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

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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

Pilze sind omnipräsent. Sie sind essentiell für funktionsfähige Böden und damit unabdingbar für den Erhalt der enormen Lebensvielfalt auf unserem Planeten. Allerdings können einige bodenassoziierte Pilze, oder allgemeiner einige Pilze unserer Umwelt, Gesundheitsprobleme bei Menschen hervorrufen. Naturgemäß werden solche Zusammenhänge in erster Linie aus Richtung der medizinischen Wissenschaften untersucht. Jedoch muss eine umfassende Behandlung des Themas auch die Lebensweise der beteiligten Organismen im Kontext ihrer Umwelt berücksichtigen. In dieser Dissertation wurden drei aktuelle, mit Pilzen verbundene Gesundheitsfragen aus einem mikrobiellen und ökologischen Blickwinkel betrachtet, um ihren bekannten ökologischen Kontext zu erweitern und unser Verständnis zu möglichen Gesundheitsbeeinträchtigungen zu verbessern.

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 volatilen organischen Verbindungen, was Einschätzungen zum von diesen Substanzen ausgehenden Risiko für die menschliche Gesundheit ungewiss macht, bis weitere mikrobiologische Grundlagenforschung zu diesem Thema vorliegt.

Das zweite Problem wurde auf einer räumlich und ökologisch größeren Skala angegangen, d.h. der Blick wurde auf das allergene Potential von Pilzen in landwirtschaftlicher Umgebung gelegt. Nach Pollen sind Pilze die zweite wichtige Ursache für Allergien im Außenraum, doch ihre Verteilung und Häufigkeit sind kaum beschrieben. Es existiert kein etabliertes Vorgehen, um allergene Potenziale von Pilzen zu messen und auch direkte Bestimmungen der Allergene

von Pilzen sind problembehaftet. Um diese Situation zu verbessern, wurde ein Protokoll zur Quantifizierung der relevantesten allergenen Pilze aus verschiedenen Umweltmaterialen im Außenraum entwickelt und auf Getreidefelder angewandt. Dabei erwies sich Pflanzenmaterial zur Erntezeit als Hauptquelle. Individuelle Bedingungen einzelner Felder hatten starken Einfluss auf die Mengen getesteter allergener Pilze. Trotz dadurch verursachter Schwankungen blieben die Mengen der wichtigsten Allergieverursacher, *A. alternata* und *Cladosporium cladosporioides*, in allen Fällen hoch. Dies lässt vermuten, dass hohe Mengen allergener Pilze ein unvermeidlicher Nebeneffekt der Kultivierung einiger Feldfrüchte sein könnten.

Die dritte Forschungsfrage beschäftigte sich mit dem Verhältnis zwischen mikrobieller Umwelt im Innenraum und ihren Effekten auf kindliche Immunsysteme. Diese Frage wurde unter einem weiten Blickwinkel angegangen, mittels einer einwohnerbasierten Geburtskohorte. Zu diesem Zweck wurden molekulare Fingerabdrücke von Pilzen und Bakterien in Innenraumstaub aus Wohnungen mit Kindern analysiert. Eine epidemiologisch relevante Anzahl von Proben ermöglichte es, eine Verbindung zwischen der Pilzgemeinschaft in Staub während der frühen Kindheit und späteren allergischen Symptomen herzustellen. Außerdem wurden Einflussfaktoren auf die Zusammensetzung des Mikrobioms im Staub von Wohnräumen identifiziert. Unter anderem wurde eine starke Assoziation zwischen der Zusammensetzung des Staubmikrobioms und dem jahreszeitlichen (Pilze) oder phänologischen (Bakterien) Verlauf festgestellt, mit Auswirkungen auf das Design zukünftiger Studien zum Innenraum-Mikrobiom.

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

II Weikl F, Radl V, Munch JC, Pritsch K. 2015. Targeting allergenic fungi in agricultural environments aids the identification of major sources and potential risks for human health. *Science of the Total Environment* 529: 223-230.
DOI 10.1016/j.scitotenv.2015.05.056

III Tischer C*, Weikl F*, Probst AJ, Standl M, Heinrich J, Pritsch K. Urban dust microbiome: Impact on later atopy and wheezing. Article submitted to *Environmental Health Perspectives* on November 20, 2015
*contributed equally to this work

IV Weikl F, Tischer C, Probst AJ, Heinrich J, Markevych, I, Jochner S, Pritsch K. Fungal and bacterial communities in indoor dust follow different environmental determinants. Article submitted to *PLOS ONE* on November 23, 2015

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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ABBREVIATIONS AND ACRONYMS

ANOVA	analysis of variance
BVOC	biogenic volatile organic compound
CD	cluster of differentiation
DG18	dichloran-agar with 18% glycerol
DOI	digital objects identifier
EPA	Environmental Protection Agency (of the United States of America)
EPS	extracellular polysaccharides
GEE	general estimation equation
HLA	human leucocyte antigen
IFN	interferon
IgE	immunoglobulin E
IL	interleukin
INSD	international nucleotide sequence database
ITS	internal transcribed spacer
LISAplus	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
MHC	major histocompatibility complex
MRPP	multi-response permutation procedure
MSQPCR	mould specific quantitative polymerase chain reaction
MVOC	microbial volatile organic compound
NGS	next generation sequencing
OPLS	orthogonal partial least squares
PCA	principal component analysis
PCo	principal coordinate
PCoA	principal coordinate analysis
qPCR	quantitative polymerase chain reaction
RAST	radioallergosorbent test
rDNA	ribosomal deoxyribonucleic acid
SBS	sick building syndrome
SEA	soil extract agar
T cell	lymphocyte expressing a T-cell-receptor
Th	T-helper lymphocytes
TNF	tumour necrosis factor
tRFLP	terminal restriction fragment polymorphism
VOC	volatile organic compound
WHO	World Health Organization

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 hypersensivity 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 CO_2 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 C_8 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 neuro-degeneration 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: focussing 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 / m^3) and *Cladosporium* (3000 spores / m^3) 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, Kespohl et al. 2013). A recent example for the dilemma was given by Kespohl 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.

[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” (Crameri 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[2,3], up to 44% among atopics[4] and 80% among asthmatics[5]. The incidence of mould allergy within asthmatic children is 45% whereas it is 70% in asthmatic adults[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 *Alternaria, Aspergillus, Cladosporium,* and *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-

[2] Tariq et al. (1996)
[3] Salvaggio and Aukrust (1981)
[4] Corey et al. (1997)
[5] Lopez and Salvaggio (1985)
[6] Hsieh and Shen (1988)

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 focussing 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

22, 2015). 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**).

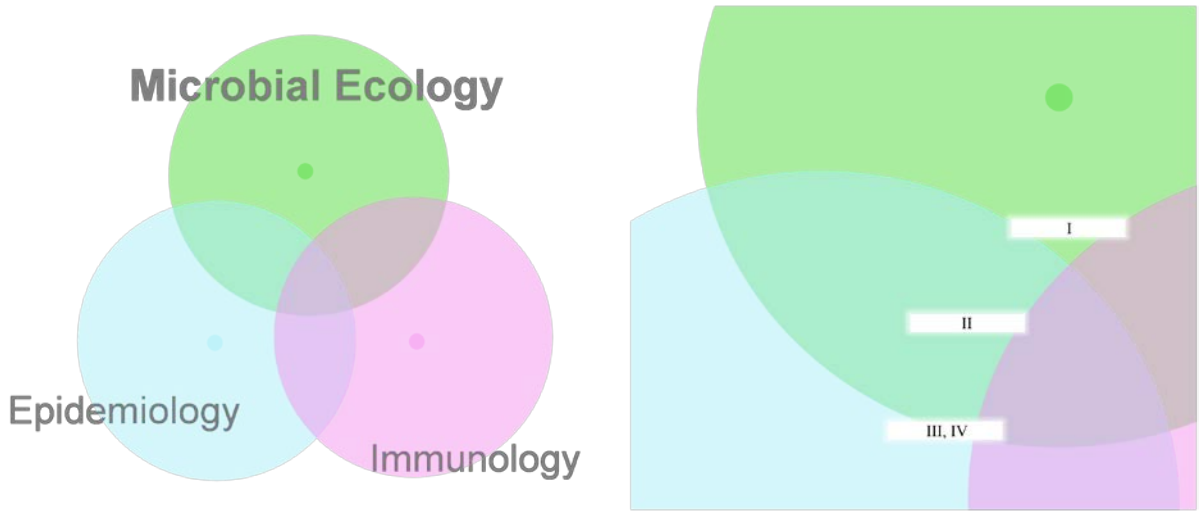

Figure 1 The author's opinion on locating projects I, II, III, and IV in the space of established research disciplines. Left: Microbial Ecology and two disciplines with common intersections to the projects. Right: The degree of overlap for subtopics of the thesis.

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, Mülheim 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 ($E^{-\Delta\Delta CT}$ 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 cm^2 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 cm^2 each of straw and root material were randomly selected per field, and from these pieces, one piece < 1 mm^2 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 δ) 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 1st 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 µm 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**).

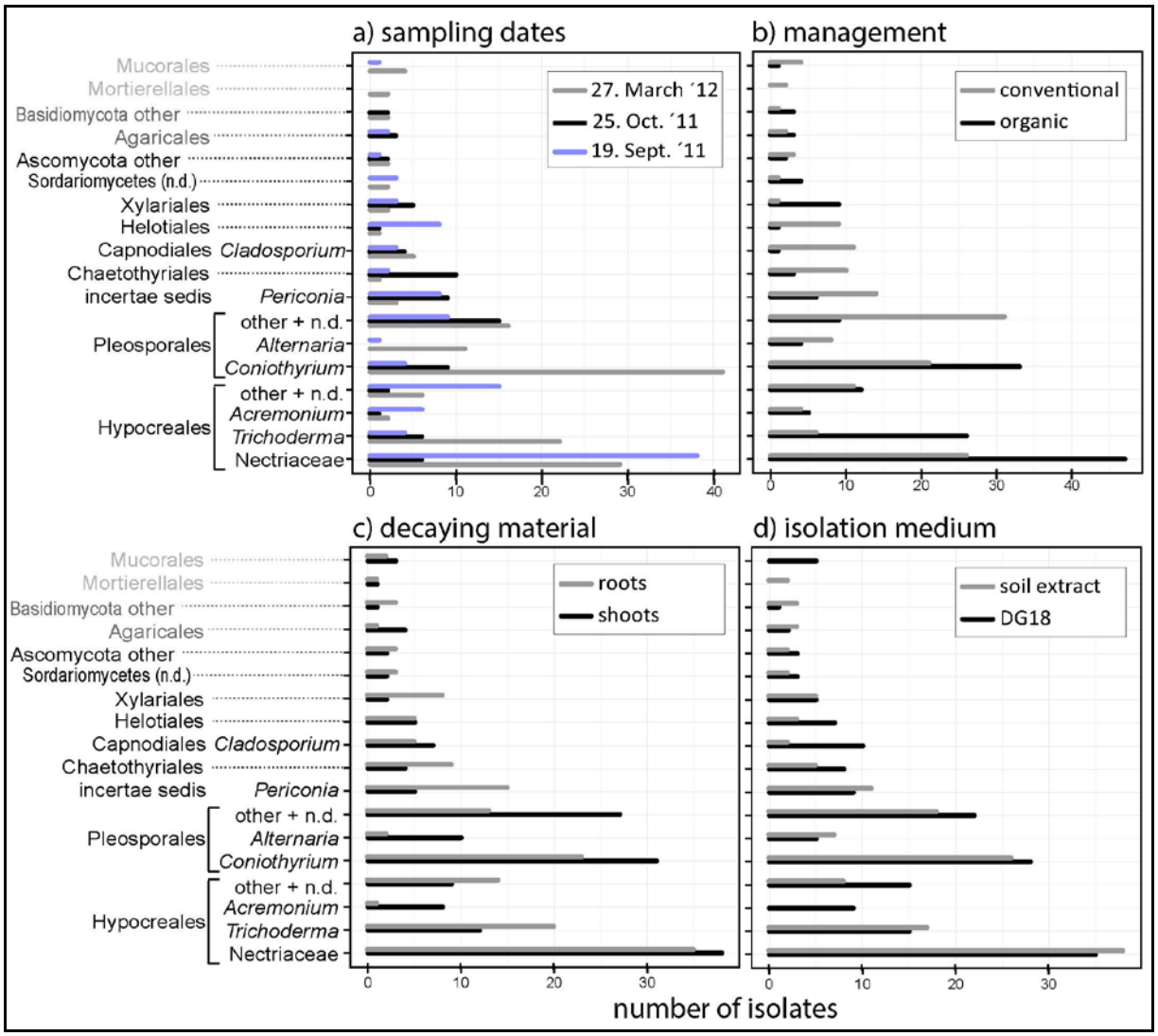

Figure 2 Numbers of identified isolates from project **eII** shown with different separation parameters (**a**, **b**, **c**, **d**). Taxa written in black: Ascomycota, taxa in grey: Basidiomycota, taxa in light grey: Zygomycota. Taxonomic representation is simplified with composite groups: 'Basdiomycota other' = Cantharellalles (1x), Tremellales [*Cryptococcus*] (1x), not determined (2x); 'Ascomyctoa other = incertae sedis [*Minimidochium*] (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: bold values: Adonis $P < 0.05$.

	Fungi		Bacteria	
atopic health outcomes	***P***	***R^2***	***P***	***R^2***
Current asthma at 6 years	**0.032**	0.020	0.275	0.008
Current asthma at 10 years	0.778	0.002	0.679	0.002
Ever wheezing until 10 years	0.291	0.007	0.937	0.001
Eczema at 1 year	**0.004**	0.035	0.369	0.006
IgE at 6 years: total levels	0.373	0.018	**0.017**	0.043
IgE at 6 years: inhalant allergens	0.222	0.008	0.171	0.010
IgE at 10 years: total levels	0.943	0.012	0.987	0.007
IgE at 10 years: inhalant allergens	0.901	0.002	0.176	0.011

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 $P <$

0.05) 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.

	Fungi		**Bacteria**	
Leucocyte measure	***P***	***R^2***	***P***	***R^2***
leucocytes / µl [blood]	0.284	0.056	0.088	0.112
lymphocytes / µl [blood]	**0.006**	0.189	0.282	0.057
share of sub-populations on leucocytes				
granulocytes	**0.048**	0.121	**0.008**	0.234
monocytes	**0.033**	0.127	0.337	0.053
lymphocytes	**0.018**	0.153	**0.013**	0.206
share of sub-populations on lymphocytes				
B lymphocytes (CD19+)	0.151	0.079	0.501	0.033
natural killer cells (CD16+ CD56+)	0.159	0.077	0.565	0.027
T lymphocyte (CD3+ HLA-DR+)	0.105	0.091	0.992	0.002
cytotoxic T cell (CD3+ CD8+)	0.629	0.030	0.304	0.054
T lymphocytes (CD3+)	0.656	0.028	0.634	0.023
T helper cell (CD3+ CD4+)	0.738	0.024	0.566	0.027
activated T lymphocyte (CD25+ CD3+)	0.132	0.082	0.858	0.011
share of sub-populations on T lymphocytes (CD3+)				
IL4+	0.953	0.015	0.965	0.005
IL2+	0.547	0.034	0.397	0.043
IFNγ+	0.788	0.022	0.640	0.023
TNFα+	0.802	0.021	0.981	0.004
share of sub-populations on cytotoxic T cells (CD3+ CD8+)				
IL4+	**0.022**	0.145	0.217	0.070
IL2+	0.649	0.029	0.840	0.012
IFNγ+	0.942	0.015	0.574	0.028
TNFα+	0.600	0.031	0.844	0.013
share of sub-populations on T helper cells (CD3+ CD4+)				
IL4+	0.224	0.065	0.993	0.003
IL2+	0.446	0.042	0.820	0.013
IFNγ+	0.487	0.038	0.969	0.004
TNFα+	0.253	0.060	0.963	0.005
share of CD25+ lymphocytes on CD3- lymphocytes	0.063	0.108	0.158	0.085
share of TNFα+ cells on non T cells	0.169	0.075	0.474	0.035
share of IFNγ+ cells on non T cells	**0.032**	0.134	0.645	0.022
CD3+ CD4+ / CD3+ CD8+ cells	0.945	0.014	0.288	0.057
CD3+ CD45RA+ (T naive) / CD3+ CD45RO+ (T memory) cells	0.135	0.082	0.665	0.021

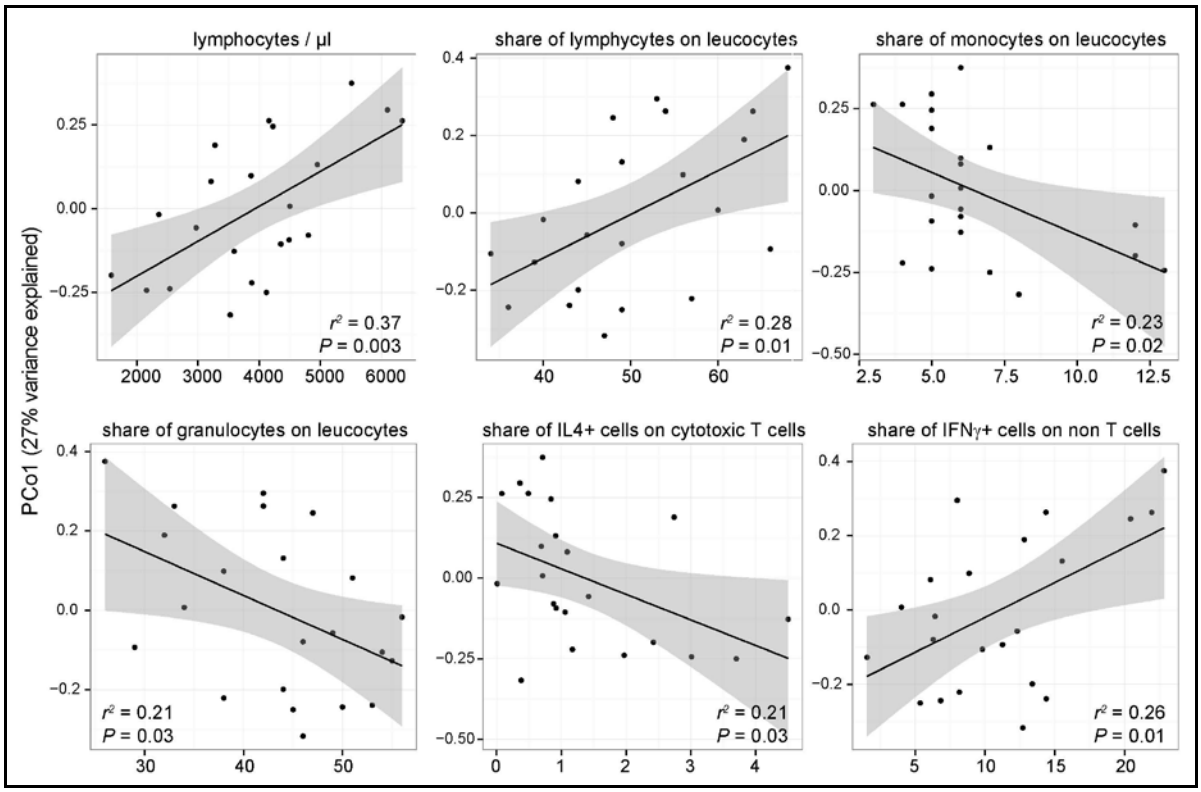

Figure 3 The fungal community in dust in correlation to infants' leucocyte measures. Different measures of leucocytes in the blood of two-year-old infants are plotted against the main part of community dissimilarity between the fungal communities in dust of the children's homes three months after birth. Leucocytes measures used in this analysis were selected for their significance in Adonis tests ($P < 0.05$) (**Table 2**). PCo1: 1st 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. *ρ*: Spearman correlation coefficient, *P*: significance level for the true correlation being not equal to 0, bold values: *P* (Spearman) < 0.05.

white blood cell measures	**Simpson index (1-D)**		**Shannon index (H')**		**Pielou evenness (E)**	
	P	***ρ***	***P***	***ρ***	***P***	***ρ***
leucocytes / µl [blood]	0.306	-0.23	0.228	-0.27	0.204	-0.28
lymphocytes / µl [blood]	**0.007**	-0.57	**0.004**	-0.59	**0.003**	-0.61
share of sub-populations on leucocytes						
granulocytes	**0.002**	0.63	**0.003**	0.61	**<0.001**	0.66
monocytes	**0.018**	0.50	**0.011**	0.53	**0.035**	0.45
lymphocytes	**<0.001**	-0.69	**<0.001**	-0.69	**<0.001**	-0.72
share of IL4+ cells on cytotoxic T cells	**<0.001**	0.76	**<0.001**	0.69	**<0.001**	0.73
share of IFNγ+ cells on non T cells	**0.008**	-0.56	**0.017**	-0.51	**0.037**	-0.45

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, a result of 18% glycerol) compared to most other media ($a_w \approx 0.999$ 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 **II**) 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 C_8 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 a*nd *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 (Crameri et al. 2014, Simon-Nobbe et al. 2008).

As with all forms of allergy, avoiding exposure is still the best treatment (Crameri 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 (Crameri 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 10^7 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

Abarenkov, K., Henrik Nilsson, R., Larsson, K.-H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Ursing, B.M., Vrålstad, T., Liimatainen, K., Peintner, U., Kõljalg, U., 2010. The UNITE database for molecular identification of fungi – recent updates and future perspectives. New Phytologist 186, 281-285.

Adams, R.I., Miletto, M., Lindow, S.E., Taylor, J.W., Bruns, T.D., 2014. Airborne bacterial communities in residences: similarities and differences with fungi. PloS One 9, e91283.

Adams, R.I., Miletto, M., Taylor, J.W., Bruns, T.D., 2013. Dispersal in microbes: fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. ISME Journal 7, 1262-1273.

Agger, S., Lopez-Gallego, F., Schmidt-Dannert, C., 2009. Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. Molecular Microbiology 72, 1181-1195.

Andersson, M.A., Nikulin, M., Koljalg, U., Andersson, M.C., Rainey, F., Reijula, K., Hintikka, E.-L., Salkinoja-Salonen, M., 1997. Bacteria, molds and toxins in water-damaged building materials. Applied and Environmental Microbiology 63, 387-393.

Arrieta, M., Stiemsma, L.T., Dimitriu, P.A., Thorson, L., Russell, S., Yurist-Doutsch, S., Kuzeljevic, B., Gold, M.J., Britton, H.M., Lefebvre, D.L., Subbarao, P., P., M., Becker, A., McNagny, K.M., Sears, M.R., Kollmann, T., CHILD Study Investigators, Mohn, W.W., Turvey, S.E., Finlay, B.B., 2015. Early infancy microbial and metabolic alterations affect risk of childhood asthma. Science Translational Medicine 7, 1-14.

Bazemore, R.A., Feng, J., Cseke, L., Podila, G.K., 2012. Biomedically important pathogenic fungi detection with volatile biomarkers. Journal of Breath Research 6, 016002.

Bennett, J.W., 2015. Silver linings: a personal memoir about Hurricane Katrina and fungal volatiles. Frontiers in Microbiology 6, 206.

Beusse, R., Choung, B., Hatfield, J., 2013. Public may be making indoor mold cleanup decisions based on EPA tool developed only for research applications. EPA, Office of inspector general. Report No. 13-P-0356. [www.epa.gov/oig/reports/2013/ 20130822-13-P-0356.pdf, retrieved 10/2014]

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

Bitas, V., Kim, H.S., Bennett, J.W., Kang, S., 2013. Sniffing on microbes: diverse roles of microbial volatile organic compounds in plant health. Molecular Plant-Microbe Interactions Journal 26, 835-843.

Blackwell, M., 2011. The fungi: 1, 2, 3 ... 5.1 million species? American Journal of Botany 98, 426-438.

Bousquet, J., Lockey, R., Malling, H., members, W.p., 1998. Allergen immunotherapy: Therapeutic vaccines for allergic diseases. A WHO position paper. Journal of Allergy and Clinical Immunology 102, 558-562.

Burge, H.A., Rogers, C.A., 2000. Outdoor allergens. Environmental Health Perspectives 108, 653-659.

Burge, P.S., 2004. Sick building syndrome. Occupational and Environmental Medicine 61, 185-190.

Burr, M.L., Matthews, I.P., Arthur, R.A., Watson, H.L., Gregory, C.J., Dunstan, F.D., Palmer, S.R., 2007.

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

Cabral, J.P.S., 2010. Can we use indoor fungi as bioindicators of indoor air quality? Historical perspectives and open questions. Science of the Total Environment 408, 4285-4295.

Camarinha-Silva, A., Wos-Oxley, M.L., Jauregui, R., Becker, K., Pieper, D.H., 2012. Validating T-RFLP as a sensitive and high-throughput approach to assess bacterial diversity patterns in human anterior nares. FEMS Microbiology Ecology 79, 98-108.

Casas, L., Tischer, C., Wouters, I.M., Valkonen, M., Gehring, U., Doekes, G., Torrent, M., Pekkanen, J., Garcia-Esteban, R., Hyvarinen, A., Heinrich, J., Sunyer, J., 2013. Endotoxin, extracellular polysaccharides, and beta(1-3)-glucan concentrations in dust and their determinants in four European birth cohorts: results from the HITEA project. Indoor Air 23, 208-218.

Chapman, M.D., Pomes, A., Breiteneder, H., Ferreira, F., 2007. Nomenclature and structural biology of allergens. Journal of Allergy and Clinical Immunology 119, 414-420.

Corey, J.P., Kaiseruddin, S., Gungor, A., 1997. Prevalence of mold-specific immunoglobulins in a Midwestern allergy practice. Otolaryngology-Head and Neck Surgery 117, 516-520.

Crameri, R., Garbani, M., Rhyner, C., Huitema, C., 2014. Fungi: the neglected allergenic sources. Allergy 69, 176-185.

Demyttenaere, J.C.R., Moriña, R.M., De Kimpe, N., Sandra, P., 2004. Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. Journal of Chromatography A 1027, 147-154.

Denning, D.W., O'Driscoll, B.R., Hogaboam, C.M., Bowyer, P., Niven, R.M., 2006. The link between fungi and severe asthma: a summary of the evidence. European Respiratory Journal 27, 615-626.

Denning, D.W., Pashley, C., Hartl, D., Wardlaw, A., Godet, C., Del Giacco, S., Delhaes, L., Sergejeva, S., 2014. Fungal allergy in asthma-state of the art and research needs. Clinical and Translational Allergy 4, 14.

Domsch, K.H., Gams, W., Anderson, T.-H., 1993. Compendium of soil fungi. Authorized Reprint (1980), IHW-Verlag, Eching, Germany

Effmert, U., Kalderas, J., Warnke, R., Piechulla, B., 2012. Volatile mediated interactions between bacteria and fungi in the soil. Journal of Chemical Ecology 38, 665-703.

Ege, M., Mayer, M., Normand, A.-C., Genuneit, J., Cookson, W.O.C.M., Braun-Fahrländer, C.-B., Heederik, D., Piarroux, R., von Mutius, E., 2011. Exposure to Environmental Microorganisms and Childhood Asthma. New England Journal of Medicine 364, 701-709.

Esch, R.E., 2004. Manufacturing and standardizing fungal allergen products. Journal of Allergy and Clinical Immunology 113, 210-215.

Fahlbusch, B., Heinrich, J., Groß, I., Jäger, L., Richter, K., Wichmann, H.E., 1999. Allergens in house-dust samples in Germany: results of an East-West German comparison. Allergy 54, 1215-1222.

Farag, M.A., Zhang, H., Ryu, C.M., 2013. Dynamic chemical communication between plants and bacteria through airborne signals: induced resistance by bacterial volatiles. Journal of Chemical Ecology 39, 1007-1018.

Fernández-Rodríguez, S., Tormo-Molina, R., Maya-Manzano, J.M., Silva-Palacios, I., Gonzalo-Garijo, Á., 2014. Outdoor airborne fungi captured by viable and non-viable methods. Fungal Ecology 7, 16-26.

Fischer, G., Dott, W., 2003. Relevance of airborne fungi and their secondary metabolites for environmental, occupational and indoor hygiene. Archives of Microbiology 179, 75-82.

Frankland, J.C., Dighton, J., Boddy, L., 1990. Methods for studying fungi in soil and forest litter. Methods in Microbiology 22, 343-404.

Gadd, G.M. (Ed.), 2006. Fungi in biogeochemical cycles. Cambridge University Press. Cambridge, UK.

Gardes, M., Bruns, T.D., 1993. ITS primers with enhanced specifity for basidiomycetes - application to the identification of mycorrhizae and rusts. Molecular Ecology 2, 113-118.

Gehring, U., Heinrich, J., Hoek, G., Giovannangelo, M., Nordling, E., Bellander, T., Gerritsen, J., de Jongste, J.C., Smit, H.A., Wichmann, H.E., Wickman, M., Brunekreef, B., 2007. Bacteria and mould components in house dust and children's allergic sensitisation. European Respiratory Journal 29, 1144-1153.

Górny, R.L., 2004. Filamentous microorganismns and their fragments in indoor air – a review. Annals of Agricultural and Environmental Medicine 11, 185-197.

Górny, R.L., Reponen, T., Willeke, K., Schmechel, D., Robine, E., Boissier, M., Grinshpun, S.A., 2002. Fungal fragments as indoor air biocontaminants. Applied and Environmental Microbiology 68, 3522-3531.

Gouy, M., Guindon, S., Gascuel, O., 2010. SeaView version 4: A multiplatform graphical user interface for sequence alignment and phylogenetic tree building. Molecular Biology and Evolution 27, 221-224.

Gravesen, 1979. Fungi as a cause of allergic disease. Allergy 34, 134-154.

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.

Guarro, J., Gené, J., Stchigel, A., Figueras, M., 2012. Atlas of soil ascomycetes. CBS-KNAW Fungal Biodiversity Centre.

Haugland, R., Vesper, S., 2002. Method of identifiying and quantifying specific fungi and bacteria. U.S. Environmental Protection Agency, Washington, DC (US), USA.

Heal, G., 2000. Nature and the marketplace: capturing the value of ecosystem services. Island Press, Washington, DC (US), USA.

Heddergott, C., Calvo, A.M., Latgé, J.P., 2014. The Volatome of *Aspergillus fumigatus*. Eukaryotic cell 13, 1014-1025.

Heinrich, J., Bolte, G., Holscher, B., Douwes, J., Lehmann, I., Fahlbusch, B., Bischof, W., Weiss, M., Borte, M., Wichmann, H.E., 2002. Allergens and endotoxin on mothers' mattresses and total immunoglobulin E in cord blood of neonates. European Respiratory Journal 20, 617-623.

