<td>(0.52-0.86)</td>
<td>(1.36-2.66)</td>
<td>(0.52-0.86)</td>
<td>(1.36-2.66)</td>
<td></td>
</tr>
<tr>
<td>one/several times a day (long)</td>
<td>(0.83-0.94)</td>
<td>(2.78-3.6)</td>
<td>(0.62-0.86)</td>
<td>(1.6-2.71)</td>
<td>(0.62-0.86)</td>
<td>(1.6-2.71)</td>
<td></td>
</tr>
<tr>
<td>Type of living room floor</td>
<td>0.30</td>
<td>3.35</td>
<td>0.78</td>
<td>2.14</td>
<td>0.78</td>
<td>2.14</td>
<td></td>
</tr>
<tr>
<td>carpet</td>
<td>(0.86-0.94)</td>
<td>(2.95-3.64)</td>
<td>(0.51-0.85)</td>
<td>(1.35-2.58)</td>
<td>(0.51-0.85)</td>
<td>(1.35-2.58)</td>
<td></td>
</tr>
<tr>
<td>smooth</td>
<td>0.90</td>
<td>3.09</td>
<td>0.81</td>
<td>2.32</td>
<td>0.81</td>
<td>2.32</td>
<td></td>
</tr>
<tr>
<td>smooth with rugs</td>
<td>(0.81-0.94)</td>
<td>(2.78-3.6)</td>
<td>(0.58-0.87)</td>
<td>(1.46-2.71)</td>
<td>(0.58-0.87)</td>
<td>(1.46-2.71)</td>
<td></td>
</tr>
<tr>
<td>Position of the home</td>
<td>0.92</td>
<td>3.20</td>
<td>0.80</td>
<td>2.36</td>
<td>0.80</td>
<td>2.36</td>
<td></td>
</tr>
<tr>
<td>ground floor</td>
<td>(0.83-0.93)</td>
<td>(2.74-3.61)</td>
<td>(0.68-0.87)</td>
<td>(1.84-2.81)</td>
<td>(0.68-0.87)</td>
<td>(1.84-2.81)</td>
<td></td>
</tr>
<tr>
<td>1st floor</td>
<td>0.15</td>
<td>3.3</td>
<td>0.81</td>
<td>2.35</td>
<td>0.81</td>
<td>2.35</td>
<td></td>
</tr>
<tr>
<td>2nd floor</td>
<td>(0.84-0.94)</td>
<td>(2.81-3.66)</td>
<td>(0.64-0.88)</td>
<td>(1.73-2.8)</td>
<td>(0.64-0.88)</td>
<td>(1.73-2.8)</td>
<td></td>
</tr>
<tr>
<td>3rd floor or higher</td>
<td>0.92</td>
<td>3.45</td>
<td>0.83</td>
<td>2.41</td>
<td>0.83</td>
<td>2.41</td>
<td></td>
</tr>
<tr>
<td>2nd floor</td>
<td>(0.85-0.95)</td>
<td>(2.93-3.67)</td>
<td>(0.63-0.86)</td>
<td>(1.71-2.74)</td>
<td>(0.63-0.86)</td>
<td>(1.71-2.74)</td>
<td></td>
</tr>
<tr>
<td>NDVI (100 m buffer)</td>
<td>0.89</td>
<td>3.06</td>
<td>0.78</td>
<td>2.21</td>
<td>0.78</td>
<td>2.21</td>
<td></td>
</tr>
<tr>
<td>1st tertile (0.06-0.22)</td>
<td>(0.83-0.92)</td>
<td>(2.74-3.43)</td>
<td>(0.57-0.82)</td>
<td>(1.62-2.37)</td>
<td>(0.57-0.82)</td>
<td>(1.62-2.37)</td>
<td></td>
</tr>
<tr>
<td>2nd tertile (0.27-0.33)</td>
<td>0.89</td>
<td>3.1</td>
<td>0.75</td>
<td>2.12</td>
<td>0.75</td>
<td>2.12</td>
<td></td>
</tr>
<tr>
<td>3rd tertile (0.33-0.59)</td>
<td>(0.82-0.93)</td>
<td>(2.72-3.58)</td>
<td>(0.45-0.85)</td>
<td>(1.25-2.64)</td>
<td>(0.45-0.85)</td>
<td>(1.25-2.64)</td>
<td></td>
</tr>
</tbody>
</table>

Variables with significant (Wilcoxon signed rank test or Kruskall–Wallis test P < 0.05)

associations to diversity changes (Simpson Index (1-D), Shannon Index (H')) for fungi and bacteria. p25-p75: interquartile ranges; bold: P ≤ 0.05. Values for all insignificant (P > 0.05) variables are given in S2 Table.

