Bioinformatic analyses of biogenic aerosol particles by classical and high-throughput sequencing: diversity, seasonal dynamics, and characterization of airborne microbial communities

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Abstract

The airborne microbiome, that is the totality of microbes in the atmosphere as a defined environment, influences a broad range of processes that have positive or negative consequences for the atmosphere and/or biosphere. Patterns of archaea and bacteria diversity in the atmosphere are likely caused by the spatial and temporal variation of emission sources and also influenced by a wide range of environmental and meteorological factors, e.g. radiation, precipitation events or rapid changes in temperatures. The overall diversity of the microbiome is not only driven by nature but additionally by many anthropogenic influences. Studies of all these phenomena are still in their infancy, especially research on the overall airborne microbiome lacks until today. In this thesis the presence, diversity and properties of archaea, bacteria and fungi as part of the airborne microbiome, were revealed by using DNA based sequencing approaches. Mainz, Germany, was chosen as an example site for continental boundary layer air. Taken together, the findings show a higher diversity and relative abundance for bacteria compared to archaea. Additionally, the size fraction influenced the bacterial diversity significantly as the species richness in bioaerosols larger than 3 μm was found to be higher by more than 25 % than the fine fraction, which is dominated by spore-forming taxa. The results also point to the diverse effects of natural as well as anthropogenic influences on the diversity of the microbiome of the atmosphere, e.g. fertilization with live stock manure or biogas substrates that affect the diversity of archaea. This thesis may be seen as the first attempt to collate research on different groups of organisms for one specific sampling location to get insights into the entire airborne microbiome.

Zusammenfassung

Das Mikrobiom der Luft, definiert als die Gesamtheit aller Mikroorganismen in einem bestimmten Lebensraum, hat Einfluss auf eine Reihe von atmosphärischen Prozessen und kann in Atmo- und/oder Biosphäre sowohl positive als auch negative Effekte ausüben. Repetitive Muster der Archaeen und Bakteriendiversität werden häufig durch eine Reihe von umweltbedingten oder meteorologischen Stressfaktoren wie z.B. Strahlung, Niederschlag oder schnelle Temperaturschwankungen, hervorgerufen. Die Gesamtdiversität der Atmosphäre wird zusätzlich durch eine Vielzahl anthropogener Faktoren beeinflusst. Viele Zusammenhänge sind hier noch unbekannt, besonders im Hinblick auf das gesamte Mikrobiom der Luft als Kollektiv fehlen tiefergehende Einblicke. In der vorliegenden Arbeit wurden für den Standort Mainz das Vorkommen, die Diversität und die relativen Verhältnisse von Archaeen und Bakterien mit Hilfe von 16s-RNA-Gen Sequenzierung untersucht. Fasst man die Ergebnisse zusammen, so zeigen die Luftproben eine diverse Zusammensetzung mit einem deutlich höheren Anteil an Bakterien im Vergleich zu Archaeen. Die Partikelgrößenfraktionierung zeigt einen signifikanten Einfluss auf die Bakteriendiversität, so zeigt sich der Artenreichtum im Grobstaub, Partikel größer als 3 μm, um mehr als ein Viertel höher als in der Fraktion des Feinstaubs, welcher von Sporenbildnern dominiert wird. Die meisten Ergebnisse zeigen, wie unterschiedliche, teils natürliche, teils anthropogene Effekte die Diversität des Luftbioms beeinflussen. Als Beispiel für anthropogene Quellen lassen sich das Düngen mit Wirtschaftsdüngern also Stallmist, Jauche, Gülle und Gärsubstraten aus Biogasanlagen, aufzeigen, dies beeinflusste besonders die Diversität von Archaeen. Die Besonderheit dieser Arbeit ist der Versuch, verschiedene Organismengruppen eines Sammelstandortes detailliert aufzuarbeiten und zusammenzufassen, um so ein Bild der mikrobiologischen Gesamtheit in der Luft zu erhalten.

Introduction

1.1 Air microbiome - using DNA to identify communities in the atmosphere

Primary biogenic aerosol particles (PBAPs) are biological particles directly emitted from the biosphere including living and dead prokaryotes, e.g. archaea, and bacteria, gametophytes like pollen and all kind of spores as well as animal, plant and fungal fragments and many smaller compounds. The estimated concentrations of bacteria and fungal spores (10^4 to 10^6 cells m^{-3}) on the one hand and archaea (1 to 10 cells m^{-3}) on the other hand differ widely [Burrows et al., 2009, Després et al., 2012, Fröhlich-Nowoisky et al., 2009, Fröhlich-Nowoisky et al., 2014, Fröhlich-Nowoisky et al., 2016].
Since the potential relevance of PBAPs for atmospheric processes, human health and agriculture was recognized, researchers try to understand the concentration, abundances of PBAPs as well as the drivers for their dispersal. Despite the fact that for example the emission of plant, animal and human pathogens as well as allergens have a threatening impact on human health and agriculture [Brodie et al., 2007, Després et al., 2012, Fröhlich-Nowoisky et al., 2009, Fröhlich-Nowoisky et al., 2016, Kellogg and Griffin, 2006] the identity, diversity, abundance and microbioal variations are still understudied as recently reviewed by Zhai et al., [2018].
Furthermore, the understanding of the major influences of PBAPs on marine as well as terrestrial environments lack up to day [Fröhlich-Nowoisky et al., 2016].
Bacteria, pollen and fungal spores are under special intense research lately, as they have been discovered to be excellent cloud condensation nuclei (CCN) and ice crystals (IN). Reproductive units of fungi and plants as well as microorganisms can be transported over long distances [Burrows et al., 2009, Després

et al., 2012, Fröhlich-Nowoisky et al., 2016, Womack et al., 2010] and influence the biodiversity of faraway environments by spreading organisms. They thus enable gene transfer over geographical barriers. The potential of transporting genetically modified organisms at least for plant pollen is conceivable but still under discussion [Folloni et al., 2012]. First studies for fungi have concentrated on composition and diversity grouping the fungi in the classical way using the biological taxonomy [Fröhlich-Nowoisky et al., 2009]. However, also the lifestyle of fungi might explain their appearance in the atmosphere, as different taxonomic groups, which have adopted to the same habitat, might be emitted more likely together than with taxonomic close relatives. Comparable theories can be made for other bioarosols as well, as some bacteria have for example the opportunity to change into a dormant spore stadium which might increase the chance to survive the stress pressure of the atmosphere. Other bacteria might use entirely different means to survive, e.g. by being shielded in a colony or a soil particle. These hypotheses have not yet been included in most analyses.

During their resistance time in the atmosphere PBAPs undergo different kinds of stresses as well as biological aging. Especially UV-radiation can damage nucleic acids [Rastogi et al., 2010], which have been used in this study for the identification of the PBAPs (Fig.1). For organisms, which have to keep their metabolisms running even while aerosolised, namely most archaea and bacteria, not only UV light but also changes in temperatures and precipitation are additional strong stresses.

However, there have been no controlled studies dealing with all key airborne organisms and particles for one specific sampling location. The most noticeable gap in research on the airborne microbiome seems to be the poor understanding of the abundance, diversity and overall presence of archaea. Although they have been studied rather well in the microbiomes of soil and marine environments, almost no studies exist for archaea of the atmosphere, likely due to their low number concentrations. More research here is needed. Different from fungi spores and pollen, the presence of bacteria and archaea in the atmosphere depends not only on seasonality and meteorological factors. Their presence is additionally driven by many anthropogenic influences, which especially act like sources (Fig1.).

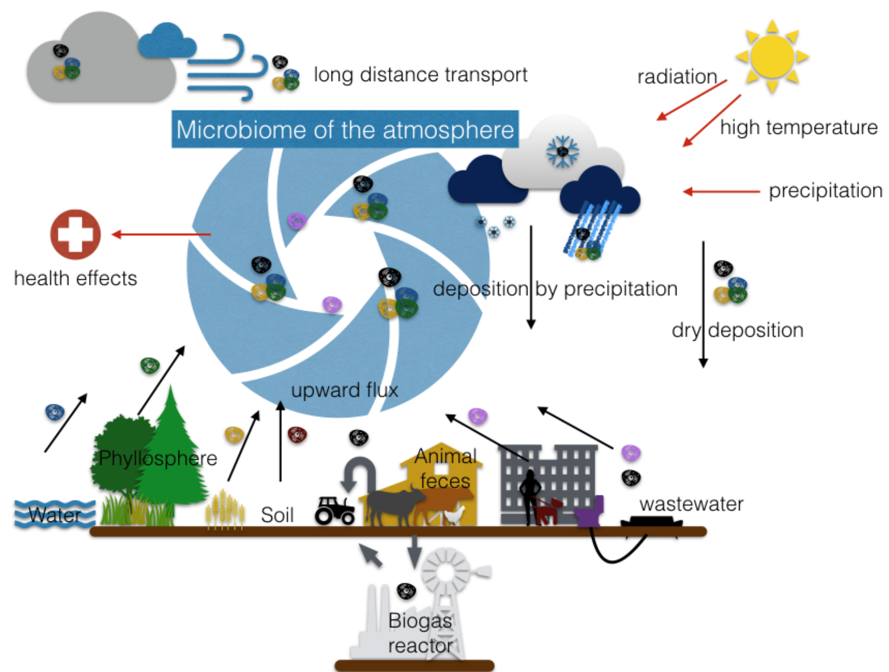

Figure 1.1: Microbiome of the atmosphere - biosphere and anthropogenic activities as source and sink of airborne microorganisms.

1.2 Next generation sequencing for the characterization of the airborne microbiome by using QIIME

The traditional way to identify airborne microorganisms was based on direct culturing from air samples during the pioneer years of research on airborne microbes. Later new techniques enabled the culture-independent Sanger sequencing. For many years full-length 16S-rRNA gene sequencing became fashion and the golden standard to perform diversity research for airborne samples (reviewed in Fröhlich-Nowoisky et al., 2016). The effectiveness of Sanger approaches was always limited by its time consuming character and high costs per sequence. However, although this approach is basically outdated nowadays for understanding whole communities, it is still a helpful tool in finding und determine single organism on deep taxonomic levels, as the long sequence length enables a finer taxonomic identification even on species level.
The understanding of complex microbiological community structures, distributions and abundances, that is the microbiome, was revolutionized with the invention of the so called next generation sequencing (NGS) approaches. These newer sequencing technologies made it possible to generate millions of 16s-rRNA sequences per sample, what opened up the possibility to detect rare taxa with a higher probability in comparison to classical Sanger sequencing approaches, where only a small number of sequences usually could be studied [Caporaso et al., 2011]. From the early days of Sanger based research the 16s-rRNA gene region has been the main target for identifying prokaryotic organisms. Its benefits compared to other sequencing targets are mainly: its presence in every prokaryotic cell, including archaea, and its structure in uniting highly conserved regions as well as very variable regions. Especially the second point is valuable because conserved motifs allow target specific primer design, whereas the variable regions code for the phylogenetic information of interest. As all eukaryotes have conserved 16s-rRNA gene regions in their mitochondrias and all plants additional in several types of plastids, the 16s-rDNA gene, as a sequencing target, is however not suitable for eukaryotic diversity research. The nuclear ribosomal internal transcribed spacer (ITS) regions have

been shown to be a comparable target region for fungi [Schoch et al., 2012], like the 16s-rRNA gene region is for bacteria and archaea.
Although the sequences in the first NGS runs were only 70-100 bp long, they still give as shown by Liu et al. accurate phylogenetic and taxonomic information, though possibly only on the phyla, class, and family level. Deeper taxonomic identification, however, may be possible with Sanger sequences, where the sequences can be up to 900 bp long (2007). In addition, to merely identifying individual taxa as usually done with Sanger sequences, due to the low sequence numbers and costs, an NGS run allows also to map an entire community as listed in an appropriate reference database [Liu et al., 2007]. Both methods have advantages and disadvantages and a combination can be very helpful for answering different kinds of questions.
Although NGS technique led to an increase in studies of PBAPs, one important issue has not yet been addressed: how to control for the quality of the data, that is, how to ensure that the DNA analysed truly is derived from the sampled air and not from surrounding contaminants like the sampler device, the filter themselves, the researcher etc. The usage of blank filters as a quality control in bioaerosol research is at least for NGS approaches not well established, possibly no blanks exist [Bowers et al., 2009], or no OTUs are removed [Yooseph et al., 2013], or the blanks are not sequenced along with the samples and just tested for DNA content and free DNA [Bowers et al., 2011, Bowers et al., 2012]. Even the question whether chloroplast and mitochondrial sequences should be deleted from the datasets is still open.

1.3 Thesis aims

To characterize and understand the diversity of airborne microorganisms such as fungi, oomycetes, bacteria and archaea, it is necessary to get as broad as possible insights into the individual groups of the airborne microbiome. Thus, the main objectives of this thesis were to analyse on the one hand the diversity, composition and abundances of these groups and on the other hand their seasonal variations, main discrepancies between coarse and fine particulate matter and the influence of meteorological parameters on the same.

Methodological objectives

1) To establish the analysis of high-throughput sequencing methods as well as methodologies of community analysis and detecting patterns in the composition and diversity for airborne microbial communities. (Appendix C1, C2, C3 and C4)

2) To launch new approaches to a) assure quality of short sequence data and b) implement environmental blank controls in NGS technology. (Appendix C1 and C2)

Research objectives

3) Based on a solid NGS dataset to get first insights into airborne archaea community characteristics specifically also in fine particulate matter. (Appendix C1)

4) Investigate the composition, abundance and diversity of microorganisms in the atmosphere, focussing on differences within particle size fractions. (Appendix C1 and C2)

5) Characterize seasonal shifts and identify possible causes shaping the structure of archaea, bacteria, fungi and oomycetes communities. (Appendix C1, C2, C3 and C4)

Results and conclusion

2.1 Methodological objectives

1. With around 9.5*10^6 sequences the Illumina dataset sampled in 2007 and sequenced in 2011 to this day is the largest DNA based dataset acquired and analysed for the sampling point in Mainz. To analyse such large amounts of data, the QIIME (Quantitative Insights Into Microbial Ecology; http://www.qiime.org) analysis pipeline has been established. QIIME is an open-source pipeline to analyse, especially, high-throughput sequence data from fungal, viral, bacterial and archaeal communities [Caporaso et al., 2011]. The modular set-up of the QIIME pipeline gives the possibility to change nearly every implemented software and all adjustments therein (Fig.2).
Within the community based analysis different estimations of taxonomic diversity e.g. species richness and diversity estimators, as well as beta-diversity approaches have been established to monitor differentiations among especially size fractions but also seasonal shifts (Fig2). (Appendix C1 Wehking et al., 2018a, C2 Wehking et al., 2018b, C3 Laang et al., 2018 and C4 Pickersgill et al., 2018)

2. As the Illumina dataset consists of very short reads (70 bases) it is of special importance to choose the right way in cluster and identify the OTUs. The QIIME analysis pipeline presents, for example, several approaches for picking operational taxonomic units (OTU)[Stackebrandt and Goebel, 1994], from which the most suitable must be selected by the analyser, depending on the data of interest. For the presented work all sequences were clustered against a reference sequence collection (closed reference approach). In detail,