Heinrich, J., Bruske, I., Schnappinger, M., Standl, M., Flexeder, C., Thiering, E., Tischer, C., Tiesler, C.M., Kohlbock, G., Wenig, C.M., Bauer, C.P., Schaaf, B., von Berg, A., Berdel, D., Kramer, U., Cramer, C., Lehmann, I., Herbarth, O., Behrendt, H., Ring, J., Kuhnisch, J., Koletzko, S., 2012. [Two

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

Heinrich, J., Holscher, B., Douwes, J., Richter, K., Koch, A., Bischof, W., Fahlbusch, B., Kinne, R.W., Wichmann, H.E., 2003. Reproducibility of allergen, endotoxin and fungi measurements in the indoor environment. Journal of Exposure Analysis and Environmental Epidemiology 13, 152-160.

Hocking, A.D., Pitt, J.I., 1980. Dichloran-glycerol medium for enumeration of xerophilic fungi from low-moisture foods. Applied and Environmental Microbiology 39, 488-492.

Hooper, D.U., Chapin, F.S., Ewel, J.J., Hector, A., Inchausti, P., Lavorel, S., Lawton, J.H., Lodge, D.M., Loreau, M., Naeem, S., Schmid, B., Setälä, H., Symstad, A.J., Vandermeer, J., Wardle, D.A., 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs 75, 3-35.

Hsieh, K.-H., Shen, J.-J., 1988. Prevalence of Childhood Asthma in Taipei, Taiwan, and Other Asian Pacific Countries. Journal of Asthma 25, 73-82.

Humphries, C., 2012. Indoor Ecosystems. Science 335, 648-650.

Hung, R., Lee, S., Bennett, J.W., 2014. The effects of low concentrations of the enantiomers of mushroom alcohol (1-octen-3-ol) on *Arabidopsis thaliana*. Mycology 5, 73-80.

Hung, R., Lee, S., Bennett, J.W., 2015. Fungal volatile organic compounds and their role in ecosystems. Applied Microbiology and Biotechnology 99, 3395-3405.

Inamdar, A.A., Bennett, J.W., 2014. A common fungal volatile organic compound induces a nitric oxide mediated inflammatory response in *Drosophila melanogaster*. Scientific Reports 4, 3833.

Inamdar, A.A., Hossain, M.M., Bernstein, A.I., Miller, G.W., Richardson, J.R., Bennett, J.W., 2013. Fungal-derived semiochemical 1-octen-3-ol disrupts dopamine packaging and causes neurodegeneration. Proceedings of the National Academy of Sciences USA 110, 19561-19566.

Inamdar, A.A., Moore, J.C., Cohen, R.I., Bennett, J.W., 2012b. A model to evaluate the cytotoxicity of the fungal volatile organic compound 1-octen-3-ol in human embryonic stem cells. Mycopathologia 173, 13-20.

Inamdar, A.A., Zaman, T., Morath, S.U., Pu, D.C., Bennett, J.W., 2012a. *Drosophila melanogaster* as a model to characterize fungal volatile organic compounds. Environmental Toxicology 29, 829-836.

Insam, H., Seewald, M.S.A., 2010. Volatile organic compounds (VOCs) in soils. Biology and Fertility of Soils 46, 199-213.

Junker, R.R., Tholl, D., 2013. Volatile organic compound mediated interactions at the plant-microbe interface. Journal of Chemical Ecology 39, 810-825.

Kelley, S.T., Gilbert, J.A., 2013. Studying the microbiology of the indoor environment. Genome Biology 14, 202.

Kespohl, S., Maryska, S., Zahradnik, E., Sander, I., Bruning, T., Raulf-Heimsoth, M., 2013. Biochemical and immunological analysis of mould skin prick test solution: current status of standardization. Clinical and Experimental Allergy 43, 1286-1296.

Kesselmeier, J., Staudt, M., 1999. Biogenic volatile organic compounds (VOC): an overview on emission, physiology and ecology. Journal of Atmospheric Chemistry 33, 23-88.

Kettleson, E.M., Adhikari, A., Vesper, S., Coombs, K., Indugula, R., Reponen, T., 2015. Key determinants of the fungal and bacterial microbiomes in homes. Environmental Research 138C, 130-135.

Kinross, J.M., Darzi, A.W., Nicholson, J.K., 2011. Gut microbiome-host interactions in health and disease. Genome Medicine 3:14

Knutsen, A.P., Bush, R.K., Demain, J.G., Denning, D.W., Dixit, A., Fairs, A., Greenberger, P.A., Kariuki, B., Kita, H., Kurup, V.P., Moss, R.B., Niven, R.M., Pashley, C.H., Slavin, R.G., Vijay, H.M., Wardlaw, A.J., 2012. Fungi and allergic lower respiratory tract diseases. Journal of Allergy and Clinical Immunology 129, 280-291.

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.

Köhler, J.R., Casadevall, A., Perfect, J., 2015. The spectrum of fungi that infects humans. Cold Spring Harbor Perspectives in Medicine 5, a019273.

Kõljalg, U., Larsson, K.-H., Abarenkov, K., Nilsson, R.H., Alexander, I.J., Eberhardt, U., Erland, S., Høiland, K., Kjøller, R., Larsson, E., Pennanen, T., Sen, R., Taylor, A.F.S., Tedersoo, L., Vrålstad, T., 2005. UNITE: a database providing web-based methods for the molecular identification of ectomycorrhizal fungi. New Phytologist 166, 1063-1068.

Korpi, A., Jarnberg, J., Pasanen, A.L., 2009. Microbial volatile organic compounds. Critical Reviews in Toxicology 39, 139-193.

Kramer, R., Abraham, W.-R., 2012. Volatile sesquiterpenes from fungi: what are they good for? Phytochem Rev 11, 15-37.

Kuske, M., Romain, A., Nicholas, J., 2005. Microbial volatile organic compounds as indicators of fungi. Can an electronic nose detect fungi in indoor environments? Building and Environment 40, 824-831.

Lax, S., Smith, D.P., Hampton-Marcell, J., Owens, S.M., Handley, K.M., Scott, N.M., Gibbons, S.M., Larsen, P., Shogan, B.D., Weiss, S., Metcalf, J.L., Ursell, L.K., Vásquez-Baeza, Y., Van Treuren, W., Hasan, N.A., Gibson, M.K., Colwell, R., Dantas, G., Knight, R., Gilbert, J.A., 2014. Longitudinal analysis of microbial interaction between humans and the indoor environment. Science 345, 1048-1052.

Legatzki, A., Rosler, B., von Mutius, E., 2014. Microbiome diversity and asthma and allergy risk. Current Allergy and Asthma Reports 14, 466.

Lemfack, M.C., Nickel, J., Dunkel, M., Preissner, R., Piechulla, B., 2014. mVOC: a database of microbial volatiles. Nucleic Acids Research 42, D744-D748.

Lopez, M., Salvaggio, J.E., 1985. Mold-Sensitive Asthma. Clinical Reviews in Allergy 3, 183-196.

Lynch, S.V., Wood, R.A., Boushey, H., Bacharier, L.B., Bloomberg, G.R., Kattan, M., O'Connor, G.T., Sandel, M.T., Calatroni, A., Matsui, E., Johnson, C.C., Lynn, H., Visness, C.M., Jaffee, K.F., Gergen, P.J., Gold, D.R., Wright, R.J., Fujimura, K., Rauch, M., Busse, W.W., Gern, J.E., 2014. Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. Journal of Allergy and Clinical Immunology 134, 593-601 e512.

MacDougall, A.S., McCann, K.S., Gellner, G., Turkington, R., 2013. Diversity loss with persistent

human disturbance increases vulnerability to ecosystem collapse. Nature 494, 86-89.

Martin, R., Miquel, S., Langella, P., Bermudez-Humaran, L.G., 2014. The role of metagenomics in understanding the human microbiome in health and disease. Virulence 5, 413-423.

Martinez, F.D., Holt, P.G., 1999. Role of microbial burden in aetiology of allergy and asthma. The Lancet 354, SII12-SII15.

McDonald, J.H., 2014. Handbook of biological statistics, 3 ed., Sparky House Publishing, Baltimore, Maryland, USA.

Miller, D.J., Allemann, R.K., 2012. Sesquiterpene synthases: passive catalysts or active players? Natural Product Reports 29, 60-71.

Morath, S.U., Hung, R., Bennett, J.W., 2012. Fungal volatile organic compounds: A review with emphasis on their biotechnological potential. Fungal Biology Reviews 26, 73-83.

Moschandreas, D., Pagilla, K.R., Storino, L.V., 2003. Time and space uniformity of indoor bacteria concentrations in Chicago area residences. Aerosol Science and Technology 37:11, 899-906.

Moularat, S., Hulin, M., Robine, E., Annesi-Maesano, I., Caillaud, D., 2011. Airborne fungal volatile organic compounds in rural and urban dwellings: detection of mould contamination in 94 homes determined by visual inspection and airborne fungal volatile organic compounds method. Science of the Total Environment 409, 2005-2009.

Naeem, S., Li, S., 1997. Biodiversity enhances ecosystem reliability. Nature 390, 507-509.

NESCent working group on the evolutionary biology of the built environment, Martin, L.J., Adams, R.I., Bateman, A., Bik, H.M., Hawks, J., Hird, S.M., Hughes, D., Kembel, S.W., Kinney, K., Kolokotronis, S.O., Levy, G., McClain, C., Meadow, J.F., Medina, R.F., Mhuireach, G., Moreau, C.S., Munshi-South, J., Nichols, L.M., Palmer, C., Popova, L., Schal, C., Taubel, M., Trautwein, M., Ugalde, J.A., Dunn, R.R., 2015. Evolution of the indoor biome. Trends in Ecology and Evolution 30, 223-232. [TREE-1909, http://dx.doi.org/10.1016/j.tree.2015.02.001]

Nevalainen, A., Taubel, M., Hyvarinen, A., 2015. Indoor fungi: companions and contaminants. Indoor Air 25, 125-156.

Nilsson, R.H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.H., Kõljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PloS one e59, 1-4.

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

Okada, H., Kuhn, C., Feillet, H., Bach, J.F., 2010. The 'hygiene hypothesis' for autoimmune and allergic diseases: an update. Clinical and Experimental Immunology 160, 1-9.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H., 2013. vegan: Community Ecology Package., version 2.0-10 ed.

Olszak, T., An, D., Zeissig, S., Pinilla Vera, M., Richter, J., Franke, A., Glickman, J.N., Siebert, R., Baron, R.M., Kasper, D.L., Blumberg, R.S., 2012. Microbial exposure during early life has persistent effects on natural killer T cell function. Science 336, 489-493.

O'Malley, M.A., 2007. The nineteenth century roots of ‘everything is everywhere’. Nature Reviews

Microbiology 5, 647-651.

Pawankar, R., Canonica, G.W., Holgate, S.T., Lockey, R.F. (Eds.), 2011. WAO White Book on Allergy. World Allergy Organization, Milwaukee, USA.

Pasanen, A.L., 2001. A review: Fungal exposure assessment in indoor environments. Indoor Air 11, 87-98.

Pedrós-Alió, C., 2012. The rare bacterial biosphere. Annual review of marine science 4, 449-466.

Penuelas, J., Asensio, D., Tholl, D., Wenke, K., Rosenkranz, M., Piechulla, B., Schnitzler, J.P., 2014. Biogenic volatile emissions from the soil. Plant, cell and environment 37, 1866-1891.

Pflughoeft, K.J., Versalovic, J., 2012. Human microbiome in health and disease. Annual Reviews in Pathology 7, 99-122.

Pilloni, G., Granitsiotis, M.S., Engel, M., Lueders, T., 2012. Testing the limits of 454 pyrotag sequencing: reproducibility, quantitative assessment and comparison to T-RFLP fingerprinting of aquifer microbes. PloS One 7, e40467.

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

Poll, C., Brune, T., Begerow, D., Kandeler, E., 2010. Small-scale diversity and succession of fungi in the detritusphere of rye residues. Microbial Ecology 59, 130-140.

Prakash, O., Pandey, P.K., Kulkarni, G.J., Mahale, K.N., Shouche, Y.S., 2014. Technicalities and Glitches of Terminal Restriction Fragment Length Polymorphism (T-RFLP). Indian Journal of Microbiology 54, 255-261.

Quin, M.B., Flynn, C.M., Schmidt-Dannert, C., 2014. Traversing the fungal terpenome. Natural Product Reports 31, 1449-1473.

R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

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.

Redecker, D., Kodner, R., Graham, L.E., 2000. Glomalean fungi from the Ordovician. Science 289, 1920-1921.

Reponen, T., Seo, S.C., Grimsley, F., Lee, T., Crawford, C., Grinshpun, S.A., 2007. Fungal Fragments in Moldy Houses: A Field Study in Homes in New Orleans and Southern Ohio. Atmospheric Environment 41, 8140-8149.

Rintala, H., Pitkäranta, M., Toivola, M., Paulin, L., Nevalainen, A., 2008. Diversity and seasonal dynamics of bacterial community in indoor environment. BMC Microbiology 8, 56.

Rosenbrock, P., 1997. Untersuchungen zum mikrobiellen Abbau von polychlorierten Dibenzo-p-dioxinen und Dibenzofuranen (PCDD/F) in Böden mittels reduktiver Dechlorierung und aerober Mineralisierung. Technische Universität Braunschweig, Braunschweig, Germany.

Ryan, T.J., Beaucham, C., 2013. Dominant microbial volatile organic compounds in 23 US homes.

Chemosphere 90, 977-985.

Sadaka, N., Ponge, J.-F., 2003. Fungal colonization of phyllosphere and litter of *Quercus rotundifolia* Lam. in a holm oak forest (High Atlas, Morocco). Biology and Fertility of Soils 39, 30-36.

Sahlberg, B., Gunnbjornsdottir, M., Soon, A., Jogi, R., Gislason, T., Wieslander, G., Janson, C., Norback, D., 2013. Airborne molds and bacteria, microbial volatile organic compounds (MVOC), plasticizers and formaldehyde in dwellings in three North European cities in relation to sick building syndrome (SBS). Science of the Total Environment 444, 433-440.

Saijo, Y., Kanazawa, A., Araki, A., Morimoto, K., Nakayama, K., Takigawa, T., Tanaka, M., Shibata, E., Yoshimura, T., Chikara, H., Kishi, R., 2011. Relationships between mite allergen levels, mold concentrations, and sick building syndrome symptoms in newly built dwellings in Japan. Indoor Air 21, 253-263.

Salvaggio, J., Aukrust, L., 1981. Mold-induced asthma. Journal of Allergy and Clinical Immunology 68, 327-346.

Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C., B., A., 2010. Food and indoor fungi. CBS-KNAW Fungal Biodiversity Centre, Untrecht, Netherlands.

Schaub, B., Lauener, R., von Mutius, E., 2006. The many faces of the hygiene hypothesis. Journal of Allergy and Clinical Immunology 117, 969-977.

Schleibinger, H., Laussmann, D., Bornehag, C.G., Eis, D., Rueden, H., 2008. Microbial volatile organic compounds in the air of moldy and mold-free indoor environments. Indoor Air 18, 113-124.

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., Garbeva, P., 2015. Volatile affairs in microbial interactions. ISME Journal 9, 2329–2335.

Schoch, C.L., Seifert, K.A., Huhndorf, S., Robert, V., Spouge, J.L., Levesque, C.A., Chen, W., 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. Proceedings of the National Academy of Science USA 109, 6241-6246.

Schröder, P., Huber, B., Reents, H.J., Munch, J.C., Pfadenhauer, J., 2008. Outline of the Scheyern project., in: Schröder, P., Pfadenhauer, J., Munch, J.C. (Eds.), Perspectives for Agroecosystem Management., Amsterdam, Netherlands.

Simon-Nobbe, B., Denk, U., Poll, V., Rid, R., Breitenbach, M., 2008. The spectrum of fungal allergy. International Archives of Allergy and Immunology 145, 58-86.

Sironi, M., Clerici, M., 2010. The hygiene hypothesis: an evolutionary perspective. Microbes and Infection 12, 421-427.

Six, J., Frey, S.D., Thiet, R.K., Batten, K.M., 2006. Bacterial and fungal contributions to carbon sequestration in agroecosystems. Soil Science Society of America Journal 70, 555.

Smits, L.S., Letz, K.L., Evans, S.T., J.K., G., 2003. Evaluating the response of patients undergoing both allergy skin testing and in vitro allergy testing with the immunoCAP technology system. Journal of the American Academy of Nurse Practitioners 15, 415-423.

Spiteller, P., 2015. Chemical ecology of fungi. Natural product Reports 32, 971-993.

Stolwijk, J.A., 1991. Sick-building syndrome. Environmental Health Perspectives 95, 99-100.

Strachan, D.P., 1989. Hay fever, hygiene, and household size. British Medical Journal 299, 1259-1260.

Tariq, S.M., Matthews, S.M., Stevens, M., Hakim, E.A., 1996. Sensitization to *Alternaria* and *Cladosporium* by the age of 4 years. Clinical and Experimental Allergy 26, 794-798.

Tischer, C., Casas, L., Wouters, I.M., Doekes, G., Garcia-Esteban, R., Gehring, U., Hyvarinen, A., Oldenwening, M., Kerkhof, M., Sunyer, J., Standl, M., Thiering, E., Torrent, M., Heinrich, J., group, H.s., 2015. Early exposure to bio-contaminants and asthma up to 10 years of age: results of the HITEA study. European Respiratory Journal 45, 328-337.

Tischer, C., Gehring, U., Chen, C.M., Kerkhof, M., Koppelman, G., Sausenthaler, S., Herbarth, O., Schaaf, B., Lehmann, I., Kramer, U., Berdel, D., von Berg, A., Bauer, C.P., Koletzko, S., Wichmann, H.E., Brunekreef, B., Heinrich, J., 2011. Respiratory health in children, and indoor exposure to (1,3)-beta-D-glucan, EPS mould components and endotoxin. European Respiratory Journal 37, 1050-1059.

Townsend, C.R., Harper, J.L., Begon, M., 2002. Ökologie. Springer, Berlin, Germany.

Twaroch, T.E., Curin, M., Valenta, R., Swoboda, I., 2015. Mold allergens in respiratory allergy: from structure to therapy. Allergy, Asthma and Immunology Research 7, 205-220.

Valkonen, M., Wouters, I.M., Taubel, M., Rintala, H., Lenters, V., Vasara, R., Genuneit, J., Braun-Fahrlander, C., Piarroux, R., von Mutius, E., Heederik, D., Hyvarinen, A., 2015. Bacterial exposures and associations with atopy and asthma in children. PloS One 10, e0131594.

van Dijk, E.L., Auger, H., Jaszczyszyn, Y., Thermes, C., 2014. Ten years of next-generation sequencing technology. Trends in Genetics 30, 418-426.

Van Oost, K., Govers, G., de Alba, S., Quine, T.A., 2006. Tillage erosion: a review of controlling factors and implications for soil quality. Progress in Physical Geography 30, 443-466.

Vesper, S., 2011. Traditional mould analysis compared to a DNA-based method of mould analysis. Critical Reviews in Microbiology 37, 15-24.

von Mutius, E., Vercelli, D., 2010. Farm living: effects on childhood asthma and allergy. Nature Reviews Immunology 10, 861-868.

Vuorio, R., Andersson, M.A., Rainey, F.A., Kroppenstedt, R.M., Kämpfer, P., Busse, H.-J., Vilijanen, M., Salkinoja-Salonen, M., 1999. A new rapidly growing mycobacterial species, *Mycobacterium murale* sp. nov., isolated from the indoor walls of a children's day care centre. International Journal of Systematic Bacteriology 49, 25-35.

Wenke, K., Kai, M., Piechulla, B., 2010. Belowground volatiles facilitate interactions between plant roots and soil organisms. Planta 231, 499-506.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR Protocols: A Guide to Methods and Applications. Academic Press, London, UK, pp. 315-322.

Yazdanbakhsh, M., P.G., K., van Ree, R., 2002. Allergy, parasites, and the hygiene hypothesis. Science 296, 490-494.

Zhang, X., Sahlberg, B., Wieslander, G., Janson, C., Gislason, T., Norback, D., 2012. Dampness and moulds in workplace buildings: associations with incidence and remission of sick building

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

Zhang, Z., Yang, M.J., Pawliszyn, J., 1994. Solid-phase microextraction. A solvent-free alternative for sample preparation. Analytical Chemistry 66, 844A-853A.

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 ($C_{15}H_{24}$). They function as infochemicals[1,2] and play crucial roles in plant-to-plant, plant-to-microbe/animal and microbe-to-microbe interactions[3]. Industrially, sesquiterpenes can act as precursors of advanced biofuels with properties similar to petroleum-based fuels[4-7]. Sesquiterpenes can affect atmospheric chemistry and impact climate in a similar manner to other volatile terpenoids[8,9], although – due to analytical difficulties – a large uncertainty exists about sesquiterpene emissions[10]. Microorganisms and especially fungi have recently been recognized as potentially important sources of volatile organic compounds (VOCs)[3]. Thus, studying microbial volatile terpenoids is both industrially valuable and essential to understand biotic and biosphere-atmosphere interactions.

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

In addition to its role as a plant pathogen[11] and producer of mycotoxins[14], *A. alternata* is a major fungal allergen source[18], which has led to it becoming one of the most thoroughly studied fungi. Still, the microbial VOC database[19] lists only one compound (6-methoxyheptanol) for *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*[20] and two compounds ((+)-β-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

69 fungal VOCs i.e., sesquiterpenes related to the different lifestyles and for applied approaches
70 such as the use of sesquiterpene biomarkers for fungi.
71
72 **Results**
73 *Growth characteristics*
74 Under nutrient-rich conditions, the mycelia of both fungi grew similarly with growth ending
75 between day 14 (co-cultivation) and day 21 (solitary cultivation). Neither fungus was able to
76 overgrow the other and cultures were aged after day 28, as indicated by partly collapsed and
77 hyaline hyphae.
78 Under nutrient poor conditions, *F. oxysporum* grew faster than *A. alternata*; however, the
79 growth of *F. oxysporum* under co-cultivation slowed between day 14 and day 21. Between
80 days 21 and 28, *F. oxysporum* started to overgrow peripheral parts of *A. alternata* mycelia. At
81 day 35, both fungi were still growing when cultivated alone.
82 (Fig. 1). Fungal morphology is shown in Supplementary Figs. S2 and S3 online.
83
84 *Overview of VOC emission diversity and strength*
85 The chemical compounds in VOC emissions were quantitatively and qualitatively diverse,
86 and the quantity was higher for *A. alternata* compared to *F. oxysporum*. Altogether, we
87 detected 27 different volatile compounds of unambiguous fungal origin, 25 sesquiterpenes
88 and two alcohols (Table 1). From *A. alternata*, 26 compounds were emitted – in total up to
89 183.5 ± 82.3 pmol cm^{-2} h^{-1} (day 3, nutrient rich), while *F. oxysporum* emitted 10 compounds
90 – in total up to 6.7 ± 4.7 pmol cm^{-2} h^{-1} (day 3, nutrient rich). All VOCs from *F. oxysporum*
91 were also detected in *A. alternata*, except for δ-elemene (Table 2).
92 All 27 substances were detected when both fungi grew in direct contact (direct confrontation
93 plates) under nutrient rich conditions. Fewer different VOC were detected when the fungi
94 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).

121

122 *Emission of specific compounds*

123 Under all nutrient conditions and at all time-points, the sesquiterpenes thujopsene and β-
124 cedrene were the most abundant VOCs of *A. alternata*. Together, they constituted more than
125 two-thirds of the total emissions (Fig. 2). The next highest emissions generally were an
126 unknown SQT (sesquiterpene) #1, a mixture of thujopsane-2β-ol plus unknown SQT #4, α-
127 himachalene, and β-acoradiene (Table 1, Supplementary Fig. S4 online). While the general
128 emission patterns of *A. alternata* did not differ notably with culture age, the contributions of
129 thujopsene and β-cedrene varied by up to 25% between sampling time-points (Fig. 2).
130 The emission pattern of *F. oxysporum* was more variable (detection was approximately at the
131 limit of quantification for some substances) and the overall emission rate was more than 100-
132 fold lower at most time-points than for *A. alternata* (Fig. 2, Supplementary Fig. S5 online).
133 For example, at day 3 under nutrient rich conditions, nine compounds were detected for *F.*
134 *oxysporum* (highest: δ-elemene, 2-methyl-1-butanol, and α-himachalene), while three weeks
135 later (day 28), only two compounds (δ-elemene and α-himachalene) remained detectable.
136 Three substances (γ-curcurmene, germacrene D, and an unknown SQT #2) were only detected
137 in trace amounts (< 0.003 pmol cm^{-2} h^{-1}) on one or two sampling dates.
138 Common to all cultures was a much more rapid decline in the emission rate of alcohols
139 compared to sesquiterpenes. By day 14, only traces of alcohols were detected (e.g., 0.04 ±
140 0.008 pmol cm^{-2} h^{-1} 2-methyl-1-butanol emitted from solitary cultures of *A. alternata*).
141 Amounts of compounds under nutrient poor cultivation resembled those of the nutrient rich
142 cultivations for *A. alternata* (except for the absent compounds, Table 2). For *F. oxysporum*,
143 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
145 individual compounds from *F. oxysporum* were inferred for nutrient poor conditions.

146 Almost all individual compounds from *A. alternata* were found in lower amounts on split-
147 plates compared to solitary cultivation (Fig. 4). When grown together with *F. oxysporum,*
148 some sesquiterpenes of *A. alternata* were significantly less abundant, e.g., β-cedrene (days 14
149 and 21; t-tests $P = 0.001$) and thujopsene (days 7 and 14; t-tests $P = 0.005$).
150
151 *Multivariate analyses and correlation matrices*
152 VOC profiles strongly differed in multivariate analyses (PCA and OPLS) – in quality and
153 quantity – between *A. alternata* and *F. oxysporum* cultures growing alone, as shown by the
154 large distance and separation between *A. alternata* and *F. oxysporum* samples in the first
155 significant principal component in a PCA, which explained 94% of the total VOC variance
156 (Fig. 5a).
157 Furthermore, VOC emissions from co-cultivated fungi could be separated from those emitted
158 from cultures containing only one fungus, as demonstrated by the separation of the second
159 significant principal component. PCA indicated differences in emissions between fungi
160 growing in direct contact or on split plates, but these were rather marginal.
161 Overall, high emission rates of sesquiterpenes were negatively correlated in solitary cultures
162 of *F. oxysporum* (PCA: Fig. 5b, OPLS: Supplementary Fig. S6 online). Only the
163 sesquiterpene δ-elemene, unique to *F. oxysporum*, was highly and negatively correlated to
164 solitary cultures of *A. alternata* with a scaled and centered regression coefficient of −0.82 in
165 the OPLS model (Supplementary Fig. S6 online).
166 The OPLS model described changes of VOC emissions from the different fungal cultures
167 very well; the cumulative R^2(X) and Q^2(cum) were 98.6% and 70.1% respectively, using 3
168 predictive components. The analysis of variance testing the cross-validated predictive
169 residuals (CV-ANOVA) indicated that the OPLS model discriminated the cultures of a single
170 fungus in a highly reliable manner (*P*-values: *A. alternata* $< 2 \times 10^{-7}$, *F. oxysporum* $< 4 \times 10^{-14}$, 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 δ-elemene and α-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[20]. 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* (~ 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

197 study at day 3 was more than 250 times higher for *A. alternata* and ten times higher for *F.*
198 *oxysporum,* compared to the earlier data on *Trichoderma viride.*
199 In plants – a global major source of terpenes[10] – lower sesquiterpene emission rates were
200 often reported[27] than the emission rate from *A. alternata* found here. *A. alternata* is
201 geographically widespread and abundant in nature[12]. Therefore, *A. alternata* might potentially
202 contribute significantly to the emission of sesquiterpenes into the atmosphere, as generally
203 hypothesized for fungi[3].
204
205 This study expanded the sesquiterpenes of *A. alternata* described from two major
206 compounds[20] to a complex variety of substances. We used a passive sampling system
207 specifically suitable to trap non-polar compounds such as sesquiterpenes but with a low
208 affinity for relatively polar compounds such as alcohols, ketones, and aldehydes. Therefore,
209 their contribution to the total VOC emitted from *A. alternata* in the present analysis cannot be
210 completely assessed. Physicochemical differences in the VOC collection system and trapping
211 material can explain why previous studies found different chemical compounds for
212 *Alternaria*[16].
213 We detected oSQT as minor constituents in the *A. alternata* emission profile. Few oSQT have
214 been described for fungi so far[28]. To the best of our knowledge, this is the first report on the
215 production of widdrol, thujopsane-2β-ol and cedren-13-ol, 8 in fungi, while isomers of 10-
216 epi-γ-eudesmol have been described for freshwater fungi (*Beltrania rhombica*)[29] and
217 mushrooms (*Inonotus obliquus*)[30]. Our most predominant VOC, β-cedrene, has only been
218 reported in fungal odor profiles from *A. alternata*[20] and *Penicillium decumbens*[31]. The
219 structurally related di-epi-α-cedrene and α-cedrene have been found in some other
220 ascomycetes[28]. The second most abundant substance in our study, thujopsene, has been
221 reported in various fungi including *Penicillium decumbens*[31]. This fungus emitted thujopsene
222 in addition to β-cedrene and other compounds, which included substances that were also

223 detected in this study for *A. alternata,* e.g., (*E*)-β-farnesene, β-acoradiene, β-chamigrene, and
224 α-chamigrene. Thujopsene was found to have an auto-regulatory function for the growth of *P.*
225 *decumbens* and inhibitory effects on several ascomycetes[31].
226 Compared to earlier studies on fungal VOCs, the number of sesquiterpenes from *A. alternata*
227 was relatively high. This confirmed results from recent measurements showing that many
228 fungi emit a suite of many different sesquiterpenes[21,31-33]. Our attempt to relate sesquiterpene
229 synthases (SQTS) to this multitude of sesquiterpenes by using emission correlation matrices,
230 as has been shown recently[34], gave ambiguous results and suggested that either the same
231 enzyme was responsible for the formation of all sesquiterpenes or that different SQTS were
232 biochemically active in concert. The formation of multiple products by single sesquiterpene
233 synthases has been documented in fungi, but multiple SQTS homologs were also found in
234 single fungi of different groups[35]. The high number of different sesquiterpenes is generally
235 mirrored in the *Alternaria* genome: A search of the *A. alternata* genome library (*Alternaria*
236 *alternata,* SRC1lrK2f v1.0) revealed 30 putative terpene synthase genes
237 (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Altal1, accessed
238 04/11/2015).
239 VOC emissions from our *F. oxysporum* strain differed – except for acoradiene[23] – from other
240 strains described so far[22-24], indicating that VOC profiles are strain-dependent in *F.*
241 *oxysporum.* The lower number of compounds for *F. oxysporum* resulted in a much simpler
242 correlation matrix compared to *A. alternata.* On the basis of this correlation matrix, we
243 speculate that the same SQTS synthesized δ-elemene as the primary product along with
244 several additional minor compounds. In accord with the lower number of detectable
245 sesquiterpenes, only five putative terpene synthase genes were annotated in the *Fusarium*
246 *oxysporum* genome (http://genome.jgi.doe.gov/pages/search-for-genes.jsf?organism=Fusox1,
247 accessed 04/11/2015).