Fig 2. Temporal dynamics of microbial diversity. Points: dust samples from different homes.

Diversity indices of the dust samples are sorted by sampling date, (a) for fungi (n = 286) and
(b) for bacteria (n = 283). Regression: a locally weighted polynomial fit with 95% confidence
interval.

Discussion

This study considered both the fungal and the bacterial microbiomes in indoor dust, in more
than 250 households in a metropolitan area. We observed that the environmental factors
that affected the composition of the fungal microbiome were different than those that
affected the bacterial microbiome. However, the time of the year (season or phenological
period) was the most decisive of the parameters tested for both fungi and bacteria.

Fungal community variation and diversity were influenced by several indoor environmental
factors. In contrast, bacterial community variation was significantly affected only by
behaviour regarding ventilation, and bacterial diversity was significantly affected only by the
type of living room floor. Similar results have been recently reported [6, 7], and our findings
are in accord with studies on the building environment microbiome, which emphasize the
importance of the architectural design (including ventilation) and human behaviour itself as
decisive factors for the biogeography of bacterial communities in indoor environments [11,
15]. In a recent study [6], the fungal community composition in settled dust samples from
various indoor locations seemed to be mainly driven by fungi associated with the outdoor
environment, while for the bacterial community, the composition reflected the taxa
released from the residents, an observation that had also been made earlier [13]. In the
same study [6], the authors did not detect any effect of the house or residential
characteristics on the bacterial microbiome, which is in accord with our study. However, we
found an effect of two determinants that were kept uniform in their study [6]. The
ventilation behaviour showed an effect on the bacterial community variation, as was also
previously reported [15]. Additionally, the type of living room floor affected the bacterial
diversity. It is apparent that small-scale structured floors (i.e., smooth with rugs) can
harbour a higher diversity than homogenous surfaces.
Fungal community characteristics were not affected by dampness or water leakage but were
affected by the presence of mould. In a study comparing 17 homes with low mouldiness
(evaluated with “Environmental Relative Moldiness Index”, [44]) with 18 homes that had
high mouldiness, fungal Richness was significantly associated with the relative humidity but
not Evenness or the Shannon or Simpson indices [45]. These findings suggest that dampness
alone might be a relatively weak determinant of the indoor fungal community,
notwithstanding that the growth of mould is often associated with dampness [46] because
different individual sites have different reactions to moisture, and the assessment of
dampness and the moisture status is complex [47].
The ventilation strategy in a university building has been identified as one of the strongest
factors affecting bacterial community variation [11]. In this study, the association between
ventilation habits and bacterial community variation was also significant, but its likely
impact (MRPP $A$) was more than 10-fold smaller than the impact of the outdoor phenology
(i.e., the sampling time). The probable reason for this difference may be the different
environments sampled in the study of Meadow et al. (2014) [11] and the present work: air
sampling versus settled dust, mechanic ventilation versus non-mechanic ventilation through
windows, etc. In this study, tight windows (which implied a lower air exchange rate) were
also significantly associated with fungal community variation and higher fungal diversity.
Along with building structures and residential characteristics, the presence of pets has also
been found to influence the house-associated microbial community [48]. In dust samples
taken from nine different locations within the home in 40 US households, more diverse bacterial communities were observed in homes where dogs had been present compared to homes without dogs [49]. Differently, in our study, the presence of pets at the time of dust sampling did not show a significant effect on the fungal or on the bacterial microbiome.