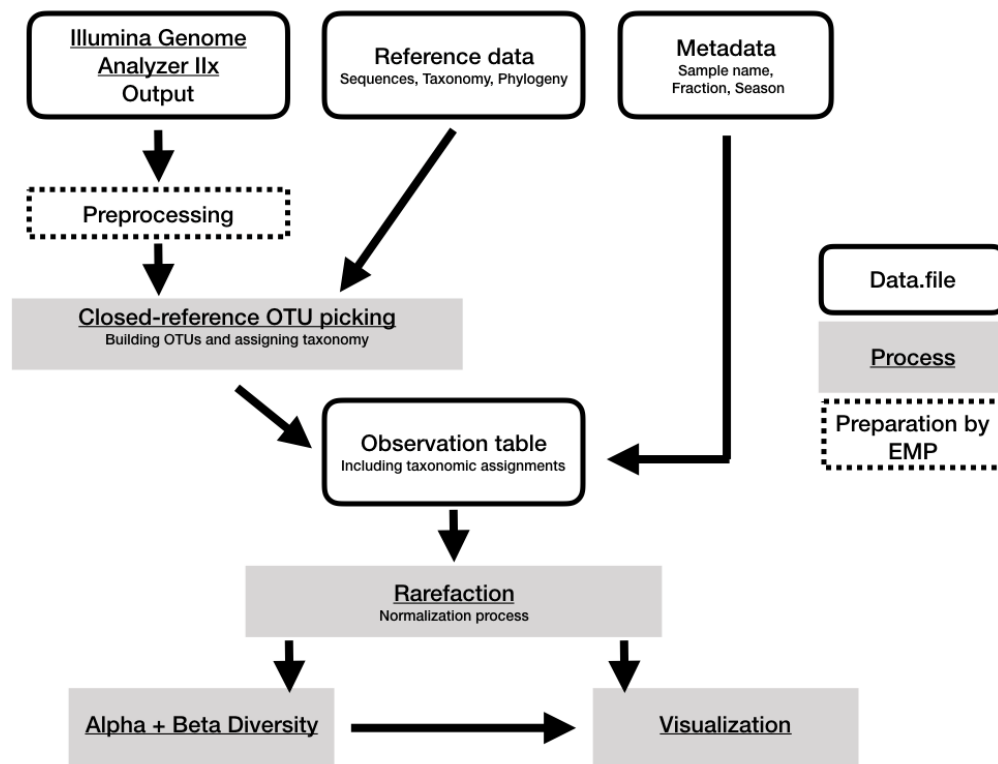

Figure 2.1: Workflow for the next generation sequencing projects - All shown steps performed with the QIIME (Quantitative Insights Into Microbial Ecology) work tool.

the sequences of the air filter samples are compared to the preclustered OTU reference set, whereas the similarity threshold can be varied but was chosen at 97%. For the actual identification process a taxonomy map corresponding to the OTU reference set was used. Sequences that do not match any reference-set OTU are discarded before the downstream analysis. This leads, on the one hand to a possible bias when high sequence counts do not match the used reference database [Adams et al., 2015], but, on the other hand, gives a further quality check, as bad reads are discarded likewise. A second advantage of this approach is the fact that it can run on workstations and does not need big computer clusters. It works very fast as it can be fully parallelized, so it is very suitable for large datasets. The usage of sequenced blank controls and the deletion of corresponding taxonomic groups or OTUs has been established for the first time. (Appendix C1 Wehking et al., 2018a, C2 Wehking et al., 2018b)

2.2 Research objectives

3. Airborne archaea were shown to be a ubiquitous, diverse part of the atmospheric microbiome in Mainz, Germany. Archaea seem to be rare in the atmosphere in general: the relative abundance was about 0.01% but still detectable. However, especially reports on archaea in fine particulate matter have so far been lacking completely. First insights were gained in this thesis with the Cenarchaeaceae, Nitrososphaeraceae, Methanosarcinales, Thermoplasmata and the genus Nitrosopumilus as the main taxa found in particles smaller than 3 μm. (Appendix C1 Wehking et al., 2018a)

4. The composition of the airborne bacterial community was significantly influenced by the size fraction (ANOSIM Global R = 0.44, P $<$ 0.001). As the fine fraction is dominated by spore forming taxa, the mean of the calculated species richness of the coarse fraction is more than a quarter higher (Chao1 - 10.000 subsampled seq/sample). A possible explanation for this might be that larger carrier particles protect living organisms from stressful influences (i.e. UV radiation) and serve as carbon and energy substrates for running a metabolism. (Appendix C1 Wehking et al., 2018a, C2 Wehking et al., 2018b)

5. Additionally, the bacterial communities in both size fractions show different seasonalities for their composition and relative abundances, for example the summer is the most diverse season in fine and least diverse in coarse particulate matter. The high diversity in the fine filter fraction throughout the summer, might be caused by heat or dry stress-induced spore building groups, which may be more easily emitted to the atmosphere than normal cell states. The results of research done on archaea seasonality indicate that Euryarchaeota occur more often through spring and fall, whereas Thaumarchaeota have been found over the entire year. The seasonal shifts of Euryarchaeota occurrence might be seen as one example of anthropogenic influences caused by fertilisation processes with, for example, livestock manure as this group appears throughout

the main fertilisation seasons. (Appendix C1 Wehking et al., 2018a, C2 Wehking et al., 2018b, C3 Laang et al., 2018 and C4 Pickersgill et al., 2018).

Understanding the patterns in the atmospheric microbiome is necessary for many kinds of research e.g. health care, biogeo- or crop-sciences. A fundamental understanding of the ecology as a basis for recognizing changes coming along with climate change and anthropogenic influences is essential, while the airborne microbes and fungi may be an excellent marker to monitor these influences. Monitoring these changes is only feasible, if as many PBABs of the atmospheric microbiome as possible (at least fungi, bacteria and archaea) are considered as a whole community, where the mutual dependencies needs to be seen as a jigsaw. Finally, the pieces of knowledge collected in Mainz for all groups individually (e.g. fungi, bacteria, archaea, oomycetes and plant particles) need to be assembled into one over-all picture. Taken together, the findings of the here presented studies suggest a huge impact of carrier particles on the diversity of the microbiome of the atmosphere. A next step could be to correlate changes in the diversity of archaea or/and bactaria in coarse PM with findings of different plant particles to monitor for possible dependences. On a personal view this is the most promising advantage of the Mainz Bioaerosol Laboratory (MBAL) for the future. As most PBABs have been analysed for this sampling location and the data implemented in the MySQL based Bioaerosol Database (BADAB) a greater focus on the dependencies between the majour groups could produce interesting findings that account to answer the question whether the atmosphere is a real habitat or not.

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List of abbreviations

PBAP - Primary biogenic aerosol particle

NGS - Next generation sequencing

ITS - Ribosomal internal transcribed spacer

bp - Base pair

OTU - Operational taxonomic unit

CCN - cloud condensation nuclei

DNA - deoxyribonucleic acid

QIIME - Quantitative Insight Into Microbial Ecology

Personal list of publications

Journal Articles

Community composition and seasonal changes of archaea in coarse and fine air particulate matter

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Size dependence of bacterial diversity in air particulate matter

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On the constraints and influences on the temporal and size fraction occurrences of airborne fungi

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Diversity, abundance, and seasonal dynamics of plant-pathogen Oomycetes in continental air

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High diversity of bioaerosols in the southern tropical Indian region

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Datasets

Illumina sequences of 16S-rRNA from atmospheric coarse and fine particulate matter samples

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Selected publications

C.1) Community composition and seasonal changes of archaea in coarse and fine air particulate matter (Wehking et. al., 2018a - Biogeosciences, https://doi.org/10.5194/bg-15-4205-2018)

C.2) Size dependence of bacterial diversity in air particulate matter (Wehking et. al., 2018b - to be submitted)

C.3) On the constraints and influences on the temporal and size fraction occurrences of airborne fungi (Pickersgill et. al., 2017 - Biogeosciences Discuss., https://doi.org/10.5194/bg-2017-452 - to be resubmitted)

C.4) Diversity, abundance, and seasonal dynamics of plant-pathogen Oomycetes in continental air (Lang-Yona et. al. 2018 -under revision in Frontiers in Microbiology - Abstract accepted.)

C.1 Community composition and seasonal changes of archaea in coarse and fine air particulate matter

Wehking J.[1,2], Pickersgill D.A. [1,2], Bowers R.M.[3,4], Teschner D. [1], Fröhlich-Nowoisky J.[2], Pöschl U. [2], Després V.R. [1,2]

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Community composition and seasonal changes of archaea in coarse and fine air particulate matter

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Abstract. Archaea are ubiquitous in terrestrial and marine environments and play an important role in biogeochemical cycles. Although air acts as the primary medium for their dispersal among different habitats, their diversity and abundance is not well characterized. The main reason for this lack of insight is that archaea are difficult to culture, seem to be low in number in the atmosphere, and have so far been difficult to detect even with molecular genetic approaches. However, to better understand the transport, residence time, and living conditions of microorganisms in the atmosphere as well as their effects on the atmosphere and vice versa, it is essential to study all groups of bioaerosols. Here we present an in-depth analysis of airborne archaea based on Illumina sequencing of 16S rRNA genes from atmospheric coarse and fine particulate matter samples and show seasonal dynamics and discuss anthropogenic influences on the diversity, composition, and abundance of airborne archaea.

The relative proportions of archaea to bacteria, the differences of the community composition in fine and coarse particulate matter, and the high abundance in coarse matter of one typical soil related family, the Nitrososphaeraceae, point to local phyllosphere and soil habitats as primary emission sources of airborne archaea.

We found comparable seasonal dynamics for the dominating Euryarchaeota classes and Crenarchaeota orders peaking in summer and fall. In contrast, the omnipresent Cenarchaeales and the Thermoplasmata occur only throughout summer and fall. We also gained novel insights into archaeal composition in fine particulate matter (< 3 µm), with Cenarchaeaceae, Nitrososphaeraceae, Methanosarcinales, Thermoplasmata, and the genus *Nitrosopumilus* as the dominating taxa.

The seasonal dynamics of methanogenic Euryarchaeota point to anthropogenic activities, such as fertilization of agricultural fields with biogas substrates or manure, as sources of airborne archaea. This study gains a deeper insight into the abundance and composition of archaea in the atmosphere, especially within the fine particle mode, which adds to a better understanding of the overall atmospheric microbiome.

1 Introduction

In addition to bacteria and eukaryotes, archaea constitute one of the three independent domains of life (Woese et al., 1990). In the beginning of archaeal research in the 1880s, primarily methanogenic archaea were discovered and cultivated, so the belief arose that archaea are exclusively extremophiles (Cavicchioli, 2011; Farlow, 1880; Schleper et al., 2005). However, during the last decades, cultivation- and culture-independent methods, such as DNA sequencing, have substantially improved the understanding of archaea and proved that they are also abundant in various environments such as marine or soil habitats, where they can represent more than 10 % of the microbial community (Buckley et al., 1998; Cao et al., 2012; Cavicchioli, 2011; Delong, 1998; Robertson et al., 2005; Yilmaz et al., 2016).

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So far, diversity studies for archaea have mainly concentrated on the major habitats also known for bacteria such as marine and soil environments (Bintrim et al., 1997; Buckley et al., 1998; DeLong, 1992; Ochsenreiter et al., 2003). In the global marine environment the abundance of archaea is approximately 1×10^{28} archaeal compared to 3×10^{28} bacterial cells (Karner et al., 2001), with archaea accounting for 2–10 % in surface waters and for 20–40 % in deep ocean water (Massana et al., 1997).

The abundance and composition of archaea in soil vary between different soils types (Bates et al., 2011). All cultivated methanogens belong to the kingdom Euryarchaeota and are strictly dependent on anaerobic conditions with low redox potentials (Le Mer and Roger, 2010); thus, they are only present in small numbers in many soils. The fertilization with livestock manure adds anaerobically adapted organisms to the surface of agriculturally used soils. Thus, even in aerated soils, core anaerobic populations seem to survive albeit in low number (Angel et al., 2012). Another issue influencing the abundance and composition of archaea in soil is – as also observed in water columns – the depth (Karner et al., 2001). Analyses of soil depth profiles revealed changing diversity patterns with depth (Bundt et al., 2001; Pesaro and Widmer, 2002) in composition and number.

Next to the well-established major habitats, the atmosphere is another environment in which microorganisms can be detected; however, it remains unclear whether the atmosphere can be considered a natural habitat or whether it only represents a medium of dispersal for terrestrial and marine microorganisms and their spores (Bowers et al., 2009, 2011, 2012, 2013; Smith et al., 2013; Womack et al., 2010; Yooseph et al., 2013). For airborne bacteria and archaea the main known emission sources are surface waters and the surface layer of soils (Womack et al., 2010). Therefore, the different abundances and composition of archaea within water and soil columns are of special interest to understand possible emission sources for airborne archaea. For bacteria, which are abundant in air, the concentration of 16S rRNA gene copies quantified using qPCR in soil was 10^{11} to 10^{12} gene copies kg^{-1} and for archaea 10^9 to 10^{11} gene copies kg^{-1} (Cao et al., 2012; Kemnitz et al., 2007). In ocean surface waters the concentration is lower but estimated to be 10^8 to 10^9 gene copies L^{-1} for bacteria and 10^6 to 10^7 gene copies L^{-1} for archaea (Kemnitz et al., 2007; Yin et al., 2013), whereas only 10^4 to 10^6 bacterial gene copies m^{-3} air have been detected (Cao et al., 2012; Fröhlich-Nowoisky et al., 2014; Kemnitz et al., 2007; Yin et al., 2013). Interestingly, in contrast to bacteria, it seems challenging to detect, amplify, and analyze archaea in air, as their concentration of 100 ppm is much lower than the abundance of bacteria (Cao et al., 2012; Fröhlich-Nowoisky et al., 2014). Until now, it remains unclear whether these observations are biased by technical obstacles or reflect the true abundances. The largest study on airborne archaea is to our knowledge by Fröhlich-Nowoisky et al. (2014) and is based on Sanger sequencing. However, in Fröhlich-Nowoisky et al. (2014) the number of sequences were low, the observations had little statistical support, and the analysis of the microbiome of aerosolized archaea was difficult. Therefore, we present an in-depth next-generation sequencing study of airborne archaea collected on coarse and fine particulate matter filters over 1 year in Mainz, Germany. We attempt to compare the composition, diversity, and abundance to the same characteristics as in other habitats, which also allows an inference about the primary emission sources of airborne archaea.

2 Material and methods

2.1 Aerosol sampling

As described in Fröhlich-Nowoisky et al. (2009), in total 24 pairs of air filter samples (i.e., 20 filter pairs of one fine and one coarse particle filter sample each, 2 pairs of start-up air filter blanks, and 2 pairs of mounting filter blanks) were analyzed within this dataset. The air filters were installed on an in-house-built high-volume dichotomous sampler (Solomon et al., 1983). The whole sampling campaign lasted 1 year in Mainz, Germany (March 2006–April 2007). The rotary vane pump (Becker VT 4.25) worked with a flow rate of $\sim 0.3\,m^3\,min^{-1}$. The particles were split according to their aerodynamic diameter by a virtual impactor. Particles with an aerodynamic diameter larger than the nominal cut-off of $\sim 3\,\mu m$ and, due to the sampling device, an additional 10 % of the fine particles were sampled in line with the inlet on one glass fiber filter (flow rate: $\sim 0.03\,m^3\,min^{-1}$) representing the coarse fraction. The fine particles were collected on a second glass fiber filter perpendicular to the inlet ($\sim 0.27\,m^3\,min^{-1}$) which was essentially free from coarse particles (Solomon et al., 1983). To get a representative dataset for the whole year, five random samples, consisting of a coarse and fine filter, were analyzed for each of the four seasons of the sampling campaign. The sampling period of a single filter pair was generally 7 days except for filter pairs MZ 11 (24 h), MZ 15 (5 days), and MZ 31 (5 days; Table S1 in the Supplement). The sampled air masses represent a mixture of urban and rural continental air, as the sampler was positioned on the roof of the Max Planck Institute for Chemistry on the campus of the University of Mainz (49°59′31.36″ N, 8°14′15.22″ E). To reduce the sampling of particles emitted from the ground, the sampling device was on a mast about 5 m above the flat roof of the three-story building.

2.2 Extraction, amplification, and sequencing

The DNA extraction and sequencing was part of the Earth Microbiome Project (EMP – http://www.earthmicrobiome.org/, last access: 4 November 2016) using the MoBio PowerMag Soil DNA Isolation kit and the Illumina GAIIx sequencer with the sequencing by synthesis technology. As shown before, this technology is suitable for analyzing mi-

crobial communities in soil, water, and human skin (Caporaso et al., 2011).

For the PCR amplifications the 515f/806r primer set (Fwd: GTGCCAGCMGCCGCGGTAA; Rev: GGACTACHVGGGTWTCTAA) described in Caporaso et al. (2011) proved to be suitable, as shown by Bates et al. (2011). It covers the conserved flanking regions ideal for amplifying bacteria and archaea over the V4 region of the 16S rRNA gene (Bowers et al., 2013; Huse et al., 2008; Muyzer et al., 1993). In addition, the primer pair is preferred for this amplification as it exhibits only few biases against individual bacterial taxa. As suggested in Caporaso et al. (2011) each DNA extract was amplified in triplicate. These triplicates were combined and purified using a 96-well PCR clean-up kit from MO BIO. The utilized PCR reaction was performed; amplicons were purified and sequenced using the GAIIx.