248 To translate the observed sesquiterpene emission pattern of both fungi to a molecular basis,
249 future work will focus on the heterologous expression of the putative terpene synthase genes,
250 genetic differences between strains[36] and their functional biochemical characterization.
251 Our results clearly demonstrated highest sesquiterpene emissions in very young (3 days old)
252 mycelia in both fungi. Contradictory to our findings, sesquiterpene emissions have been
253 attributed to a later growth phase[20,28]. However, according to our literature survey, *Aspergillus*
254 *versicolor* was the only truly indicative example for strongly increased sesquiterpene
255 emissions with age, showing a 10^3-fold increase between day 3 and 14 of incubation[37]. The
256 early peak of emission in our study suggests that sesquiterpene formation is associated with
257 rapid growth in *A. alternata* and *F. oxysporum*, at least on nutrient rich medium. For cultures
258 on nutrient poor medium, we started VOC sampling when *A. alternata* cultures reached a size
259 of 3 cm^2, which occurred only after 14 days of incubation. Therefore, no information on
260 emissions at early growth stages under nutrient poor cultivation conditions is available.
261 Nonetheless, growth on nutrient poor media before day 14 was slower than during the
262 following two weeks. If emissions are related to rapid growth as found under nutrient rich
263 conditions, the growth pattern suggests no intensely increased emissions under nutrient poor
264 conditions at an earlier time. However, this still needs to be studied further.
265 The general VOC pattern for *A. alternata* was stable during aging, except for an overly rapid
266 decline in the alcohol emissions. This temporal emission pattern for small hydrocarbons is in
267 accordance with other works. For example, in *Penicillium expansum,* C_8 compounds were
268 emitted mainly between an age of 3 and 9 days[20]. In contrast to the stable pattern found here,
269 the VOC diversity of *Trichoderma atroviride* increased by 24 substances (three
270 sesquiterpenes) in a comparison between 5- and 14-day-old cultures[38]. However, the effect of
271 aging mycelia on sesquiterpene emissions has rarely been intentionally investigated and
272 knowledge of the prevalence of sesquiterpene emissions in aging fungi remains fragmentary.
273

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[32]. 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[33], or when several *Fusarium* spp. were cultivated on different substrates[21].

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

300 total emissions from split-plates compared with the solitary cultivation of *A. alternata* was
301 only significant at day 14, when possible effects of headspace interactions (e.g., change in
302 emission profiles or amounts, adsorption of VOC by *F. oxysporum*) could not be separated
303 from effects triggered by physical contact or exudates. However, during co-cultivation on
304 split-plates, the headspace concentration of nearly all compounds was already lower very
305 early at day 3 and 7 compared to the concentration found in solitary cultures of *A. alternata.*
306 Because border effects were similar for both setups during this early stage, an active/passive
307 adsorption of compounds in *F. oxysporum* mycelia is probable, especially because strains of
308 *F. oxysporum* can transform terpenes[39,40] and various fungi are known to grow with terpenes
309 or similar compounds as a sole carbon source[41]. Another possibility is the reduced production
310 of sesquiterpenes by *A. alternata* after recognition of *F. oxysporum* volatiles (i.e., δ-elemene).
311 Although the exact mechanism must be elucidated in future studies, our experiment suggests a
312 VOC-mediated interaction between the two fungal species.
313
314 The multivariate analysis clearly separated the sesquiterpene profiles of both fungi,
315 suggesting that the profile might serve as a very good biomarker to discriminate between the
316 tested strains of *A. alternata* and *F. oxysporum*. Such specific sesquiterpene profiles could
317 also be used in ambient air to detect fungal growth. An earlier study[42] concluded that
318 sesquiterpene biomarkers may be the best approach to detect indoor fungal growth, after
319 *Aspergillus*, *Chaetomium*, and *Epicoccum* were identified from water-damaged buildings via
320 their sesquiterpene profiles. Examples of the use of different classes of VOCs as fungal
321 biomarkers include screening for fungal causes of lung diseases[43] and ecotyping[26]. The
322 peculiar sesquiterpene profile of *A. alternata* is an ideal target for detecting those allergenic
323 fungi in indoor environments.
324 The available data on VOC emissions of different *F. oxysporum* strains[21-24] suggests that
325 sesquiterpenes are useful biomarkers on the sub-species level for this species, as has been

proposed earlier[21]. The stability of sesquiterpene emissions by *A. alternata* under various conditions suggests that such emissions are especially good biomarkers for the growth of the species. However, intraspecific variability in *A. alternata* has not been assessed; therefore more *A. alternata* strains must be chemotyped. Because strains of *Alternaria* are known to produce different terpenoids (bi- and tricyloaleranenes) and other classes of toxins[14], it may be speculated that a pathotype relation with sesquiterpene profiles could be established in a similar manner as has been demonstrated for *F. oxysporum*[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 *A. alternata*. If this observation holds true for different isolates of *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 *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.

Methods

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[44]. To minimize the volatile background, 15 g L^{-1} gelrite (SERVA electrophoresis, Heidelberg, Germany) were used for solidification instead of agar. The poor medium contained (L^{-1}): $KHPO_4$ 1 g, KNO_3 1 g, $MgSO_4 \times 7\ H_2O$ 0.5 g, KCl 0.5 g, glucose (D+) 0.2 g,

352 sucrose 0.2 g, $ZnSO_4 \times 7\ H_2O$ 0.01 g, $CuSO_4 \times 5\ H_2O$ 0.005 g; the rich medium contained (L^{-1}):
353 malt extract 20 g, $ZnSO_4 \times 7\ H_2O$ 0.01 g, $CuSO_4 \times 5\ H_2O$ 0.005 g. The media were poured
354 (20 ml $plate^{-1}$) into sterile degreased glass petri-dishes without division (direct confrontation)
355 or with a glass-barrier of 7 mm height dividing the plate into two equal halves (split-plate).
356 The split-plates ensured that only VOC-mediated interactions were possible during early
357 growth, while on direct confrontations exudates into the medium and early exploratory
358 hyphae were additional routes for interactions. After solidification, each dish of nutrient poor
359 medium was provided with four cellulose filters (30 mm, No. 1001-329, Whatman GE,
360 Dassel, Germany) that served as a polymer carbon source and a supporting matrix for mycelia
361 to approximate the situation in a withering plant. Sterile culture plates were stored at least one
362 week at 4 °C in odorless roasting tubes (Toppits Cofresco, Minden, Germany) before use.
363
364 *Strains and cultivation*
365 The experimental plates were inoculated with 3 × 50 conidial spores from spore-solutions
366 made from *Alternaria alternata* (Fr.) Keissler (DSMZ 62006) or *Fusarium oxysporum* f.
367 *aechmeae* (Fr.) Schltdl. (DSMZ 62297) cultures grown as recommended[44]. For the rich
368 medium, the spores were applied in three droplets onto one half of the plate and
369 approximately 1 cm from the rim with equal spacing between the droplets. For the poor
370 medium, two droplets were applied on half of the cellulose-filter next to the rim of the dish
371 and one droplet was placed in between the filters. The plates were sealed with Parafilm M
372 (Bemis, Oshkosh, WI, USA) and incubated at 20 °C in the dark.
373 On both media, five growth setups were tested in five replicate plates each: (1) plates with
374 sterile media for sampling the VOC-background, (2) *A. alternata* on one half of a split-plate
375 with the second half containing sterile medium ('solitary *A. alternata*'), (3) *F. oxysporum* on a
376 half of a split-plate with the second half containing sterile medium ('solitary *F. oxysporum*'),
377 (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[26], 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 cm^2 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[26], 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

404 + 10 m DG, Agilent Technologies). The TDU-GC-MS was run as described previously[45,46].
405 Calibration was achieved by injecting pure standard mixtures dissolved in hexane at seven
406 different concentrations ranging from ~ 20 to 800 pmol μl^{-1}. Each mixture was made
407 independently in triplicate for each concentration and measured in duplicate. The resulting
408 MS signal responses were found to be linear with an increasing standard concentration with
409 $R^2 > 0.99$. Non-isothermal Kovats retention indices were calculated according to generally
410 accepted standards[47], based on chromatography retention times of a saturated alkane mixture
411 ($C_7 - C_{40}$; Sigma-Aldrich, Taufkirchen, Germany) and other alkanes ($< C_7$) occurring in the
412 chromatogram background.
413 Limits of detection (LOD) were set to twice σ, and the limit of quantification (LOQ) to 10-
414 folder of LOD. Emission rates were calculated on a fungal mycelium area (pmol cm^{-2} h^{-1}) or
415 plate (pmol h^{-1}) bases.
416
417 *Statistical analysis*
418 Relationships between VOC emissions from *A. alternata* and *F. oxysporum* growing solitary
419 or in co-cultures in nutrient rich medium were analyzed using principal component analysis
420 (PCA) and orthogonal partial least squares discriminant analysis (OPLS) (SIMCA-P v13,
421 Umetrics, Umeå, Sweden). For this, day 7 was chosen because all VOCs were detected and
422 the highest plate-based emission rates were measured at this date. Using plate-based emission
423 rates allowed the inclusion of data for the co-cultivation setups. Established procedures to
424 analyses and evaluate MS data were followed in a similar manner as previously described[45,48-
425 53]. Prior to analysis, all x-variables were logarithmically (log10) transformed, centered, and
426 each type of data was scaled block-wise with 1 sd^{-1}. Calculated significant principal
427 components were validated using 'full cross-validation', with 95% confidence level on
428 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[54] using algorithms of the Hmisc package[55] (v3.10-1). Hierarchical clustering (single linkage) of the results was performed with the corrplot package[56] (v0.73).

References

1 Ditengou, F. A. *et al.* Volatile signalling by sesquiterpenes from ectomycorrhizal fungi reprogrammes root architecture. *Nat Commun* **6**, 6279, doi:10.1038/ncomms7279 (2015).

2 Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J. & Garbeva, P. Volatile affairs in microbial interactions. *Isme J*, doi:10.1038/ismej.2015.42 (2015).

3 Penuelas, J. *et al.* Biogenic volatile emissions from the soil. *Plant Cell Environ* **37**, 1866-1891, doi:10.1111/pce.12340 (2014).

4 Martin, V. J., Pitera, D. J., Withers, S. T., Newman, J. D. & Keasling, J. D. Engineering a mevalonate pathway in *Escherichia coli* for production of terpenoids. *Nat Biotechnol* **21**, 796-802 (2003).

5 Rude, M. A. & Schirmer, A. New microbial fuels: a biotech perspective. *Curr Opin Microbiol* **12**, 274-281, doi:10.1016/j.mib.2009.04.004 (2009).

6 Peralta-Yahya, P. P., Zhang, F., del Cardayre, S. B. & Keasling, J. D. Microbial engineering for the production of advanced biofuels. *Nature* **488**, 320-328, doi:10.1038/nature11478 (2012).

7 Peralta-Yahya, P. P. *et al.* Identification and microbial production of a terpene-based advanced biofuel. *Nat Commun* **2**, 483, doi:10.1038/ncomms1494 (2011).

8 Claeys, M. *et al.* Formation of secondary organic aerosols through photooxidation of isoprene. *Science* **303**, 1173-1176 (2004).

9 Ehn, M. *et al.* A large source of low-volatility secondary organic aerosol. *Nature* **506**, 476-479 (2014).

10 Guenther, A. Biological and chemical diversity of biogenic volatile organic emissions into the atmosphere. *J Atmos Sci* **2013**, 1-27, doi:10.1155/2013/786290 (2013).

11 Thomma, B. P. *Alternaria* spp.: from general saprophyte to specific parasite. *Mol Plant Pathol* **4**, 225-236, doi:10.1046/j.1364-3703.2003.00173.x (2003).

12 Vázquez de Aldana, B. R., Bills, G. & Zabalgogeazcoa, I. Are endophytes an important link between airborne spores and allergen exposure? *Fungal Divers.* **60**, 33-42, doi:10.1007/s13225-013-0223-z (2013).

13 Dang, H. X., Pryor, B., Peever, T. & Lawrence, C. B. The *Alternaria* genomes database: a comprehensive resource for a fungal genus comprised of saprophytes, plant pathogens, and allergenic species. *BMC Genomics* **16**, 239, doi:10.1186/s12864-015-1430-7 (2015).

14 Ostry, V. *Alternaria* mycotoxins: an overview of chemical characterization, producers, toxicity, analysis and occurrence in foodstuffs. *World Mycotoxin J* **1**, 175-188 (2008).

15 Takaoka, S. *et al.* Complex regulation of secondary metabolism controlling pathogenicity in the phytopathogenic fungus *Alternaria alternata*. *New Phytol* **202**, 1297-1309, doi:10.1111/nph.12754 (2014).

16 Effmert, U., Kalderas, J., Warnke, R. & Piechulla, B. Volatile mediated interactions between bacteria and fungi in the soil. *J Chem Ecol* **38**, 665-703, doi:10.1007/s10886-012-0135-5

(2012).
17 Lou, J., Fu, L., Peng, Y. & Zhou, L. Metabolites from *Alternaria* fungi and their bioactivities. *Molecules* **18**, 5891-5935, doi:10.3390/molecules18055891 (2013).
18 Simon-Nobbe, B., Denk, U., Poll, V., Rid, R. & Breitenbach, M. The spectrum of fungal allergy. *Int Arch Allergy Immunol* **145**, 58-86, doi:10.1159/000107578 (2008).
19 Lemfack, M. C., Nickel, J., Dunkel, M., Preissner, R. & Piechulla, B. mVOC: a database of microbial volatiles. *Nucleic Acids Res* **42**, D744-D748, doi:10.1093/nar/gkt125010.5772/52050 (2014).
20 Schuchardt, S. & Kruse, H. Quantitative volatile metabolite profiling of common indoor fungi: relevancy for indoor air analysis. *J Basic Microbiol* **49**, 350-362, doi:10.1002/jobm.200800152 (2009).
21 Demyttenaere, J. C. R., Moriña, R. M., De Kimpe, N. & Sandra, P. Use of headspace solid-phase microextraction and headspace sorptive extraction for the detection of the volatile metabolites produced by toxigenic *Fusarium* species. *J Chrom A* **1027**, 147-154, doi:10.1016/j.chroma.2003.08.105 (2004).
22 Minerdi, D., Bossi, S., Gullino, M. L. & Garibaldi, A. Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. *Environ Microbiol* **11**, 844-854, doi:10.1111/j.1462-2920.2008.01805.x (2009).
23 Freire, E. S. *et al.* Volatile Substances Produced by *Fusarium oxysporum* from Coffee Rhizosphere and Other Microbes affect Meloidogyne incognita and Arthrobotrys conoides. *J Nematol* **44**, 321-328 (2012).
24 Zhang, Q. *et al.* Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of *Verticillium* wilt of cotton. *Plant Soil* **392**, 101-114, doi:10.1007/s11104-015-2448-y (2015).
25 Hung, R., Lee, S. & Bennett, J. W. Fungal volatile organic compounds and their role in ecosystems. *Appl Microbiol Biotechnol* **99**, 3395-3405, doi:10.1007/s00253-015-6494-4 (2015).
26 Muller, A. *et al.* Volatile profiles of fungi--chemotyping of species and ecological functions. *Fungal Genet Biol* **54**, 25-33, doi:10.1016/j.fgb.2013.02.005 (2013).
27 Duhl, T. R., Helmig, D. & Guenther, A. Sesquiterpene emissions from vegetation: a review. *Biogeosciences* **5**, 761-777 (2008).
28 Kramer, R. & Abraham, W.-R. Volatile sesquiterpenes from fungi: what are they good for? *Phytochem Rev* **11**, 15-37, doi:10.1007/s11101-011-9216-2 (2012).
29 Rukachaisirikul, V., Kaewbumrung, C., Phongpaichit, S. & Hajiwangoh, Z. Eudesmane sesquiterpenes from the aquatic fungus *Beltrania rhombica*. *Chem Pharm Bull* **53** (2005).
30 Ayoub, N., Schultze, W. & Lass, D. Volatile constituents of the medicinal fungus chaga *Inonotus obliquus* (Pers.: Fr.) Pilát (Aphyllophoromycetideae). **11**, 55-60, doi:10.1615/IntJMedMushr.v11.i1.60 (2009).
31 Polizzi, V. *et al.* Autoregulatory properties of (+)-thujopsene and influence of environmental conditions on its production by *Penicillium decumbens*. *Microb Ecol* **62**, 838-852, doi:10.1007/s00248-011-9905-9 (2011).
32 Heddergott, C., Calvo, A. M. & Latgé, J. P. The volatome of *Aspergillus fumigatus*. *Eukaryot Cell* **13**, 1014-1025 (2014).
33 Polizzi, V. *et al.* Influence of various growth parameters on fungal growth and volatile metabolite production by indoor molds. *Sci Total Environ* **414**, 277-286, doi:10.1016/j.scitotenv.2011.10.035 (2012).
34 Oster, M., Beck, J. J., Furrow, R. E., Yeung, K. & Field, C. B. In-field yellow starthistle (Centaurea solstitialis) volatile composition under elevated temperature and CO2 and implications for future control. *Chemoecol*, doi:10.1007/s00049-015-0200-y (2015).
35 Agger, S., Lopez-Gallego, F. & Schmidt-Dannert, C. Diversity of sesquiterpene synthases in the basidiomycete *Coprinus cinereus*. *Mol Microbiol* **72**, 1181-1195, doi:10.1111/j.1365-2958.2009.06717.x (2009).
36 Ma, L.-J. *et al.* Comparative genomics reveals mobile pathogenicity chromosomes in

Fusarium. *Nature* **464**, 367-373 (2010).

37 Matysik, S., Herbarth, O. & Mueller, A. Determination of volatile metabolites originating from mould growth on wall paper and synthetic media. *J Microbiol Methods* **75**, 182-187, doi:10.1016/j.mimet.2008.05.027 (2008).

38 Lee, S., Hung, R., Yap, M. & Bennett, J. W. Age matters: the effects of volatile organic compounds emitted by *Trichoderma atroviride* on plant growth. *Arch Microbiol* **197**, 723-727, doi:10.1007/s00203-015-1104-5 (2015).

39 Molina, G., Bution, M. L., Bicas, J. L., Dolder, M. A. & Pastore, G. M. Comparative study of the bioconversion process using R-(+)- and S-(-)-limonene as substrates for *Fusarium oxysporum* 152B. *Food Chem* **174**, 606-613, doi:10.1016/j.foodchem.2014.11.059 (2015).

40 Maróstica, M. R. & Pastore, G. M. Production of R-(+)-α-terpineol by the biotransformation of limonene from orange essential oil, using cassava waste water as medium. *Food Chem* **101**, 345-350 (2007).

41 Prenafeta-Boldu, F. X., Summerbell, R. & Sybren de Hoog, G. Fungi growing on aromatic hydrocarbons: biotechnology's unexpected encounter with biohazard? *FEMS Microbiol Rev* **30**, 109-130, doi:10.1111/j.1574-6976.2005.00007.x (2006).

42 Polizzi, V. *et al.* Identification of volatile markers for indoor fungal growth and chemotaxonomic classification of *Aspergillus* species. *Fungal Biol* **116**, 941-953, doi:10.1016/j.funbio.2012.06.001 (2012).

43 Bazemore, R. A., Feng, J., Cseke, L. & Podila, G. K. Biomedically important pathogenic fungi detection with volatile biomarkers. *J Breath Res* **6**, 016002, doi:10.1088/1752-7155/6/1/016002 (2012).

44 Samson, R. A., Houbraken, J., Thrane, U., Frisvad, J. C. & B., A. *Food and indoor fungi*. (CBS-KNAW Fungal Biodiversity Centre, 2010).

45 Ghirardo, A., Heller, W., Fladung, M., Schnitzler, J. P. & Schroeder, H. Function of defensive volatiles in pedunculate oak (*Quercus robur*) is tricked by the moth *Tortrix viridana*. *Plant Cell Environ* **35**, 2192-2207, doi:10.1111/j.1365-3040.2012.02545.x (2012).

46 Kreuzwieser, J. *et al.* The Venus flytrap attracts insects by the release of volatile organic compounds. *J Exp Bot* **65**, 755-766, doi:10.1093/jxb/ert455 (2014).

47 van den Dool, H. & Kratz, P. D. A Generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography. *J Chromatog* **11**, 463-471 (1962).

48 Velikova, V. *et al.* Knocking down of isoprene emission modifies the lipid matrix of thylakoid membranes and influences the chloroplast ultrastructure in poplar. *Plant Physiol* **168**, 859-870, doi:10.1104/pp.15.00612 (2015).

49 Kaling, M. *et al.* UV-B mediated metabolic rearrangements in poplar revealed by non-targeted metabolomics. *Plant Cell Environ* **38**, 892-904, doi:10.1111/pce.12348 (2015).

50 Jud, W. *et al.* Effects of heat and drought stress on post illumination bursts of volatile organic compounds in isoprene emitting and non-emitting poplar. *Plant Cell Environ*, doi:10.1111/pce.12643 (2015).

51 Vanzo, E. *et al.* S-nitroso-proteome in poplar leaves in response to acute ozone stress. *PloS one* **9**, e106886, doi:10.1371/journal.pone.0106886 (2014).

52 Way, D. A. *et al.* Increasing atmospheric CO2 reduces metabolic and physiological differences between isoprene- and non-isoprene-emitting poplars. *New Phytol* **200**, 534-546, doi:10.1111/nph.12391 (2013).

53 Ghirardo, A., Sorensen, H. A., Petersen, M., Jacobsen, S. & Sondergaard, I. Early prediction of wheat quality: analysis during grain development using mass spectrometry and multivariate data analysis. *Rapid Commun Mass Spectrom* **19**, 525-532, doi:10.1002/rcm.1823 (2005).

54 Team, R. C. *R: A language and environment for statistical computing.*, (R Foundation for Statistical Computing, 2013).

55 Hmisc: Harrell Miscellaneous (2012).

56 corrplot: Visualization of a correlation matrix. (2013).

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581

582 **Author Contributions Statement**

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

588

589 **Additional Information**

590 *Competing financial interests*

591 The authors declare no competing financial interests.

592 *Supplementary Information*

593 Supplementary Materials File

594

595 **Figure Legends**

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

600

601 **Figure 2** Time-course of VOC emission rates from fungi growing under nutrient rich
602 conditions. (**a**) Total VOC emission rates under different cultivation conditions; **F|**: *Fusarium*
603 *oxysporum* alone, **|A**: *Alternaria alternata* alone, **F|A**: split-plate with both fungi, **FA**: direct
604 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*

631 (blue) are depicted with circles; direct confrontations are indicated by beige triangles and split
632 plates by grey triangles; the ellipse indicates the tolerance based on Hotelling's T^2 with a
633 significance level of $\alpha = 0.05$. (**b**) Correlation scaled loading plot: white squares:
634 sesquiterpenes, black dots: alcohols; the outer and inner ellipses indicate 100% and 75%
635 explained variance, respectively.

636

637 **Tables**

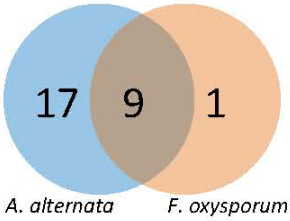
638 **Table 1** VOC analysis using stir bar sorptive extraction (SBSE) and GC-MS. Means of VOC
639 emission rates ± s.e. (n = 5) from 7 days old *Alternaria alternata* and *Fusarium oxysporum*
640 co-cultures (confrontation plates) growing on nutrient rich medium (malt extract gelrite).

Compounds	RT (min)	Kovats' RI	CAS	LOD (pmol plate^{-1} h^{-1})	emission (pmol plate^{-1} h^{-1})
alcohols					
Isobutanol	6.827	619	78-83-1	< 0.01	4.88 ± 0.97
2-Methyl-1-butanol	8.695	724	137-32-6	< 0.01	8.44 ± 1.92
sesquiterpenes					
δ-Elemene	32.864	1335	20307-84-0	< 0.01	13.39 ± 1.03
β-Elemene	35.067	1391	515-13-9	0.05	4.98 ± 0.34
Di-epi-α-Cedrene	35.822	1410	50894-66-1	< 0.01	11.67 ± 0.62
α-Cedrene	36.422	1427	11028-42-5	< 0.01	14.3 ± 0.7
β-Cedrene	36.861	1439	546-28-1	< 0.01	421.31 ± 20.92
Thujopsene	37.252	1449	470-40-6	< 0.01	206.11 ± 18.37
(E)-β-Farnesene[a]	37.355	1452	18794-84-8	0.02	6.18 ± 0.34
β-Acoradiene	38.083	1472	24048-44-0	< 0.01	24.36 ± 1.29
unknown SQT[b] #1	38.304	1478	n.a.	0.06	92.5 ± 5.73
α-Himachalene	38.44	1482	3853-83-6	< 0.01	22.49 ± 1.52
unknown SQT #2	38.684	1488	n.a.	< 0.01	2.38 ± 0.49
β-Chamigrene	38.92	1495	18431-82-8	< 0.01	16.91 ± 0.78
allo-Aromadendrene	39.138	1501	25246-27-9	0.14	14.65 ± 0.94
γ-Curcumene	39.322	1508	28976-68-3	< 0.01	15.08 ± 2.06
Germacrene D	39.559	1517	23986-74-5	< 0.01	20.66 ± 1.22
Eremophilene	39.86	1529	10219-75-7	< 0.01	6.35 ± 0.43
α-Chamigrene	40.257	1545	19912-83-5	< 0.01	6.78 ± 0.41
γ-Cadinene	40.323	1548	1460-97-5	< 0.01	10.76 ± 0.58
unknown SQT #3	41.478	1593	n.a.	0.03	2.76 ± 0.22
oxygenated sesquiterpenes					
Thujopsane-2β-ol + unknown SQT #4[c]	41.761	1606	150737-93-2 n.a.	< 0.01	33.07 ± 3.95
Cedren-13-ol, 8-	42.399	1641	18319-35-2	< 0.01	1.35 ± 0.14
Widdrol	42	1619	6892-80-4	< 0.01	3.47 ± 0.31
10-epi-γ-Eudesmol	42.247	1632	15051-81-7	< 0.01	3.14 ± 0.26

[a]verified by authentical standards, otherwise tentatively identified; [b]SQT: sesquiterpene; [c]co-eluted peaks

641

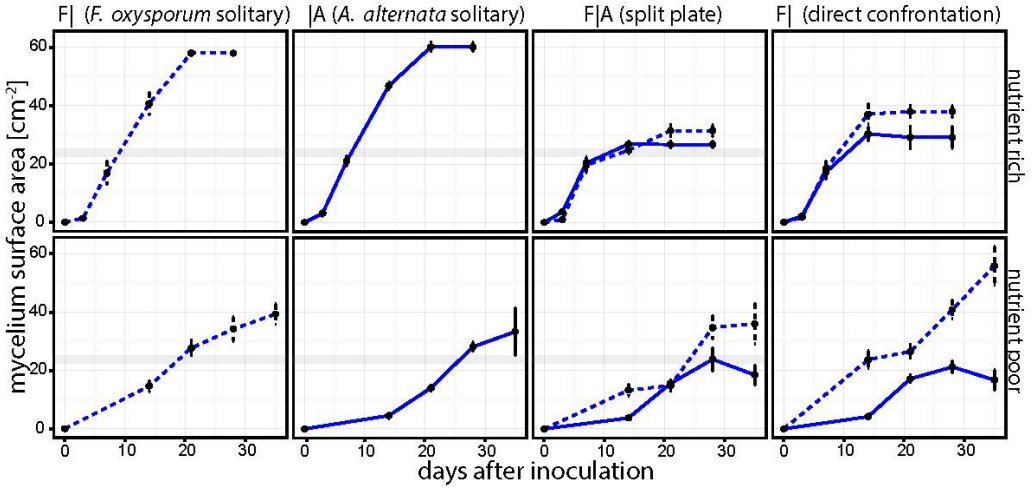
642 **Table 2** Fungal origin of volatile organic compounds (VOC) (above the limit of
643 quantification) collected from solitary cultures of *Alternaria alternata* and *Fusarium*
644 *oxysporum* grown on nutrient rich medium (malt extract gelrite).