The outdoor environment decisively influences the indoor environment with residential and building characteristics mediating the association [6, 13, 50]. In this context, various types of land use have been found to significantly shape the bacterial signature in outdoor air [51, 52]. In our urban environment, we could not confirm that the closer neighbourhood (< 500 m, Table 1 “Outdoor characteristics”) is directly associated with the indoor bacterial community. However, we observed an association with the position of the home. It can be speculated that fewer bacteria are carried from outdoors by the occupants into the flats at a higher level above ground. However, it is also possible that the direct influx of airborne bacteria through the windows varies with the elevation above ground, although such a relationship has been only been found for cultured bacteria in a study that investigated a large elevation difference (238 m) [53]. Future work is necessary to confirm this result by accounting for the building height.

We observed an association of the fungal community with the greenery surrounding the homes, the grade of urbanization and level of airborne particulate matter. Correlations between particulate matter and airborne fungi were previously observed [54], and the fungi themselves may make up a significant fraction of the airborne particulate matter [55, 56]. Additionally, plants are a major source of airborne fungi [57], which may explain the influence of the surrounding greenery we observed for the fungi in indoor dust. In a study on a continental scale in the USA, urbanization was not generally associated with changes in
the microbial community of external household surfaces compared to rural areas; however, it tended to lead to a more homogenous community composition [58]. Nevertheless, the number of comparable investigations involving urbanization, exhaust, and greenery is limited, and further studies are necessary in order to confirm their impact on the composition of the fungal microbiome of indoor dust. The results from such studies might identify key environmental characteristics concerning the closer neighbourhood with the potential to create surroundings that promote healthy living [59].

The microbial community structure followed a temporal pattern, which was the major factor affecting the variables considered for fungal and bacterial communities in living room dust. For fungi, semi-annual patterns in their quantity in the indoor environment have previously been repeatedly found by spore counts and cultivation, which were correlated with outdoor concentrations [3]. Additionally, molecular studies on smaller time windows suggested such patterns in different geographical regions [4, 5]. For bacteria, the influence of the sampling time was determined to be not very important in earlier studies [6-9]. Our finding that bacterial communities are considerably influenced by seasons is probably because of the large number of individual samples that were analysed in this study. Almost daily sampling allowed the delineation of temporal dynamics, overcoming inevitable individual variation between locations. A recent investigation on one housing complex with 11 units sampled during one month in summer and in winter found a strong seasonality of the indoor fungal community but little evidence for the same in the bacterial community [6]. However, the authors presumed that a seasonal relationship for bacteria was obscured in their study because of large amounts of human-associated bacteria in the dust samples analysed. Additionally, studies that compare different time windows during the year might also
overlook bacterial indoor seasonality. For example, using our data, a comparison of all of the July-August samples with the December-January samples would have underestimated the total annual influence of seasonal change, and for a period of approximately 100 days during phenological winter we would have found almost no effect of this parameter at all (cf. Figs 1,2).

The above mentioned seasonal patterns of community change were dissimilar for fungi and bacteria. However, the varying taxonomic resolution of the fingerprinting method restricts an explanation of the relationship between changes in community variation and diversity to a general level. For fungi, a decrease in diversity during summer suggests an influx of high amounts of a few OTUs, such as Alternaria and Cladosporium [21, 22]. For bacteria, the rapid and strong change in community variation and reduced diversity during the spring suggest a substantial and rapid influx of interrelated OTUs during the early plant flowering (i.e., the full spring phenological period).

In this study, the indoor bacterial community variation and the phenological period in which the samples were taken were strongly associated. However, the fingerprinting technique used did not allow the identification of microbial taxa, and so we could not explore whether these shifts were caused by plant-related taxa. Recently, sources for bacteria in particulate matter of outdoor aerosols from Colorado, USA, were tracked to leaves, soils and cow faeces [25]. In Milan, Italy, plant-derived microbes dominated outdoor airborne bacteria during the summer, while spore-forming bacteria dominated in the winter [26]. Outdoor airborne bacteria sampled during the spring in northern France were also mainly plant-derived [60]. All of this evidence suggests that plant phenology was indeed the cause for the association we found between a change in the bacterial indoor dust community and the full
Conclusions

On the scale of an urban metropolitan area, we confirmed that the fungal microbiome in indoor dust is more strongly affected than the bacterial microbiome by both, indoor and especially outdoor factors.