2.3 Grouping of sequences into OTUs and taxonomic identification

The sequences were analyzed using the Quantitative Insight Into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010). To assign sequences to operational taxonomic units (OTUs), we used QIIME's closed reference OTU picking script, which uses Uclust (Edgar, 2010) and the Greengenes reference database (gg_13_8_otus/rep_set/97_otus.fasta, last update 15 August 2013; McDonald et al., 2012) with 97 % similarity. For the actual identification process a corresponding taxonomy map provided by the Greengenes database was used. Sequences, which did not match to any Greengenes reference set OTU, were discarded for the downstream analysis.

2.4 Controls

Prior to the sampling procedure all filters were baked in sealed aluminum foil bags overnight at 500 °C. To best conserve the DNA of the collected bioaerosols, after the sampling procedure all filter samples were stored at −80 °C until analysis. To detect possible contaminants from the sampling device and the filter handling, blank filters were taken at 4-week intervals. Contamination-free, prebaked filter pairs were mounted on the sampler as for regular sampling, but the pump was not turned on at all (mounting blanks). In addition, small environmental samples were taken to collect air exclusively around and from the interior of the sampling device by turning the pump on for 5 s only (start-up filter blanks). A detailed list of all analyzed air and blank filter samples with their individual sampling details can be found in the Supplement in Tables S1 and S2.

The DNA of the blank filters was extracted and quantified in parallel to the actual filter samples. Often, the detected DNA concentrations on blanks can be too small to be quantified or to build usable sequencing libraries (Cao et al., 2014). However, as the start-up blanks were briefly exposed to environmental air, they also could contain DNA. Within this study we controlled the actual filter changing process by sequencing two mounting blanks, i.e., MZ 23 and MZ 73. Two sequences were obtained from the fine particle filter of MZ 23 and 408 archaeal sequences (371 sequences on the coarse particle blank filter and 37 sequences on the fine particle blank filter) were detected on MZ 73. On the coarse particle filter of MZ 23 no archaeal sequences were detected. Minimal DNA amounts are to be expected, as the mounting blanks were briefly exposed to the air during the mounting process. The sequences on the mounting blanks were assigned to five archaeal families (Cenarchaeaceae, Methanobacteriaceae, Methanoregulaceae, Methanosaetaceae, Methanomassiliicoccaceae). The handling of the sequences obtained with next-generation sequencing techniques, e.g., for amplicon sequencing of environmental air sample controls, is neither well established nor standardized. To ensure that all contaminants were removed comprehensively from the dataset, we decided to omit all identified families from the data if present in more than 1 % of all detected archaeal sequences of the mounting blanks.

The subsequently deleted families (from 404 sequences) Methanoregulaceae (8.5 %, 3 OTUs), Methanomassiliicoccaceae (17.6 %, 3 OTUs), and the largest family of the Methanobacteriaceae (72.4 %, 4 OTUs) all belonged to the Euryarchaeota (see also Table S2). In total 2341 sequences remained for the downstream analysis.

Likewise, two pairs of start-up air filter blanks were sequenced. But as they sampled the air for 5 s the obtained sequences were not treated like the mounting blanks. On these four filter samples 709 archaeal sequences were found, distributed with 328 sequences on MZ 22 (326 sequences on coarse, 2 sequences on fine) and 381 sequences on MZ 72 (3 sequences on coarse, 378 sequences on fine).

2.5 Statistical analysis

All data management and most of the analyses were performed using a MySQL database and R statistics if not stated otherwise (R-Team, 2011).

To characterize the biodiversity of the archaea community and thus to approximate the likely diversity, several statistical parameters were calculated: species richness estimators, rarefaction curves, and community diversity indices using the software tool EstimateS (Colwell et al., 2012).

2.6 Meteorological analysis

As a possible correlation between the abundance of taxonomic ranks in an air mass and meteorological parameters can be either following a monotone or specifically a linear relationship, in this study the Pearson product-moment correlation coefficient (r_K) testing for a linear regression and the Spearman's rank (r_R) for fine, coarse, and total suspended particles (TSPs) were used. The meteorological parameters

tested were wind speed in $m\,s^{-1}$ (average and maximum), temperature in °C (range and maximum), relative humidity in %, and the sum of precipitation in mm. The meteorological data were provided in hourly data for wind speed and half-hourly values for all other meteorological parameters by the ZIMEN Luftmessnetz of the Landesamt für Umwelt Wasserwirtschaft und Gewerbeaufsicht of Rhineland-Palatinate. All averages were calculated for the exact sampling periods (Table S1). The correlation analysis using the Pearson product-moment correlation coefficient (r_K) and Spearman's rank (r_R) were calculated for different taxonomic levels, i.e., kingdom, phylum, and class level. Only results with r_K or r_R over 0.5 or under −0.5 were interpreted. However, no significant correlations between the relative abundance and the meteorological factors were found.

3 Results and discussion

3.1 Overall diversity

To determine the archaeal diversity in air, 20 air filter pairs were sampled and analyzed for 1 year in Mainz, Germany. Each filter pair consists of one filter collecting particles with aerodynamic diameters smaller than 3 µm (fine particulate matter) and one collecting primarily coarse particles, which are larger than 3 µm. On 39 (97.5 %) of the 40 analyzed filters (20 air filter pairs) archaeal DNA could be detected. In total 2341 sequences could be assigned to archaea (Table 1). More archaeal sequences were detected on coarse particle filters (109 sequences on average per sample) than on fine particle filters (8 sequences on average per sample) for which the number of sequences ranged from 0 to 42. On all but one fine particle filter, MZ 81 sampled in December 2006, archaeal sequences were discovered. The highest number of sequences, i.e., 601, were detected on the coarse particle filter MZ 74 from November 2006. The 2341 archaeal sequences were assigned to 52 OTUs. Out of these OTUs, 17 OTUs were found in coarse as well as in fine particulate matter. As listed in Table 1 the coarse particle filters comprised 2180 sequences distributed among 41 OTUs, whereas only 161 sequences assigned to 28 OTUs were identified on the fine particle filters.

In total only 7 % of all archaeal sequences stem from fine particle filters, whereas 93 % stem from coarse particle filters. Specifically, on 75 % of the coarse particle filters 20 or more archaeal sequences were found, while on 70 % of the fine particle filters less than 6 archaeal sequences could be detected.

The community structures of both size fractions differ remarkably in composition (Fig. 1). In the fine fraction the genus *Nitrosopumilus* is the dominant taxon. This Thaumarchaeota genus shows a relative abundance of 33.5 % over all archaea sequences found on all samples in fine particulate matter. The cultivable *Nitrosopumilus maritimus* is

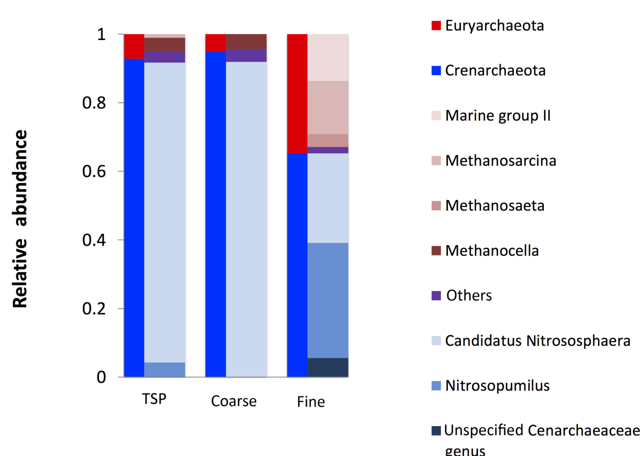

Figure 1. Archaeal community composition for total suspended (TSP), the same airstream split into coarse, and fine particulate matter on the level of phyla (red/blue) and genera (pastel colors).

a well-known representative of the genus *Nitrosopumilus*. These chemolithoautotrophic nitrifying archaea have primarily been sampled from marine sources. They form straight rods with a diameter of 0.17–0.22 µm and a length of 0.5–0.9 µm (Könneke et al., 2005) and are thus one of the smallest organism known today. With this size even long-distance transport from marine sources might be conceivable. The same can be said for species of marine group II. However, *N. maritimus* and species of marine group II have been found in soil samples (Leininger et al., 2006; Treusch et al., 2005) – in contrast to the coarse fraction, where only the genera *Methanocella* and the *Candidatus Nitrososphaera* were found with relative proportions of more than 3 %. Due to the much higher number of sequences isolated from the coarse particle fraction in comparison to the fine fraction, the TSP composition resembles that of the coarse particle fraction (Fig. 1).

Taking the relative distribution over the entire course of the year into account, on class level the Thaumarchaeota also dominate the fine particle fraction, except fort two fine filters sampled during fall where the Euryarchaeota even have a higher relative abundance than the Thaumarchaeota (93 and 92 %).

The Crenarchaeota, primarily represented by Thaumarchaeota (99 %), are the dominating phylum in the coarse particle mode. Next to Thaumarchaeota a single OTU of the miscellaneous Crenarchaeotal group (MCG; Kubo et al., 2012) representing seven sequences was found on a single coarse spring filter sample. No Euryarchaeota were observed on 65 % of the fine particle filters and 50 % of the coarse particle filters. A closer look at taxonomic assignments and the contribution of sequences to individual families reveals that most sequences within the coarse particle fraction belong to the Nitrososphaeraceae family. While this family is only present in 10 % of the fine particle filters it was identified on 75 %

Table 1. Number of sequences and indices estimating the archaeal diversity in Mainz for coarse and fine particle filter samples and total suspended particles (TSPs).

Size fraction	n (Samples)	Sq (Sequences)	Sq / n	OTU (operational taxonomic unit)	S_{Chao1} (Chao1)	H (Shannon)	D (Simpson)
Coarse	20	2180	109	41	64	3.09	0.83
Fine	20	161	8.1	28	41	3.65	0.88
TSP	20	2341	117.1	52	63	3.36	0.84
Fröhlich-Nowoisky et al. (2014)	47	435	9.3	57	137	3.32	0.82

of the coarse particle filters. In soil surveys the I.1.b group of Crenarchaeota has constantly been found (Ochsenreiter et al., 2003), with the Nitrososphaeraceae being one of the most abundant archaea families therein. Thus, the aerosolization of soil and soil dust as a primary source can be hypothesized for this family. Within this family the genus *Nitrososphaera* is an abundant taxon specifically in agricultural soils (Zhalnina et al., 2013). The landscape of the surrounding area of the sampling location is dominated by agricultural fields and the emitted soil particles are thus likely to contain the genus *Nitrososphaera*. Soil and soil dust are classically discussed as primary emission sources for airborne bacteria (Després et al., 2007, 2012; Fierer et al., 2008). Therefore, when attached to large soil particles these organisms should be mainly collected in the coarse particle fraction. To our knowledge, the only cultivated *Nitrososphaera* species, *Nitrososphaera viennensis*, has a much smaller diameter (irregular cocci with a diameter of 0.6–0.9 µm; Stieglmeier et al., 2014), which should be, if in single cell status, collected in the fine particle mode. The hypothesis that soil particles identified through Nitrososphaeraceae are mainly collected on coarse particles is also strengthened by the results of community analysis of the fine particle filters. The observed increase of the relative abundance of the Euryarchaeota could also be interpreted as the decline of Nitrososphaeraceae as soil particles are less frequent in the fine mode. On the phylum level the Nitrososphaeraceae family forms the main difference between the two size fractions.

The diversity estimator Chao1 (Table 1) and the rarefaction curves (Fig. 2) predict a relatively low diversity for archaea in Mainz air (S_{Chao1}; 64 and 41 for coarse and fine, respectively). On the other hand, the relative abundances of the OTUs and the diversity calculated by Shannon (H) or Simpson (D) (Table 1) are slightly higher for the fine particle fraction. This might be because of the small sequence number but is surely driven by the relative dominance of Nitrososphaeraceae sequences in the coarse particulate matter (Fig. 1).

Most results of this study are in agreement with the previous Sanger-sequencing-based study by Fröhlich-Nowoisky et al. (2014), which analyzed 47 air filter pairs including

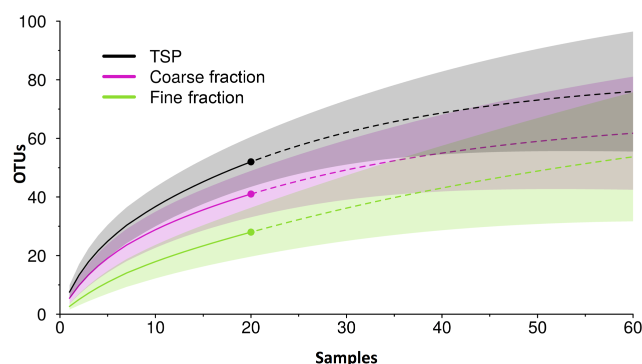

Figure 2. Rarefaction curve of species richness for total suspended (TSP), coarse, and fine particulate matter. TSP is a single airstream split into coarse and fine particulate matter. The solid curves represent the interpolated number of OTUs as a function against the number of samples. The dashed lines represent the extrapolations and the dots the sample size of this study. The colored areas represent the 95 % confidence intervals.

the 20 filter pairs we focussed on in this study. However, in Fröhlich-Nowoisky et al. (2014), only a limited number of clones were sequenced, resulting in a total of 435 sequences, as compared to 2341 sequences obtained from the current study (Table 1). Fröhlich-Nowoisky et al. (2014) concluded that archaea occur far more often in coarse particulate matter than in fine particulate matter as archaeal DNA could only be detected on 21 % of the fine particle filters, which is consistent with the results of this study. Another consistency compared to the study of Fröhlich-Nowoisky et al. (2014) is the high abundances for Group I.1.b on coarse particle samples. This can be explained by the higher relative abundance of Nitrososphaeraceae in the coarse particle fraction discovered in this study.

The main difference between the Sanger and the Illumina approach is the estimated species richness, with 137 species from Sanger estimating almost double the amount compared to the Illumina approach, which estimates 63 species. This can be caused by several issues: first, a possible lack of taxonomical depth caused by the shorter sequences compared to the Sanger approach and the usage of different primer pairs;

second, by the closed-reference-based taxonomic assignment and a possible lack of taxonomical depth in the used reference dataset; and, third and most likely, by the smaller number of sequences from more filter samples used in Fröhlich-Nowoisky et al. (2014).

As the used primers also amplified bacterial sequences, the following observation could be made. The ratio of archaea and bacteria suggests a very low proportion of airborne archaea in comparison to airborne bacteria (Fig. 3). In total, 0.07 % of the total reads could be assigned to archaea, while the rest (5.7×10^6 reads) consists of bacterial, mitochondrial, and plasmid DNA. After the sequences of mitochondria and plastids are eliminated, the ratio of archaea to bacteria increases only to 0.1 %, which is widely different from the ratios discovered in the soil and marine environment.

This extremely low ratio is an interesting phenomenon as in most possible emission sources the proportion of archaea is higher than in air.

Several studies, focusing on airborne bacteria and archaea, found that archaeal DNA in air is extremely low (Cao et al., 2014; Yooseph et al., 2013; Smith et al., 2013). Cao et al. (2014) found a proportion of 0.8 % of archaea when compared to bacteria in PM_{10} and $PM_{2.5}$ using Illumina HiSeq data (2014). Yooseph et al. (2013), who analyzed the urban prokaryotic metagenome of New York, on a multistep approach based on taxonomic classifications for their metagenomic reads and assigned to the different organism groups, found that 0.48 % of their sequences were archaeal, with roughly 80 % Euryarchaeota and 20 % Crenarchaeota and Thaumarchaeota. Both studies therefore agree with the 0.1 % archaeal sequences found in Mainz air.

Next to comparisons of species diversity and composition, the ratio of bacteria to archaea might be an indicator of the possible emission sources, as the aerosolization process is likely to equally affect all microorganisms from an emission source. We therefore compared the detected ratios with ratios of possible emission sources such as soils, surface water, and the phyllosphere reported in literature (Fig. 3).

We found that compared to soil the microbial habitat, which is often discussed as the primarily emission source, differs strongly from our and other air studies. Although archaeal abundance in aerated soils increases with depth (Kemnitz et al., 2007), the proportion known for surface soil is still much higher than the proportions in air. Thus, soil alone seems an unlikely emission source. Also in sea water their abundance increases with depth reaching up to 39 % (Karner et al., 2001). As Mainz is not close to oceans, emission from sea water seems unlikely as a primary source. The only larger emission surface from water might be the river Rhine which is very likely one of the primary sources in the study area.