Venn plot	VOC unique to *Alternaria alternata* (17)	VOC detected from both species (9)	VOC unique to *Fusarium oxysporum* (1)
	Di-epi-α-Cedrene		
	α-Cedrene		
	Thujopsene		
	(E)-β-Farnesene		
	unknown Sqt #1	Isobutanol[a]	
	β-Chamigrene[a]	1-Butanol,2-methyl[a]	
	allo-Aromadendrene	β-Elemene	
	Eremophilene[a]	β-Cedrene	
	α-Chamigrene	β-Acoradiene	δ-Elemene
	γ-Cadinene	α-Himachalene	
	unknown Sqt #2	unknown Sqt #2[b]	
	unknown Sqt #3	γ-Curcumene	
	Thujopsane-2β-ol	Germacrene D[b]	
	(+ unknown Sqt #4)		
	Cedren-13-ol, 8-[a]		
	Widdrol		
	10-epi-γ-Eudesmol		

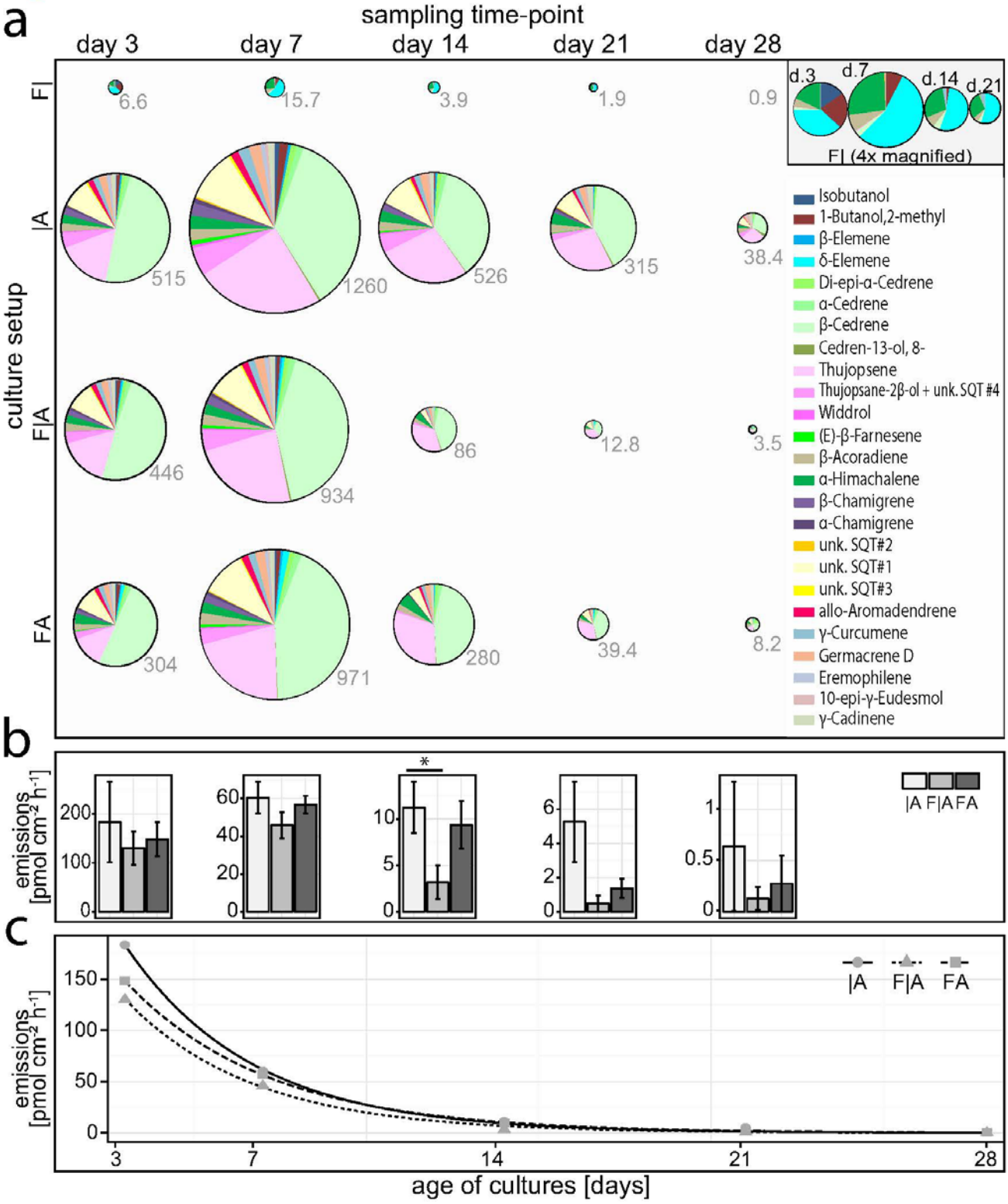
[a]absent on nutrient poor medium, [b]on nutrient poor medium only detected for *A. alternata*

645

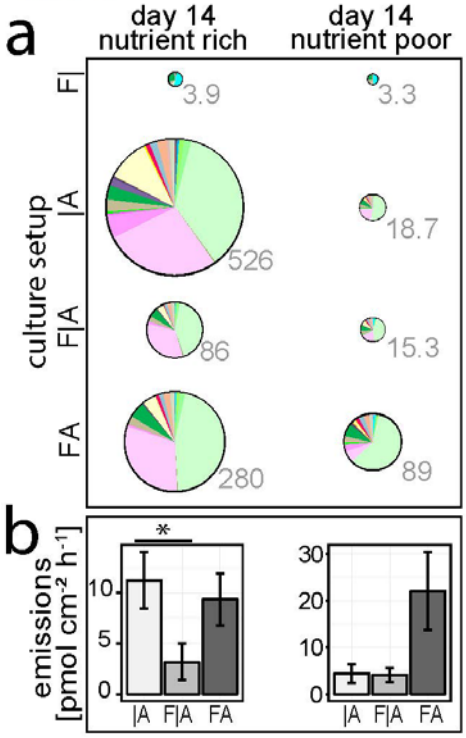
<Figure 1>


F| (F. oxysporum solitary)
|A (A. alternata solitary)
F|A (split plate)
F| (direct confrontation)
nutrient rich
nutrient poor
mycelium surface area [cm-2]
days after inoculation
0
20
40
60
0
10
20
30

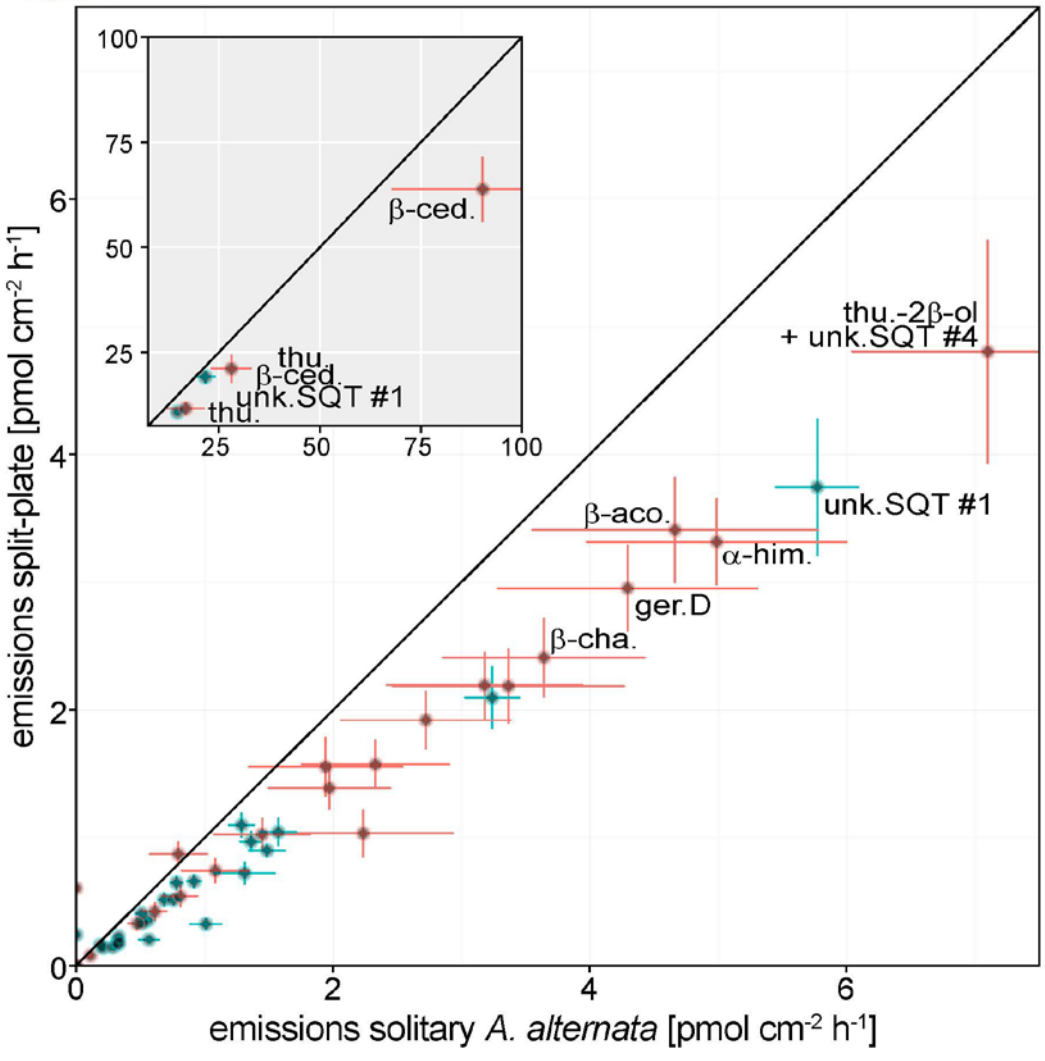
<Figure 2>


a
sampling time-point
day 3
day 7
day 14
day 21
day 28
culture setup
F|
|A
F|A
FA
6.6
15.7
3.9
1.9
0.9
515
1260
526
315
38.4
446
934
86
12.8
3.5
304
971
280
39.4
8.2
d.3
d.7
d.14
d.21
F| (4x magnified)
Isobutanol
1-Butanol,2-methyl
β-Elemene
δ-Elemene
Di-epi-α-Cedrene
α-Cedrene
β-Cedrene
Cedren-13-ol, 8-
Thujopsene
Thujopsane-2β-ol + unk. SQT #4
Widdrol
(E)-β-Farnesene
β-Acoradiene
α-Himachalene
β-Chamigrene
α-Chamigrene
unk. SQT#2
unk. SQT#1
unk. SQT#3
allo-Aromadendrene
γ-Curcumene
Germacrene D
Eremophilene
10-epi-γ-Eudesmol
γ-Cadinene
b
emissions [pmol cm-2 h-1]
*
|A F|A FA
c
emissions [pmol cm-2 h-1]
|A
F|A
FA
age of cultures [days]

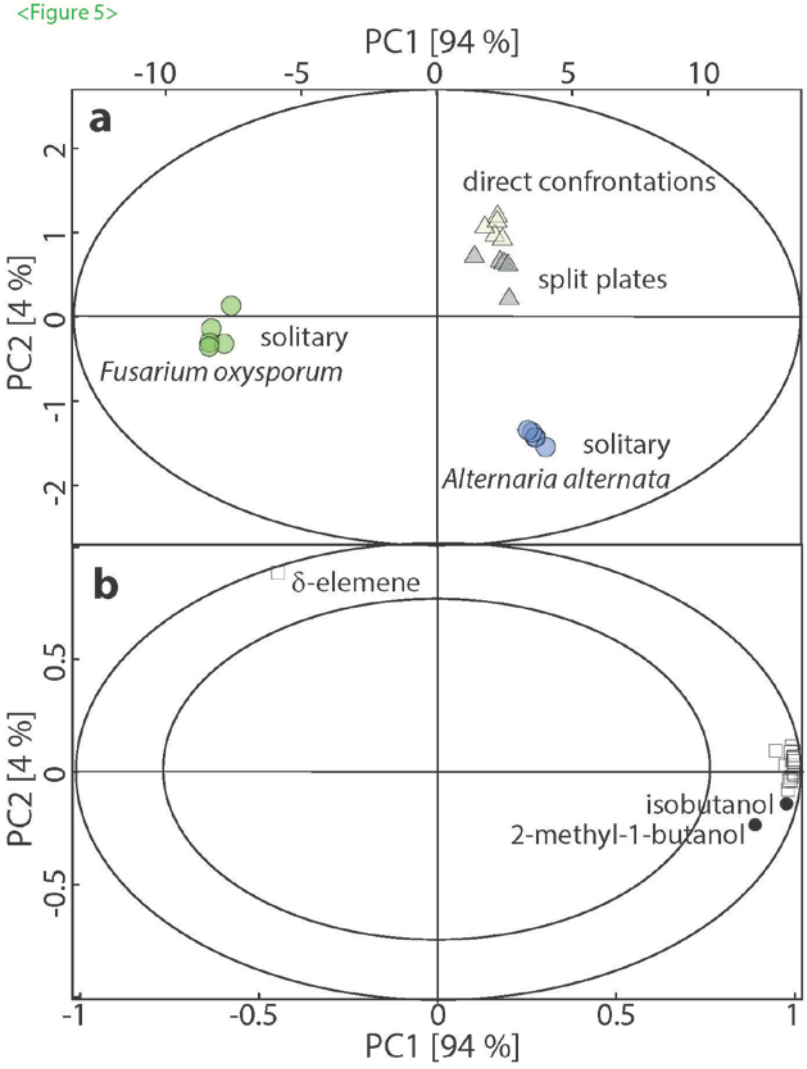
<Figure 3>



<Figure 4>



<Figure 5>


PC1 [94 %]
-10
-5
0
5
10
a
direct confrontations
split plates
solitary
Fusarium oxysporum
solitary
Alternaria alternata
PC2 [4 %]
2
1
0
-1
-2
b
δ-elemene
isobutanol
2-methyl-1-butanol
PC2 [4 %]
0.5
0
-0.5
-1
-0.5
0
0.5
1
PC1 [94 %]

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

Contents:

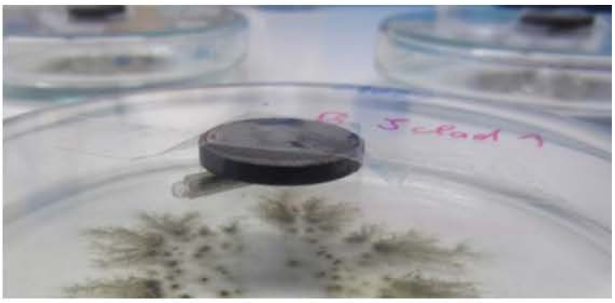


Figure S1 Example of a PDMS-twister (polydimethylsiloxane coated stir bar) attached to the inside of a glass culture-dish's lid and held in place by a magnet on the outside of the dish (pre-test with a *Cladosporium* isolate).

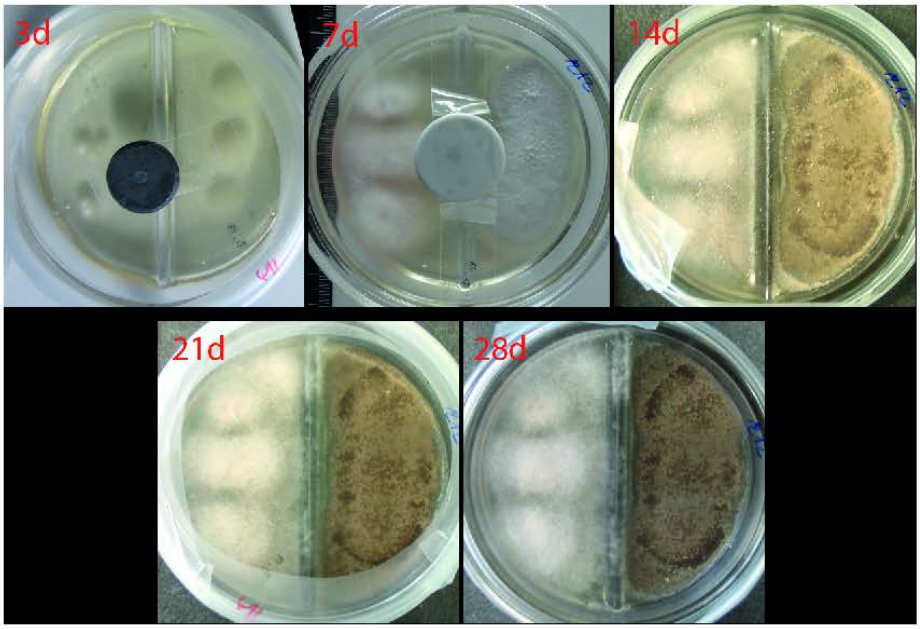


Figure S2 Time series (dates of VOC samplings) on the morphology of a shared split-plate with nutrient rich conditions. Left halves: *Fusarium oxysporum*, right halves: *Alternaria alternata*. d: days after inoculation.

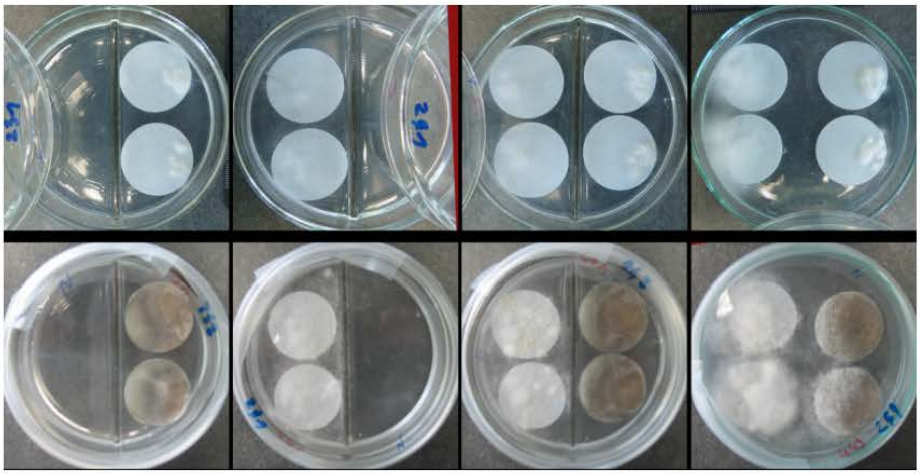


Figure S3 Culture morphology under nutrient poor conditions. Upper panel: at the first VOC sampling (day 14 after inoculation); lower panel: at the last VOC-sampling (day 35). From left to right: *Alternaria alternata* solitary, *Fusarium oxysporum* solitary, shared split-plate (left half *F. oxysporum*, 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).

nutrient poor setup	nutrient poor emissions (pmol cm^{-2} h^{-1}) ± s.e. [n = 5]	nutrient rich setup	nutrient rich emissions (pmol cm^{-2} h^{-1}) ± s.e. [n = 5]	*P*-values (t-test)
14 d solitary *A. alternata*	4.41 ± 1.97	3 d solitary *A. alternata*	183.48 ± 82.29	0.061
14 d solitary *A. alternata*	4.41 ± 1.97	14 d solitary *A. alternata*	11.23 ± 2.78	0.080
14 d split-plate	4.12 ± 1.55	3 d split-plate	130.08 ± 34.04	0.006
14 d split-plate	4.12 ± 1.55	14 d split-plate	3.17 ± 1.8	0.699
14 d confrontation	22.05 ± 8.33	3 d confrontation	148.15 ± 35	0.008
14 d confrontation	22.05 ± 8.33	14 d confrontation	9.36 ± 2.58	0.183
14 d solitary *F. oxysporum*	0.24 ± 0.15	3 d solitary *F. oxysporum*	6.74 ± 4.7	0.205
14 d solitary *F. oxysporum*	0.24 ± 0.15	14 d solitary *F. oxysporum*	0.1± 0.03	0.367

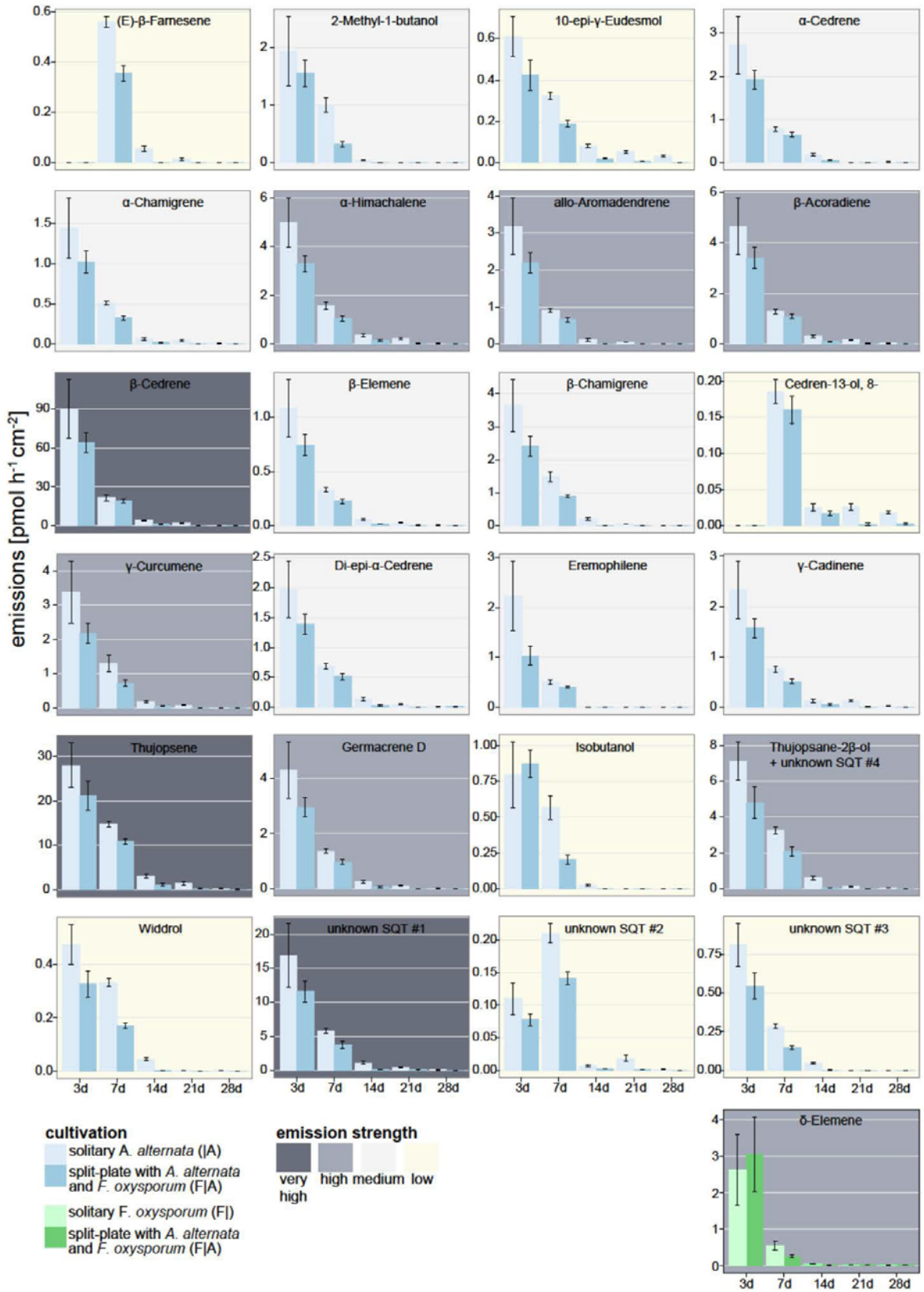


Figure S4 Comparisons between solitarily grown *Alternaria alternata* (solitary *Fusarium oxysporum* in the case of δ-elemene) and split-plates with *A. alternata* and *F. oxysporum* on nutrient rich medium. Emission rates of each compound calculated based on *A. alternata* mycelium area, except for δ-elemene, for which emission rates were calculated based on *F. oxysporum* mycelium area; error bars: s.e. (n = 5).

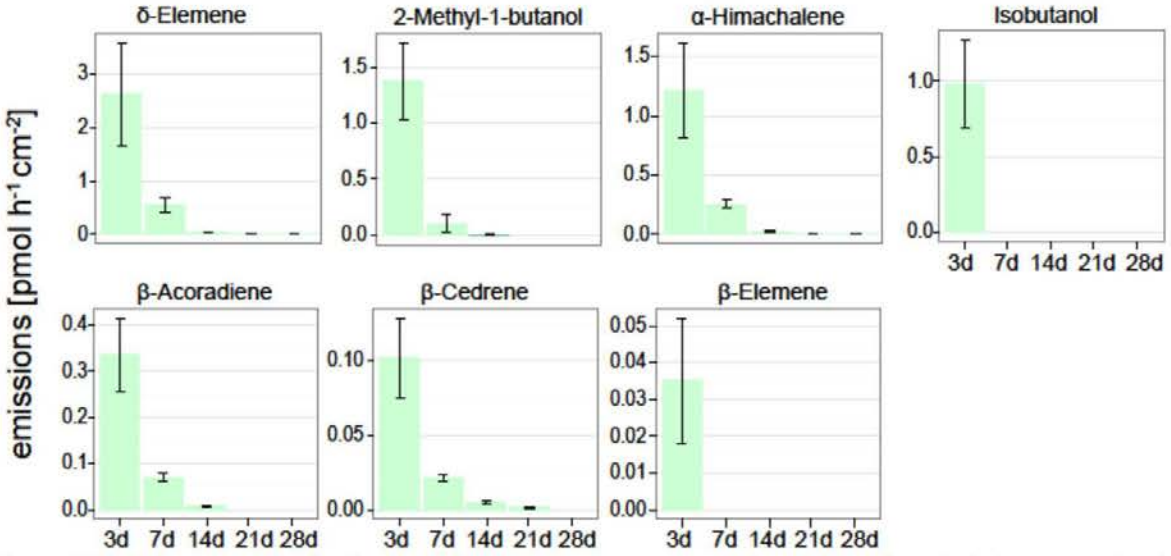


Figure S5 Emission rates of solitarily grown *Fusarium oxysporum* on nutrient rich medium. Emission rates of each compound calculated based on *F. oxysporum* mycelium area; error bars: s.e. (n = 5). The substances γ-curcurmene, germacrene-D, and unknown SQT #2 were only detected in low traces (< 0.003 pmol cm^{-2} h^{-1}) and are not displayed.

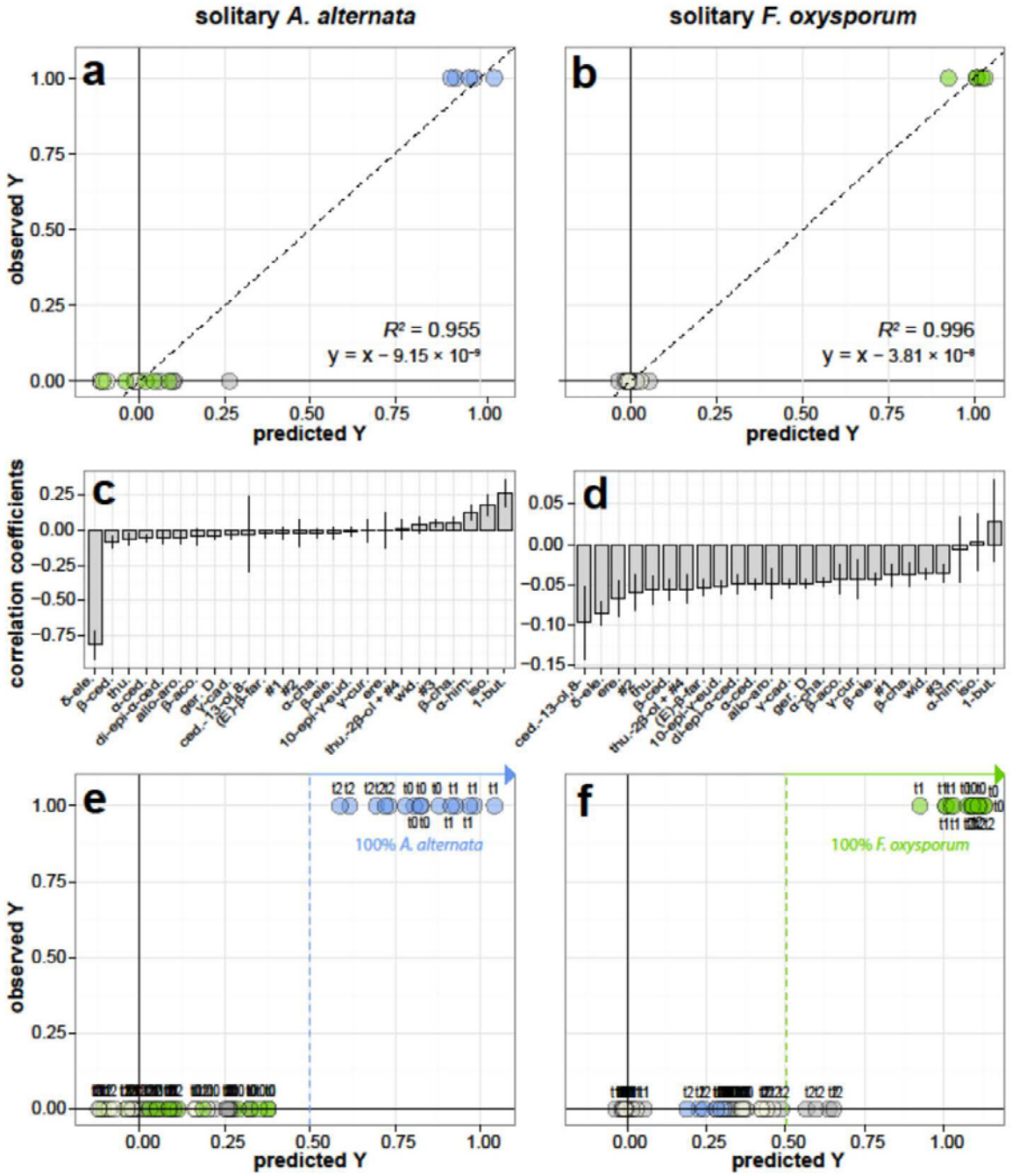


Figure S6 Observed versus predicted Y-values of orthogonal partial least square (OPLS) analysis referred to *Alternaria alternata* (a) and *Fusarium oxysporum* (b) modeled using VOC profile data from 7 days old culture and growing with nutrient rich media, and corresponding correlation coefficient plots (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^2 = 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), $P < 2 \times 10^{-7}$; *F. oxysporum* $P < 4 \times 10^{-14}$. Partly overplotted text (e, f): t1 (day 3 VOC sampling), t1 (day 7), t2 (day 14)

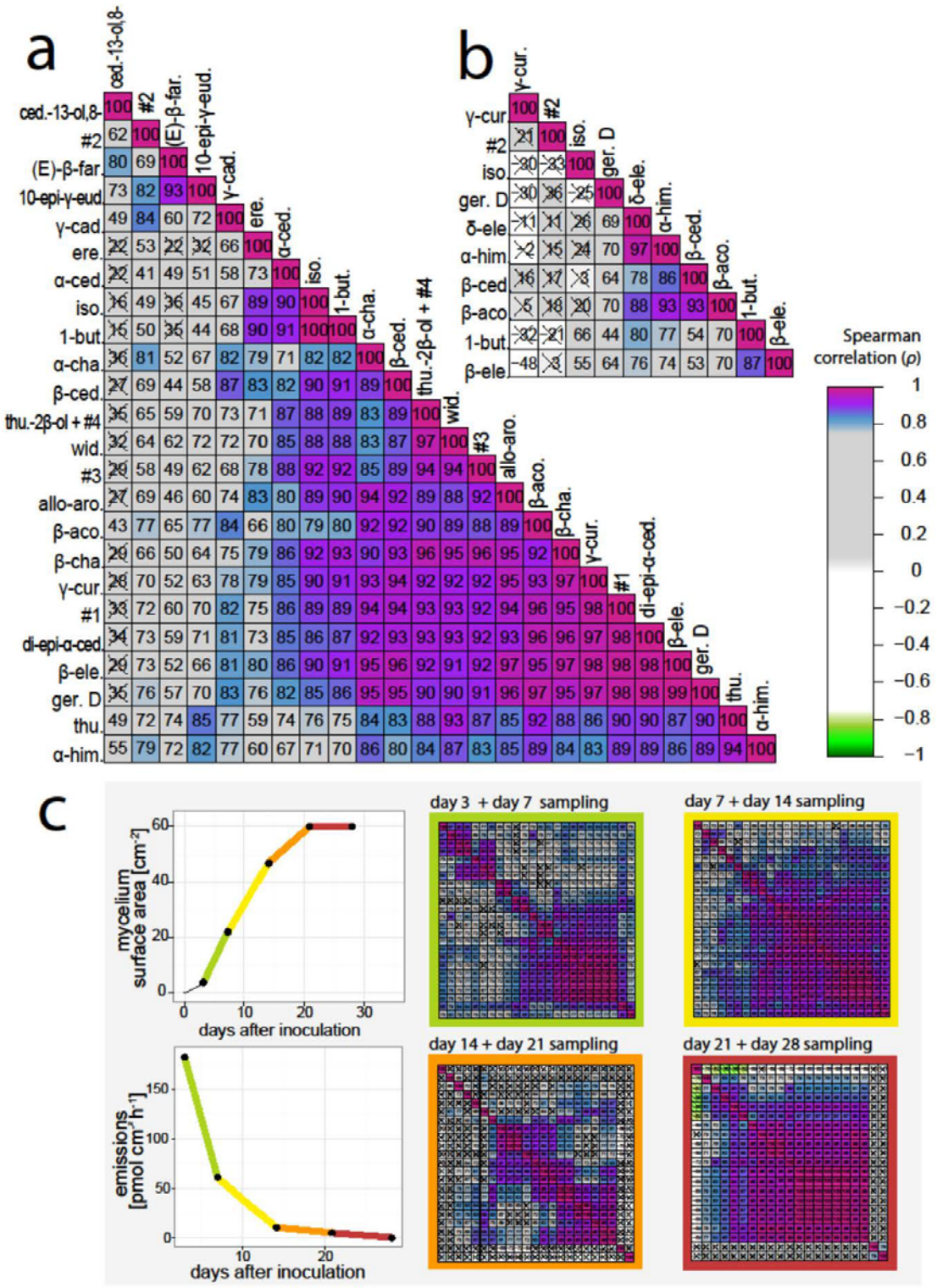


Figure S7 Correlation matrices for emissions form solitary cultures on nutrient rich medium. (a,b): Lower triangle correlation matrices including data from all time-points and replicates. Compounds ordered by hierarchical clustering (single linkage). Numbers: Spearman correlation coefficient (ρ) expressed 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.