Samples from studies similar to ours, i.e., studies that had independent sampling locations on a regional scale and a time-frame of several months, could be used to confirm the unexpectedly strong effect on indoor bacteria that we found for the sampling time during the year.

The semi-annual cycle for the fungal indoor community that we inferred from our samples is similar to the well-explained seasonal change in indoor and outdoor fungal propagules. In contrast, changes in indoor bacteria must be elucidated in future studies. The local plant phenology, particularly at the annual onset of the flowering period, may well be a major driver of temporal change in the indoor bacterial microbiome in many geographical regions.

Acknowledgements

The authors wish to thank Andrea Obersteiner for practical advice, Barbara Zehentner for help at the bench, and Jean Charles Munch for institutional support.

References


10. Amend AS, Selfert KA, Samson R, Bruns TD. Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. PNAS. 2010;107(31):13748–53.


49. Dunn RR, Fierer N, Henley JB, Leff JW, Menninger HL. Home life: factors structuring the bacterial diversity found within and between homes. PLOS ONE 8(5): e64133. doi:10.1371/journal.pone.0064133

Supporting Information

S1 Supporting Information. Distribution of samples.

S2 Supporting Information. Environmental characteristics.
S1 Table. Community variation.

S2 Table. Significance of associations with diversity – all variables.

S1 Fig. Amount of fungi in the samples.
<Figure 2>
S1 Supporting Information. Distribution of samples in the Munich urban area.

The sampling scheme for house dusts can be considered as random single time-point sampling, which was originally done for a population based cohort study targeting newborns in urban environments (1). Hereby, the random factor was defined by the date of birth of a child in an obstetrical clinic in Munich (2) and the location of the households in which the newborns lived until the date of dust sampling, which was in the third month after birth. The map below illustrates the result of this random sampling by showing the distribution of all sampling locations and seasons across the Munich metropolitan area. It covers the biggest part of the ‘Larger Urban Zone’ (3) of Munich and includes the position of 280 sampled households located within a radius of 35 km from the center of Munich (98% of all samples).
S1 Supporting Information References


S2 Supporting Information. Environmental characteristics.

INDOOR characteristics
For the current investigation we focused on signs of dampness and mould, ventilation habits, type of living room floor, exposure to pets, smoking of tobacco in the flat, number of people living in the home, and renovation measures. All the characteristics were questionnaire-derived.

OUTDOOR characteristics
For questionnaire-based outdoor environmental characteristics, we had information regarding the age of the building, the position of the home (ground floor or higher levels), building density of the neighborhood, whether there were traffic jams around the home during rush hour and if there were facilities with noticeable air pollution near the residency.

Annual average concentrations of particulate matter with an aerodynamic diameter of less than 2.5 μm (PM2.5) and less than 10 μm (PM10), of between 2.5 μm and 10 μm (PMcoarse; coarse particulate matter), PM2.5 absorbance (PM2.5 absorbance, a proxy of black carbon), nitrogen dioxide (NO₂), and nitrogen oxides NO and NO (NOₓ) were estimated at the residential addresses at birth by land-use regression (LUR) models developed as part of the European Study of Cohorts for Air Pollution Effects (http://www.escapeproject.eu) (1, 2).

Greenness refers to vegetation level and was defined by the Normalized Difference Vegetation Index (NDVI). Since it is known that plants strongly absorb visible light (from approximately 0.4 μm to 0.7 μm) for use in photosynthesis while strongly reflecting near-infrared light (from 0.7 μm to 1.1 μm) to prevent overheating, NDVI formulae is based on the difference of surface reflectance in these wavelengths (3).

In this study, we used cloud-free Landsat 5 TM satellite images at a resolution of 30 m (http://earthexplorer.usgs.gov/), obtained during vegetation rich months (14th of July and 24th of August) to obtain maximum exposure contrasts; NDVI was calculated as the mean value in circular 30m, 100 m and 500 m buffers around the residential address at birth (4, 5).