In a review by Vorholt (2012) it is convincingly shown that the abundance of archaea in the phyllosphere is less than 1 % of the total microorganism load (Fig. 4), which is similar to the 0.1 % we found. With a total area of 10^9 km^2 of the upper and smaller leaf surface, the phyllosphere surface habitat is approximately twice the size of the land surface and is supposed to comprise up to 10^{26} cells worldwide (Vorholt, 2012); therefore, it could present a significant emission source (Woodward and Lomas, 2004) in the studied area. Thus, the phyllosphere might be the local primary emission source.

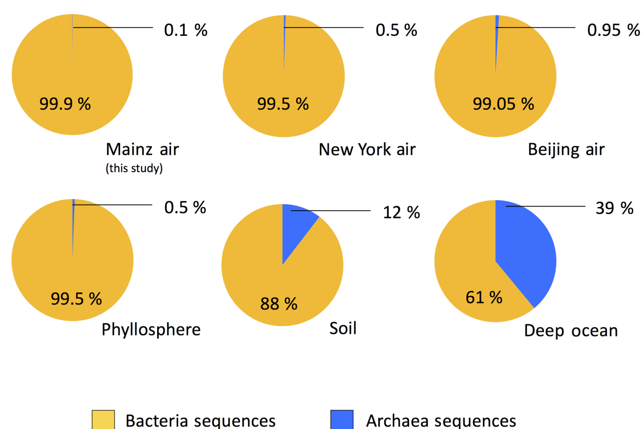

Figure 3. Relative proportions of archaeal (blue) and bacterial (yellow) sequences detected in environmental samples. Proportions for soil are based on Kemnitz et al. (2007), for the deep ocean on Karner et al. (2001), and for the phyllosphere on Delmotte et al. (2009) and Knief et al. (2012). The proportions of the Mainz air are based on this study. The data for the New York air are published in Yooseph et al. (2013) and the data of Beijing are based on Cao et al. (2014).

The situation might, however, differ for individual groups found in the air filter samples, such as the Nitrososphaera family. This family includes typical soil microorganisms, which would point to soil as a primary emission source. The presence of this family in the air might be, on the one hand, caused by the diversity of the phyllosphere. Especially for annual plants, the microorganism diversity of the phyllosphere is primarily driven by soil and the soil microbiome surrounding the sampling site (Knief et al., 2010). On the other hand, the explanation especially for the findings in the coarse fraction is that larger soil particles carry many typical soil archaea. Thus, based on the proportions of bacteria and archaea, the most likely interpretation is that the microbiome detected in the Mainz air primarily originates from the phyllosphere and is complemented by small soil particles, which add a large amount of typical soil archaea. Unfortunately, there is a lack of literature on archaea of the phyllosphere; thus, the identification of the emission source based on the composition cannot be answered for certain.

Based on the identified genera, however, the phyllosphere and the soil can both be the primary emission source. But as the microbiome of the soil drives the composition of the phyllosphere, comparing taxonomy alone will not lead to a final answer.

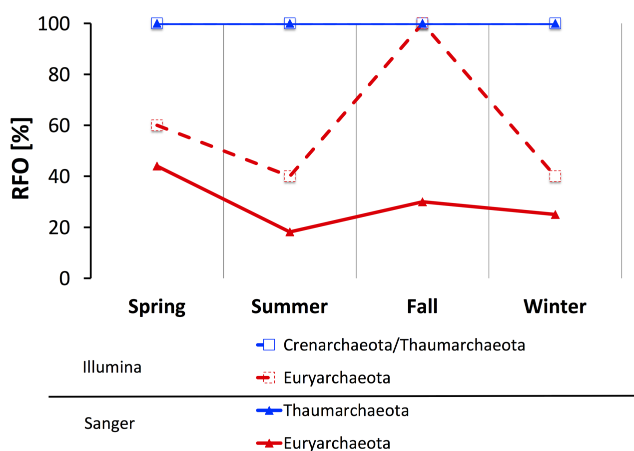

Figure 4. Seasonal variation in the relative frequency of occurrence (RFO) of airborne archaea on the phylum level. The relative frequency of occurrence – the proportion of samples in which these taxa were detected – is given for both phyla, i.e., Thaumarchaeota and Euryarchaeota. The graph based on Sanger sequencing represents the data published in Fröhlich-Nowoisky et al. (2014), whereas the remaining data comprises the results of this study.

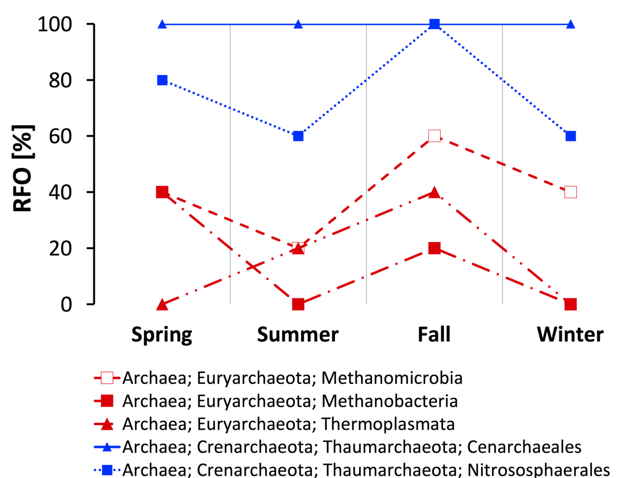

Figure 5. Seasonal variation in the relative frequency of occurrence of dominating Euryarchaeota classes and Crenarchaeota orders within this study.

3.2 Seasonal dynamics

To better understand the seasonal dynamics of archaea in the atmosphere the availability of emission sources over different seasons per year can be analyzed. As mentioned, from the 2341 archaeal sequences 168 could be assigned to Euryarchaeota. Based on their relative frequencies of occurrence (RFO), Thaumarchaeota are present all year, whereas Euryarchaeota are less abundant and their RFO values show seasonal peaks in spring and fall (Fig. 4).

Although the seasonal increasing or decreasing trends of the RFO values over the year are similar to Fröhlich-Nowoisky et al. (2014), overall they are higher.

Fröhlich-Nowoisky et al. (2014) suggested the nearby river Rhine as a potential permanent source for Methanomicrobiales and Thermoplasmatales as they are known to be present in river water throughout the year (Auguet et al., 2009; Cao et al., 2013). The RFO values of the orders shown in Fig. 5 present a slightly different picture: Methanomicrobia were observed in every season with RFO values around 40 %; thus, the Rhine could contribute continuously to the aerosolized Methanomicrobia. However, the Thermoplasmata group was exclusively found in summer and fall samples, arguing against an emission from an omnipresent source like the Rhine.

Alternatively to the Rhine, potential emission sources for several groups of Euryarchaeota – especially in agricultural areas as around Mainz – are biogas substrates and livestock fertilization methods (Fröhlich-Nowoisky et al., 2014). Figure 5 shows that Methanomicrobia and Methanobacteria both have their highest relative RFO during fall and another increase during the springs in 2006 and 2007. This supports the hypothesis, of livestock manure being a possible emission source, as both classes are commonly known to be present in the microbiome of livestock and the typical times for fertilization of fields with manure are in spring and fall (Nicol et al., 2003; Radl et al., 2007). As all methanogen groups, they have been reported in biogas reactors, too (Jaenicke et al., 2011). For the Thermoplasmata the peaks in summer and fall might be linked to the usage of biogas reactor substrates, which are also applied to agricultural fields as fertilizer. The differing RFO values of Thermoplasmata and other Euryarchaeota might be caused by their sensitivity to temperature and especially to pH, which only allows their survival in moderate to high temperatures and low pH environments.

The hypothesis that aerosolized archaea are linked to agricultural activities is also supported by the seasonal variation of the RFO of the order of the Nitrososphaerales within the Thaumarchaeota that is also present in the Euryarchaeota classes as discussed. Nitrososphaerales were found in agricultural soil samples close to the sampling area of our study (Ochsenreiter et al., 2003; Zhalnina et al., 2013) and thus can be considered a typical agricultural soil microorganism.

4 Conclusions

This study gains a deeper insight into the diversity of airborne archaea. The overall abundance of archaea in the atmosphere compared to bacteria is very low, which is comparable to the ratio found for the phyllosphere. We found the Nitrososphaeraceae family out of the I.1.b group of Crenarchaeota to be the major archaeal family in coarse particulate matter. The groups Cenarchaeaceae, Nitrososphaeraceae, Methanosarcinales, and Thermoplasmata as well as the genus *Nitrosopumilus* could be observed within the fine particulate matter.

The observed seasonal dynamics for the dominating Euryarchaeota classes and Crenarchaeota orders, which peak in summer and fall, might be a result of agriculture in the surrounding area. Therefore, anthropogenic activities like fertilization with livestock manure or substrates of biogas reactors might influence the diversity of airborne archaea as their occurrence is increased during the main fertilization seasons.

This combination of findings provides support for the conceptual premise that the occurrence of archaea in air might be driven by the microbiota of the phyllosphere but the influence of livestock manure gains an edge over the phyllosphere through the fertilization seasons. Additionally, groups emitted with soil as carrier particles seem to have a major influence on the community composition. For a further understanding of the dependencies of airborne microorganisms on their sources, future studies should additionally explore possible source habitats to gain as complete a picture as possible.

We conclude that the understanding of the seasonality, diversity, and composition of airborne archaea as one very small fraction within the bioaerosols is an important contribution to the understanding of the patterns driving the whole atmospheric microbiome.

Data availability. The post-library-split sequence dataset is available from the Edmond digital repository at: https://doi.org/10.17617/3.11 (Wehking et al., 2018).

The Supplement related to this article is available online at https://doi.org/10.5194/bg-15-4205-2018-supplement.

Author contributions. JW and VRD wrote the paper. VRD, UP, JF-N, DAP, JW, RMB, and DT designed the research. JF-N and RMB performed the sample collection and laboratory work. JW performed downstream analysis. JW and DAP performed correlations with meteorological data. All co-authors discussed the results, read and contributed to the manuscript.

Competing interests. The authors declare that they have no conflict of interest.

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C.2 Size dependence of bacterial diversity in air particulate matter

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Size dependence of bacterial diversity in air particulate matter

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Abstract. Airborne bacteria are a ubiquitous, diverse part of the atmospheric microbiome. They cause a broad range of processes and can act in a beneficial as well as a pathogenic way. The patterns influencing the bacterial diversity in the atmosphere are likely caused by the spatial and temporal variation of emission sources and also influenced by a wide range of environmental and meteorological factors, e.g. radiation, precipitation events or rapid changes in temperatures. Especially the bacterial diversity in different size fractions and different seasons are challenging research tasks and information on this is still lacking for central Europe.

We found the composition of the airborne bacterial community to be significantly different within coarse and fine air particulate matter. Samples of continental boundary layer air consisting of coarse and fine particulate matter fractions (cut-off 3µm) were taken over the course of one year in Mainz, Germany. The 16S rRNA gene was targeted using an Illumina sequencing approach. We found *Actinobacteria, Bacteroidetes, Firmicutes,* and *Proteobacteria* to be the dominant phyla over the sampling period. Spore-forming taxa dominated in the fine fraction, whereas the order of *Clostridiales* shows the largest proportional differences between the two size fractions. The distinct bacterial communities in the size fractions show different seasonalities in composition and relative abundances. Viewing the seasonalities in the size fractions separately, summer is the most diverse season in fine and least diverse in coarse particulate matter. As the bacterial community in the coarse fraction is more diverse, it may be an explanation that larger carrier particles protect living organisms from environmental stressors (i.e. UV radiation) and serve as carbon and energy substrates for microbial metabolism.

1 Introduction

The steady increase of research on the airborne microbiome in recent years has led to the generally accepted concept that the atmosphere contains a diverse and variable microbial community. Ongoing research focuses specifically on understanding the bacterial diversity and on the influences of atmospheric processes on microbial community structures (Delort et al., 2010; Kourtev et al., 2011; Morris et al., 2011). Furthermore, the effects of airborne microorganisms on the health of humans, plants, and animals has been investigated previously (e.g., Legionnaires' disease; Langer et al., 2012).

Traditionally, airborne microbial ecology has been surveyed through culture based approaches (for instance Shaffer and Lighthart, 1997), however as sequencing has become increasingly inexpensive, culture independent approaches have become the state of the art (Després et al., 2012; Fröhlich-Nowoisky et al., 2016 and references therein). A typical complex airborne microbial community (airborne microbiome) consists on phylum level mainly of representatives of *Actinobacteria, Bacteroidetes, Firmicutes* and *Proteobacteria*. (Bowers et al., 2011, 2012; Brodie et al., 2007; Pearce et al., 2010; Robertson et al., 2013; Yooseph et al., 2013).The density of microorganisms in the atmosphere quantified via qPCR is very low with around 10^4 -10^6 gene copies * m^3 in comparison to its density in soil with 10^{11} gene copies * kg^{-1} or marine environments with 10^8 to 10^9 gene copies per L (Cao et al., 2012; Després et al., 2012; Fröhlich-Nowoisky et al., 2014, 2016; Yin et al., 2013).

Nevertheless, airborne bacteria often show very diverse community structures, which are likely derived from local emission sources. Within these local emission sources various subgroups have been compared, namely emission from soils, water surfaces, and the phyllosphere (Bowers et al., 2012, 2013; Cao et al., 2014; Franzetti et al., 2011; Gandolfi et al., 2013).

The community structures of airborne bacteria show considerable temporal variability as they depend on several additional meteorological and anthropogenic influences (Bertolini et al., 2013; Bowers et al., 2011a and b, 2013; Lighthart, 2000). Moreover, significantly different communities have been reported according to sampling locations and land-use types (agricultural, forest, urban and suburban environments; Bowers et al., 2011a, 2011b). Taken together, these studies indicate that local sources might even play a larger role in shaping airborne bacterial communities than meteorological variations do. According to a possible seasonality of these sources, the composition of airborne microorganisms changes over the course of the year, but these effects are still not totally understood.

In aerosol research often a size categorization at a cut-off of 2.5µm is used (fine particulate matter (PM) = nominal mean aerodynamic diameter ≤ 2.5 µm) to differentiate between the fine PM, which can be inhaled deeply into the tracheobronchial and alveolar regions of the lung, and coarse PM, which can only reach the upper airways of the head (Brook et al., 2004). Thus the particle size has been found to be the key trigger in causing health problems of PM (Brown et al. 2013) and several health effects of the fine particle fraction have been reported (Strak et al., 2012). So far, much research has been done on the physical and chemical properties of different sizes of PM pollutants but relatively little is known about the bacterial diversity of fine PM, as in most studies on airborne microbes the size fractions have not been analysed separately.

As most bioaerosol research has focussed on total suspended particle (TSP), only few studies that used two size fractions exist in literature. Cao et al., (2014) found microbiological allergens and potential pathogens during a smog event in Beijing using a metagenomic approach. Another study dealing with coarse and fine filter samples in a metaproteomic approach found differences in the composition between the fractions (Liu et al., 2016). Bowers et al., found significantly higher bacterial taxonomic richness in the coarse fraction (PM10−2.5; 2013). Dealing with different size fractions and cumulative three month samples for summer and winter Franzetti et al., 2011 found seasonal differences in community structures mainly driven by an increase of *Actinomycetales* in winter samples. A systematic understanding of how the bacterial diversity of different PM changes over the course of the year is, to our knowledge still lacking for central Europe.

There are two primary aims of this study to contribute to the growing area of bioaerosol research: We used a high-throughput sequencing approach of 16S-ribosomal DNA on a GaIIx from Illumina to first investigate potential differences of bacterial diversity between two size fractions (cut off 3 µm). To ascertain the temporal variability in microbial communities over the four seasons of an entire year in the rural-urban Rhein-Main, we performed a detailed analysis of bacterial diversity in the boundary layer air sampled during the years 2006 and 2007 in Mainz, Germany. Finally, we combined both and show differences in the seasonal behaviour of the bacterial composition for the two investigated size fractions.