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

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[c] Helmholtz Zentrum München – German Research Center for Environmental Health, Neuherberg, Germany

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.
- Crops at harvest, particularly cover crops, show high allergenic fungal loads.

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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 aims were 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.

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 increasing 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 surroundings 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 mold-specific quantitative PCR (MSQPCR, Haugland and Vesper, 2002), which is based upon analyzing house dust as a time-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

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0048-9697/

dominated by the local outdoor airborne community (Amend et al., 2010; Adams et al., 2013).

For monitoring allergenic 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 allergenic 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 (Katial et al., 1997), thunderstorms (Nasser and Pulimood, 2009), farming operations (Friesen 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 allergenic 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/koningii* 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 allergenic fungi in these environmental samples. Therefore, we analyzed agricultural field samples with the aim of detecting 1) major sources of allergenic 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* (Fresen.) G.A. de Vries (DSMZ 62121), *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 sporulation were 'potato carrot agar' for *A. alternata*, 'synthetischer nährstoffarmer agar' (supplemented with cellulose filters (Ø 3 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 (asexual 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 4×10^5 for *P. roqueforti*, 2×10^5 for *C. cladosporioides* and *T. viride*, and 1×10^5 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 m^2 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 490 m asl (section 1) to a depression of 470 m asl (section 4) with soil conditions changing from sandy-to-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
Overview of the field samplings.

Experiment	Sampling dates	Plot sizes (plot n)	Plot type	Source materials	Subsamples per source and plot
Accuracy of different calculation approaches	10/25/11	Spot samples (4)	Harvested wheat fields	Bulk soil [spiked with conidia]	12 [conidia dilution series of each fungus]
Reproducibility of whole procedure	10/25/11	Spot sample (1)	Harvested wheat fields	Bulk soil, decaying roots, decaying shoots	6 [3 spiked with conidia of target fungi]
Postharvest time series	08/17/11 09/19/11 10/25/11 03/27/12	0.29 ha–3.6 ha (8)	Harvested wheat fields[a]	Bulk soil, 'root/soil', decaying roots, decaying shoots	10 [merged to 'field sample']
Within-field heterogeneity	10/25/11	0.7 ha (4)	Sections of 1 field[b]	Bulk soil, decaying shoots	10 [merged to a 'field-section sample'][c]
Preharvest test of individual plants	07/23/14	0.01 ha (1)[d]	Fields with mature crops[d]	Lower part of plants, upper part of plants, seed heads	4 plants [individuals not merged]
Preharvest field trial	07/23/14	0.014 ha (39)	Field trial plots: mature crops[e]	Lower part of plants, seed heads	25 [merged to a 'plot-sample']

[a] 4 organic, 4 conventional.
[b] Quarters of the most heterogeneous field from the postharvest time series.
[c] Divided into 3 replicates per subsample before merging to test reproducibility.
[d] Different fields for each species: wheat, rye, barley, oat, pea, field bean.
[e] Plots for organic wheat (n = 15), organic rye (n = 15), conventional wheat (n = 9).

shoot samples, respectively. Samples were collected anew for the heterogeneity test and in the same manner as 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 cm above the soil, and seed heads), which were separately analyzed.

All samples were frozen onsite on 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-htp 96 Well Soil DNA Isolation Kit (Mo-Bio Laboratories Inc., Carlsbad, USA) was most suitable in comparison with the suitability of 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 lysis step. Here, for each extraction plate, 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 calibrator 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, Karcher AG, Birkenfeld, Germany) was precooled with dry ice, loaded with 4–6 g of frozen sample 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 TaqMan® assay for the calibrator (Suppl. 2). The following assays (original names) were used: Proqu (*P. roqueforti*), C.clad1 (*C. cladosporioides*), Aaltr (*A. alternata*), Tviri (*T. viride/atroviride/koningii*) from Haugland and Vesper (2002), and the assay of *F. culmorum* from Cullen et al. (2005). Oligonucleotides were synthesized by MWG-Eurofins 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-template, reagents, concentrations, 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 on 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. roqueforti*) assay (Eq. (ii)):

$$N = N_0 \times E^{-\Delta CT} \quad \text{i}$$

$$N = N_0 \times E^{-\Delta\Delta CT} \quad \text{ii}$$

where N_0 = the number of target fungus conidia in the calibrator; $\Delta CT = CT_{sample,target\ fungus} - CT_{calibrator,target\ fungus}$; $\Delta\Delta CT = \Delta CT_{sample} - \Delta CT_{calibrator}$; $\Delta CT_{sample} = CT_{sample,target\ fungus} - CT_{sample,reference\ fungus}$; $\Delta CT_{calibrator} = CT_{calibrator,target\ fungus} - CT_{calibrator,reference\ fungus}$; and E = 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 ΔCT between dilution steps) were used. With our dilution steps, the linear range usually ended around 0.00098x (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 $CT_{sample,target\ fungus}$ 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 5×10^2 CE/g is recommended (see Discussion).

2.6. Tests for accuracy and sensitivity

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

Accuracies of different calculation approaches near the detection limit were assessed with six extractions from an artificial conidial mixture, containing 1×10^3 conidia of *A. alternata* and *F. culmorum*, 2×10^3 of *C. cladosporioides* and *T. viride*, and 4×10^5 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 ($E^{-\Delta CT}$) exhibited a tendency to underestimate conidial counts for large supplements (max.: 0.33-fold for 2×10^6 *C. cladosporioides* conidia from microscopical counts). The $E^{-\Delta\Delta CT}$ setup exhibited a tendency to overestimate conidial counts for small supplements (max.: 1.99-fold for 2×10^3 counted conidia of *C. cladosporioides*). The ratios of calculated to counted conidia for individual extracts were between 0.28–1.16 ($E^{-\Delta CT}$, median: 0.62, mean 0.63) and 0.82–2.5 ($E^{-\Delta\Delta CT}$, median: 1.26, mean 1.36). If the samples spiked with 2×10^3 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 ($E^{-\Delta CT}$) and 0.82–1.96 ($E^{-\Delta\Delta CT}$). 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 ntDNA ($p < 0.05$, *t* tests with $E^{-\Delta CT}$ 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 subplots of one wheat field (s.d. <31% 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*: $p = 0.03$). 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*), $p = 0.018$ (*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)

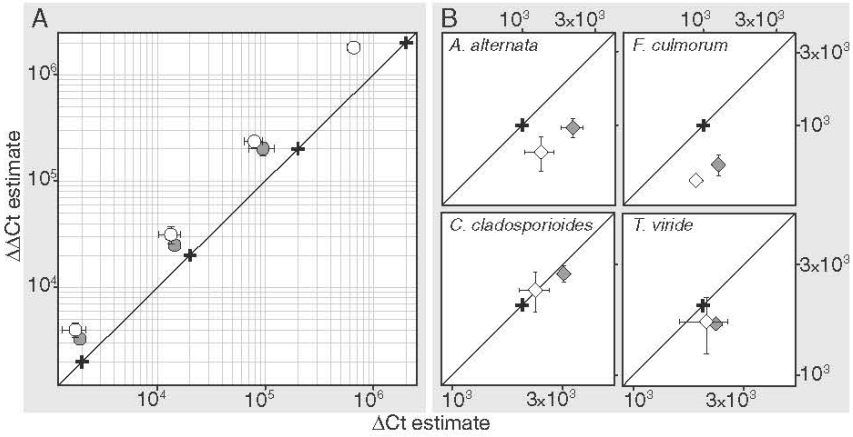

Fig. 1. Estimates of conidial equivalents by $E^{-\Delta CT}$ vs. $E^{-\Delta\Delta CT}$ calculations (means +/− standard deviation). The diagonal line indicates equal results for both calculations and crosses indicate numbers of added conidia, as determined by spore counts; Fig. 1A: results from different soil samples (n = 4) spiked with 2×10^3–2×10^6 target conidia of *C. cladosporioides* (white circles) and 2×10^3–2×10^5 target conidia of *T. viride* (gray circles); Fig. 1B: results from DNA extracts of conidial suspensions with (gray rhombs) or without (white rhombs) addition of ntDNA (n = 3, each).

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 (99% of samples $>10^6$ CE/g for *C. cladosporioides* and 95% of samples $>5 \times 10^3$ CE/g for *A. alternata*). Quantities of *C. cladosporioides* were commonly 10–100 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 5×10^2 CE/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* ($>10^8$/g) and *A. alternata* ($>10^5$/g) over all plant compartments. Levels for cereals (wheat, rye, oat, and barley) were lower in most cases (Fig. 4), yet at substantial levels ($>10^7$/g or $>10^4$/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).

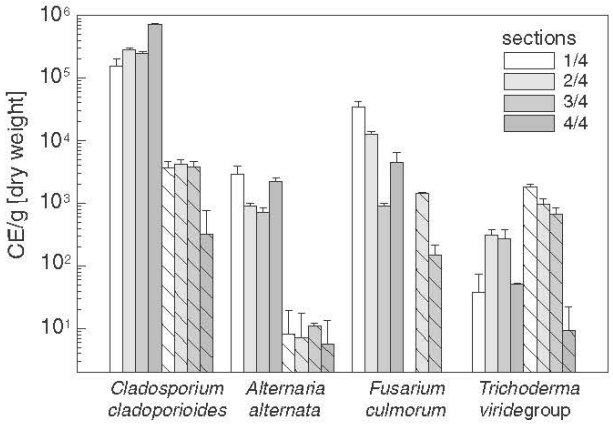

Fig. 2. Within-field variability (in conidial equivalents, CE) of the tested allergenic fungi in late autumn. Four sections of a heterogeneous field sampled in replicates with standard deviation (n = 3); open: shoots; hatched: bulk soil.

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 MSQPCR, with the ratios of calculated to counted conidia falling in a small range when compared, for example, with the ratios 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.

$E^{-\Delta CT}$ calculations tended to underestimate large amounts of conidia and $E^{-\Delta\Delta CT}$ 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 Vesper (2002), such a resemblance should be verified before the $E^{-\Delta\Delta CT}$ approach may be discontinued in favor of the $E^{-\Delta CT}$ method. Consequently, we recommend utilizing the cheaper and faster $E^{-\Delta CT}$ 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-specificity 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 ntDNA 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

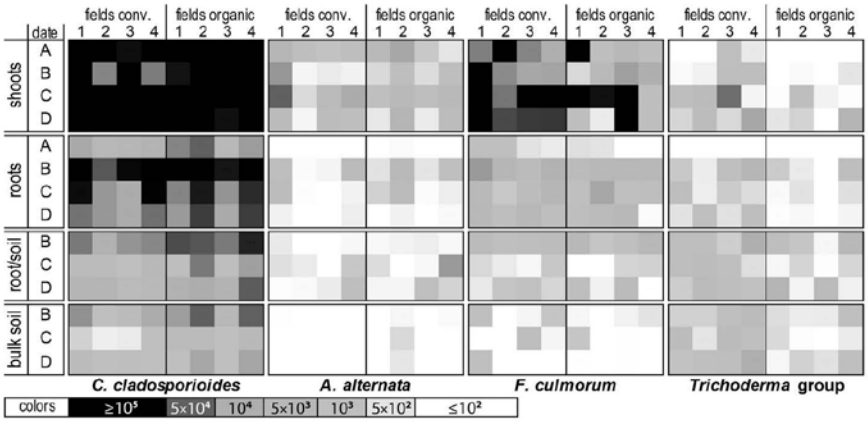

Fig. 3. Postharvest time series. Fungal concentrations as conidial equivalents (CE) per gram (g) of material (dry-weight) coded in grayscale for each fungal qPCR assay. Each row represents one time point for one source material, and each column represents one fungal assay for one individual field. Dates: (A) after harvest in late summer, (B) pretillage in autumn, (C) late autumn, and (D) before sowing in spring; CE: conidial equivalents. The exact values underlying each square are available in Suppl. 5.

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 (Peccoud 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 5×10^2 CE/g is a straightforward strategy to overcome such issues. It could be used in analogy to the threshold estimated for air samples with 1×10^2 spores of *Alternaria* per m^3 air and 3×10^3 spores of *Cladosporium* per m^3 air (Gravesen, 1979).

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 Kitchen et al. (2010) and Bengtsson et al. (2008), 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 *A. alternata*, *C. cladosporioides* and *F. culmorum* 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 *C. cladosporioides*. 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 *A. alternata* 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,

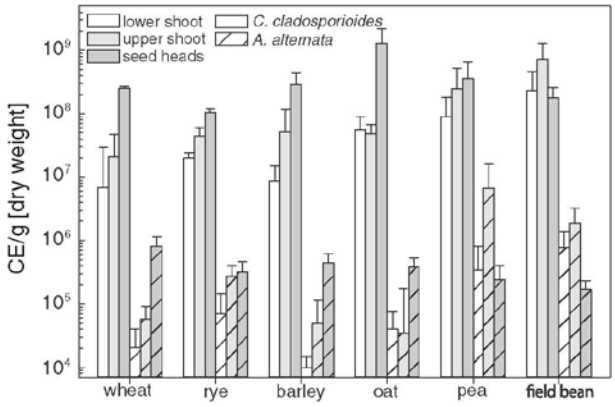

Fig. 4. Amounts (in conidial equivalents, CE) of *C. cladosporioides* (solid columns) and *A. alternata* (hatched columns) in samples of various crops at maturity. The mean of four plants +/− standard deviation.

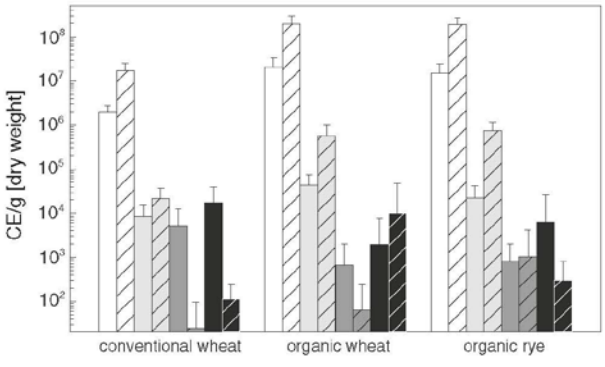

Fig. 5. Quantities (in conidial equivalents, CE) of allergenic fungi on cereal crops cultivated in conventional and organic fields at maturity of the crops; white: *C. cladosporioides*, light gray: *A. alternata*, dark gray: *Trichoderma* group, and black: *F. culmorum*; solid bars: lower shoots (0–30 cm) and diagonally lined bars: ears; n = 9 (conventional), n = 15 (organic wheat), and n = 15 (organic rye); error bars: standard deviation.

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 managements were strongly superposed 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 allergenic 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 practice 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 ranges were 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 spot sampling, for example, on grids or gradients at a defined spatial scale, would be a sufficient strategy for larger surveys. Strongly elevated amounts 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., Meklin 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

Adams, R.I., Miletto, M., Taylor, J.W., Bruns, T.D., 2013. Dispersal in microbes: fungi in indoor air are dominated by outdoor air and show dispersal limitation at short distances. ISME J. 7, 1262–1273.
Amend, A.S., Seifert, K.A., Samson, R., Bruns, T.D., 2010. Indoor fungal composition is geographically patterned and more diverse in temperate zones than in the tropics. Proc. Natl. Acad. Sci. U. S. A. 107, 13748–13753.
Andersson, M.A., Nikulin, M., Köljalg, U., Andersson, M.C., Rainey, F., Reijula, K., Hintikka, E.-L., Salkinoja-Salonen, M., 1997. Bacteria, molds and toxins in water-damaged building materials. Appl. Environ. Microbiol. 63, 387–393.
Arts, J.H.E., Mommers, C., de Heer, C., 2006. Dose–response relationships and threshold levels in skin and respiratory allergy. Crit. Rev. Toxicol. 36, 219–251.
Barta, J., Belmonte, J., Torres-Rodriguez, J.M., Cistero-Bahima, A., 2009. Sensitization to *Alternaria* in patients with respiratory allergy. Front. Biosci. 14, 3372–3379.
Bengtsson, M., Hemberg, M., Rorsman, P., Stahlberg, A., 2008. Quantification of mRNA in single cells and modelling of RT-qPCR induced noise. BMC Mol. Biol. 9, 63.
Burge, H.A., 2002. An update on pollen and fungal spore aerobiology. J. Allergy Clin. Immunol. 110, 544–552.
Burge, H.A., Rogers, C.A., 2000. Outdoor allergens. Environ. Health Perspect. 108, 653–659.
Corden, J.M., Millington, W.M., 2001. The long-term trends and seasonal variation of the aeroallergen *Alternaria* in Derby, UK. Aerobiologia 17, 127–136.
Corden, J.M., Millington, W.M., Mullins, J., 2003. Long-term trends and regional variation in the aeroallergen. *Alternaria* in Cardiff and Derby UK — are differences in climate and cereal production having an effect? Aerobiologia (Bologna) 19, 191–199.
Cullen, D.W., Toth, I.K., Pitkin, Y., Boonham, N., Walsh, K., Barker, I., Lees, A.K., 2005. Use of quantitative molecular diagnostic assays to investigate *Fusarium* dry rot in potato stocks and soil. Phytopathology 95, 1462–1471.
Denning, D.W., O'Driscoll, B.R., Hogaboam, C.M., Bowyer, P., Niven, R.M., 2006. The link between fungi and severe asthma: a summary of the evidence. Eur. Respir. J. 27, 615–626.
Fernandez-Rodriguez, S., Tormo-Molina, R., Maya-Manzano, J.M., Silva-Palacios, I., Gonzalo-Garijo, A., 2014. Outdoor airborne fungi captured by viable and non-viable methods. Fungal Ecol. 7, 16–26.
Friesen, T.L., De Wolf, E.D., Francl, L.J., 2001. Source strength of wheat pathogens during combine harvest. Aerobiologia (Bologna) 17, 293–299.
Gravesen, S., 1979. Fungi as a cause of allergic disease. Allergy 34, 135–154.
Hagn, A., Pritsch, K., Schloter, M., Munch, J.C., 2003. Fungal diversity in agricultural soil under different farming management systems, with special reference to biocontrol strains of *Trichoderma* spp. Biol. Fertil. Soils 38, 236–244.
Haugland, R., Vesper, S., 2002. Method of Identifying and Quantifying Specific Fungi and Bacteria. U.S. Environmental Protection Agency, Washington, DC, USA.
Haugland, R.A., Varma, M., Wymer, L.J., Vesper, S.J., 2004. Quantitative PCR analysis of selected *Aspergillus, Penicillium* and *Paecilomyces* species. Syst. Appl. Microbiol. 27, 198–210.
Katial, R.K., Zhang, Y., Jones, R.H., Dyer, P.S., 1997. Atmospheric mold spore counts in relation to meteorological parameters. Int. J. Biometeorol. 41, 17–22.
Kitchen, R.R., Kubista, M., Tichopad, A., 2010. Statistical aspects of quantitative real-time PCR experiment design. Methods 50, 231–236.
Kurup, V.P., Shen, H.D., Banerjee, B., 2000. Respiratory fungal allergy. Microbes Infect. 2, 1101–1110.

Levetin, E., 2004. Methods for aeroallergen sampling. Curr. Allergy Asthma Rep. 4, 376–383.
Lopez, M., Salvaggio, J.E., 1985. Mold-sensitive asthma. Clin. Rev. Allergy 3, 183–196.
Meklin, T., Reponen, T., McKinstry, C., Cho, S.H., Grinshpun, S.A., Nevalainen, A., Vepsalainen, A., Haugland, R.A., Lemasters, G., Vesper, S.J., 2007. Comparison of mold concentrations quantified by MSQPCR in indoor and outdoor air sampled simultaneously. Sci. Total Environ. 382, 130–134.
Nasser, S.M., Pulimood, T.B., 2009. Allergens and thunderstorm asthma. Curr. Allergy Asthma Rep. 9, 384–390.
Pawankar, R., Canonica, G.W., Holgate, S.T., Lockey, R.F. (Eds.), 2011. WAO White Book on Allergy. World Allergy Organization, Milwaukee, USA.
Peccoud, J., Jacob, C., 1996. Theoretical uncertainty of measurements using quantitative polymerase chain reaction. Biophys. J. 71, 101–108.
Pitkäranta, M., Meklin, T., Hyvärinen, A., Paulin, L., Auvinen, P., Nevalainen, A., Rintala, H., 2008. Analysis of fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl. Environ. Microbiol. 74, 233–244.
R Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria 3-900051-07-0 (URL http://www.R-project.org).
Reponen, T., Singh, U., Schaffer, C., Vesper, S., Johansson, E., Adhikari, A., Grinshpun, S.A., Indugula, R., Ryan, P., Levin, L., Lemasters, G., 2010. Visually observed mold and moldy odor versus quantitatively measured microbial exposure in homes. Sci. Total Environ. 408, 5565–5574.
Roe, J.D., Haugland, R.A., Vesper, S.J., Wymer, L.J., 2001. Quantification of *Stachybotrys chartarum* conidia in indoor dust using real time, fluorescent probe-based detection of PCR products. J. Expo. Anal. Environ. Epidemiol. 11, 12–20.
Samson, R.A., Houbraken, J., Thrane, U., Frisvad, J.C., Andersen, B. (Eds.), 2010. Food and Indoor Fungi. CBS-KNAW Fungal Biodiversity Centre, Utrecht, Netherlands.
Sautour, M., Sixt, N., Dalle, F., L'Ollivier, C., Fourquenet, V., Calinon, C., Paul, K., Valvin, S., Maurel, A., Aho, S., Couillault, G., Cachia, C., Vagner, O., Cuisenier, B., Caillot, D., Bonnin, A., 2009. Profiles and seasonal distribution of airborne fungi in indoor and outdoor environments at a French hospital. Sci. Total Environ. 407, 3766–3771.
Schneider, S., Hartmann, M., Enkerli, J., Widmer, F., 2010. Fungal community structure in soils of conventional and organic farming systems. Fungal Ecol. 3, 215–224.
Schröder, P., Huber, B., Reents, H.J., Munch, J.C., Pfadenhauer, J., 2008. Outline of the Scheyern Project. In: Schroder, P., Pfadenhauer, J., Munch, J.C. (Eds.), Perspectives for Agroecosystem Management. Elsevier B.V., Amsterdam, Netherlands, pp. 3–16.
Simon-Nobbe, B., Denk, U., Poll, V., Rid, R., Breitenbach, M., 2008. The spectrum of fungal allergy. Int. Arch. Allergy Immunol. 145, 58–86.
Ward, M.D., Chung, Y.J., Copeland, L.B., Doerfler, D.L., 2010. A comparison of the allergic responses induced by *Penicillium chrysogenum* and house dust mite extracts in a mouse model. Indoor Air 20, 380–391.

Supplementary Information

Contents:

SI.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-Mareckova 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 $CaCO_3$ to the soil (Sagova-Mareckova et al., 2008). Based on the data shown in suppl. table 1, method PS was chosen for all further analyses.

Method	DNA / µg [0,5 g dry soil]	Absorbance 260 nm / 280 nm	Absorbance 260 nm / 230 nm	Fragmentation DNA	PCR* from undiluted DNA	Handling	Costs	Total score
PCS	37.2 (59) 3	1.39 (8) 2	1.94 (7) 1	6 - 3 kbp 2	weak 1	2	4	15
SV	158 (43) 5	1.74 (4) 4	2.07 (24) 1	8 - 3 kbp 3	strong 3	2	4	22
PS	83.2 (42) 4	1.74 (3) 4	-1.00 (57) 4	8 - 6 kbp 4	overload 5	4	2	27
MV	38.1 (15) 3	1.60 (5) 3	-0.18 (16) 4	> 10 kbp 5	overload 5	2	2	24

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)

SI.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 ModelInspector (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 "Proqu" 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.

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.

Target organism	original name of assay	Oligo-sequences 5'-3': F (forward), R (reverse), P (probe)
Alternaria alternata	Aaltr*	F GGCGGGCTGGAACCTC R GCAATTACAAAAGGTTTATGTTTGTCGTA P TTACAGCCTTGCTGAATTATTCACCCTTGTCTTT
Cladosporium cladosporioides svar.1	Cclad1*	F CATTACAAGTGACCCCGGTCTAAC R CCCCGGAGGCAACAGAG P CCGGGATGTTCATAACCCTTTGTTGTCC
Trichoderma viride/atroviride/koningi	Tviri*	F CCCAAACCCAATGTGAACCA R TCCGCGAGGGGACTACAG P CCAAACTGTTGCCTCGGCGGG
Fusarium culmorum	Fcul**	F TTGGTGTTGGGAGCTGCA R CTATGGAAGCTCGACGTGACC P CCTGCTGCACTCCCCAAATACATTGG
Penicillium roqueforti	Proqu*	F CGGGCCCGCCTTAAC R TTAAATAATTTATATTTGTTCTCAGACTGCAT P CGCGCCCGCCGAAGACA
Fungi	5.8*	F AACTTTCAACAACGGATCTCTTGG R GCGTTCAAAGACTCGATGATTCAC P CATCGATGAAGAACGCAGCGAAATGC

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

SI.3 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).

Assay	E (R2 ≥ 0,998)	Independent runs of assay	Highest provoked false positives [CE / extract]
Aaltr	1.76 ± 0.02	12	66
Cclad1	1.92 ± 0.02	8	17
Tviri	1.84 ± 0.,01	5	277
Fcul	1.87 ± 0.00	7	50
Proqu	1.69 ± 0.03	27	29 (unprovoked: non-template control)
Uni	1.93 ± 0.02	7	not applicable

Suppl. table 3: general properties of the used assays during validation of the qPCR assays

SI.4 Statistical analyses

SI.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
                  Df Sum Sq Mean Sq F value   Pr(>F)
timepoint          3   5.36   1.786  26.320 3.04e-12 ***
material           3  58.10  19.367 285.422  < 2e-16 ***
manag.             1   2.67   2.671  39.357 1.29e-08 ***
plot               6   6.45   1.076  15.852 2.89e-12 ***
timepoint:material 7   7.33   1.047  15.434 4.97e-13 ***
material:manag.    3   0.70   0.234   3.451   0.0199 *
Residuals         88   5.97   0.068
---
A. alternata
                   Df Sum Sq Mean Sq F value   Pr(>F)
material            3  60.46  20.155  57.377  < 2e-16 ***
manag.              1   2.03   2.031   5.783   0.0183 *
plot                6  14.52   2.421   6.891 5.07e-06 ***
material:timepoint 10  15.39   1.539   4.381 5.45e-05 ***
material:manag.     3   2.35   0.785   2.234   0.0899 .
Residuals          88  30.91   0.351
---
F. culmorum
                  Df Sum Sq Mean Sq F value   Pr(>F)
material           3  81.23  27.078  70.725  < 2e-16 ***
manag.             1   3.92   3.924  10.248   0.0019 **
plot               6   5.74   0.956   2.497   0.0281 *
material:timepoint 10  18.36   1.836   4.796 1.74e-05 ***
material:manag.    3   3.72   1.241   3.242   0.0258 *
Residuals          88  33.69   0.383
---
Trichoderma viride/atroviride/koningii
            Df Sum Sq Mean Sq F value   Pr(>F)
timepoint    3 19.872   6.624  29.613 1.03e-13 ***
material     3  2.017   0.672   3.005   0.0340 *
manag.       1  9.113   9.113  40.742 5.80e-09 ***
plot         6  3.686   0.614   2.746   0.0165 *
Residuals   98 21.921   0.224
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
```

2. Multivariate analyses of the post-harvest data

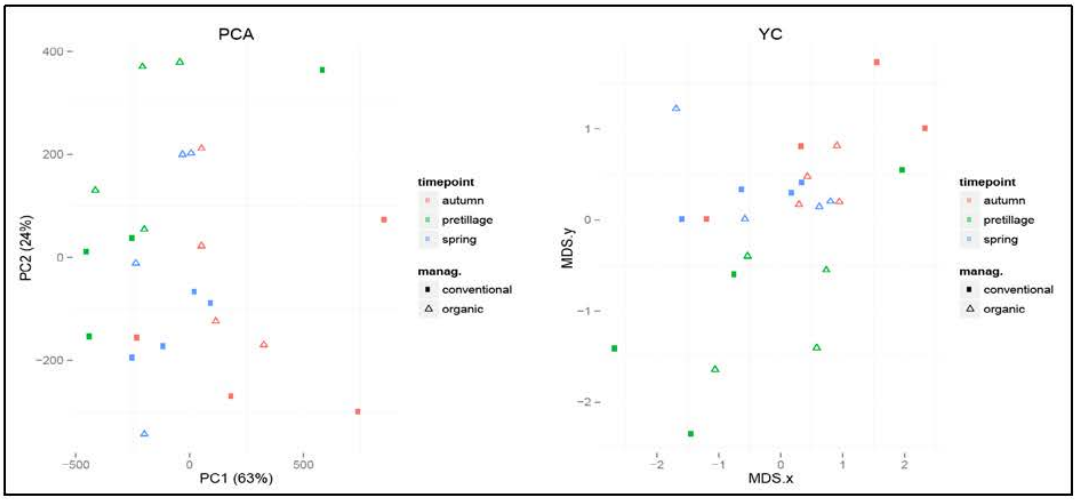
QPCR results were ordered in a way that allowed the fungal group and sampling material to be treated as effect-variables and analyzed with the Adonis function (Permutational Multivariate Analysis of Variance Using Distance Matrices) using Bray-Curtis dissimilarities and 10000 permutations both implemented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

```
           Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
timepoint   2    1.0200 0.51001  4.3775 0.28865  4e-05 ***
manag.      1    0.1837 0.18367  1.5765 0.05197  4e-05 ***
Residuals  20    2.3301 0.11651         0.65938
Total      23    3.5338                 1.00000
---
Signif. codes:  0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

          Df SumsOfSqs MeanSqs F.Model      R2 Pr(>F)
timepoint  2    1.0200 0.51001  6.1999 0.28865  4e-05 ***
field      7    1.3621 0.19459  2.3655 0.38546  4e-05 ***
Residuals 14    1.1517 0.08226         0.32590
Total     23    3.5338                 1.00000
---
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 prcomp (stats-package 2.15.3) with square-root transformation and YC calculated as in (http://www.mothur.org/wiki/Thetayc, April 2014) with non-metric multidimensional scaling using isoMDS (MASS package 7.3-23).