In addition to air pollution from traffic and greenness, we also considered urbanization grade (urban index) at the place of residence. The urban index was calculated as the proportion of land use with predominantly sealed soil (according to CORINE land cover data, EEA 2006) within a radius of 2 km (6).
<table>
<thead>
<tr>
<th>Table: S2 Supporting Information. Environmental characteristics.</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>INDOOR environmental characteristic</strong></td>
<td></td>
</tr>
<tr>
<td>N° of rooms within the flat (excluding bathroom)</td>
<td></td>
</tr>
<tr>
<td>1-3 rooms</td>
<td>51/277 (18%)</td>
</tr>
<tr>
<td>≥ 4 rooms</td>
<td>226/277 (82%)</td>
</tr>
<tr>
<td>Dampness</td>
<td>18/279 (6%)</td>
</tr>
<tr>
<td>Mould at home</td>
<td>102/279 (37%)</td>
</tr>
<tr>
<td>Water leakage</td>
<td>34/279 (12%)</td>
</tr>
<tr>
<td>Tightness of the windows</td>
<td></td>
</tr>
<tr>
<td>dense</td>
<td>207/273 (76%)</td>
</tr>
<tr>
<td>less dense</td>
<td>66/273 (24%)</td>
</tr>
<tr>
<td>Ventilation living room through windows - summer</td>
<td></td>
</tr>
<tr>
<td>seldom/never/via another room</td>
<td>4/273 (1%)</td>
</tr>
<tr>
<td>once/several times a day (short)</td>
<td>51/273 (19%)</td>
</tr>
<tr>
<td>once/several times a day (long)</td>
<td>148/273 (80%)</td>
</tr>
<tr>
<td>Ventilation living room through windows - winter</td>
<td></td>
</tr>
<tr>
<td>seldom/never/via another room</td>
<td>15/273 (5%)</td>
</tr>
<tr>
<td>once/several times a day (short)</td>
<td>245/273 (90%)</td>
</tr>
<tr>
<td>once/several times a day (long)</td>
<td>13/273 (5%)</td>
</tr>
<tr>
<td>Heating in the home (no central heating)</td>
<td>44/286 (15%)</td>
</tr>
<tr>
<td>Renovation measures last 12 months</td>
<td>194/281 (69%)</td>
</tr>
<tr>
<td>Pets</td>
<td>49/281 (17%)</td>
</tr>
<tr>
<td><strong>Type of living room floor</strong></td>
<td></td>
</tr>
<tr>
<td>carpet</td>
<td>116/279 (42%)</td>
</tr>
<tr>
<td>smooth</td>
<td>50/279 (18%)</td>
</tr>
<tr>
<td>smooth with rugs</td>
<td>113/279 (41%)</td>
</tr>
<tr>
<td><strong>Smoking of tobacco in the flat</strong></td>
<td>35/277 (13%)</td>
</tr>
<tr>
<td><strong>OUTDOOR environmental characteristics at birth</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Age of the building</strong></td>
<td></td>
</tr>
<tr>
<td>Built before 1945</td>
<td>47/277 (17%)</td>
</tr>
<tr>
<td>Built after 1945</td>
<td>230/277 (83%)</td>
</tr>
<tr>
<td><strong>Position of the home</strong></td>
<td></td>
</tr>
<tr>
<td>Ground floor</td>
<td>90/279 (32%)</td>
</tr>
<tr>
<td>1&lt;sup&gt;st&lt;/sup&gt; floor</td>
<td>78/279 (28%)</td>
</tr>
<tr>
<td>2&lt;sup&gt;nd&lt;/sup&gt; floor</td>
<td>40/279 (14%)</td>
</tr>
<tr>
<td>3&lt;sup&gt;rd&lt;/sup&gt; floor or higher</td>
<td>71/279 (25%)</td>
</tr>
<tr>
<td><strong>Residential density of the neighborhood</strong></td>
<td></td>
</tr>
<tr>
<td>high</td>
<td>115/273 (42%)</td>
</tr>
<tr>
<td>average</td>
<td>141/273 (52%)</td>
</tr>
<tr>
<td>low</td>
<td>17/273 (6%)</td>
</tr>
<tr>
<td><strong>Traffic jams in rush hour</strong></td>
<td>36/273 (13%)</td>
</tr>
<tr>
<td>NDVI (500-m buffer), median (p25%-p75%)</td>
<td>0.32 (0.27-0.37) 281</td>
</tr>
<tr>
<td>NDVI (100-m buffer), median (p25%-p75%)</td>
<td>0.30 (0.24-0.35) 281</td>
</tr>
<tr>
<td>NDVI (30-m buffer), median (p25%-p75%)</td>
<td>0.29 (0.22-0.36) 281</td>
</tr>
<tr>
<td><strong>Urban index</strong></td>
<td></td>
</tr>
<tr>
<td>Urban (≥ 0.7)</td>
<td>140/281 (50%)</td>
</tr>
<tr>
<td>Semi-urban (≥ 0.3 and &lt; 0.7)</td>
<td>80/281 (28%)</td>
</tr>
<tr>
<td>Rural (≤ 0.3)</td>
<td>61/281 (22%)</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt; μg/m³, median (p25%-p75%)</td>
<td>21.78 (17.59-26.71) 281</td>
</tr>
<tr>
<td>NO&lt;sub&gt;x&lt;/sub&gt; μg/m³, median (p25%-p75%)</td>
<td>35.66 (29.55-42.99) 281</td>
</tr>
</tbody>
</table>
S2 Supporting Information references