2 Material and methods

2.1 Aerosol sampling

As described in detail in Wehking et al., (2018) in total 24 pairs of air filter samples (i.e., 20 filter pairs consisting of one fine and one coarse particle filter sample each, two pairs of start-up air filter blanks and two pairs of mounting filter blanks as described below) were sampled over one year in Mainz, Germany (May 2006 - April 2007). The air filters were installed on a self-built high-volume-dichotomous sampler (Solomon et al., 1983) working with a rotary vane pump (Becker VT 4.25) with a total flow rate of ~0.3 m^3 min^{-1}. All particles with an aerodynamic diameter larger than ~3 µm and 10% of the fine particles were collected on one glass fiber filter (~0.03 m^3 min^{-1}) representing the coarse fraction. The fine particles from the same air mass were collected on the corresponding second glass fiber filter (~0.27 m^3 min^{-1}) which was essentially free of coarse particles (Solomon et al., 1983). To conserve the DNA of the collected bioaerosols after the sampling procedure all filter samples were stored at -80° C until analysis. Except for filter pairs MZ11 (24 h) MZ15 and MZ 31 (5 d), all filter pairs were sampling particles over a 7-day-period. The sampled air masses represent a mixture of urban and rural continental air, as the sampler was positioned on the roof of the Max Planck Institute for Chemistry on the campus of the University of Mainz (49°59’31.36’’N, 8°14’15.22’’E). To reduce the sampling of particles emitted from the ground, the sampling device was on a mast about 5 m above the flat roof of the three-story building, roughly 14 m above ground level. Prior to the sampling procedure all filters were baked in sealed aluminium foil bags overnight at 500° C.

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2.2 Extraction, amplification, and sequencing

The DNA extraction and sequencing were performed as a part of the Earth Microbiome Project (EMP - http://www.earthmicrobiome.org/) using the Illumina GAIIx sequencer and can be found in detail in Wehking et al. (2018). For the PCR amplifications the 515f/806r primer set (Fwd:GTGCCAGCMGCCGCGGTAA; Rev:GGACTACHVGGGTWTCTAA) described in Caporaso et al., (2011), amplifying bacteria and archaea over the V4 region of the 16S rRNA gene, was used (Bowers et al., 2013; Huse et al., 2008; Muyzer et al., 1993).

2.3 Taxonomic identification

The downstream processing of the sequences was performed using the Quantitative Insight Into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010). To identify corresponding

OTUs, a closed reference OTU script was used comparing the air filter sequences to the preclustered greengenes reference set (gg_13_8_otus/rep_set/97_otus.fasta, last update 08/15/2013; McDonald et al., 2012) and the corresponding taxonomy map were used.

A total of 9,222,533 partial 16s rRNA gene sequences were generated out of twenty pairs of coarse and fine filter samples as well as two pairs of blanks. As only sequences can be identified with this method, which hit a greengenes reference sequence can be identified with this method, not all sequences have been identified. Thus, the total number of identified sequences was reduced to 5,678,317. These sequences have been clustered into 20,683 different OTUs (i.e., "artificial species" on 97 % identity level; Stackebrandt and Goebel, 1994).

As the filters varied in sequence number, from sometimes as few as 1,500 to a maximum of 250,965 sequences, the samples have been rarefied for an unbiased analysis of their diversity. This was done by picking randomly 10,000 sequences per filter sample and using these for all downstream analyses. Only a few filter samples did not reach the 10,000 sequences needed for the rarefication, thus, five fine and two coarse filter samples were lost for all following analysis steps.

2.4 Controls

Quality assurance procedures by implementing regular controls in next generation sequencing techniques, e.g., for amplicon sequencing of environmental air samples, are neither well established nor standardized. What may be a real problem in low biomass samples, like in this study, is the presence of microbial communities in commercial DNA extraction kits and other laboratory reagents (Salter et al., 2014). The DNA of the blank filters was extracted and quantified in parallel to the actual filter samples. Often, the detected DNA concentrations on blanks can be too small to be quantified or to build usable sequencing libraries (Cao et al., 2014). Within this study we controlled the actual filter changing process by sequencing two mounting blanks, i.e., MZ 23 und MZ 73. A minimal DNA amount is likely, as the filters are shortly exposed to the air of the sampling site during the mounting process. For quality assurance, all OTUs that were found on handling blanks were deleted on all other samples as well. A detailed list of all analyzed air and blank filter samples with the statistics of the removed contaminants can be found in Table S1.

2.5 Statistical analysis

In general, all data management and analyses were performed using the QIIME workflow (Caporaso et al., 2010), and the R-Project if not stated otherwise (R-Team, 2011).

To characterize the biodiversity of a microbial community and thus to approximate the likely diversity of the entire community several statistical parameters can be calculated: species richness estimators, rarefaction curves, and community diversity indices.

3 Results and discussion

3.1 Community structure

Figure 1 (A) shows the Chao1 (for details see supplements) estimated species richness and (B) the Shannon-index of the coarse compared to the fine particle fraction. The mean of the species richness calculated by the Chao1 of the coarse fraction is more than a quarter higher than the mean of the species richness of the fine fraction (coarse=1264 OTUs; fine=1007 OTUs). Thus, the bacteria sampled on the coarse fraction >3 µm are more diverse than the inhalable fine fraction bacteria. As a single bacterial cell in general has the size of ~1 µm this raises the question why any bacteria are present at all on the coarse particle fraction.

One explanation might be that several bacteria are sampled on coarse particle filters due to the size of the particles they are transported with. On the one hand they can either be attached to or even enclosed by bigger particles, such as soil crumbs, plant or fungal fragments. On the other hand some bacteria, even though small when being single, live continuously in colonies, thus are attached to other bacteria during their life. These colonies can form aggregates and thus will often, though not exclusively, be sampled in the coarse particle fraction. While chains can also, due to their aerodynamic behaviour, be sampled on fine particle filters, lumps will almost exclusively be found on coarse particle filters. Some bacteria are also able to form spores when aerosolized, as a reaction to a stressful environment. Such spores will in general be detected on fine particle filters. Due to the construction of the sampling device, 10 % of fine particles are, however, sampled in the coarse particle fraction as well.

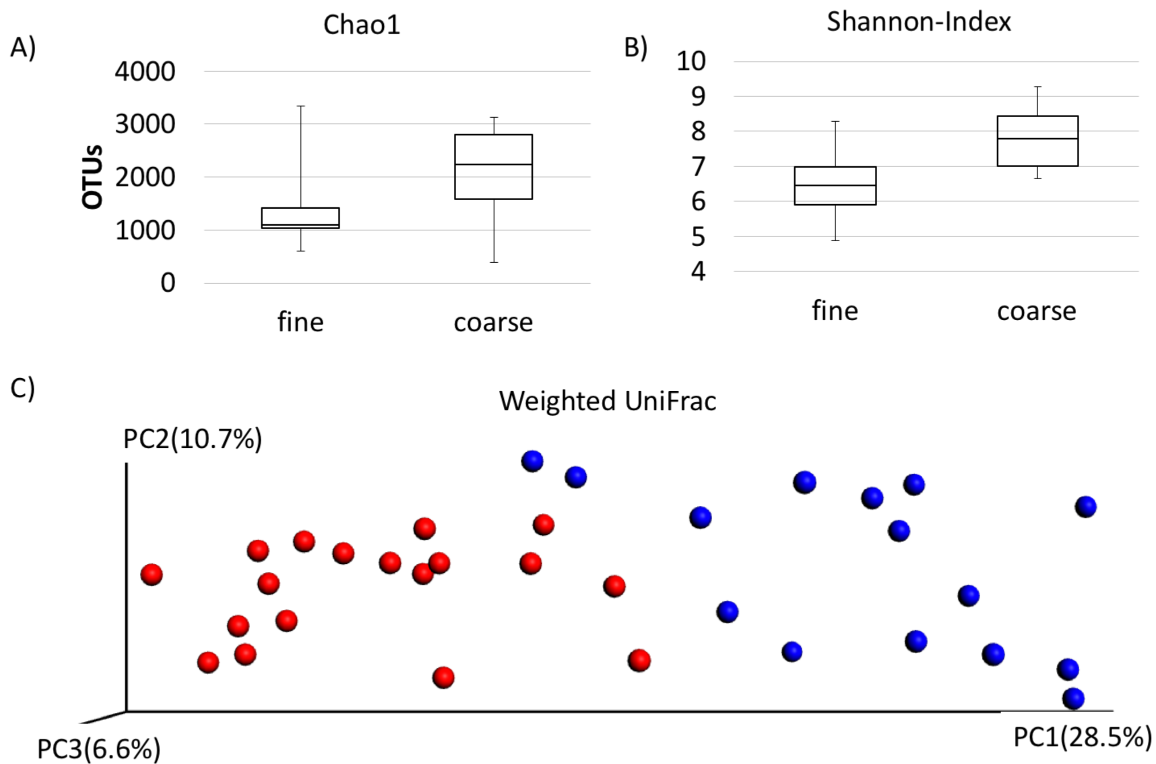

Figure 1. Bacterial diversity measures. (A) Chao1 estimated species richness (B) Shannon index (C) Principal coordinates analysis (PCoA) of the pair-wise distances using the weighted UniFrac algorithm, points indicating individual filter samples (15 fine (blue) and 18 coarse (red) filter samples). The principal coordinate axes (PC1-PC3) indicate the amount of variation captured along. Filter samples which appear closer together have smaller pair-wise distances and are more similar to each other. The composition of the airborne bacterial community was significantly different between coarse and fine particulate matter (ANOSIM Global R = 0.44, P < 0.001). All samples were subsampled at 10,000 randomly selected sequences each.

Higher diversity levels in the coarse PM was, for airborne bacteria, described first by Bowers et al., (2013) using a $PM_{2,5}$ cut-off and a different sequencing technique, who found the bacterial taxonomical richness in the coarse fraction to be roughly 80% higher than in the fine particle fraction. Additionally it is known from water habitats like rivers or open sea, that microbial communities attached to particles show higher levels of diversity compared to free plankton (Ortega-Retuerta et al., 2013).

Bacteria, which are attached or enclosed in soil or leaf fragments, might benefit from their enclosure while aerosolized, as they are likely better protected against environmental stresses, e.g., UV radiation, than bacteria which are aerosolized individually. Additionally, the sheltered bacteria might profit from their carrier particle, as these may serve as carbon and energy substrates for running the bacterial metabolism. Finally, the diversity of a soil particle is high

compared to the air and that means with one grain of soil a small community is aerosolised at once.

Interestingly, principal coordinates analysis (PCoA) of weighted unifrac pair-wise distances visualized by EMPeror (Vázquez-Baeza et al., 2013), shows, for the here presented data set, that the airborne bacterial community differs significantly between coarse and fine particulate matter samples (Fig. 1 C; ANOSIM, Global R = 0.44, P < 0.001). Thus, both size fractions have striking differences in their composition and relative abundance of bacteria even though all sampled bacteria were simultaneously in the air when sampled.

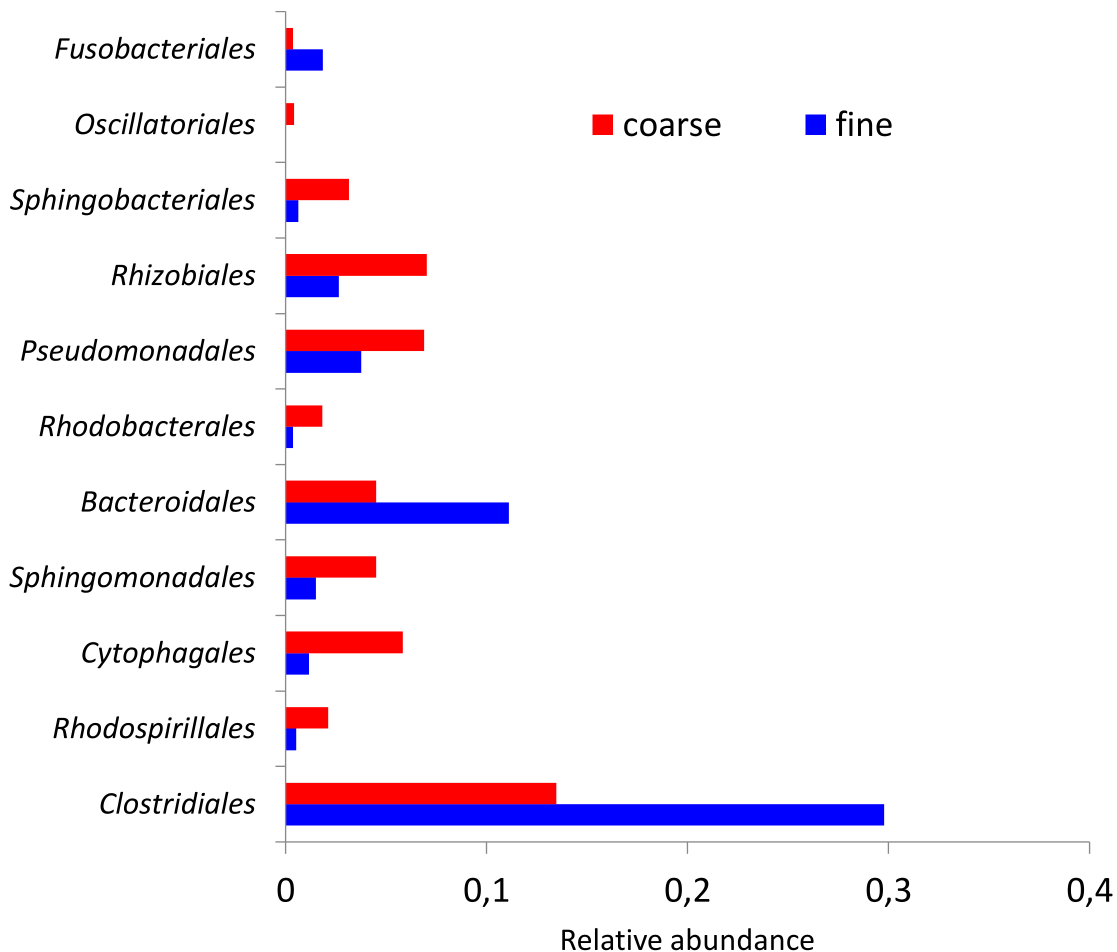

Figure 2. Relative abundance of bacterial orders with significantly different relative abundances across coarse and fine particulate matter (ANOVA overall FDR_P < 0.05). All samples have been subsampled at 10,000 randomly selected sequences and grouped together in coarse as well as fine particulate matter samples.

As the taxonomic composition between the size fractions varies significantly, Fig. 2 illustrates the bacterial orders, which occurred in both size fractions but with significantly (ANOVA (FDR_P < 0.05) different relative abundances. Members of the *Bacteroidetes, Cyanobacteria, Firmicutes, Fusobacteria* and *Proteobacteria* were found to be primarily responsible for the community separation between the two particle size fractions. *Clostridiales*

were found to be the most influential order with higher relative abundances in fine particulate matter (29.8 %) than in the coarse fraction (13.5 %; Fig. 2).

Comparable observations have been made by Bowers et al., (2013) for an urban as well as an rural environment. In their discussion about the bacterial groups having a major influence on their findings, Bowers et al., (2013) found the *Clostridiales* to be one of the most influential orders, too. However, they were observed in higher relative abundances in the coarse not in the fine particle fraction. Within another study, dealing with the comparison of different size fractions, Franzetti et al., (2011) found *Firmicutes* with *Clostridiales* therein in higher relative abundance coarse PM, too, but performed no significance tests. Both authors linked this higher presence in coarse PM to possible bacterial aggregates additionally associated to larger particles as discussed above. These rather contradictory results for *Clostridiales* influencing the community in the fine particle fraction may be explained by different conditions for Mainz compared to both other studies which might influence the bacteria before being aerosolized, e.g. meteorological parameters. It is possible to hypothesise, that conditions in Mainz drove the *Clostridiales* to build up small non-dividing spores (Fig. 4), which are normally reduced in size compared to the vegetative cells. Bacteria change from an active metabolic to a non-dividing state to increase their potential resistance against non-optimal conditions (Delort et al., 2010). Most *Firmicutes* groups can survive physical pressure by forming spores or cell wall modification. To support this hypothesis, the highest relative abundance of *Clostridiales* in fine particulate matter was found in the sample with the highest maximum windspeed, what could lead to easier aerosolization.

Compared to the fine filter fraction, that is dominated by the *Firmicutes* (40 %) with the *Clostridiales* therein, in the collated coarse particle filter fraction *Proteobacteria* are the most abundant phylum (38 %), as visualized in Fig. 3. The proportions of *Bacteriodetes* and *Actinobacteria* are very similar in both size fractions (fine 15.4 % and coarse 17 %; 13.7 % and 15.4 %, respectively). When comparing our results on phylum level to the major findings in the literature, it becomes clear that the coarse particle fraction is similar to the results published for TSP samples in general.