1. Overview of pre-harvest ANOVA analyses

assay	factor	n / category (n with N = 0)	Df	mean SSq	F value	P
conventional plots						
C. cladosporioides	plant-section	9	1	3.781	106.797	< 0.001
A. alternata.	plant-section	9	1	1.152	7.756	0.015
	tillage	6	2	0.563	3.788	0.048
Trichoderma group	plant-section	9 (12)	1	13.035	6.15	0.027
F. culmorum	plant-section	9 (4)	1	17.739	17.56	< 0.001
	tillage	6 (4)	2	3.871	3.832	0.047
organically managed plots						
C. cladosporioides	plant-section	30	1	17.995	230.270	< 0.001
A. alternata	plant-section	30	1	29.059	280.932	< 0.001
	fertilizer	> 24	1	0.502	4.849	0.032
	IA plant-section:crop	30	1	0.381	3.682	0.032
wheat plots						
C. cladosporioides	management	> 18	1	11.046	152.99	< 0.001
	plant-section	24	1	12.018	166.46	< 0.001
A. alternata	management	> 18	1	14.172	102.337	< 0.001
	plant-section	24	1	10.253	74.039	< 0.001
	IA management:plant-section	> 18	1	1.2604	9.102	0.004
Trichoderma group	plant-section	24 (35)	1	13.052	8.105	0.007
F. culmorum	management	> 18 (21)	1	14.037	8.082	0.007
	IA managment:plant-section	> 18 (21)	1	9.521	5.482	0.004