### S1 Table. Community variation.
Significance of associations between environmental determinants and microbiome community variation (Adonis $P$, MRPP $\delta$) with respective $R^2$ (Adonis) and $A$ (MRPP chance corrected between groups agreement) values which are not shown in the paper itself; bold: $P$ or $\delta \leq 0.05$

<table>
<thead>
<tr>
<th>Environmental characteristics</th>
<th>Fungi</th>
<th></th>
<th>Bacteria</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$P$</td>
<td>$R^2$</td>
<td>$\delta$</td>
<td>$A$</td>
</tr>
<tr>
<td>N° of rooms within the flat</td>
<td>0.75</td>
<td>0.001</td>
<td>0.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Dampness</td>
<td>0.59</td>
<td>0.002</td>
<td>0.29</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Mould at home</td>
<td>0.04</td>
<td>0.012</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td>Water leakage</td>
<td>0.81</td>
<td>0.001</td>
<td>0.85</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Tightness of the windows</td>
<td>0.03</td>
<td>0.013</td>
<td>0.04</td>
<td>0.04</td>
</tr>
<tr>
<td>Ventilation living room through windows - summer</td>
<td>0.27</td>
<td>0.009</td>
<td>0.24</td>
<td>0.01</td>
</tr>
<tr>
<td>Ventilation living room through windows - winter</td>
<td>0.67</td>
<td>0.005</td>
<td>0.64</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Heating within the home</td>
<td>0.03</td>
<td>0.013</td>
<td>0.02</td>
<td>0.05</td>
</tr>
<tr>
<td>Renovation measures last 12 months</td>
<td>0.44</td>
<td>0.003</td>
<td>0.81</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pets</td>
<td>0.27</td>
<td>0.004</td>
<td>0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Type of living room floor</td>
<td>&lt; 0.001</td>
<td>0.057</td>
<td>&lt; 0.001</td>
<td>0.02</td>
</tr>
<tr>
<td>Smoking of tobacco in the flat</td>
<td>0.42</td>
<td>0.003</td>
<td>0.41</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Age of the building</td>
<td>0.01</td>
<td>0.017</td>
<td>0.01</td>
<td>0.05</td>
</tr>
<tr>
<td>Position of the home</td>
<td>0.49</td>
<td>0.010</td>
<td>0.87</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Building density of the neighborhood</td>
<td>0.52</td>
<td>0.006</td>
<td>0.59</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Traffic jams in rush hour</td>
<td>0.83</td>
<td>0.001</td>
<td>0.83</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Facility with noticeable air pol. within 50 and 100 m</td>
<td>0.58</td>
<td>0.002</td>
<td>0.72</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Facility with noticeable air pollution within 50 m</td>
<td>0.33</td>
<td>0.004</td>
<td>0.28</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NDVI (500 m buffer)</td>
<td>0.72</td>
<td>0.004</td>
<td>0.63</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>NDVI (100 m buffer)</td>
<td>0.05</td>
<td>0.017</td>
<td>0.006</td>
<td>0.008</td>
</tr>
<tr>
<td>NDVI (30 m buffer)</td>
<td>0.06</td>
<td>0.016</td>
<td>0.01</td>
<td>0.007</td>
</tr>
<tr>
<td>Urban index</td>
<td>0.02</td>
<td>0.021</td>
<td>0.01</td>
<td>0.007</td>
</tr>
<tr>
<td>$\text{NO}_2$</td>
<td>0.23</td>
<td>0.010</td>
<td>0.06</td>
<td>0.004</td>
</tr>
<tr>
<td>$\text{NO}_x$</td>
<td>0.06</td>
<td>0.016</td>
<td>0.03</td>
<td>0.005</td>
</tr>
<tr>
<td>PM$_{2.5}$</td>
<td>0.004</td>
<td>0.028</td>
<td>0.005</td>
<td>0.009</td>
</tr>
<tr>
<td>PM$_{10}$</td>
<td>0.54</td>
<td>0.006</td>
<td>0.32</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>PM$_{2.5}$ absorbance</td>
<td>0.04</td>
<td>0.018</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>PM$_{2.5}$ absorbance</td>
<td>0.07</td>
<td>0.015</td>
<td>0.06</td>
<td>0.004</td>
</tr>
</tbody>
</table>