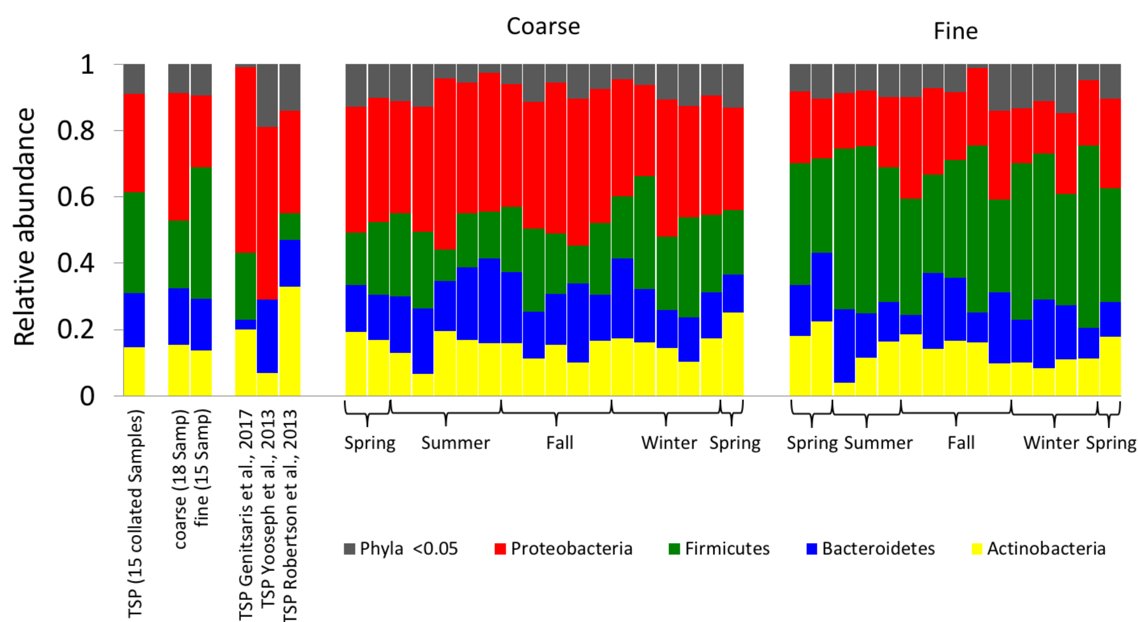

Figure 3. Bacterial community composition for total suspended, coarse, and fine particulate matter on the level of phyla. The bars within the seasons give the relative abundances of the bacterial taxa for individual filter samples. All individual samples were subsampled at 10,000 randomly selected sequences and subsequently collated, seen in the columns TSP, coarse and fine.

Yooseph et al., found for example that in New York outdoor air (2013) *Proteobacteria* are the dominating phylum next to *Bacteriodetes* and *Actinobacteria*. Genitsaris et al., who sampled TSP over a comparable time of one year found comparable relative abundances (2017) with *Proteobacteria* (56 %) as the dominating phylum and *Actinobacteria* as well as *Firmicutes* with a relative number of sequences of 20 % each (Fig. 2). Our TSP dataset collated of the fine and coarse samples consists of *Actinobacteria, Bacteroidetes*, *Firmicutes* and *Proteobacteria*. This composition of dominating taxa is comparable to findings within most other studies dealing with bacteria in the atmosphere (Bowers et al., 2011b, 2012; Brodie et al., 2007; Genitsaris et al., 2017; Pearce et al., 2010; Robertson et al., 2013; Yooseph et al., 2013). Within the TSP all six existing classes of *Proteobacteria* were found in the Mainz air, but the relative abundances and compositions differ widely between the analysed size fractions: the *Proteobacteria* on coarse level are mainly composed of *Alphaproteobacteria* (18 %), whereas the fine filter fraction shows a higher ratio of *Gammaproteobacteria* (7 %), with almost the same amounts

for *Alpha-* and *Betaproteobacteria* (6 % and 5 %). *Zetaproteobacteria* are rare and only found in the coarse particle mode on two filters MZ31 and MZ47 with very low abundances. One of the most discussed influence on the climate by bioaerosols in general and bacterial groups in detail is their influence on cloud formation, precipitation and eco-system interactions (Delort et al., 2010; Després et al., 2012; Fröhlich-Nowoisky et al., 2016; Huffman et al., 2013). Until now, bacteria are the best studied microorganism group which can act as nuclei for ice crystallisation (IN) in clouds (Fröhlich-Nowoisky et al., 2016). Many strains of the family *Pseudomonadaceae* for example, have IN-active proteins on their outer-membrane (Kozloff et al., 1991). Especially in the coarse fraction more than 4 % of all sequences have been assigned to the *Pseudomonadaceae* family (Fig. 4).

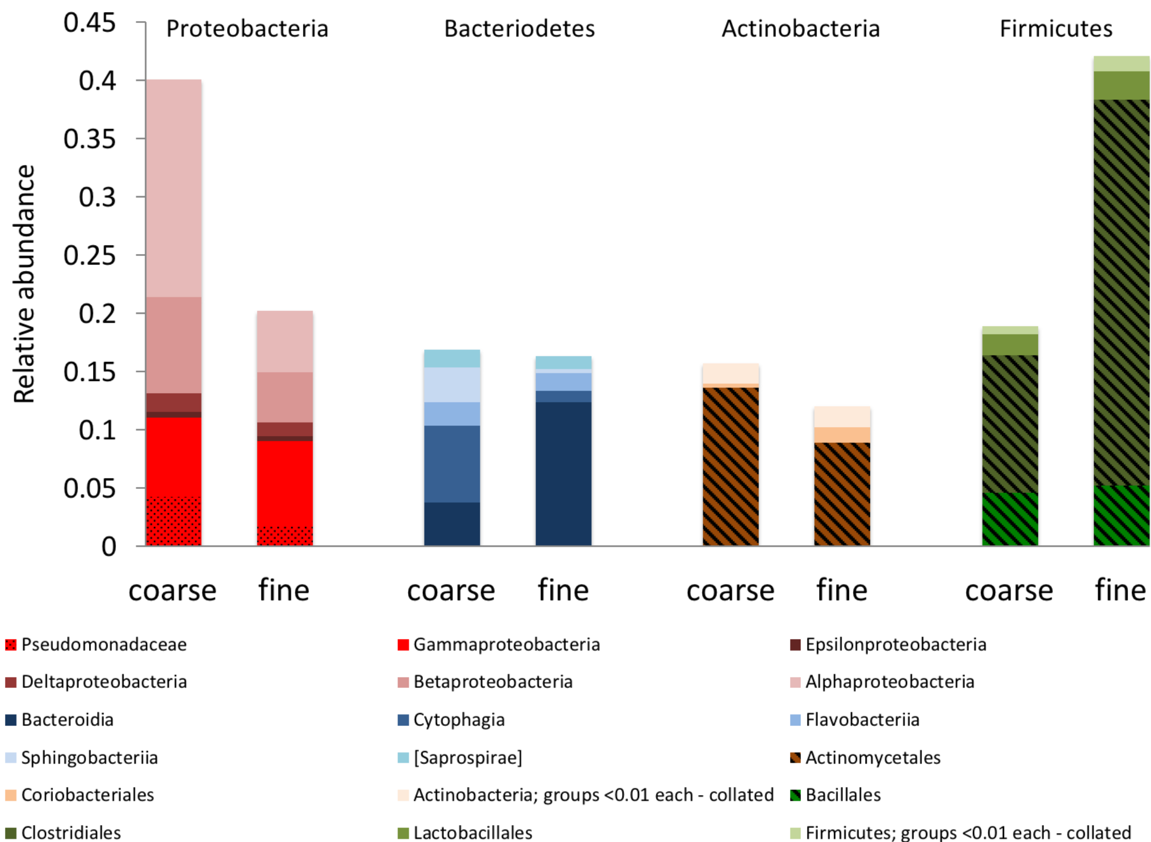

Figure 4. Bacterial community composition for the four main phyla of the Mainz dataset on deeper taxonomic levels in coarse and fine particulate matter. Additionally, the proportion of spore-forming groups are marked via slashes and the Pseudomonadaceae family via dots.

The phylum *Firmicutes* has been observed on every analysed filter sample of the fine filter fraction (Fig. 4). The dominating phyla on class level were the Clostridia (13.7 %, coarse and 30 %, fine) and the Bacilli (6.3 % and 9 %). The *Erysipelotrichi* were found in both size fractions, whereas the smallest class of *Firmicutes* the OPB54 group was found exclusively on

coarse filters. Figure 4 shows the four main phyla found in the dataset, additionally split into spore and nonspore-forming groups. Whereas the *Firmicutes* consist mainly of Clostridia and the Bacilli, the main orders there in, the *Clostridiales* and the Bacillales can build endospores. Whereas the *Lactobacillales*, as the other major order within the Bacilli, do not (Fig. 4). Especially the endospore forming *Firmicutes* groups dominated the fine filter fraction and whereas the *Clostridiales* have been shown to be the most significant driver of the community separation (Fig. 2).

The Actinomycetales were the most abundant representative of the Actinobacteria phyla in both size fractions (fine= 10 %, coarse= 13 %). On deeper taxonomic levels the *Acidimicrobiia*, *Actinobacteria*, *Coriobacteriia*, OPB41, *Rubrobacteria*, *Thermoleophilia* were found with relative proportions over 1% of all *Actinobacteria* sequences either in coarse or in fine filter fractions. The classes OPB41, MB-A2-108 and *Nitriliruptoria* were observed but in smaller proportions. Together with the *Firmicutes* the *Actinobacteria* are the second major bacteria group of gram-positive bacteria. As a ubiquitously distributed group, which occurs aquatic and terrestrial ecosystems, they are of great importance for agriculture, biotechnology, and medicine. Most *Actinobacteria* can rest during suboptimal conditions as semidormant spores.

The group of *Bacteroidetes* is in smaller amounts represented in the dataset. On class level the fine fraction is dominated by sequences of the *Bacteroidia* order with 11 % while in the coarse fraction only 4.5 % of all Bacteroidetes sequences belong to this taxon. In the coarse fraction the members of the *Cytophagia* with a relative abundance of around 5.8 % are the most abundant class of the Bacteroidetes group. The superphyla *Cytophaga-Flavo-Bacteroidetes* has often been the most common observed group during early years of bioaerosol research (Després et al., 2012).

On seven fine and one coarse filter samples no Cyanobacteria sequences have been found after removing all chloroplast sequences (MZ31b, MZ47b, MZ59b, MZ67b, MZ74b, MZ81b, MZ88b, MZ81a). The remaining 14,545 sequences were formed into 153 OTUs. Five classes show relative proportions of more than 1% of all *Cyanobacteria* sequences.

Thus, all these findings suggest a larger role of spore-forming taxa in promoting the significant community separation between coarse and fine particulate matter. According to these observations it can be hypothesized that spore-forming taxa are found more often in fine particulate matter because they possibly build up smaller aggregates, are less often attached to carrier particles or even aerosolised in single-cell-state, overall they are smaller than vegetative cells.

3.2 Seasonal dynamics

For the typical four seasons of the sampling site, namely spring, summer, fall and winter the species richness was estimated for the previously described dataset of the 10,000 randomly subsampled sequences. The observed species richness in spring was highest with around 750 OTUs, whereas the richness during the other seasons was estimated around 200 OTUs less (Fig.5).

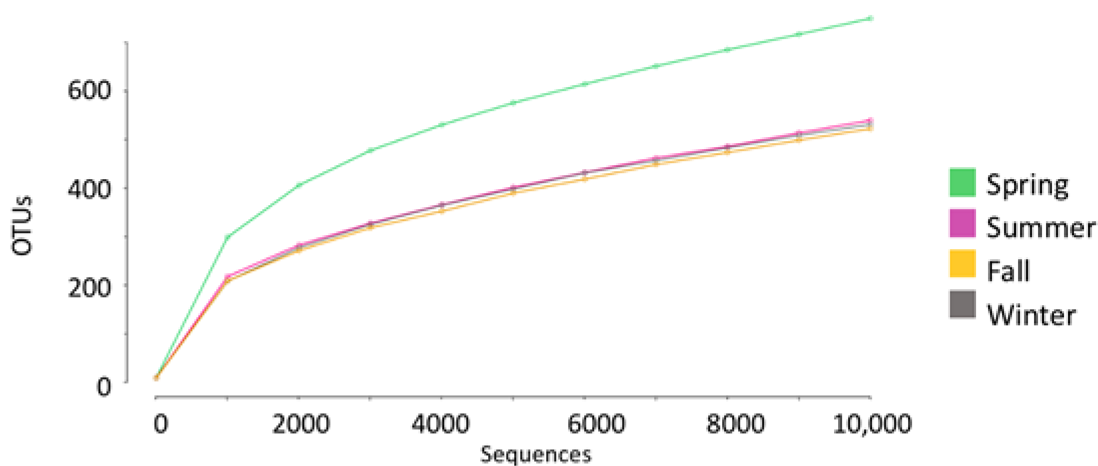

Figure 5. Rarefaction curves of the observed bacterial species richness of TSP for the four seasons.

This seasonal variation might be caused by the presence and absence of possible sources for specific bacteria groups: The microorganisms sampled in Mainz air primarily originate from the surrounding rural area, and thus represent as a mixture of the soil and phyllosphere microbiome. Additional influences might be caused by the more than 34,000 students of the year 2006/2007 on the campus of the University of Mainz and the nearby river Rhine.

As already proposed for other sampling locations seasonal effects on the source environments, e.g. changes in vegetation or flowering periods over the course of the year lead to differences in bacterial diversity between seasons (Bertolini et al., 2013; Bowers et al., 2012a; Huffman et al., 2013). In Mainz, most plants start growing from the late winter (February) until flowering period over the summer season. Summer and Fall are harvesting seasons in agricultural areas, so the plant surface decreases during this period. After the harvesting process the soil surface is still partially covered with stubble, so aerosolization processes from soil might be suppressed. The increase in plant biomass and surface during spring might be the key driver for the high species richness during season (Fig. 5). Different stress levels caused by UV

light or the availability of water are likewise discussed to lead to changes in diversity and in addition to the usage of survival strategies of some specialized bacterial taxa, e.g., spore formation (Franzetti et al., 2011).

For microorganisms which are aerosolized, air is a constantly changing environment. Air masses are not stationary but continuously moving, thus bioaerosols are exposed to the changes caused by such movements, e.g., changes in temperature, relative humidity, air pressure etc. These changes might introduce stresses on the aerosolized organism. In addition, aerosolized microorganisms sediment either fast with wet precipitation or more slowly by dry sedimentation. The different sedimentation rates and thus residential times in air affect also the composition of aerosolized bacteria. Thus, the bacterial composition changes often depending on the active local emission sources, current weather conditions or their individual residential time in the atmosphere. All these conditions change drastically with seasons and therefore should lead to drastically seasonal changes in the composition of bacterial populations.

Nevertheless, we found members of all four phyla, which were dominating the whole dataset (Fig.2) in high relative abundances constantly over the whole year, namely *Actinobacteria, Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Every season was represented by evenly spread 5 filter pairs. Next to these four bacterial phyla we also detected – in every season though not in all filters- two archaea and nine other bacterial phyla. All these other phyla show relative abundances in lower amounts than 1%, but were nevertheless found constantly. On an OTU based approach 2,854 different OTUs were found in three seasons over the year and 1,369 (6.6%) of the 20,683 overall found OTUs could be detected in every season. This is a small value compared to an urban mediterranean area in Greece, where 13.6% of all OTUs where found in all seasons, nevertheless they found less OTU richness in their study (Genitsaris et al., 2017). A possible explanation for this discrepancy might be, that more species can survive in all seasons in Greece due to the more constant climate, compared to central Germany, where the climatic conditions between summer and winter vary widely. This means that nevertheless the air is a constantly changing setting, that argues in itself against a constant, core microbiome, as it is already known for other environments as soils or human skin (Bjork et al., 2017; Huse et al., 2012; Pershina et al., 2018). Interestingly, also the air seems to have a core microbiome, thus a number of OTUs, which are present in the air all the time, independent of seasons, sources or stresses.