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.

```
C. cladosporioides (1)
              Df Sum Sq Mean Sq  F value    Pr(>F)
plant_section  1 3.7808  3.7808 106.7974 6.207e-08 ***
tillage        2 0.0869  0.0435   1.2279    0.3226
Residuals     14 0.4956  0.0354

A. alternata (1)
              Df Sum Sq Mean Sq F value  Pr(>F)
plant_section  1 1.1518 1.15183  7.7559 0.01461 *
tillage        2 1.1250 0.56250  3.7876 0.04844 *
Residuals     14 2.0791 0.14851

T. viride/atroviride/koningii (1)
              Df  Sum Sq Mean Sq F value  Pr(>F)
plant_section  1 13.0345 13.0345  6.1502 0.02647 *
tillage        2  1.8319  0.9160  0.4322 0.65746
Residuals     14 29.6712  2.1194

F. culmorum (1)
              Df  Sum Sq Mean Sq F value    Pr(>F)
plant_section  1 17.7392 17.7392 17.5601 0.0009072 ***
tillage        2  7.7423  3.8712  3.8321 0.0470662 *
Residuals     14 14.1428  1.0102

C. cladosporioides (2)
              Df Sum Sq Mean Sq F value Pr(>F)
plant_section  1 17.995  17.995 230.270 <2e-16 ***
fertilizer     1  0.229   0.229   2.929 0.0925 .
Residuals     56  4.376   0.078

A. alternata (2)
                   Df  Sum Sq Mean Sq  F value  Pr(>F)
plant_section       1 29.0588 29.0588 280.9320 < 2e-16 ***
fertilizer          1  0.5015  0.5015   4.8488 0.03188 *
plant_section:crop  2  0.7617  0.3809   3.6822 0.03157 *
Residuals          55  5.6891  0.1034

T. viride/atroviride/koningii (2)
             Df Sum Sq Mean Sq F value  Pr(>F)
fertilizer    1  4.280  4.2805  2.8279 0.09852 .
tillage:crop  5 16.136  3.2273  2.1321 0.07577 .
Residuals    53 80.223  1.5136

F. culmorum (2)
          Df  Sum Sq Mean Sq F value  Pr(>F)
tillage    2  11.979  5.9895  3.1197 0.05179 .
```

```
Residuals 57 109.434  1.9199

C. cladosporioides (3)
              Df  Sum Sq Mean Sq F value    Pr(>F)
management     1 11.0461 11.0461  152.99 6.427e-16 ***
plant_section  1 12.0183 12.0183  166.46 < 2.2e-16 ***
Residuals     44  3.1768  0.0722

A. alternata (3)
                         Df  Sum Sq Mean Sq  F value    Pr(>F)
management                1 14.1718 14.1718 102.3365 7.939e-13 ***
plant_section             1 10.2531 10.2531  74.0390 8.155e-11 ***
tillage                   2  0.8889  0.4445   3.2095  0.050454 .
management:plant_section  1  1.2604  1.2604   9.1015  0.004325 **

T. viride/atroviride/koningii (3)
              Df Sum Sq Mean Sq F value   Pr(>F)
plant_section  1 13.052 13.0520  8.1051 0.006627 **
management     1  2.194  2.1943  1.3626 0.249228
Residuals     45 72.465  1.6103

F. culmorum (3)
                         Df Sum Sq Mean Sq F value   Pr(>F)
management                1 14.037 14.0369  8.0817 0.006875 **
tillage                   2  9.789  4.8944  2.8179 0.071060 .
management:plant_section  2 19.043  9.5214  5.4819 0.007668 **
Residuals                42 72.949  1.7369

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

SI.5 Supplementary table 4: qPCR measurements used for Figure 3

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

C. cladosporioides

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	221931	189596	92529	208114	689661	1029114	187376	266025
	B	1472541	31582	189434	36047	89757	253110	236146	475972
	C	1985546	166855	493060	1082916	725535	453460	518064	692371
	D	483323	126022	253562	433416	254127	625904	91385	553759
roots	A	12481	5248	6182	7367	31598	50620	4936	19375
	B	365418	54958	95696	153298	142270	440342	88190	330342
	C	93646	22022	11857	98243	32056	86610	23835	77318
	D	40810	21943	11689	42958	20404	68723	12025	68794
root/soil	B	37775	8300	23584	30223	60772	55538	36941	81454
	C	3593	3592	1366	4729	2552	37292	2322	17701
	D	6424	7006	3804	4309	8552	10718	7335	52636
bulk soil	B	25061	1384	3229	11275	18507	50987	5195	49944
	C	764	361	417	1249	4052	3782	1143	4436
	D	3104	1497	2361	1031	1444	13463	1498	14722

A. alternata

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	5925	1520	971	2374	4256	13607	874	489
	B	22471	257	410	313	659	1675	611	1801
	C	50705	646	4102	11007	1243	4558	2290	4408
	D	4032	264	2565	2251	460	6541	415	3028
roots	A	43	18	10	17	38	179	102	27
	B	2156	170	268	3339	528	4143	504	1137
	C	2683	154	27	310	765	4009	175	368
	D	317	26	14	370	21	315	49	199
root/soil	B	431	31	59	229	225	305	372	221
	C	569	395	0	901	612	0	212	20704
	D	151	203	400	1947	260	264	1362	744
bulk soil	B	137	0	21	107	106	243	20	185
	C	0	0	0	0	0	656	0	115
	D	0	0	0	64	0	472	0	77

F. culmorum

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	34210	219738	31613	10144	105792	1924	5496	1682
	B	173921	28102	13583	15358	668	4462	18048	9713
	C	372274	39838	256401	1012017	291853	90782	121335	1936
	D	120241	62446	70344	71972	1593	415	187757	2003
roots	A	1421	1009	528	405	571	520	10	9
	B	20304	5951	3430	7806	3389	4760	5044	5387
	C	4122	897	2987	5992	1031	13697	1379	2227
	D	4762	1587	5291	1582	2059	3468	4774	183
root/soil	B	3080	1101	2086	3000	5035	920	1271	5294
	C	666	420	234	906	82	94	726	770
	D	431	930	989	705	1522	337	3910	54
bulk soil	B	1491	104	264	934	245	46	308	516
	C	33	0	2473	257	923	7	138	20
	D	1127	0	101	0	130	52	134	80

Trichoderma group

env.	date	conv. 1	conv. 2	conv. 3	conv. 4	organic 1	organic 2	organic 3	organic 4
shoots	A	104	82	1122	423	10	87	55	0
	B	194	314	2172	2554	66	194	108	485
	C	1864	3311	43469	269	145	1520	297	158
	D	714	3035	673	13455	492	756	102	6873
roots	A	82	18	69	112	44	28	0	87
	B	885	389	1025	5907	854	412	108	2226
	C	1532	366	831	616	204	525	33	577
	D	535	1508	538	1537	158	224	89	268
root/soil	B	2136	1890	807	9803	1794	928	244	4897
	C	2236	2566	1487	589	243	731	145	699
	D	826	4013	1660	993	6956	289	4039	330
bulk soil	B	765	771	1148	3776	594	392	411	1287
	C	2925	505	3234	1462	656	147	61	405
	D	964	1085	1625	9815	252	774	539	1012

SI.6 Supplementary References

Bowyer, P., Fraczek, M., Denning, D.W., 2006. Comparative genomics of fungal allergens and epitopes shows widespread distribution of closely related allergen and epitope orthologues. BMC Genomics. 7, 251.

Cullen, D.W., Toth, I.K., Pitkin, Y., Boonham, N., Walsh, K., Barker, I., Lees, A.K., 2005. Use of quantitative molecular diagnostic assays to investigate Fusarium dry rot in potato stocks and soil. Phytopathology. 95, 1462-1471.

Haugland, R., Vesper, S., 2002. Method of identifying and quantifying specific fungi and bacteria, U.S. Environmental Protection Agency, Washington, DC, USA.

Nilsson, R.H., Ryberg, M., Kristiansson, E., Abarenkov, K., Larsson, K.H., Koljalg, U., 2006. Taxonomic reliability of DNA sequences in public sequence databases: a fungal perspective. PLoS ONE. 1, e59.

Oksanen, J., Blanchet, F.G., Kindt, R., Legendre, P., Minchin, P.R., O'Hara, R.B., Simpson, G.L., Solymos, P., Stevens, M.H.H., Wagner, H. (2013). vegan: Community Ecology Package. R package version 2.0-10. URL http://CRAN.R-project.org/package=vegan

R Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Sagova-Mareckova, M., Cermak, L., Novotna, J., Plhackova, K., Forstova, J., Kopecky, J., 2008. Innovative methods for soil DNA purification tested in soils with widely differing characteristics. Appl. Environ. Microbiol. 74, 2902-2907.

Towe, S., Wallisch, S., Bannert, A., Fischer, D., Hai, B., Haesler, F., Kleineidam, K., Schloter, M., 2011. Improved protocol for the simultaneous extraction and column-based separation of DNA and RNA from different soils. J. Microbiol. Methods. 84, 406-412.

Vesper, S., 2011. Traditional mould analysis compared to a DNA-based method of mould analysis. Crit. Rev. Microbiol. 37, 15-24.

White, T.J., Bruns, T., Lee, S., Taylor, J.W., 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR Protocols: A Guide to Methods and Applications, Vol. 3, Academic Press, Inc., pp. 315-322.

III Urban dust microbiome: Impact on later atopy and wheezing.

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Urban dust microbiome: Impact on later atopy and wheezing.

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Key words:

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

Short summary:

Exposure to higher fungal diversity shortly after birth is associated with a significant lower risk 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 sub-group 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 m^2 for 2 minutes for textile surfaces or 4 m^2 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 (Quiagen, Hilden, Germany) with primers ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') (Gardes and Bruns 1993) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White et al. 1990) for fungal ITS (internal transcribed spacer) DNA, or Bac27f (5'-AGAGTTTGATCMTGGCTCAG-3') (Jiang et al. 2006) and 907r (5-CCGTCAATTCMTTTGAGTTT-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 Formamid (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 (3^{rd} tertile versus 1^{st} 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 (3^{rd} tertile versus 1^{st} 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 (6 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, Miletto M, Lindow SE, Taylor JW, Bruns TD. 2014. Airborne bacterial communities in residences: similarities and differences with fungi. PLoS One 9: e91283.

Adams RI, Miletto 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.

Adams RI, Miletto M, Taylor JW, Bruns TD. 2013b. The Diversity and Distribution of Fungi on Residential Surfaces. PLoS One 8:e78866; doi:10.1371/journal.pone.0078866.

Barberán A, Dunn RR, Reich BJ, Pacifici K, Laber EB, Menninger HL, et al. 2015. The ecology of microscopic life in household dust. Proc. R. Soc. B Biol. Sci. 282:20151139; doi:10.1098/rspb.2015.1139.

Casas L, Tischer C, Wouters IM, Valkonen M, Gehring U, Doekes G, et al. 2013. Endotoxin, extracellular polysaccharides, and beta(1-3)-glucan concentrations in dust and their determinants in four European birth cohorts: results from the HITEA project. Indoor Air; doi:10.1111/ina.12017.

Chen J. 2012. GUniFrac: Generalized UniFrac distances. R package version 1.0. 2012.

Collins RE, Rocap G. 2007. REPK: An analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Res. 35:58–62; doi:10.1093/nar/gkm384.

Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH. 2009. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics 10:171; doi:10.1186/1471-2105-10-171.

Dannemiller KC, Gent JF, Leaderer BP, Peccia J. 2015. Influence of housing characteristics on bacterial and fungal communities in homes of asthmatic children. Indoor Air n/a–n/a; doi:10.1111/ina.12205.

Dannemiller KC, Mendell MJ, Macher JM, Kumagai K, Bradman a., Holland N, et al. 2014. Next-generation DNA sequencing reveals that low fungal diversity in house dust is associated with childhood asthma development. Indoor Air 24:236–247; doi:10.1111/ina.12072.

Ege MJ, Mayer M, Normand A-C, Genuneit J, Cookson WOCM, Braun-Fahrländer C, et al. 2011. Exposure to environmental microorganisms and childhood asthma. N. Engl. J. Med. 364:701–709; doi:10.1056/NEJMoa1007302.

Gardes M, Bruns TD. 1993. ITS primers with enhanced specificity for basidiomycetes--application to the identification of mycorrhizae and rusts. Mol. Ecol. 2: 113–118.

Heinrich J, Bolte G, Holscher B, Douwes J, Lehmann I, Fahlbusch B, et al. 2002. Allergens and endotoxin on mothers' mattresses and total immunoglobulin E in cord blood of neonates. Eur. Respir. J. 20: 617–623.

Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. 2006. Microbial diversity in water and

sediment of Lake Chaka, an athalassohaline lake in northwestern China. Appl. Environ. Microbiol. 72:3832–3845; doi:10.1128/AEM.02869-05.

Karvonen AM, Hyvarinen A, Rintala H, Korppi M, Taubel M, Doekes G, et al. 2014. Quantity and diversity of environmental microbial exposure and development of asthma: a birth cohort study. Allergy 69:1092–1101; doi:10.1111/all.12439.

Kembel SW, Jones E, Kline J, Northcutt D, Stenson J, Womack AM, et al. 2012. Architectural design influences the diversity and structure of the built environment microbiome. ISME J. 6:1469–79; doi:10.1038/ismej.2011.211.

Lynch S V, Wood RA, Boushey H, Bacharier LB, Bloomberg GR, Kattan M, et al. 2014. Effects of early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. J. Allergy Clin. Immunol. 134:593–601 e12; doi:10.1016/j.jaci.2014.04.018.

Mühling M, Woolven-Allen J, Murrell JC, Joint I. 2008. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J. 2:379–392; doi:10.1038/ismej.2007.97.

Oksanen J, Guillaume Blanchet F, Kindt R, Legendre P, Minchin PR, O'Hara RB, et al. 2013. Vegan: Community Ecology. Package. R package version 2.0-10.

Pakarinen J, Hyvärinen A, Salkinoja-Salonen M, Laitinen S, Nevalainen A, Mäkelä MJ, et al. 2008. Predominance of Gram-positive bacteria in house dust in the low-allergy risk Russian Karelia. Environ. Microbiol. 10:3317–3325; doi:10.1111/j.1462-2920.2008.01723.x.

R Team development core. 2012. R: A Language and Environment for Statistical Computing.; doi:10.1007/978-3-540-74686-7.

Simpson EH. 1949. Measurement of Diversity. Nature 163:688–688; doi:10.1038/163688a0.

Tischer C, Standl M, Lehmann I, Schaaf B, von Berg A, Heinrich J. 2015. Sleeping on animal fur is related to asthma outcomes in later childhood. Eur. Respir. J. 46:107–14; doi:10.1183/09031936.00204914.

Valkonen M, Wouters IM, Taubel M, Rintala H, Lenters V, Vasara R, et al. 2015. Bacterial Exposures and Associations with Atopy and Asthma in Children. PLoS One 10: e0131594.

von Mutius E, Vercelli D. 2010. Farm living: effects on childhood asthma and allergy. Nat Rev Immunol 10:861–868; doi:10.1038/nri2871.

White TJ, Bruns S, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protoc. A Guid. to Methods Appl. 3:315–322; doi:citeulike-article-id:671166.

Zutavern A, Brockow I, Schaaf B, Bolte G, von Berg A, Diez U, et al. 2006. Timing of solid food introduction in relation to atopic dermatitis and atopic sensitization: results from a prospective birth cohort study. Pediatrics 117:401–411; doi:10.1542/peds.2004-2521.

Table 1: Characteristics of the study population

	n / N (%)
Study population	**189**
Female	87/189 (**46%**)
Maternal education	
Low & Medium	60 / 187 (**32%**)
High	127 / 187 (**68%**)
Season of dust sampling	
Winter	41 / 189 (**22%**)
Spring	29 / 189 (**15%**)
Summer	57 / 189 (**30%**)
Autumn	62 / 189 (**33%**)
Fungal diversity	
1st tertile	[0.403 - 0.863)
2nd tertile	[0.863 - 0.931)
3rd tertile	[0.931 - 0.977]
Bacterial diversity	
1st tertile	[0.179 - 0.693)
2nd tertile	[0.693 - 0.835)
3rd tertile	[0.835 - 0.941]
IgE aero-allergens (≥ 0.35 kU/l) – 6 years	43/159 (**27%**)
IgE aero-allergens (≥ 0.35 kU/l) – 10 years	56/141 (**40%**)
Wheezing ever – 10 years	73 / 170 (**43%**)

Table 2: Adjusted* odds ratios (aORs 95% CI) for the association between fungal and bacterial diversity (Simpson index, tertiles) and health outcomes

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

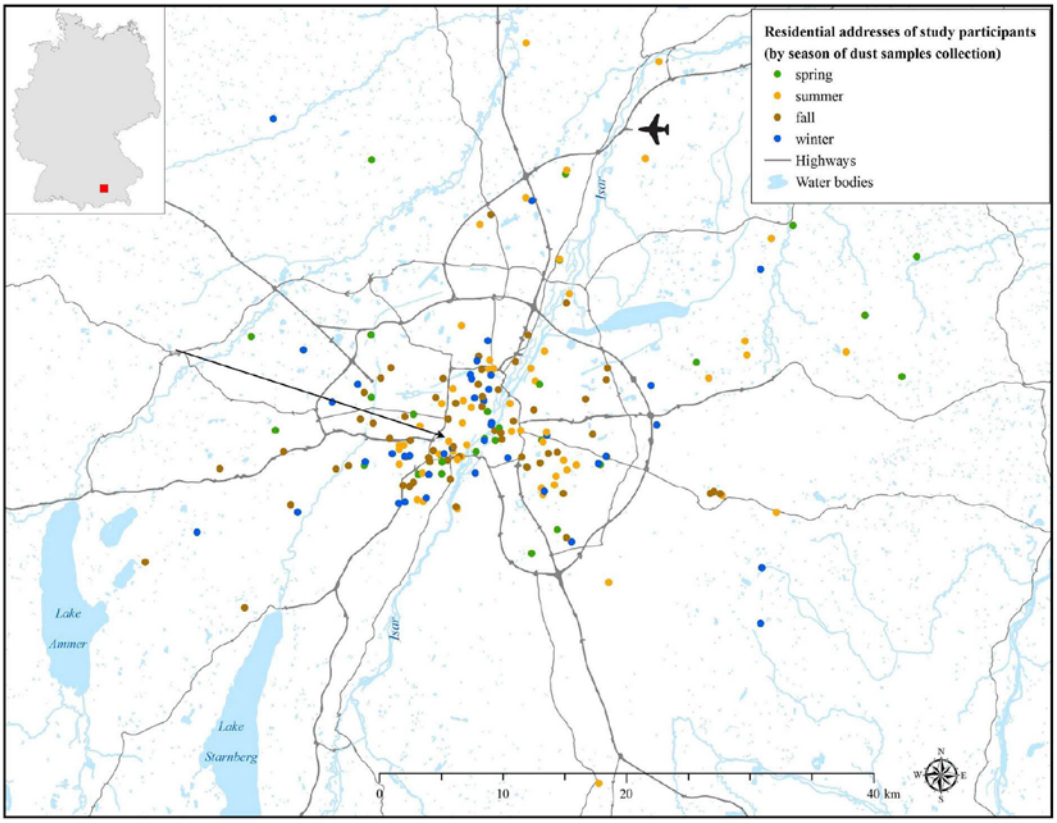

Figure 1: Map of the Munich urban area

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

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Fungal and Bacterial Communities in Indoor Dust Follow Different Environmental Determinants

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Abstract

People spend most of their time inside buildings and the indoor microbiome is a major part of our everyday environment. It affects humans' wellbeing and therefore its composition is important for use in inferring human health impacts. It is still not well understood how environmental conditions affect indoor microbial communities. Existing studies have mostly focussed on the local (e.g., building units) or continental scale and rarely on the regional scale, e.g. a specific metropolitan area.

Therefore, we wanted to identify key environmental determinants for the house dust microbiome from an existing collection of spatially (area of Munich, Germany) and temporally (301 days) distributed samples and to determine changes in the community as a function of time.

To that end, settled dust samples that had been collected from the living room floors of 286 individual households, were profiled for fungal and bacterial community variation and diversity using microbial fingerprinting techniques. The profiles were tested for their association with occupant behaviour, building characteristics, outdoor pollution, vegetation, and urbanization.

Our results showed that more environmental and particularly outdoor factors (vegetation, urbanization, airborne particulate matter) affected the community composition of indoor fungi than of bacteria. The passage of time affected fungi and, surprisingly, also strongly affected bacteria. We inferred that fungal communities in indoor dust changed semi-annually, whereas bacterial communities paralleled phenological periods. These differences in temporal dynamics cannot be fully explained and should be further investigated in future 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.

46 The potential effects of the time of the year on the composition of an indoor microbial
47 community have been analysed by sampling at different times or over short periods of time
48 [4, 5, 8]. Continuous observations (i.e., high frequency temporal sampling of indoor dust
49 over several months) have used spore counts or cultivation [3, 18, 19]. In the adjacent
50 outdoor air environment, seasonal changes have been well described in fungi [3, 20-22] and
51 have recently been observed in bacteria during 14 months of monitoring [23], confirming
52 studies that had shorter time-frames [24-26]. In addition, outdoor airborne bacteria can
53 change within few days [27, 28]. Although large knowledge gaps exist, temporal changes in
54 microbial communities seems to be common in a number of other environments as well
55 [29]. For an actual assessment on how microbial communities in indoor dust are affected by
56 the passage of time, comparing different short windows of time is not sufficient. Instead, it
57 is necessary to perform analyses over a period of several months or years with frequent
58 sampling, but such DNA-based studies are rare.
59
60 In this study, we determined the variation and diversity of the fungal and bacterial
61 microbiome in dust samples from 286 households. The samples were distributed spatially
62 (i.e., over an area of Munich, Germany) and temporally (i.e., over 10 months). We used
63 fungal and bacterial fingerprints (terminal restriction fragment length polymorphism – tRFLP
64 [30]) along with associated data sets on environmental parameters.
65 Our first objective was to identify key indoor and outdoor environmental factors that
66 affected the microbial community. Secondly, we assessed and compared the temporal
67 dynamics of the fungal and dust bacterial communities. Regarding that, we explored
68 whether the existing sample design of spatially unrelated single samples collected in a
69 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 LISAplus 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). LISAplus is an ongoing birth cohort study that has screening, recruitment and exclusion criteria that have been described elsewhere [31, 32]. LISAplus 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.

92 Samples were collected by trained inspectors using vacuum cleaners (Phillips, Hamburg,
93 Germany) equipped with ALK filter holders (ALK, Hørsholm, Denmark) containing a paper
94 filter when infants of participating families were two to three months old. A detailed
95 description of the dust sampling and processing has been previously published [31, 33]. The
96 filter boxes were stored below −20 °C.
97

98 ## Microbial fingerprinting

99 Frozen filter boxes with vacuumed dust were equilibrated to ambient conditions in a clean
100 PCR chamber with the airflow deactivated for 60 minutes. Dust was released from the filter
101 boxes, freed from obvious extraction obstacles (e.g., stones, etc.) and 100 mg were used for
102 DNA extraction with a PowerSoil-htp96 Soil DNA Isolation Kit (Mo-Bio Laboratories,
103 Carlsbad, CA, USA). For tRFLP DNA-fingerprinting, the DNA was PCR-amplified using a
104 TopTaq DNA polymerase kit (Qiagen, Hilden, Germany) with the primers ITS1F (5'-
105 CTTGGTCATTTAGAGGAAGTAA-3') [34] and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [35] for
106 fungal ITS (internal transcribed spacer) DNA, or Bac27f (5'-AGAGTTTGATCMTGGCTCAG-3')
107 [36] and 907r (5-CCGTCAATTCMTTTGAGTTT-3) [37] for bacterial 16S rRNA genes. Forward
108 primers were labelled with 6-FAM and reverse primers with 6-HEX fluorescent dyes,
109 respectively. The PCR profiles were [4 min 94 °C; 32 cycles of 60 s 94 °C, 60 s 50 °C, 90 s 72
110 °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
111 72 °C] (bacteria). Products from two PCR reactions were pooled, purified and digested with
112 the restriction enzyme HpyCH4IV (fungi) or MspI (bacteria). HpyCH4IV was selected after in
113 silico enzyme digestions using REPK v1.3 [38] against an artificial set of fungal sequences
114 commonly found in dust. Cleaned fragments were transferred to HiDi Formamide (Applied

115 Biosystems, Foster City, CA, USA) containing MapMarker 1000-ROX (1:400; Bioventures,
116 Murfreesboro, TN, USA) and separated on an ABI 3730 capillary sequencer (Applied
117 Biosystems). Raw fragment tables were built with peak-scanner 2.0 (Applied Biosystems). T-
118 REX v1.14 [39] was used for noise filtering (peak height, multiplier 1) and binning of
119 fragments (threshold 1 bp).
120

121 Statistical analyses

122 The R programming environment [40] was used for all statistical analyses. Bray—Curtis
123 dissimilarities [41] between the samples (community variation) and biodiversity indices
124 were calculated from 10^3 times randomly rarefied (to the lowest amount of signal present in
125 one sample) OTU (operational taxonomic unit) abundances using algorithms from the vegan
126 [42] and GUniFrac [43] packages. The results from forward and reverse terminal restriction
127 fragments (including labelled forward or reverse primers) were averaged. Multivariate
128 testing for the effect of environmental characteristics on the community was conducted
129 using average Bray—Curtis dissimilarity matrices with the Adonis (Permutational
130 Multivariate Analysis of Variance Using Distance Matrices) and MRPP (Multi-Response
131 Permutation Procedure) functions, each with 10^5 permutations available in the vegan
132 package. Partial Mantel tests (10^4 permutations, implemented in the vegan package) were
133 used to test for possible geographic correlations of community dissimilarities.
134 Microbial diversity was assessed with the Simpson (1–D) and Shannon (H') indices, and
135 seasonal influences were additionally evaluated with Pilou's Evenness (J) and Richness (S).
136 Details on these calculations are available in the vegan package [42]. Relationships between
137 biodiversity indices and environmental characteristics were assessed using a Wilcoxon

138 signed rank test for dichotomous variables and a Kruskall—Wallis test for variables with
139 three or more categories due to the non-normal distribution of fungal and bacterial diversity
140 (Shapiro—Wilk test $P < 0.01$).
141

142 Results

143 Community variation

144 The variation in the fungal community was more sensitive to the tested environmental
145 determinants than the variation in the bacterial community (Table 1).
146 For indoor factors, variation in the fungal community in the living room floor dust was
147 affected in particular by signs of mould inside the home, the tightness of the windows,
148 heating inside the home, and the type of living room floor (i.e., Adonis $P < 0.05$, MRPP $\delta <$
149 0.05; Table 1). In contrast, the bacterial community variation was only significantly affected
150 by ventilation (winter half year, Adonis $P = 0.05$, MRPP $\delta = 0.05$), and in tendency (i.e.,
151 Adonis P or MRPP $\delta > 0.05$ but not both tests) by the type of living room floor (Adonis $P =$
152 0.08, MRPP $\delta = 0.02$) as well as the position of the home (Adonis $P = 0.1$, MRPP $\delta = 0.05$).
153 For outdoor factors (Table 1), a significant effect on the variation in the fungal community
154 was due to the building age, the surrounding greenery within a 100 m buffer, the
155 urbanization grade (urban index), and particulate matter (< 2.5 µm and coarse particulates)
156 (Adonis $P \leq 0.05$, MRPP $\delta < 0.05$). The surrounding greenery within a 30 m buffer (Adonis $P =$
157 0.06, MRPP $\delta = 0.03$) and nitrogen oxides (NO_x) (Adonis $P = 0.06$, MRPP $\delta = 0.01$) were also
158 in tendency associated with the variation in the fungal community. The only outdoor
159 characteristic that may have affected variation in the bacterial community was the position
160 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 1. Significance of associations between environmental determinants and microbial community variation (based on Bray—Curtis dissimilarities).

	Fungi		Bacteria	
Environmental characteristics	P^a	δ^b	P^c	δ^d
Indoor characteristics				
N° of rooms within the flat	0.75	0.87	0.71	0.79
Dampness	0.69	0.29	0.16	0.39
Mould at home	**0.04**	**0.03**	0.09	0.13

Water leakage	0.81	0.85	0.57	0.62
Tightness of the windows	**0.03**	**0.04**	0.36	0.36
Ventilation living room through windows - summer	0.27	0.24	0.71	0.93
Ventilation living room through windows - winter	0.67	0.64	**0.05**	**0.05**
Heating within the home	**0.03**	**0.02**	0.36	0.41
Renovation measures last 12 months	0.44	0.61	0.65	0.65
Pets	0.27	0.28	0.62	0.75
Type of living room floor	**< 0.001**	**< 0.001**	0.08	**0.02**
Smoking of tobacco in the flat	0.42	0.41	0.71	0.78
Outdoor characteristics				
Age of the building	**0.01**	**0.01**	0.28	0.31
Position of the home	0.49	0.67	0.1	**0.05**
Building density of the neighbourhood	0.52	0.59	0.39	0.44
Traffic jams in rush hour	0.83	0.83	0.29	0.24
Facility with noticeable air pollution within 50 and 100 m	0.58	0.72	0.45	0.40
Facility with noticeable air pollution within 50 m	0.33	0.28	0.85	0.94
NDVI (500 m buffer)	0.72	0.63	084	0.94
NDVI (100 m buffer)	**0.05**	**0.006**	0.19	0.22
NDVI (30 m buffer)	0.06	**0.01**	0.33	0.30
Urban index	**0.02**	**0.01**	0.51	0.60
NO_2	0.23	0.06	0.63	0.75
NO_x	0.06	**0.03**	0.37	0.41
$PM_{2.5}$	**0.004**	**0.005**	0.51	0.44
PM_{10}	0.54	0.32	0.82	0.70
PM_{coarse}	**0.04**	**0.008**	0.41	0.46
$PM_{2.5}$ absorbance	0.07	0.06	0.37	0.42

Results from Adonis (P) and MRPP (δ), bold: P or $\delta \leq 0.05$; all R^2 (Adonis) A (MRPP chance corrected between groups agreement) values are given in S1 Fig. [a] $R^2 < 0.06$, [b] $A < 0.03$, [c] $R^2 < 0.02$, [d] $A < 0.01$.

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

	representation of sampling times	Community variation				Diversity indices			
		Adonis		MRPP		P (Kruskal–Wallis test)			
		P	R^2	δ	A	H'	1–D	J	S
fungi	**four astronomical seasons** [a]	$<10^{-5}$	0.131	$<10^{-5}$	0.076	0.0018	0.0113	0.0553	0.0001
bacteria		*$<10^{-5}$*	*0.245*	*$<10^{-5}$*	*0.151*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*
fungi	**four meteorological seasons** [b]	$<10^{-5}$	0.145	$<10^{-5}$	0.085	0.0003	0.0002	0.0006	0.0003
bacteria		*$<10^{-5}$*	*0.248*	*$<10^{-5}$*	*0.156*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*
fungi	**nine phenological periods** [c]	$<10^{-5}$	0.187	$<10^{-5}$	0.111	$<10^{-5}$	$<10^{-5}$	10^{-5}	10^{-5}
bacteria		*$<10^{-5}$*	*0.341*	*$<10^{-5}$*	*0.205*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*	*$<10^{-5}$*
fungi	**continuous (metric)**	0.0267	0.013						
bacteria		*$<10^{-5}$*	*0.228*						

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

189 (Shannon Index (H'), Simpson Index (1-D), Pielou's Evenness (J), Richness (S)). [a] e.g., spring
190 starting at March equinox, ending at June solstice; [b] e.g., spring starting March 1, ending
191 May 31; [c] for the area of Munich in 1998–1999, displayed and supported in Fig 1d.
192
193 **Fig 1. Microbial community variation and temporal dynamics.** Points: dust samples from
194 different homes. **(a, c)** first coordinates of a principal coordinate analysis (PCoA) on Bray–
195 Curtis dissimilarities of the fungal (a) and bacterial (c) communities; in brackets: explained
196 variance, colours: meteorological seasons. **(b, d)** the principal coordinate that explains most
197 of the variation for fungi (b) or bacteria (d) sorted by the sampling date; regression (b, d): a
198 locally weighted polynomial fit with 95% confidence interval; colour strip (b): meteorological
199 seasons: spring, summer, autumn, and winter; colour strip (d): phenological periods of the
200 geographic area during the time-frame of sampling: early spring, full spring, early summer,
201 midsummer, late summer, early autumn, full autumn, late autumn, and winter (dates
202 obtained from: http://www.dwd.de/, http://www.phenocal.chira.de; accessed 18 Jul 2014).
203

204 ## Diversity

205 The determinants of significantly different community variations in fungi and bacteria were
206 not associated per se with significantly different diversities (Simpson and Shannon indices,
207 Table 3: significant variables, S2 Table: all variables).
208 For indoor environmental factors, infrequent ventilation of a flat via the windows in the
209 summer half year was significantly associated with a higher diversity of less abundant fungi
210 (Shannon index P = 0.04, Kruskal—Wallis test). Homes with carpets had a lower bacterial
211 diversity than homes with smooth floors, but the highest bacterial diversity was sampled

212 from mixed floors (Shannon index P = 0.01 and Simpson index P = 0.04, Kruskal—Wallis
213 tests).
214 Regarding outdoor factors, low and high (1st and 3rd tertile) surrounding greenery within a
215 100 m buffer significantly increased the fungal diversity in the dust compared to the third of
216 samples from homes with a medium level of surrounding greenery (2nd tertile) (Simpson
217 index P = 0.03, Kruskal—Wallis test). The bacterial diversity was significantly lower for
218 homes above the 1st floor (Shannon index P = 0.04 and Simpson index P = 0.03, Kruskal—
219 Wallis tests).
220 Fungal diversity was only lower in late summer compared to the rest of the year (Fig 2a).
221 However, the effect of the season on fungal diversity indices was strongly significant when
222 the sampling times were categorized according to outdoor plant phenology or the
223 meteorological seasons (P < 0.001, Kruskal—Wallis tests on Simpson and Shannon indices,
224 Evenness, and Richness; Table 2). In contrast, the bacterial diversity precisely mirrored the
225 shift in the bacterial community variation during spring (Fig 2b). Differences between the
226 maximum and minimum diversity (determined from the localized regression shown in Fig 2)
227 were also much less for fungi (approximate difference: Shannon index 20%, Simpson index
228 11%, evenness 11%) than for bacteria (approximate difference: Shannon index 61%,
229 Simpson index 52%, evenness 42%).
230

231 **Table 3. Significant associations between environmental determinants and microbial**

232 **diversity.**

Environmental characteristics	Fungi 1-D median (p25-p75)	*P*	Fungi H' median (p25-p75)	*P*	Bacteria 1-D median (p25-p75)	*P*	Bacteria H' median (p25-p75)	*P*
Vent. living room: summer		0.08		**0.04**		0.62		0.56
seldom/never/via another room	0.91 (0.85-0.93)		3.16 (2.79-3.47)		0.71 (0.53-0.86)		2.00 (1.47-2.57)	
once/several times a day (short)	0.93 (0.88-0.94)		3.54 (3.04-3.72)		0.77 (0.52-0.86)		2.21 (1.36-2.66)	
once/several times a day (long)	0.90 (0.83-0.94)		3.20 (2.78-3.6)		0.79 (0.62-0.86)		2.26 (1.6-2.71)	
Type of living room floor		0.30		0.33		**0.04**		**0.01**
carpet	0.92 (0.86-0.94)		3.35 (2.95-3.64)		0.78 (0.51-0.85)		2.14 (1.35-2.58)	
smooth	0.90 (0.81-0.94)		3.09 (2.78-3.6)		0.81 (0.58-0.87)		2.32 (1.46-2.71)	
smooth with rugs	0.90 (0.83-0.93)		3.20 (2.74-3.61)		0.80 (0.68-0.87)		2.36 (1.84-2.81)	
Position of the home		0.15		0.09		**0.04**		**0.03**
ground floor	0.91 (0.84-0.94)		3.3 (2.81-3.66)		0.81 (0.64-0.88)		2.35 (1.73-2.8)	
1st floor	0.92 (0.85-0.95)		3.45 (2.93-3.67)		0.83 (0.63-0.86)		2.41 (1.71-2.74)	
2nd floor	0.89 (0.83-0.92)		3.06 (2.74-3.43)		0.78 (0.57-0.82)		2.21 (1.62-2.37)	
3rd floor or higher	0.89 (0.82-0.93)		3.1 (2.72-3.58)		0.75 (0.45-0.85)		2.12 (1.25-2.64)	
NDVI (100 m buffer)		**0.03**		0.11		0.14		0.11
1st tertile (0.06-0.26)	0.92 (0.86-0.94)		3.40 (2.92-3.65)		0.79 (0.63-0.86)		2.22 (1.63-2.68)	
2nd tertile (0.27-0.33)	0.88 (0.81-0.93)		3.10 (2.65-3.59)		0.75 (0.54-0.85)		2.14 (1.44-2.65)	
3rd tertile (0.33-0.59)	0.90 (0.84-0.94)		3.27 (2.85-3.67)		0.81 (0.69-0.87)		2.42 (1.84-2.74)	

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

234 associations to diversity changes (Simpson Index (1-D), Shannon Index (H')) for fungi and

235 bacteria. p25-p75: interquartile ranges; bold: $P \leq 0.05$. Values for all insignificant ($P > 0.05$)

236 variables are given in S2 Table.

237

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

239 Diversity indices of the dust samples are sorted by sampling date, (a) for fungi (n = 286) and

240 (b) for bacteria (n = 283). Regression: a locally weighted polynomial fit with 95% confidence
241 interval.
242

243 Discussion

244 This study considered both the fungal and the bacterial microbiomes in indoor dust, in more