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S2 Table. Significance of associations with diversity – all variables. Significance of associations between all environmental determinants and microbial diversity (Simpson and Shannon indices, $P$-values (Wilcoxon signed rank test or Kruskall–Wallis test)); bold: $P \leq 0.05$

<table>
<thead>
<tr>
<th>Environmental characteristics at birth</th>
<th>Fungi $P$ (Simpson 1-D)</th>
<th>Fungi $P$ (Shannon $H^*$)</th>
<th>Bacteria $P$ (Simpson 1-D)</th>
<th>Bacteria $P$ (Shannon $H^*$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N° of rooms within the flat</td>
<td>0.13</td>
<td>0.13</td>
<td>0.69</td>
<td>0.50</td>
</tr>
<tr>
<td>Dampness</td>
<td>0.10</td>
<td>0.12</td>
<td>0.17</td>
<td>0.23</td>
</tr>
<tr>
<td>Mould at home</td>
<td>0.88</td>
<td>0.71</td>
<td>0.77</td>
<td>0.57</td>
</tr>
<tr>
<td>Water leakage</td>
<td>0.60</td>
<td>0.78</td>
<td>0.27</td>
<td>0.17</td>
</tr>
<tr>
<td>Tightness of the windows</td>
<td>0.78</td>
<td>0.87</td>
<td>0.78</td>
<td>0.85</td>
</tr>
<tr>
<td>Ventilation living room through windows - summer</td>
<td>0.08</td>
<td><strong>0.04</strong></td>
<td>0.62</td>
<td>0.56</td>
</tr>
<tr>
<td>Ventilation living room through windows - winter</td>
<td>0.49</td>
<td>0.32</td>
<td>0.13</td>
<td>0.20</td>
</tr>
<tr>
<td>Heating within the home</td>
<td>0.30</td>
<td>0.31</td>
<td>0.77</td>
<td>0.91</td>
</tr>
<tr>
<td>Renovation measures last 12 months</td>
<td>0.69</td>
<td>0.71</td>
<td>0.75</td>
<td>0.52</td>
</tr>
<tr>
<td>Pets</td>
<td>0.84</td>
<td>0.74</td>
<td>0.55</td>
<td>0.91</td>
</tr>
<tr>
<td>Type of living room floor</td>
<td>0.30</td>
<td>0.33</td>
<td><strong>0.04</strong></td>
<td><strong>0.01</strong></td>
</tr>
<tr>
<td>Smoking of tobacco in the flat</td>
<td>0.13</td>
<td>0.12</td>
<td>0.75</td>
<td>0.80</td>
</tr>
<tr>
<td>Age of the building</td>
<td>0.31</td>
<td>0.49</td>
<td>0.24</td>
<td>0.36</td>
</tr>
<tr>
<td>Position of the home</td>
<td>0.15</td>
<td>0.09</td>
<td><strong>0.04</strong></td>
<td><strong>0.03</strong></td>
</tr>
<tr>
<td>Building density of the neighborhood</td>
<td>0.46</td>
<td>0.27</td>
<td>0.67</td>
<td>0.62</td>
</tr>
<tr>
<td>Traffic jams in rush hour</td>
<td>0.99</td>
<td>0.59</td>
<td>0.53</td>
<td>0.44</td>
</tr>
<tr>
<td>No facility with noticeable air pollution nearby</td>
<td>0.32</td>
<td>0.43</td>
<td>0.44</td>
<td>0.54</td>
</tr>
<tr>
<td>Facility with noticeable air poll. within 50 and 100 m</td>
<td>0.17</td>
<td>0.25</td>
<td>0.26</td>
<td>0.27</td>
</tr>
<tr>
<td>Facility with noticeable air pollution within 50 m</td>
<td>0.16</td>
<td>0.11</td>
<td>0.41</td>
<td>0.40</td>