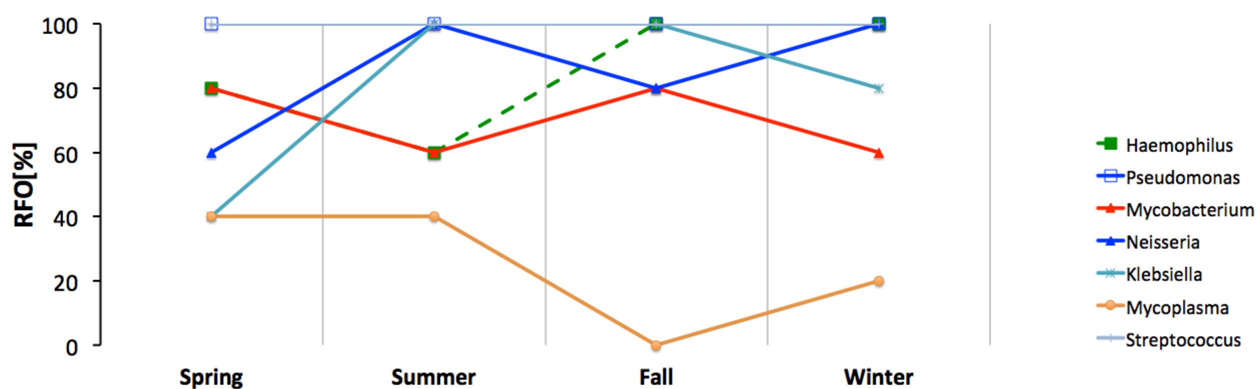

Figure 6. Seasonal variation in the relative frequency of occurrence (RFO) – the proportion of samples in which these taxa were detected - for potential human pathogenic genera of TSP air samples in Mainz, Germany.

The question whether or not there is a potential of the human pathogens in outdoor air has been addressed by Lai et al., (2009). They mention four different source areas for contributing potential pathogen organisms to outdoor air, namely natural and engineering environments, agriculture and waste treatment. Following a recently published review *Streptococcus pneumoniae, S. pyogenes, Mycoplasma pneumoniae, Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are pathogenic bacteria, which might spread as aerosol particles (Smets et al., 2016) and thus be transported and infect even over distances. Screening the dataset for corresponding genera of these potential pathogens showed that all of them could be detected. With exception for Mycoplasma all genera show relative frequencies of occurrence (RFO) of more than 50% for all for seasons (Fig.6). That means that they are constantly detectable in the outdoor atmosphere of the Mainz campus. Klebsiella and Mycobacteria were detected mainly in the coarse fraction (Tab. S2), whereas the non-sporeforming Lactobacillus family of the *Streptococacceae* occurred four times more often in the inhalable fine than in course particulate matter, what could be another hint that mainly spores are found within the fine fraction.

As the two size fractions harbour distinct communities as discussed above, it seems to be the logical consequence to recalculate the species richness for coarse and fine PM individually to compare each other (Fig.7).

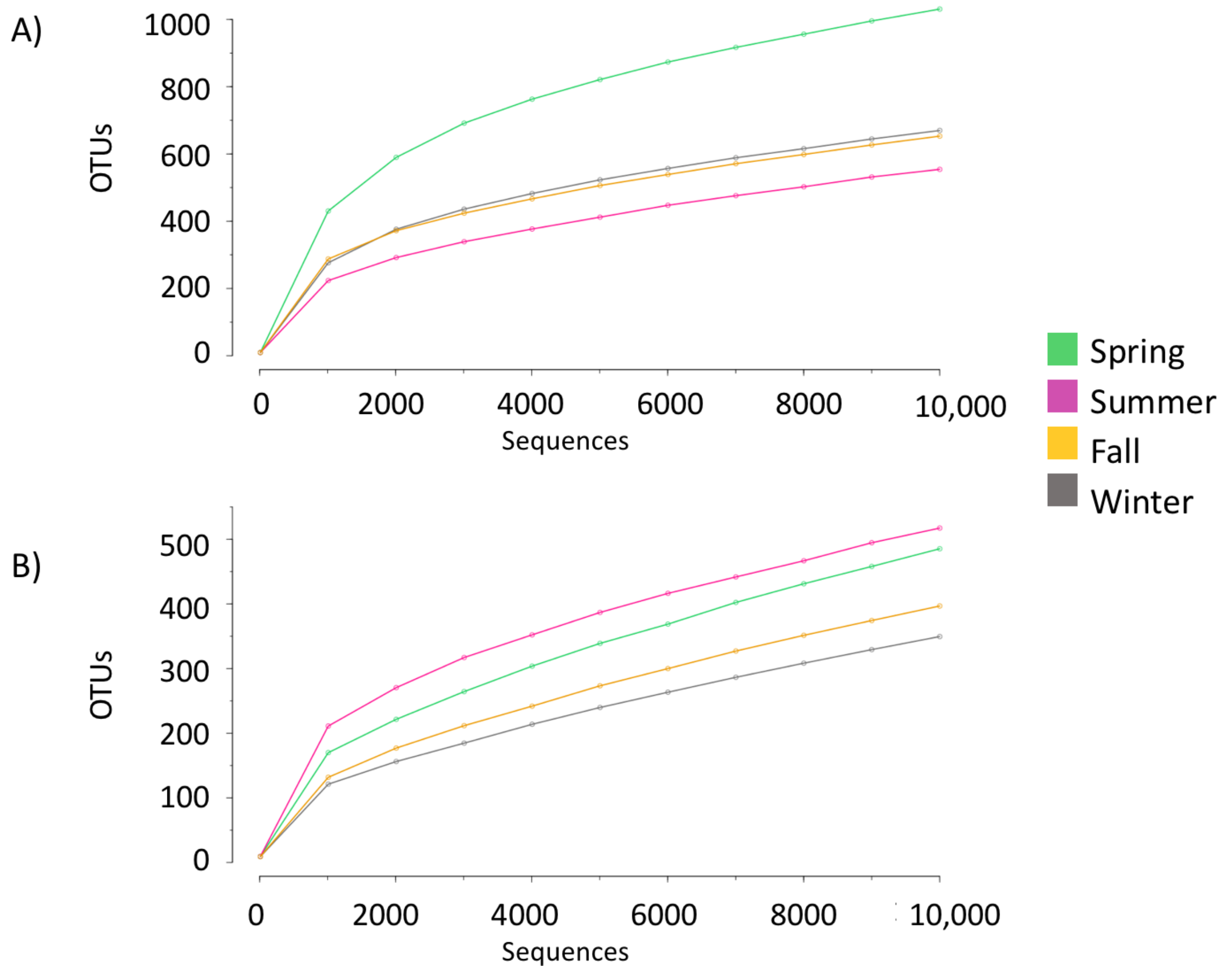

Figure 7. Rarefaction curves of the observed bacterial species richness in coarse (A) and fine (B) particulate matter samples split into four different seasons, each sample subsampled at 10,000 randomly selected sequences and collated afterwards.

Focussing on the observed species richness it is striking, that in coarse samples during spring around two times as many species can be found than in fall and winter, and thus the coarse particle fraction likely drives the trend already observed in the complete dataset (Fig. 5). In the coarse fraction the rarefaction curve of the observed species richness shows the lowest value during the summer. This might be due to the reduction in plant surfaces due to harvest, thus the presence of the already mentioned stubble but also other vegetation, which covers and supresses the aerosolisation of soil particles as carrier for soil related groups, sampled in the coarse filter fraction. When studying seasonalities over an entire year for the fine fraction – which to our knowledge, has not been done before- the summer is based on the observed species richness the season with the highest diversity, which stands in a striking difference to the behaviour detected in coarse and TSP analyses. One explanation might be that in harvest processes also bacteria are aerosolized which are not attached to bigger particles. Alternatively, the hot and

dry summer months might be stressful for some of the bacterial taxa and thus promote spore formation, which would be detected in the fine particle fraction. Furthermore, the fact that during the warm months of the year the diversity in the fine fraction increases justifies additional the idea that next to higher relative abundances of specific taxa, even higher species richness might be additional caused by spore forming groups.

4 Conclusions

The aim of the presented research was to gain deeper insight into the diversity of bacteria in different fractions of airborne particulate matter collected at different seasons. An airborne core microbiome consisting of 1,369 OTUs (6.6 % of all OTUs), was observed in every season. The four phyla *Actinobacteria, Bacteroidetes, Firmicutes*, and *Proteobacteria* were dominant within the entire dataset and in both coarse and fine size fractions, but diversity as well as species richness estimators were found to be higher in coarse than in fine particulate matter. Additionally, the communities within both size fractions were significantly different with regard to their composition and relative abundances of the found bacterial taxa. The group of *Clostridiales*, mainly represented by spore-forming taxa, was found with significantly higher relative abundance in fine particulate matter, constituting the largest difference between the bacterial communities in the two size fractions.

Both size fractions show different seasonalities regarding species richness, with summer simultaneously exhibiting the highest values for fine and the lowest for coarse particulate matter. The potential pathogenic genera *Streptococcus, Mycoplasma Haemophilus, Klebsiella,* and *Pseudomonas* occurred in in more than 50 % of all samples.

Taken together, these findings indicate different key factors to be responsible for bacterial diversity in the two size fractions. The coarse fraction can be hypothesized to contain particles carrying and shielding microorgansims from harmful stress. In the fine fraction a majority of spore-forming taxa was found. Spores are protected against stressful conditions by themselves and normally are smaller in dimension compared to the corresponding vegetative form. The remaining open question of the origin of the airborne bacterial particles should be addressed in future studies by analyzing possible environmental source communities alongside with air samples.

Data availability

The post-library-split sequence dataset is available from the Edmond digital repository at: https://doi.org/10.17617/3.1l

Competing interests

The authors declare that they have no conflict of interest.

Author contributions

JW and VRD wrote the paper. VRD, UP, HP, CO, JF-N, DAP, JW, RMB, and DT designed the research. JF-N and RMB performed the sample collection and laboratory work. JW performed downstream analysis. All co-authors discussed the results, read and contributed to the manuscript.

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Supplements

Table S1. List of air filter samples sequenced for this study and statistics of the removed contaminants. While the air masses collected on MZ 11, MZ 15, and MZ 31 were sampled over 1, 5, and 5 days, respectively, all other air filter samples were taken over a 7-day period corresponding to ~ 3000 m^3 of sampled air.

Filter	Fraction	sequences before removal of contaminants	sequences after removal of contaminants	loss of sequences in percent
Mz. 11a	coarse	1500	437	70,87
Mz. 11b	fine	22337	2825	87,35
Mz. 15a	coarse	204515	73827	63,90
Mz. 15b	fine	165641	15462	90,67
Mz. 22a	coarse	192717	36440	81,09
Mz. 22b	fine	93370	10636	88,61
Mz. 23a	coarse	87527	0	100,00
Mz. 23b	fine	44230	0	100,00
Mz. 26a	coarse	134147	51989	61,24
Mz. 26b	fine	108012	10378	90,39
Mz. 31a	coarse	165824	63330	61,81
Mz. 31b	fine	34394	7898	77,04
Mz. 41a	coarse	156922	34563	77,97
Mz. 41b	fine	171387	65512	61,78
Mz. 47a	coarse	245221	132125	46,12
Mz. 47b	fine	19992	4526	77,36
Mz. 50a	coarse	96566	44481	53,94
Mz. 50b	fine	131274	41142	68,66
Mz. 52a	coarse	193456	39326	79,67
Mz. 52b	fine	133650	38831	70,95
Mz. 54a	coarse	205570	46768	77,25
Mz. 54b	fine	68198	12514	81,65
Mz. 59a	coarse	228130	65722	71,19
Mz. 59b	fine	193519	28898	85,07

Mz. 62a	coarse	157854	45433	71,22
Mz. 62b	fine	202494	29682	85,34
Mz. 67a	coarse	164362	82867	49,58
Mz. 67b	fine	83874	14638	82,55
Mz. 72a	coarse	30289	8047	73,43
Mz. 72b	fine	53938	8594	84,07
Mz. 73a	coarse	153493	0	100,00
Mz. 73b	fine	38018	0	100,00
Mz. 74a	coarse	127628	50643	60,32
Mz. 74b	fine	60769	12086	80,11
Mz. 81a	coarse	106081	45401	57,20
Mz. 81b	fine	135405	22803	83,16
Mz. 82a	coarse	95881	41378	56,84
Mz. 82b	fine	91891	28669	68,80
Mz. 88a	coarse	142419	25837	81,86
Mz. 88b	fine	81632	9566	88,28
Mz. 90a	coarse	48327	19525	59,60
Mz. 90b	fine	108565	20105	81,48
Mz. 93a	coarse	250965	117657	53,12
Mz. 93b	fine	118161	21279	81,99
Mz. 101a	coarse	112850	28006	75,18
Mz. 101b	fine	53291	9562	82,06
Mz. 103a	coarse	124282	41757	66,40
Mz. 103b	fine	37749	10492	72,21

Table S2. Filter samples containing sequences from potential human pathogenic taxa.

	out of 20	out of 20	out of 5 TSP	out of 5 TSP	out of 5 TSP	out of 5 TSP
	coarse	fine	spring	summer	fall	winter
Haemophilus	14	15	4	3	5	5
Pseudomonas	20	18	5	5	5	5
Mycobacterium	13	5	4	3	4	3
Neisseria	15	16	3	5	4	5
Klebsiella	14	7	2	5	5	4
Mycoplasma	3	4	2	2	0	1
Streptococcus	19	19	5	5	5	5

Diversity analysis

The *Chao1* is a typical species richness estimator that approximates, by comparing singletons to doubletons etc., the total number of present species in the entire community using the number of rare taxonomic ranks per sample (Chao, 1984). In this study the *Chao1* was calculated for fine, coarse and total suspended particles on the OTU level and the sequence base. The rarefaction curve is a saturation curve in which the approximate total diversity of the entire community is estimated based on a repeated random selection process of the sampled sequences (Fisher et al., 1943). This curve enables to judge whether the sampling depth is sufficient to estimate the biodiversity in the entire community. As community diversity indices the *Shannon* (Shannon, 1948) and the *Simpson* (Simpson, 1949) indices were used to combine the species richness and the abundance in one single value reflecting the diversity evenness. The evenness is low for communities, which are dominated by a few species and high if most species are present equally often. The *Shannon* and *Simpson* indices were calculated in this approach on the OTU level for coarse, fine, and the entire air masses. Additional the ACE (abundance-based coverage estimator) was used (Lee and Chao, 1994).

C.3 On the constraints and influences on the temporal and size fraction occurrences of airborne fungi

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Contribution
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Keywords:

airborne fungi, primary bioaerosol, fungal spores, spore adaptations, plant pathogens, saprophytes

Abstract

Fungi play important roles in the environment, agriculture, and human health. Most fungal species spread by wind-driven dispersal of spores, determining their occurrence and distribution in different environments. The dynamics of airborne fungi and their dependence on lifestyle and environmental conditions, however, are not well characterized.

Here, we compare the airborne fungal composition in coarse and fine aerosol samples of continental boundary layer air, identified through isolated DNA sequences. This revealed that the composition of the airborne fungal community is temporally highly dynamic, however, not constant throughout the investigated 13-month period. We observed a high compositional identity of wintertime samples to spring and summer samples during the extremely warm winter 06/07, which may point to a very early initial spore dispersal due to warm conditions. Subsequent principal coordinates analysis revealed an ambient-temperature-dependent clustering of the samples and also revealed a separation of the coarse and fine fraction communities (cutoff 3 µm) The two communities displayed independent temporal dynamics as no discernable common patterns were identified between the coarse and fine filter samples.

To gain a deeper insight into the identified patterns, a subset containing the 25 most abundant species was formed and analyzed incorporating additional information on the fungi, including known nutritional and life-style preferences, fruiting form and spore dimensions. This allowed the identification of traits in the temporal and size fraction occurrences and correlations to meteorology, which were common amongst fungi with similar lifestyle preferences. We hypothesize the differences reflect lifestyle-dependent sporulation strategies which may facilitate and improve the assessment and forecasting of the abundance and spread of pathogenic fungi and related issues, such as crop protection, in view of land-use and climate change.