245 than 250 households in a metropolitan area. We observed that the environmental factors
246 that affected the composition of the fungal microbiome were different than those that
247 affected the bacterial microbiome. However, the time of the year (season or phenological
248 period) was the most decisive of the parameters tested for both fungi and bacteria.
249 Fungal community variation and diversity were influenced by several indoor environmental
250 factors. In contrast, bacterial community variation was significantly affected only by
251 behaviour regarding ventilation, and bacterial diversity was significantly affected only by the
252 type of living room floor. Similar results have been recently reported [6, 7], and our findings
253 are in accord with studies on the building environment microbiome, which emphasize the
254 importance of the architectural design (including ventilation) and human behaviour itself as
255 decisive factors for the biogeography of bacterial communities in indoor environments [11,
256 15]. In a recent study [6], the fungal community composition in settled dust samples from
257 various indoor locations seemed to be mainly driven by fungi associated with the outdoor
258 environment, while for the bacterial community, the composition reflected the taxa
259 released from the residents, an observation that had also been made earlier [13]. In the
260 same study [6], the authors did not detect any effect of the house or residential
261 characteristics on the bacterial microbiome, which is in accord with our study. However, we
262 found an effect of two determinants that were kept uniform in their study [6]. The

263 ventilation behaviour showed an effect on the bacterial community variation, as was also
264 previously reported [15]. Additionally, the type of living room floor affected the bacterial
265 diversity. It is apparent that small-scale structured floors (i.e., smooth with rugs) can
266 harbour a higher diversity than homogenous surfaces.
267 Fungal community characteristics were not affected by dampness or water leakage but were
268 affected by the presence of mould. In a study comparing 17 homes with low mouldiness
269 (evaluated with "Environmental Relative Moldiness Index", [44]) with 18 homes that had
270 high mouldiness, fungal Richness was significantly associated with the relative humidity but
271 not Evenness or the Shannon or Simpson indices [45]. These findings suggest that dampness
272 alone might be a relatively weak determinant of the indoor fungal community,
273 notwithstanding that the growth of mould is often associated with dampness [46] because
274 different individual sites have different reactions to moisture, and the assessment of
275 dampness and the moisture status is complex [47].
276 The ventilation strategy in a university building has been identified as one of the strongest
277 factors affecting bacterial community variation [11]. In this study, the association between
278 ventilation habits and bacterial community variation was also significant, but its likely
279 impact (MRPP *A*) was more than 10-fold smaller than the impact of the outdoor phenology
280 (i.e., the sampling time). The probable reason for this difference may be the different
281 environments sampled in the study of Meadow et al. (2014) [11] and the present work: air
282 sampling versus settled dust, mechanic ventilation versus non-mechanic ventilation through
283 windows, etc. In this study, tight windows (which implied a lower air exchange rate) were
284 also significantly associated with fungal community variation and higher fungal diversity.
285 Along with building structures and residential characteristics, the presence of pets has also
286 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

311 the microbial community of external household surfaces compared to rural areas; however,
312 it tended to lead to a more homogenous community composition [58]. Nevertheless, the
313 number of comparable investigations involving urbanization, exhaust, and greenery is
314 limited, and further studies are necessary in order to confirm their impact on the
315 composition of the fungal microbiome of indoor dust. The results from such studies might
316 identify key environmental characteristics concerning the closer neighbourhood with the
317 potential to create surroundings that promote healthy living [59].
318
319 The microbial community structure followed a temporal pattern, which was the major factor
320 affecting the variables considered for fungal and bacterial communities in living room dust.
321 For fungi, semi-annual patterns in their quantity in the indoor environment have previously
322 been repeatedly found by spore counts and cultivation, which were correlated with outdoor
323 concentrations [3]. Additionally, molecular studies on smaller time windows suggested such
324 patterns in different geographical regions [4, 5]. For bacteria, the influence of the sampling
325 time was determined to be not very important in earlier studies [6-9]. Our finding that
326 bacterial communities are considerably influenced by seasons is probably because of the
327 large number of individual samples that were analysed in this study. Almost daily sampling
328 allowed the delineation of temporal dynamics, overcoming inevitable individual variation
329 between locations. A recent investigation on one housing complex with 11 units sampled
330 during one month in summer and in winter found a strong seasonality of the indoor fungal
331 community but little evidence for the same in the bacterial community [6]. However, the
332 authors presumed that a seasonal relationship for bacteria was obscured in their study
333 because of large amounts of human-associated bacteria in the dust samples analysed.
334 Additionally, studies that compare different time windows during the year might also

335 overlook bacterial indoor seasonality. For example, using our data, a comparison of all of
336 the July-August samples with the December-January samples would have underestimated
337 the total annual influence of seasonal change, and for a period of approximately 100 days
338 during phenological winter we would have found almost no effect of this parameter at all
339 (cf. Figs 1,2).
340
341 The above mentioned seasonal patterns of community change were dissimilar for fungi and
342 bacteria. However, the varying taxonomic resolution of the fingerprinting method restricts
343 an explanation of the relationship between changes in community variation and diversity to
344 a general level. For fungi, a decrease in diversity during summer suggests an influx of high
345 amounts of a few OTUs, such as *Alternaria* and *Cladosporium* [21, 22]. For bacteria, the
346 rapid and strong change in community variation and reduced diversity during the spring
347 suggest a substantial and rapid influx of interrelated OTUs during the early plant flowering
348 (i.e., the full spring phenological period).
349 In this study, the indoor bacterial community variation and the phenological period in which
350 the samples were taken were strongly associated. However, the fingerprinting technique
351 used did not allow the identification of microbial taxa, and so we could not explore whether
352 these shifts were caused by plant-related taxa. Recently, sources for bacteria in particulate
353 matter of outdoor aerosols from Colorado, USA, were tracked to leaves, soils and cow
354 faeces [25]. In Milan, Italy, plant-derived microbes dominated outdoor airborne bacteria
355 during the summer, while spore-forming bacteria dominated in the winter [26]. Outdoor
356 airborne bacteria sampled during the spring in northern France were also mainly plant-
357 derived [60]. All of this evidence suggests that plant phenology was indeed the cause for the
358 association we found between a change in the bacterial indoor dust community and the full

359 spring period.

360

361 Conclusions

362 On the scale of an urban metropolitan area, we confirmed that the fungal microbiome in
363 indoor dust is more strongly affected than the bacterial microbiome by both, indoor and
364 especially outdoor factors.
365 Samples from studies similar to ours, i.e., studies that had independent sampling locations
366 on a regional scale and a time-frame of several months, could be used to confirm the
367 unexpectedly strong effect on indoor bacteria that we found for the sampling time during
368 the year.
369 The semi-annual cycle for the fungal indoor community that we inferred from our samples is
370 similar to the well-explained seasonal change in indoor and outdoor fungal propagules. In
371 contrast, changes in indoor bacteria must be elucidated in future studies. The local plant
372 phenology, particularly at the annual onset of the flowering period, may well be a major
373 driver of temporal change in the indoor bacterial microbiome in many geographical regions.

374

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378

379 References

380 1. NESCent Working Group on the Evolutionary Biology of the Built Environment, Martin LJ,
381 Adams RI, Bateman A, Bik HM, Hawks J, et al. Evolution of the indoor biome. Trends Ecol Evol.

382 2015;30(4):223-32. doi: 10.1016/j.tree.2015.02.001. PubMed PMID: 25770744.

383 2. Kelley ST, Gilbert JA. Studying the microbiology of the indoor environment. Genome Biol.
384 2013;14(2):202. doi: 10.1186/gb-2013-14-2-202. PubMed PMID: 23514020; PubMed Central PMCID:
385 PMC3663111.

386 3. Nevalainen A, Taubel M, Hyvarinen A. Indoor Fungi: Companions and Contaminants. Indoor
387 Air. 2014. doi: 10.1111/ina.12182. PubMed PMID: 25601374.

388 4. Adams RI, Miletto M, Taylor JW, Bruns TD. Dispersal in microbes: fungi in indoor air are
389 dominated by outdoor air and show dispersal limitation at short distances. ISME J. 2013;7(7):1262-
390 73. doi: 10.1038/ismej.2013.28. PubMed PMID: 23426013; PubMed Central PMCID: PMC3695294.

391 5. Pitkaranta M, Meklin T, Hyvarinen A, Paulin L, Auvinen P, Nevalainen A, et al. Analysis of
392 fungal flora in indoor dust by ribosomal DNA sequence analysis, quantitative PCR, and culture. Appl
393 Environ Microbiol. 2008;74(1):233-44. Epub 2007/11/06. doi: 10.1128/AEM.00692-07. PubMed
394 PMID: 17981947; PubMed Central PMCID: PMC2223223.

395 6. Adams RI, Miletto M, Lindow SE, Taylor JW, Bruns TD. Airborne bacterial communities in
396 residences: similarities and differences with fungi. PLOS ONE. 2014;9(3):e91283. doi:
397 10.1371/journal.pone.0091283. PubMed PMID: 24603548; PubMed Central PMCID: PMC3946336.

398 7. Barberan A, Dunn RR, Reich BJ, Pacifici K, Laber EB, Menninger HL, et al. The ecology of
399 microscopic life in household dust. Proc Biol Sci. 2015;282(1814). doi: 10.1098/rspb.2015.1139.
400 PubMed PMID: 26311665.

401 8. Rintala H, Pitkaranta M, Toivola M, Paulin L, Nevalainen A. Diversity and seasonal dynamics
402 of bacterial community in indoor environment. BMC Microbiol. 2008;8:56. doi: 10.1186/1471-2180-
403 8-56. PubMed PMID: 18397514; PubMed Central PMCID: PMC2323381.

404 9. Moschandreas D, Pagilla KR, Storino LV. Time and Space Uniformity of Indoor Bacteria
405 Concentrations in Chicago Area Residences. Aerosol Sci Tech. 2003;37(899-906). doi:
406 10.1080/02786820300935.

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

409 11. Meadow JF, Altrichter AE, Kembel SW, Kline J, Mhuireach G, Moriyama M, et al. Indoor
410 airborne bacterial communities are influenced by ventilation, occupancy, and outdoor air source.
411 Indoor Air. 2014;24(1):41-8. doi: 10.1111/ina.12047. PubMed PMID: 23621155; PubMed Central
412 PMCID: PMC4285785.

413 12. Lax S, Smith DP, Hampton-Marcell J, Owens SM, Handley KM, Scott NM, et al. Longitudinal
414 analysis of microbial interaction between humans and the indoor environment. Science.
415 2014;345(6200):1048-52. doi: 10.1126/science.1254529.

416 13. Täubel M, Rintala H, Pitkäranta M, Paulin L, Laitinen S, Pekkanen J, et al. The occupant as a
417 source of house dust bacteria. J Allergy Clin Immunol. 2009;124(4):834-40. e47.

418 14. Hospodsky D, Qian J, Nazaroff WW, Yamamoto N, Bibby K, Rismani-Yazdi H, et al. Human
419 occupancy as a source of indoor airborne bacteria. PLOS ONE. 2012;7(4):e34867.

420 15. Kembel SW, Jones E, Kline J, Northcutt D, Stenson J, Womack AM, et al. Architectural design
421 influences the diversity and structure of the built environment microbiome. ISME J. 2012;6(8):1469-
422 79. doi: 10.1038/ismej.2011.211. PubMed PMID: 22278670; PubMed Central PMCID: PMC3400407.

423 16. Lynch SV, Wood RA, Boushey H, Bacharier LB, Bloomberg GR, Kattan M, et al. Effects of
424 early-life exposure to allergens and bacteria on recurrent wheeze and atopy in urban children. J
425 Allergy Clin Immunol. 2014;134(3):593-601 e12. doi: 10.1016/j.jaci.2014.04.018. PubMed PMID:
426 24908147; PubMed Central PMCID: PMC4151305.

17. Valkonen M, Wouters IM, Taubel M, Rintala H, Lenters V, Vasara R, et al. Bacterial Exposures and Associations with Atopy and Asthma in Children. PLOS ONE. 2015;10(6):e0131594. doi: 10.1371/journal.pone.0131594. PubMed PMID: 26121165; PubMed Central PMCID: PMCPMC4488145.

18. Koch A, Heilemann K-J, Bischof W. Indoor viable mold spores ± a comparison between two cities, Erfurt (eastern Germany) and Hamburg (western Germany). Allergy. 2000;55:176-80.

19. Heinrich J, Holscher B, Douwes J, Richter K, Koch A, Bischof W, et al. Reproducibility of allergen, endotoxin and fungi measurements in the indoor environment. J Expo Anal Environ Epidemiol. 2003;13(2):152-60. Epub 2003/04/08. doi: 10.1038/sj.jea.7500267. PubMed PMID: 12679795.

20. Yamamoto N, Bibby K, Qian J, Hospodsky D, Rismani-Yazdi H, Nazaroff WW, et al. Particle-size distributions and seasonal diversity of allergenic and pathogenic fungi in outdoor air. ISME J. 2012;6(10):1801-11. doi: 10.1038/ismej.2012.30. PubMed PMID: 22476354; PubMed Central PMCID: PMC3446800.

21. Fernández-Rodríguez S, Tormo-Molina R, Maya-Manzano JM, Silva-Palacios I, Gonzalo-Garijo Á. Outdoor airborne fungi captured by viable and non-viable methods. Fungal Ecol. 2014;7:16-26. doi: 10.1016/j.funeco.2013.11.004.

22. Mitakakis T, Ong EK, Stevens A, Guest D, Knox RB. Incidence ofCladosporium, alternaria and total fungal spores in the atmosphere of melbourne (Australia) over three years. Aerobiologia. 1997;13(2):83-90.

23. Bowers RM, Clements N, Emerson JB, Wiedinmyer C, Hannigan MP, Fierer N. Seasonal variability in bacterial and fungal diversity of the near-surface atmosphere. Environ Sci Technol. 2013;47(21):12097-106. doi: 10.1021/es402970s. PubMed PMID: 24083487.

24. Bertolini V, Gandolfi I, Ambrosini R, Bestetti G, Innocente E, Rampazzo G, et al. Temporal variability and effect of environmental variables on airborne bacterial communities in an urban area of Northern Italy. Appl Microbiol Biotechnol. 2013;97(14):6561-70. doi: 10.1007/s00253-012-4450-0. PubMed PMID: 23053100.

25. Bowers RM, McCubbin IB, Hallar AG, Fierer N. Seasonal variability in airborne bacterial communities at a high-elevation site. Atmos Environ. 2012;50:41-9. doi: 10.1016/j.atmosenv.2012.01.005.

26. Franzetti A, Gandolfi I, Gaspari E, Ambrosini R, Bestetti G. Seasonal variability of bacteria in fine and coarse urban air particulate matter. Appl Microbiol Biotechnol. 2011;90(2):745-53. doi: 10.1007/s00253-010-3048-7. PubMed PMID: 21184061.

27. Fierer N, Liu Z, Rodriguez-Hernandez M, Knight R, Henn M, Hernandez MT. Short-term temporal variability in airborne bacterial and fungal populations. Appl Environ Microbiol. 2008;74(1):200-7. doi: 10.1128/AEM.01467-07. PubMed PMID: 17981945; PubMed Central PMCID: PMC2223228.

28. Polymenakou PN, Mandalakis M. Assessing the short-term variability of bacterial composition in background aerosols of the Eastern Mediterranean during a rapid change of meteorological conditions. Aerobiologia. 2013;29(3):429-41. doi: 10.1007/s10453-013-9295-1.

29. Shade A, Caporaso JG, Handelsman J, Knight R, Fierer N. A meta-analysis of changes in bacterial and archaeal communities with time. ISME J. 2013;7(8):1493-506. doi: 10.1038/ismej.2013.54. PubMed PMID: 23575374; PubMed Central PMCID: PMC3721121.

30. Liu WT, Marsh TL, Cheng H, Forney LJ. Characterization of Microbial Diversity by Determining Terminal Restriction Fragment Length Polymorphisms of Genes Encoding 16S rRNA. Appl Environ Microbiol. 1997;63(11):4516-22.

31. Heinrich J, Bolte G, Holscher B, Douwes J, Lehmann I, Fahlbusch B, et al. Allergens and endotoxin on mothers' mattresses and total immunoglobulin E in cord blood of neonates. European Resp J. 2002;20(3):617-23. doi: 10.1183/09031936.02.02322001.

32. Zutavern A, Brockow I, Schaaf B, Bolte G, von Berg A, Diez U, et al. Timing of solid food introduction in relation to atopic dermatitis and atopic sensitization: results from a prospective birth cohort study. Pediatrics. 2006;117(2):401-11. doi: 10.1542/peds.2004-2521. PubMed PMID: 16452359.

33. Casas L, Tischer C, Wouters IM, Valkonen M, Gehring U, Doekes G, et al. Endotoxin, extracellular polysaccharides, and beta(1-3)-glucan concentrations in dust and their determinants in four European birth cohorts: results from the HITEA project. Indoor Air. 2013;23(3):208-18. doi: 10.1111/ina.12017. PubMed PMID: 23176390.

34. Gardes M, Bruns TD. ITS primers with enhanced specifity for basidiomycetes - application to the identification of mycorrhizae and rusts. Mol Ecol. 1993;2:113-8.

35. White TJ, Bruns T, Lee S, Taylor JW. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. PCR Protocols: A Guide to Methods and Applications. 3: Academic Press, Inc.; 1990. p. 315-22.

36. Jiang H, Dong H, Zhang G, Yu B, Chapman LR, Fields MW. Microbial diversity in water and sediment of Lake Chaka, an athalassohaline lake in northwestern China. Appl Environ Microbiol. 2006;72(6):3832-45. doi: 10.1128/AEM.02869-05. PubMed PMID: 16751487; PubMed Central PMCID: PMCPMC1489620.

37. Muhling M, Woolven-Allen J, Murrell JC, Joint I. Improved group-specific PCR primers for denaturing gradient gel electrophoresis analysis of the genetic diversity of complex microbial communities. ISME J. 2008;2(4):379-92. doi: 10.1038/ismej.2007.97. PubMed PMID: 18340335.

38. Collins RE, Rocap G. REPK: an analytical web server to select restriction endonucleases for terminal restriction fragment length polymorphism analysis. Nucleic Acids Res. 2007;35(suppl 2):W58-W62.

39. Culman SW, Bukowski R, Gauch HG, Cadillo-Quiroz H, Buckley DH. T-REX: software for the processing and analysis of T-RFLP data. BMC Bioinformatics. 2009;10(1):171.

40. Team RC. R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2013. Available: http://www.R-project.org/

41. Bray JR, Curtis JT. An ordination of the upland forest communities of Southern Wisconsin. Ecological Monographs. 1957;27(4):325-49.

42. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin R, O'Hara RB, et al. vegan: Community Ecology Package. version 2.0-10 ed2013. Available: https://cran.r-project.org/web/packages/vegan/index.html

43. Chen J. GUniFrac: Generalized UniFrac distances. 1.0 ed2012. Available: https://cran.r-project.org/web/packages/GUniFrac/index.html

44. Vesper S, McKinstry C, Haugland R, Wymer L, Bradham K, Ashley P, et al. Development of an environmental relative moldiness index for US homes. J Occup Environ Med. 2007;49(8):829-33. Epub 2007/08/19. doi: 10.1097/JOM.0b013e3181255e98. PubMed PMID: 17693779.

45. Kettleson EM, Adhikari A, Vesper S, Coombs K, Indugula R, Reponen T. Key determinants of the fungal and bacterial microbiomes in homes. Environ Res. 2015;138C:130-5. doi: 10.1016/j.envres.2015.02.003. PubMed PMID: 25707017.

46. Heseltine E, Rosen J. WHO guidelines for indoor air quality: dampness and mould: WHO Regional Office Europe; 2009.

518 47. Nevalainen A, Seuri M. Of microbes and men. Indoor Air. 2005;15(s9):58-64.

519 48. Fujimura KE, Johnson CC, Ownby DR, Cox MJ, Brodie EL, Havstad SL, et al. Man's best friend?
520 The effect of pet ownership on house dust microbial communities. J Allergy Clin Immunol.
521 2010;126(2):410.

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

525 50. Kopperud RJ, Ferro AR, Hildemann LM. Outdoor versus indoor contributions to indoor
526 particulate matter (PM) determined by mass balance methods. J Air Waste Manage Assoc.
527 2004;54(9):1188-96.

528 51. Bowers RM, Sullivan AP, Costello EK, Collett JL, Jr., Knight R, Fierer N. Sources of bacteria in
529 outdoor air across cities in the midwestern United States. Appl Environ Microbiol. 2011;77(18):6350-
530 6. doi: 10.1128/AEM.05498-11. PubMed PMID: 21803902; PubMed Central PMCID: PMC3187178.

531 52. Bowers RM, McLetchie S, Knight R, Fierer N. Spatial variability in airborne bacterial
532 communities across land-use types and their relationship to the bacterial communities of potential
533 source environments. ISME J. 2011;5(4):601-12.

534 53. Li K, Dong S, Wu Y, Yao M. Comparison of the biological content of air samples collected at
535 ground level and at higher elevation. Aerobiologia. 2010;26(3):233-44.

536 54. Adhikari A, Reponen T, Grinshpun SA, Martuzevicius D, LeMasters G. Correlation of ambient
537 inhalable bioaerosols with particulate matter and ozone: a two-year study. Environ Pollut.
538 2006;140(1):16-28.

539 55. Zhang T, Engling G, Chan C-Y, Zhang Y-N, Zhang Z-S, Lin M, et al. Contribution of fungal
540 spores to particulate matter in a tropical rainforest. Environ Res Lett. 2010;5(2):024010.

541 56. Heald CL, Spracklen DV. Atmospheric budget of primary biological aerosol particles from
542 fungal spores. Geophys Res Lett. 2009;36(9).

543 57. Burge HA. An update on pollen and fungal spore aerobiology. J Allergy Clin Immunol.
544 2002;110(4):544-52. doi: 10.1067/mai.2002.128674.

545 58. Barberán A, Ladau J, Leff JW, Pollard KS, Menninger HL, Dunn RR, et al. Continental-scale
546 distributions of dust-associated bacteria and fungi. PNAS. 2015;112(18):5756-61. doi:
547 10.1073/pnas.1420815112.

548 59. Ruokolainen L, Hertzen L, Fyhrquist N, Laatikainen T, Lehtomäki J, Auvinen P, et al. Green
549 areas around homes reduce atopic sensitization in children. Allergy. 2015;70(2):195-202.

550 60. Maron P-A, Lejon DP, Carvalho E, Bizet K, Lemanceau P, Ranjard L, et al. Assessing genetic
551 structure and diversity of airborne bacterial communities by DNA fingerprinting and 16S rDNA clone
552 library. Atmos Environ. 2005;39(20):3687-95.

553

554 Supporting Information

555 **S1 Supporting Information.** Distribution of samples.

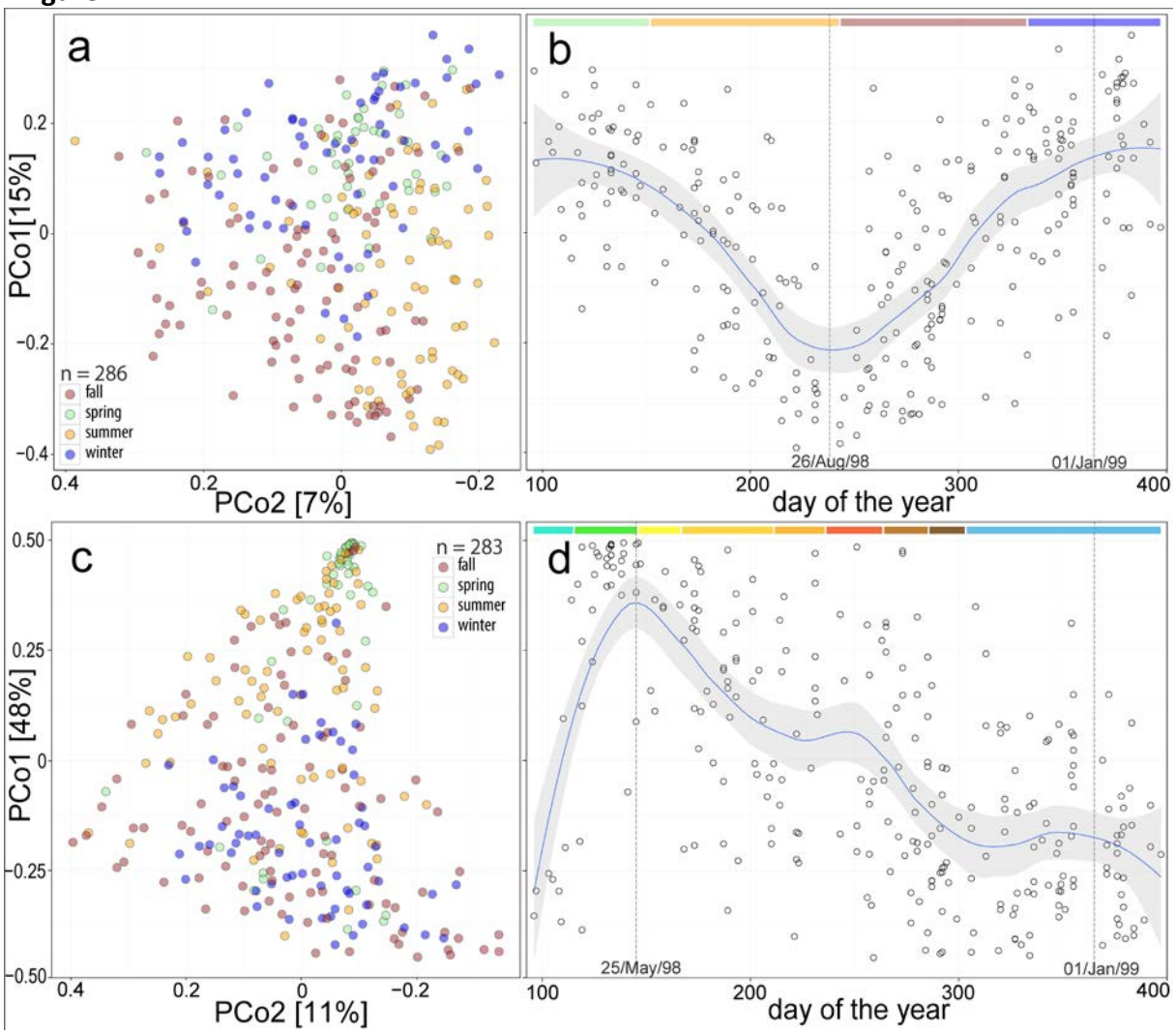
556 **S2 Supporting Information.** Environmental characteristics.

557 **S1 Table.** Community variation.

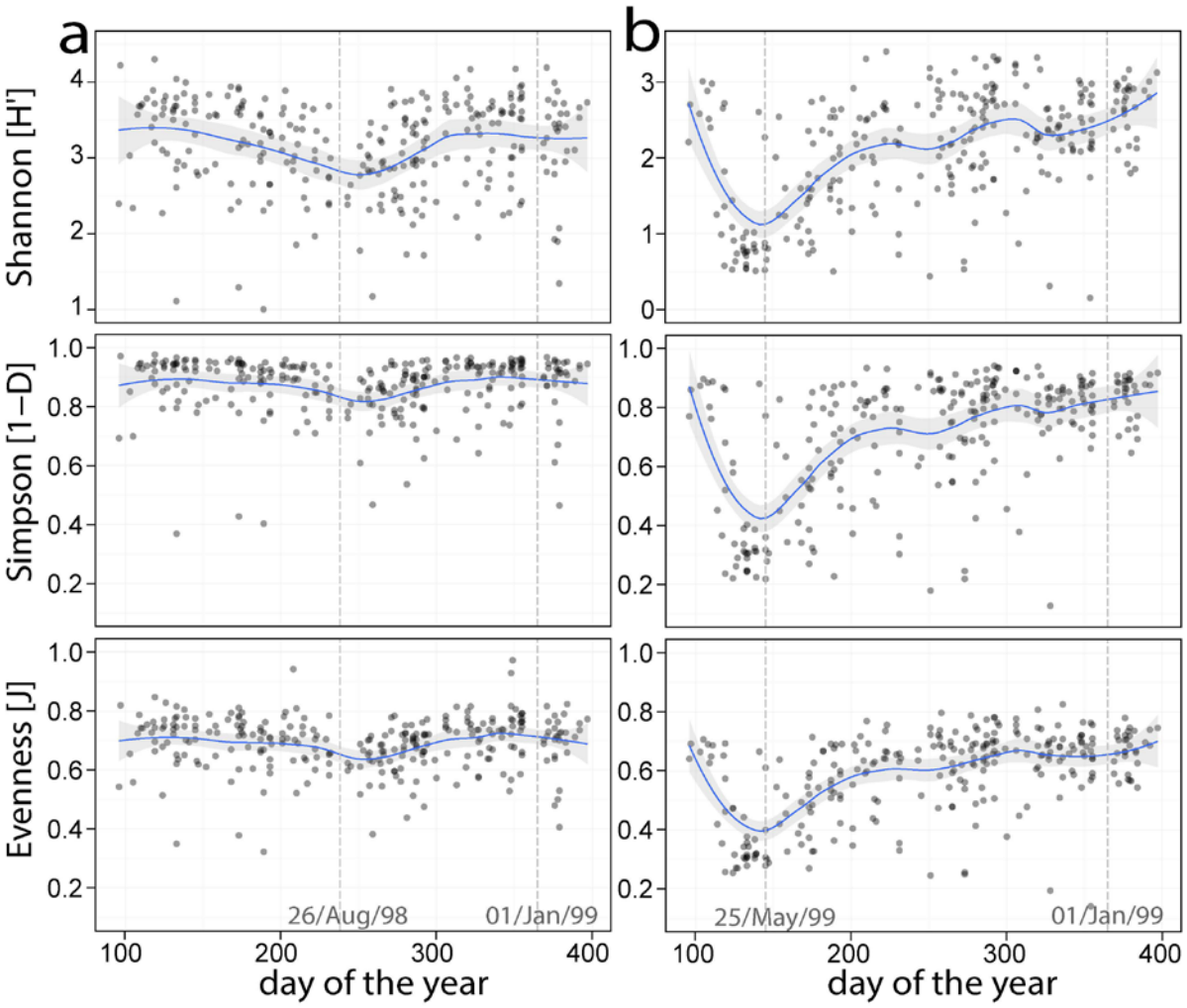
558 **S2 Table.** Significance of associations with diversity – all variables.

559 **S1 Fig.** Amount of fungi in the samples.

<Figure 1>

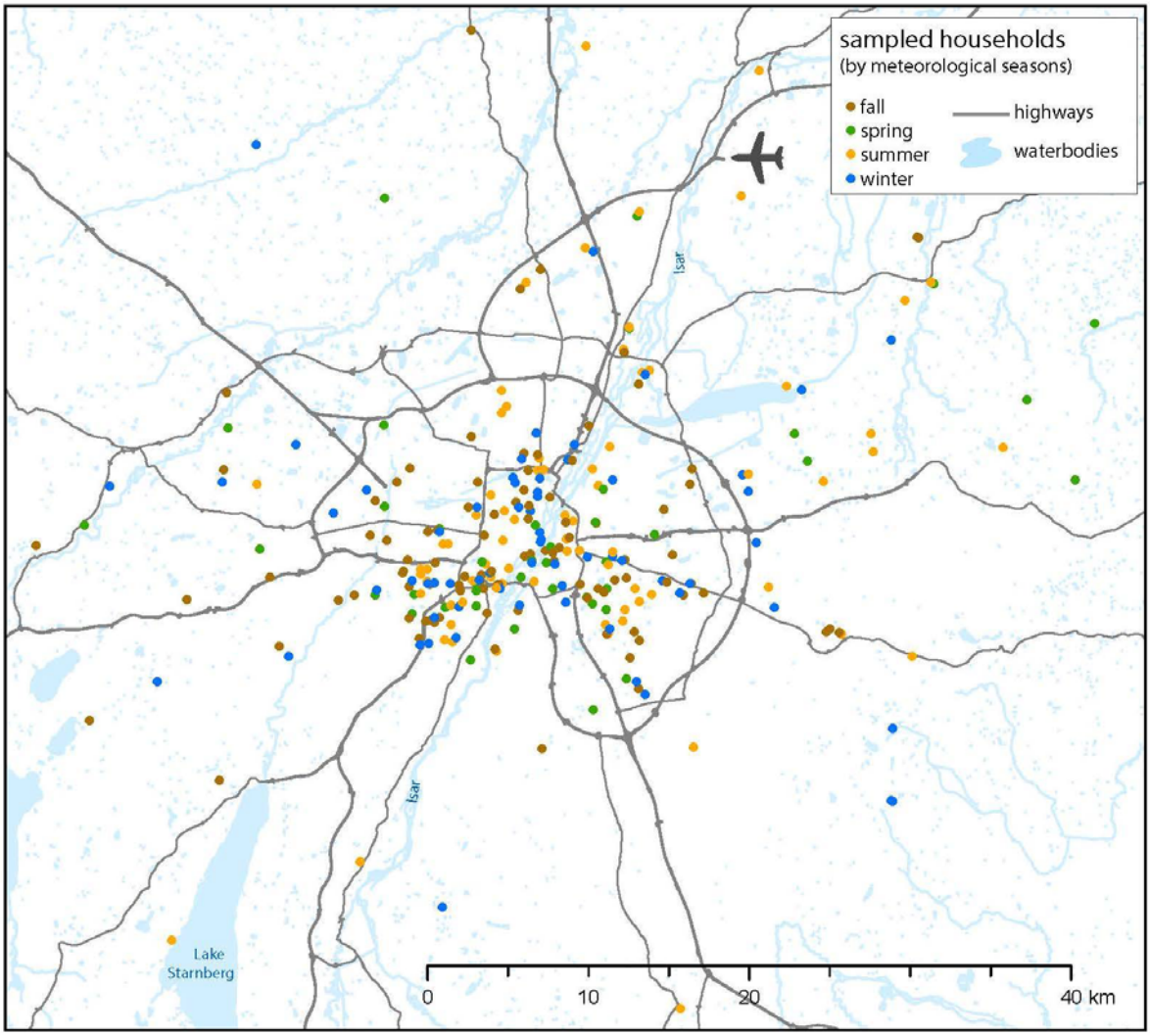

a
b
c
d
PCo1[15%]
PCo2 [7%]
n = 286
fall
spring
summer
winter
26/Aug/98
01/Jan/99
day of the year
PCo1 [48%]
PCo2 [11%]
n = 283
25/May/98

<Figure 2>


a
b
Shannon [H']
Simpson [1−D]
Evenness [J]
26/Aug/98
01/Jan/99
25/May/99
01/Jan/99
day of the year
day of the year

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

1. Helmholtz-Zentrum München, Epidemiology I: LISAplus. https://www.helmholtz-muenchen.de/en/epi1/research/research-units/research-unit-1-environmental-epidemiology/projects/lisa-plus/index.html (2015). Accessed 17 Nov 2015.

2. Heinrich J, Bolte G, Holscher B, Douwes J, Lehmann I, Fahlbusch B, et al: Allergens and endotoxin on mothers' mattresses and total immunoglobulin E in cord blood of neonates. *The European Respiratory Journal* 2002, **20:**617-623.

3. KOSIS Association Urban Audit: *The German Urban Audit - Comparison of cities in the European Statistical System*. KOSIS Association Urban Audit: Mannheim, Germany. 2013

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 (NO_2), and nitrogen oxides NO_2 and NO (NO_x) 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: S2 Supporting Information. Environmental characteristics.	n / N[1] (%)
INDOOR environmental characteristic	
N° of rooms within the flat (excluding bathroom)	
1-3 rooms	51/277 (**18%**)
≥ 4 rooms	226/277 (**82%**)
Dampness	18/279 (**6%**)
Mould at home	102/279 (**37%**)
Water leakage	34/279 (**12%**)
Tightness of the windows	
dense	207/273 (**76%**)
less dense	66/273 (**24%**)
Ventilation living room through windows - summer	
seldom/never/via another room	4/273 (**1%**)
once/several times a day (short)	51/273 (**19%**)
once/several times a day (long)	148/273 (**80%**)
Ventilation living room through windows - winter	
seldom/never/via another room	15/273 (**5%**)
once/several times a day (short)	245/273 (**90%**)
once/several times a day (long)	13/273 (**5%**)
Heating in the home (no central heating)	44/286 (**15%**)
Renovation measures last 12 months	194/281 (**69%**)
Pets	49/281 (**17%**)
Type of living room floor	
carpet	116/279 (**42%**)
smooth	50/279 (**18%**)
smooth with rugs	113/279 (**41%**)
Smoking of tobacco in the flat	35/277 (**13%**)
OUTDOOR environmental characteristics at birth	
Age of the building	
Built before 1945	47/277 (**17%**)
Built after 1945	230/277 (**83%**)
Position of the home	
Ground floor	90/279 (**32%**)
1st floor	78/279 (**28%**)
2nd floor	40/279 (**14%**)
3rd floor or higher	71/279 (**25%**)
Residential density of the neighborhood	
high	115/273 (**42%**)
average	141/273 (**52%**)
low	17/273 (**6%**)
Traffic jams in rush hour	36/273 (**13%**)
NDVI (500-m buffer), median (p25%-p75%)	0.32 (0.27-0.37) 281
NDVI (100-m buffer), median (p25%-p75%)	0.30 (0.24-0.35) 281
NDVI (30-m buffer), median (p25%-p75%)	0.29 (0.22-0.36) 281
Urban index	
Urban (≥ 0.7)	140/281 (**50%**)
Semi-urban (> 0.3 and < 0.7)	80/281 (**28%**)
Rural (≤ 0.3)	61/281 (**22%**)
NO_2 µg/m^3, median (p25%-p75%)	21.78 (17.59-26.71) 281
NO_x µg/m^3, median (p25%-p75%)	35.66 (29.55-42.99) 281

$PM_{2.5}$ μg/m^3, median (p25%-p75%)	13.42 (12.8-14.19) 281
PM_{10} μg/m^3, median (p25%-p75%)	20.47 (19.07-21.92) 281
PM_{coarse} μg/m^3, median) (p25%-p75%)	6.58 (5.66-7.85) 281
PM absorbance 10^{-5} *μg/m^3, **median (p25%-p75%)**	1.68 (1.55-1.8) 281
Facility with noticeable air pollution nearby (between 50 and 100 m)	21/277 (**8%**)
Facility with noticeable air pollution nearby (within 50 m)	12/277 (**4%**)
Season of dust sampling (sampling date)	
Winter	64 / 286 (**22%**)
Spring	45 / 286 (**16%**)
Summer	76 / 286 (**27%**)
Autumn	101 / 286 (**35%**)

[1]For most variables, data were not available for all samples, due to errors in questionnaires or measurements. N in the table represents samples with fungal fingerprints, N for bacterial fingerprints differed by 1–3 samples (286→283, 281→278, 277→274, 279→276, 273→274, or 188→189, respectively)

S2 Supporting Information references

1. Cyrys J, M. Eeftens, J. Heinrich, C. Ampe, A. Armengaud, R. Beelen , T. Bellander, T. Beregszaszi, M. Birk, G. Cesaroni, M. Cirach, K. de Hoogh, A. De Nazelle, F. de Vocht, C. Declercq, A. Dédelé, K. Dimakopoulou, K. Eriksen, C. Galassi, R. Gražulevičiené, G. Grivas, O. Gruzieva, A. Hagenbjörk Gustafsson, B. Hoffmann, M. Iakovides, A. Ineichen, U. Krämer, T. Lanki, P. Lozano, C. Madsen, K. Meliefste, L. Modig, A. Mölter, G. Mosler, M. Nieuwenhuijsen, M. Nonnemacher, M. Oldenwening, A. Peters, S. Pontet, N. Probst-Hensch, U. Quass, O. Raaschou-Nielsen, A. Ranzi, D. Sugiri, E. G. Stephanou, P. Taimisto, M.-Y. Tsai, É. Vaskövi, S. Villani, M. Wang, B. Brunekreef, G. Hoek. Variation of NO_2 and NO concentrations between and within 38 European study areas: results from the ESCPAE study. Atmos Environ. 2012;62:374-90.

2. Eeftens M, M-Y. Tsai, C, Ampe, B. Anwander, R. Beelen, T. Bellander, G. Cesaroni, M. Cirach, J. Cyrys, K. de Hoogh, A. De Nazelle, F. de Vocht, C. Declercq, A. Dédelé, K. Eriksen, C. Galassi, R. Gražulevičiené, G. Grivas, J. Heinrich, B. Hoffmann, M. Iakovides, A. Ineichen, K. Katsouyanni, M. Korek, U. Krämer, T. Kuhlbusch, T. Lanki, C. Madsen, K. Meliefste, A. Mölter, G. Mosler, M. Nieuwenhuijsen, M. Oldenwening, A. Pennanen, N. Probst-Hensch, U. Quass, O. Raaschou-Nielsen, A. Ranzi, E. Stephanou, D. Sugiri, O. Udvardy, É. Vaskövi, G. Weinmayr, B. Brunekreef, G. Hoek. Variation of PM2.5, PM10, PM2.5 absorbance and PMcoarse concentrations between and within 20 European study areas – results of the ESCAPE project. Atmos Environ. 2012;62:303-17.

3. Weier J, Herring D. Measuring Vegetation (NDVI & EVI). 2000. [http://earthobservatory.nasa.gov/Features/MeasuringVegetation/]

4. Markevych I, Thiering E, Fuertes E, Sugiri D, Berdel D, Koletzko S, et al. A cross-sectional analysis of the effects of residential greenness on blood pressure in 10-year old children: results from the GINIplus and LISAplus studies. BMC Public Health. 2014;14:477.

5. Markevych I, Fuertes E, Tiesler CM, Birk M, Bauer CP, Koletzko S, et al. Surrounding greenness and birth weight: results from the GINIplus and LISAplus birth cohorts in Munich. Health & place. 2014;26:39-46.

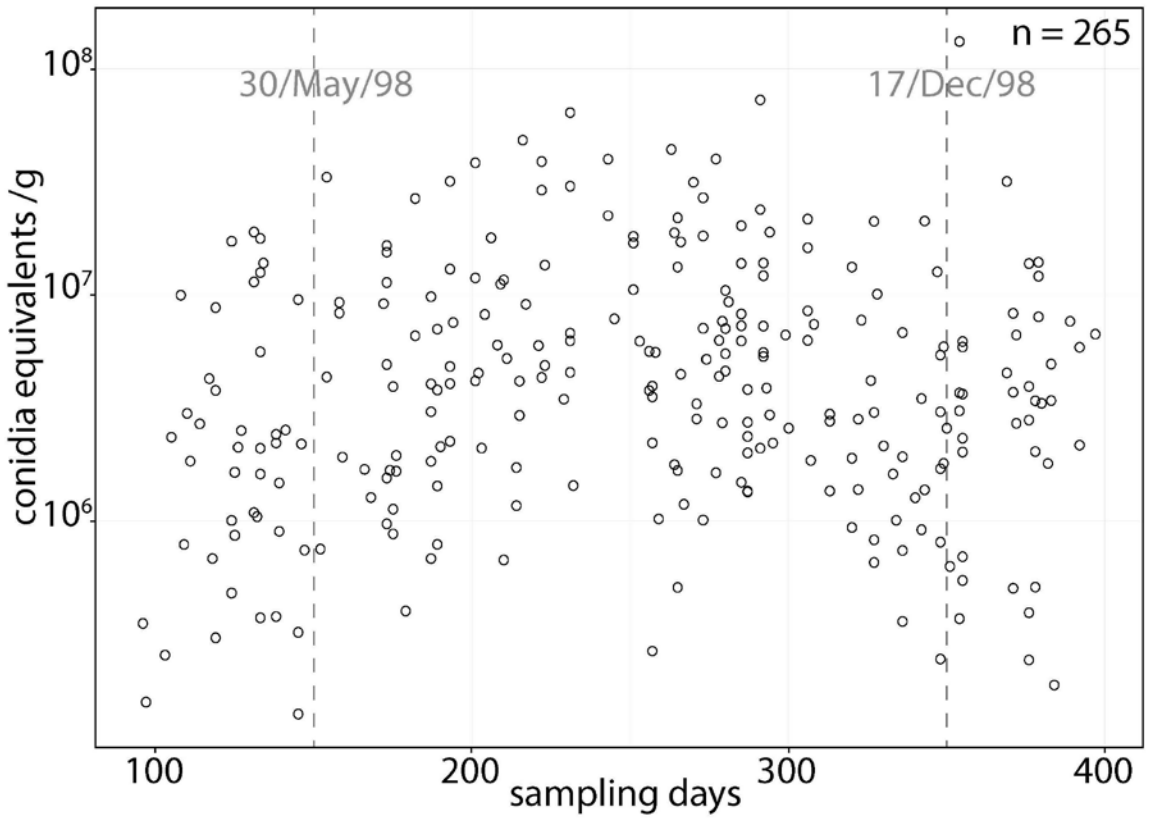
6. Jochner SC, Sparks TH, Estrella N, Menzel A. The influence of altitude and urbanisation on trends and mean dates in phenology (1980-2009). Int J Biometeorol. 2012;56(2):387-94.

S1 Table. Community variation. Significance of associations between environmental determinants and microbiome community variation (Adonis *P*, MRPP δ) 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 $\delta \leq 0.05$

	Fungi				Bacteria			
Environmental characteristics	*P*	R^2	δ	*A*	*P*	R^2	δ	*A*
N° of rooms within the flat	0.75	0.001	0.87	< 0.001	0.71	0.001	0.79	< 0.001
Dampness	0.69	0.002	0.29	< 0.001	0.16	0.007	0.39	< 0.001
Mould at home	**0.04**	0.012	**0.03**	0.004	0.09	0.009	0.13	0.002
Water leakage	0.81	0.001	0.85	< 0.001	0.57	0.002	0.62	< 0.001
Tightness of the windows	**0.03**	0.013	**0.04**	0.004	0.36	0.004	0.36	< 0.001
Ventilation living room through windows - summer	0.27	0.009	0.24	0.001	0.71	0.004	0.93	< 0.001
Ventilation living room through windows - winter	0.67	0.005	0.64	< 0.001	**0.05**	0.017	**0.05**	0.007
Heating within the home	**0.03**	0.013	**0.02**	0.005	0.36	0.004	0.41	< 0.001
Renovation measures last 12 months	0.44	0.003	0.61	< 0.001	0.65	0.002	0.65	< 0.001
Pets	0.27	0.004	0.28	< 0.001	0.62	0.002	0.75	< 0.001
Type of living room floor	**< 0.001**	0.057	**< 0.001**	0.023	0.08	0.015	**0.02**	0.009
Smoking of tobacco in the flat	0.42	0.003	0.41	< 0.001	0.71	0.001	0.78	< 0.001
Age of the building	**0.01**	0.017	**0.01**	0.005	0.28	0.005	0.31	< 0.001
Position of the home	0.49	0.010	0.67	< 0.001	0.10	0.015	**0.05**	0.007
Building density of the neighborhood	0.52	0.006	0.59	< 0.001	0.39	0.007	0.44	< 0.001
Traffic jams in rush hour	0.83	0.001	0.83	< 0.001	0.29	0.004	0.24	< 0.001
Facility with noticeable air poll. within 50 and 100 m	0.58	0.002	0.72	< 0.001	0.45	0.003	0.40	< 0.001
Facility with noticeable air pollution within 50 m	0.33	0.004	0.28	< 0.001	0.85	< 0.001	0.94	< 0.001
NDVI (500 m buffer)	0.72	0.004	0.63	< 0.001	0.84	0.003	0.94	< 0.001
NDVI (100 m buffer)	**0.05**	0.017	**0.006**	0.008	0.19	0.011	0.22	0.002
NDVI (30 m buffer)	0.06	0.016	**0.01**	0.007	0.33	0.008	0.30	< 0.001
Urban index	**0.02**	0.021	**0.01**	0.007	0.51	0.006	0.60	< 0.001
NO_2	0.23	0.010	0.06	0.004	0.63	0.005	0.75	< 0.001
NO_x	0.06	0.016	**0.03**	0.005	0.37	0.008	0.41	< 0.001
$PM_{2.5}$	**0.004**	0.028	**0.005**	0.009	0.51	0.006	0.44	< 0.001
PM_{10}	0.54	0.006	0.32	< 0.001	0.82	0.003	0.70	< 0.001
PM_{coarse}	**0.04**	0.018	**0.008**	0.008	0.41	0.007	0.46	< 0.001
$PM_{2.5}$ absorbance	0.07	0.015	**0.06**	0.004	0.37	0.008	0.42	< 0.001

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: $P \leq 0.05$

	Fungi		Bacteria	
Environmental characteristics at birth	*P* (Simpson 1-D)	*P* (Shannon H')	*P* (Simpson 1-D)	*P* (Shannon H')
N° of rooms within the flat	0.13	0.13	0.69	0.50
Dampness	0.10	0.12	0.17	0.23
Mould at home	0.88	0.71	0.77	0.57
Water leakage	0.60	0.78	0.27	0.17
Tightness of the windows	0.78	0.87	0.78	0.85
Ventilation living room through windows - summer	0.08	**0.04**	0.62	0.56
Ventilation living room through windows - winter	0.49	0.32	0.13	0.20
Heating within the home	0.30	0.31	0.77	0.91
Renovation measures last 12 months	0.69	0.71	0.75	0.52
Pets	0.84	0.74	0.55	0.91
Type of living room floor	0.30	0.33	**0.04**	**0.01**
Smoking of tobacco in the flat	0.13	0.12	0.75	0.80
Age of the building	0.31	0.49	0.24	0.36
Position of the home	0.15	0.09	**0.04**	**0.03**
Building density of the neighborhood	0.46	0.27	0.67	0.62
Traffic jams in rush hour	0.99	0.59	0.53	0.44
No facility with noticeable air pollution nearby	0.32	0.43	0.44	0.54
Facility with noticeable air poll. within 50 and 100 m	0.17	0.25	0.26	0.27
Facility with noticeable air pollution within 50 m	0.16	0.11	0.41	0.40
NDVI (500 m buffer)	0.09	0.14	0.69	0.69
NDVI (100 m buffer)	**0.03**	0.11	0.14	0.11
NDVI (30 m buffer)	0.32	0.31	0.59	0.56
Urban index	0.37	0.44	0.65	0.73
NO_2	0.25	0.35	0.54	0.51
NO_x	0.14	0.33	0.43	0.29
$PM_{2.5}$	0.72	0.60	0.74	0.81
PM_{10}	0.35	0.32	0.91	0.85
PM_{coarse}	0.10	0.15	0.62	0.66
$PM_{2.5}$ absorbance	0.66	0.49	0.62	0.75



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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