</tr>
<tr>
<td>NDVI (500 m buffer)</td>
<td>0.09</td>
<td>0.14</td>
<td>0.69</td>
<td>0.69</td>
</tr>
<tr>
<td>NDVI (100 m buffer)</td>
<td><strong>0.03</strong></td>
<td>0.11</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>NDVI (30 m buffer)</td>
<td>0.32</td>
<td>0.31</td>
<td>0.59</td>
<td>0.56</td>
</tr>
<tr>
<td>Urban Index</td>
<td>0.37</td>
<td>0.44</td>
<td>0.65</td>
<td>0.73</td>
</tr>
<tr>
<td>NO$_2$</td>
<td>0.25</td>
<td>0.35</td>
<td>0.54</td>
<td>0.51</td>
</tr>
<tr>
<td>NO$_x$</td>
<td>0.14</td>
<td>0.33</td>
<td>0.43</td>
<td>0.29</td>
</tr>
<tr>
<td>PM$_{2.5}$</td>
<td>0.72</td>
<td>0.60</td>
<td>0.74</td>
<td>0.81</td>
</tr>
<tr>
<td>PM$_{10}$</td>
<td>0.35</td>
<td>0.32</td>
<td>0.91</td>
<td>0.85</td>
</tr>
<tr>
<td>PM$_{coarse}$</td>
<td>0.10</td>
<td>0.15</td>
<td>0.62</td>
<td>0.66</td>
</tr>
<tr>
<td>PM$_{2.5}$ absorbance</td>
<td>0.66</td>
<td>0.49</td>
<td>0.62</td>
<td>0.75</td>
</tr>
</tbody>
</table>
S1 Fig. Amount of fungi in the dust samples (circles) during the time-frame of sampling shown as conidial equivalents per gram dust. Quantification followed known standards (Haugland and Vesper 2002: *Method of identifying and quantifying specific fungi and bacteria*. U.S. Environmental Protection Agency: Washington, DC, USA).
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Fabian Christopher Weikl

Born on the 24th of December 1984 in Straubing, Germany
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Education, Training, and Work Experience

05/2011 – 11/2015 Fellowship of the Helmholtz Graduate School Environmental Health for a PhD project, work group of PD Dr. Karin Pritsch, at the Institute of Soil Ecology (until 03/2015) and at the Institute of Biochemical Plant Pathology (from 04/2015), Helmholtz Zentrum München

03/2011 Intern in biological ultrastructure research, Univ. of Vienna, Austria

10/2004 – 09/2010 Study of biology at the Univ. of Regensburg, Germany
Degree: Diplom (MSc. grade 1.2, scale 1–5, 1 is best). Main subjects: microbiology, zoology, genetics. Diploma thesis (German): "The influence of desiccation, vacuum and gamma-radiation on the survival of thermophilic microorganisms"

10/2006 – 03/2010 Remission of tuition fees during advanced studies in biology due to outstanding achievements in basic studies

03/2009 – 10/2009 Working student at the German Aerospace Centre, work group for astrobiology, Cologne, Germany


07/2004 Endowed prize of a local public trust for field work on dragon- and damselflies in meadows of the river Isar, Deggendorf, Germany

09/1995 – 05/2004 Robert-Koch-Gymnasium Deggendorf Allgemeine Hochschulreife (Abitur) grade 1.9 (scale 1–6, 1 is best)