Introduction

Amongst the primary bioaerosol particles (PBAP) (Després et al. 2012) of the atmosphere, fungi are, in terms of number concentrations and impact, one of the most important groups. Typical number concentrations range from ~10^3 – 10^4 per cubic meter of air (Elbert et al. 2007; Fröhlich-Nowoisky et al. 2012), in the same orders of magnitude as bacteria, albeit with large variations depending on location, time of year, or even time of day. Fungal spores can show a large species-dependent variability in size, however, most species have spores in a size range of roughly 2 to 10 µm aerodynamic diameter (Elbert et al. 2007; Cao et al. 2014; Wang, Fang, and Lee 2008; Fröhlich-Nowoisky et al. 2009; Després et al. 2012; Huffman, Treutlein, and Pöschl 2010). As plant pathogens, fungi have a huge impact on agriculture and forestry (Strange and Scott 2005; Ostry and Juzwik 2008; Zabel and Morrell 2012) and, as pathogens and allergens, on human health (Horner et al. 1995). Their influence on atmospheric processes is also subject of ongoing discussions, as individual species have displayed ice-nucleating abilities far more efficient than non-biological aerosols and may influence cloud formation (Richard, Martin, and Pouleur 1996; Fröhlich-Nowoisky et al. 2015; Pöschl 2005; Fröhlich-Nowoisky et al. 2016).

Although wind-driven dispersal of spores is a passive process, through evolutionary adaptation to their lifestyles, fungi have heightened their sporulation success rate. These adaptations can be variations in numerous properties and will include aspects like the numbers of released spores, aerodynamic properties of spores, and the time of spore release, which we will cumulatively refer to as the "sporulation strategy". The evolutionary pressures leading to differing sporulation strategies will be factors such as the spatial distribution and abundancy of a nutrient source, for example, an obligate pathogenic fungus in comparison an unspecific generalist will have entirely different substrate constraints in the environment. In addition, the habitats to which fungal species are adapted should play a large role. On a global scale, the different seasonal climatic and vegetative cycles, will influence the presence of a nutrient sources, requiring a seasonal release of spores. On a local scale the habitats' microclimatic conditions, e.g., a sheltered forest atmosphere in comparison to open grasslands, will require differing strategies. Furthermore, meteorological factors are known to influence the fungal life cycle, from hyphal growth (van Laarhoven et al. 2015; Dowson, Boddy, and Rayner 1989; Donnelly and Boddy 1997) up to spore formation and liberation (Jones and Harrison 2004; Elbert et al. 2007), and will therefore ultimately also be major governing factors in the sporulation strategy. Moreover, insight into the influence of meteorological factors on sporulation is of critical importance to understand and estimate the impacts of climate change, which is especially important for agriculture, due to the high abundance of fungal plant pathogens. The identification of occurrence and abundancy patterns and the main environmental factors influencing them are of key importance when it comes to developing

forecast models or even large-scale global models, especially in view of the changing conditions in the Anthropocene.

As most studies dealing with temporal dynamics of the atmospheric microbiome focus on the taxonomic relations within the community as the foundation of analysis (Franzetti et al. 2011; Bowers et al. 2013, 2012; Yooseph et al. 2013; Fröhlich-Nowoisky et al. 2009, 2014, 2012), in this study, we aimed to focus more on the lifestyle-dependency of fungal atmospheric dynamics. To achieve this, we initially identify patterns in the temporal and size fraction occurrences and influence of meteorology in a dataset from a previous study by Fröhlich-Nowoisky et al., 2009, "High diversity of fungi in air particulate matter". The identified patterns are then explored in more detail by forming a subset of the 25 most abundant species and incorporating additional information, including nutritional and life-style preferences, fruiting form and spore dimensions. This allowed the identification of common patterns found amongst fungi adapted to similar lifestyles.

Material and methods

This study is based on the data obtained from a study by Fröhlich-Nowoisky et al., 2009. The following description is to be regarded as a short overview. For a detailed description of the applied laboratory methods please refer to the original publication.

Aerosol sampling

Sampling was conducted with a high-volume dichotomous sampler [self-built based on Solomon et al., "High-Volume Dichotomous Virtual Impactor for the Fractionation and Collection of Particles According to Aerodynamic Size" (Solomon, Moyers, and Fletcher 1983)] using sterilized glass fiber filters (PALL Corporation, Type A/A, 102-mm, sterilized at 500 °C for 12 h). The sampler was operated with a rotary vane pump (Becker VT.25) with a flow rate of 300 L min^{-1}, which corresponds to a cut-off of approx. 3 µm aerodynamic diameter, separating the aerosols in the air sample onto separate coarse and fine particulate matter filters. The coarse particles (> 3 µm) were collected through a virtual impactor, in line with the inlet, with a flow rate 30 L min^{-1} while the fine particles (< 3 µm) were collected perpendicular to the inlet, with a flow rate of 270 L min^{-1}. Furthermore, it should be mentioned that an estimated 10 % of fine particulate matter, due to sampler design, is collected in the coarse fraction (Solomon, Moyers, and Fletcher 1983).

The sampling site was in Mainz (130 m a.s.l), Germany, on the roof of the old 3-storey Max Planck Institute for Chemistry building on the campus of the Johannes Gutenberg University (49°59´31.36´´

N, 8°14´15.22´´ E). The sampler was attached to a mast approximately 5 m in height above the roof top.

The surroundings in the direct vicinity of the sampling location are predominately urban to the north and east, whilst strongly dominated by agriculture to the south and west. The first small forests, approximately 2-5 km^2 in size, can be found approximately 3.5-5 km in distance to the north-west and south west. The first large forests, of over 100 km^2 can be found to the north and east at a range of 10-15 km.

The samples were collected over a 13-month period (March 2006-April 2007). The individual sampling durations were generally seven days, corresponding to an air volume of approximately 3000 m^3, with a few exceptions which had durations of 1-5 days (~400-2000 m^3). Altogether 42 sets of coarse and fine particulate filters were analyzed, which amount to a combined sampling time of ~37 weeks in the 13-month period. Information concerning the individual samples can be seen in table S1 in the supporting information of Fröhlich-Nowoisky et al. (Fröhlich-Nowoisky et al. 2009). To rule out contaminations during the sampling procedure, additional controls were performed. Blank samples comprised filters that were not used for sampling but only sterilized and extracted alongside the actual samples ("extraction blanks") to monitor the quality of the DNA extraction process. Two sets of filters were used to control the sampling procedure, where one filter set was mounted in the sampler and the pump turned on for 5 s ("start-up blanks") and the other filter sample set was equally mounted in the sampler but the pump was not turned on ("mounting blanks"). The sampling blanks were collected at regular intervals (~4 weeks) after a thorough cleaning of the sampler, and extraction blanks were included in each extraction process. None of the tested blanks contained detectable DNA.

DNA extraction and sequence analysis

Total DNA was extracted from filter aliquots (1/8–1/4) using a commercial soil extraction kit (LysingMatrixE, FastDNASpin Kit for Soil, MP Biomedicals). The extracted DNA was amplified via PCR with multiple primer pairs that target the ITS1-5.8S-ITS2 and 18S regions of the rRNA region (see Fröhlich-Nowoisky et al. (Fröhlich-Nowoisky et al. 2009). The amplification products were cloned using the TOPO TA Cloning Kit (Invitrogen) and ~12 to 24 colonies were randomly selected for further analysis, based on blue-white selection. A PCR was then performed on the selected colonies, using the vector specific primers M13F-40 and M13R (Sigma-Aldrich). To monitor possible contaminations, PCR blanks were included in all runs. The isolated cloned fragments were then further processed by a restriction fragment length polymorphism (RFLP) analysis to avoid unnecessary sequencing of identical sequences. The Max Planck-Genome-centre Cologne, Germany (http://mpgc.mpipz.mpg.de/home/) performed the sequencing of the selected amplification products. In total 1513 sequences were

determined, of which 17 were removed due to chimeric results, resulting in 1496 sequences. The sequences were then grouped into 368 operational taxonomic units (OTUs) based on a 97 % sequence identity (OTU reference sequences accession numbers: FJ820489-FJ820856). Here, an OTU represents a hypothetical fungal species, as the intra-species variation of the ITS regions is usually below 3 % while the inter-species variation is on average 37 % (O'Brien et al. 2005; Schoch et al. 2012). The taxonomic affiliation of the OTUs was assessed using a BLAST search of the NCBI, whereby the OTUs were allocated to the lowest possible taxonomic level. The post-editing representative OTU sequences were on average ~630 base pairs in length. The relatively long sequences allowed Fröhlich-Nowoisky et al. to robustly identify the OTUs down to low taxonomic ranks (Approximately 30 % identified to species level, 25 % to genus level, remaining 45 % to family level or higher).

Dataset, subset, and additional information

Dependent on the performed analysis either the entire OTU dataset from Fröhlich-Nowoisky et al., 2009, as used or a subset of OTUs was used. The subset consisted OTUs found on at least 5 samples (roughly 10 % of the total samples) that were taxonomically identified to species- or genus-level. Furthermore, in the cases where multiple OTUs belonged to the genus and couldn't be differentiated on species-level, they were grouped indicated by *spp* (*species pluralis*) used in the used binomial name. The resulting OTU subset contained the most abundant 28 of the original 368 OTUs which as 25 distinct taxa, which will be referred to as species due to the nature of the ITS region. A table listing the subset fungi along with the number of contained OTUs and the abundancy in the coarse and fine filter samples can be found in the supplementary table S1.

The low taxonomic ranks within the subset allowed additional information on the species or their genera to be gathered from literature (details in supplementary table S1):

The most prominent fruiting type was defined. This resulted in five categories. Mold-like (9 species), yeast-like (9 species) fungi, for primarily asexually reproducing species, and corticoid (7 species), bracket-forming (4 species), apothecium-forming (1 taxon) fungi for primarily sexually reproducing fungi.

It was differentiated whether the fungi are primarily ligninolytic (11 species), i.e. white-rot fungi or primarily non-ligninolytic fungi (14 species) dependent on other non-woody carbon sources.

It was assessed if the species are known saprophytes or are known at least to have saprophytic life-stages (19/25 species). Also, if the species are known to contain obligate or opportunistic pathogens (19/25 species; No differentiation was made between necrotrophic or biotrophic pathogens), or whether the species are known to be surface inhabitants or symbionts of plants (2/25 species).

Finally, spore dimensions were gathered. In cases where the species are known to produce both sexual and asexual spores, the dimensions of the asexual spores were used as these, to our knowledge, usually outweigh the sexual spore in terms of number concentrations.

Statistical analysis

For the foundation of presented analysis, a relational database was created with MySQL (MySQL Community Server Version 5.6.29). In a first step, all the entire fungal dataset along with associated information, such as meteorological and literature data, were evaluated and a database structure created to store all relevant information. The database allowed the storage of all data relevant for analysis, including sequence information along with an OTU grouping and hierarchical taxonomic tree structure using the nested set model (Celko 2004). Furthermore, sampling information, including the sampling times, sampling volume, sampler type and sampler size fractioning, the location, was included.

Next to all data presented in Fröhlich-Nowoisky et al., 2009, and the additional information on the subset fungi, meteorological data from a weather station in Mainz-Mombach, provided by the ZIMEN Luft Messnetz of the Landesamt für Umwelt Wasserwirtschaft und Gewerbeaufsicht, were used. The meteorological data contain ½ h values for temperature, relative humidity, and precipitation and 1 h values for wind speed and direction for the entire 13-month sampling duration. For a unified taxonomic affiliation the higher taxonomic ranks of the species and genera were extracted from the catalogue of life (Roskov et al. 2015).

All calculations were performed either by direct database queries or using the script languages Python (Version 2.7; DB connector: MySQLdb module) or R Statistics (Version 3.1.0; DB connector: RMySQL Package).

A full list the formula described in the following, along with associated abbreviations, can be found in supplementary table S2.

To compare the OTU composition of the entire dataset between different air samples (β-diversity) the Bray Curtis Dissimilarity Index (*BC*) was, firstly, calculated between all TSP samples (total suspended particles: pooled OTUs from coarse and fine filter samples), and secondly, calculated between the samples subdivided into OTUs identified on coarse and fine fraction filter samples. A two-dimensional principle coordinate analysis (PCoA) was then performed on the resulting BC.

To compare the coarse and fine fractions occurrences of the subset fungi with their respective spore sizes a coarse-fine ratio ($\bar{S}$) was calculated:

$$\bar{S} = \frac{S_c - S_f}{S_{tot}} \qquad (1)$$

Here, S_{tot} is the total number of samples a taxon was found in, while S_c and S_f are the number of coarse or fine fraction occurrences, respectively. $\bar{S}$ can therefore vary between +1 and -1. A value of +1 indicates that a taxon was exclusively found in the coarse fraction, while -1 indicates occurrences exclusive to the fine fraction. A value of zero shows an even distribution between the size fractions. In Figure 4 the scale was replaced with fine (-1), even (0) and coarse (+1).

To compare $\bar{S}$ to the taxon-specific spore sizes, we calculated a shape-corrected theoretical aerodynamic diameter (d_a) for each subset taxon. To simplify calculations, non-spherical spores were treated as ellipsoids, which was true in most cases. Using the lengths and widths of the non-spherical spores the volumetric equivalent diameter (d_{vol}) was calculated (supplementary table S2).

The length to width ratio (q) allowed the calculation of the dynamic shape correction factor (κ) assuming the spore polar axis is orientated horizontal to the airflow (Davis and Schweiger 2002). A horizontal orientation should be true for settling spores of homologous density in still air, as the horizontal orientation maximizes air resistance. Using d_{vol} and κ the theoretical aerodynamic diameter (d_a) of the elliptical spores could then be calculated:

$$d_a = \sqrt{\frac{\rho_{spore}}{\rho_0 \kappa}} d_{vol} \qquad (2)$$

With ρ_0 being the unit density (1 g cm^{-2}) and ρ_{spore} being the spore density: as the species and genus spore densities could not be found in literature and are known to vary between species, a density of 1 g cm^{-2} was uniformly used. Therefore, for spherical spores $d_a = d_{vol} = d$ and for non-spherical spores $d_a = (1/\kappa)^{1/2} d_{vol}$

Monthly relative frequency of species occurrences (RFO = Number of samples species was found on / the total number of analyzed samples in the month) were calculated for the subset. Samples on the border of two months were allocated to the month which was sampled longest.

To assess whether there are observable correlations between RFOs and meteorological parameters, the Spearman's Rank coefficients were calculated, along with p-values which describe the probability of random variables producing the observed correlation.

The meteorological data within the individual sampling periods were also pooled into months. The monthly sampling period averages, standard deviation, maximum and minimum values for temperature, humidity, and wind speed were calculated. For precipitation, the sum precipitation was

calculated, along with an approximation of the duration of precipitation by summing every half hour with a precipitation larger than 0 mm and approximated precipitation strength (sum of precipitation / approximated duration).

Results and discussion

In the period from March 2006 to April 2007 in Mainz, Germany, 368 operational taxonomic units (OTU) isolated from the 42 analyzed air samples, comprising coarse and fine fraction particulate filters, were identified and discussed in Fröhlich-Nowoisky et al., 2009(Fröhlich-Nowoisky et al. 2009). Although the isolated DNA can potentially stem both from fungal spores and aerosolized hyphae. The hyphal fragments have, however, been found to be, at least, an order of magnitude lower in number concentration than spores (~10^2 m^{-3})(Pady and Gregory 1963). The discussed sequences can therefore be assumed to stem primarily from spores, which is also strengthened by size fraction occurrences discussed below.

To gain an overview of the temporal dynamics of the atmospheric fungal community, Figure 1 shows the total suspended particles (TSP), i.e., combined coarse and fine fraction, inter-sample variability in composition of the entire dataset using the Bray-Curtis-Dissimilarity-Index. In general, air samples in close temporal vicinity (near the diagonal) display a higher similarity when compared to samples taken half a year later, demonstrating a clear seasonality in the composition of atmospheric fungal community. However, the short-term sample-to-sample variability in OTU composition is not constant throughout the 13-month period. On the one hand the plot regions A and C, corresponding to spring until late summer 2006 and winter 2006 to spring 2007, display long periods of relatively high sample-to-sample similarity (<0.75), with winter 2006 showing the highest consistency compared to the other seasons. On the other hand, the plot region B, corresponding to late summer and autumn of 2006, displays a low sample-to-sample consistency (predominately >0.75), which indicates a period of high diversity and dynamic variability in the atmospheric fungal composition. These findings coincide with the findings in Fröhlich-Nowoisky et al., 2009, which showed the absolute fungal species richness when grouped into meteorological seasons was highest in autumn and lowest in winter, while spring was slightly more diverse than summer.

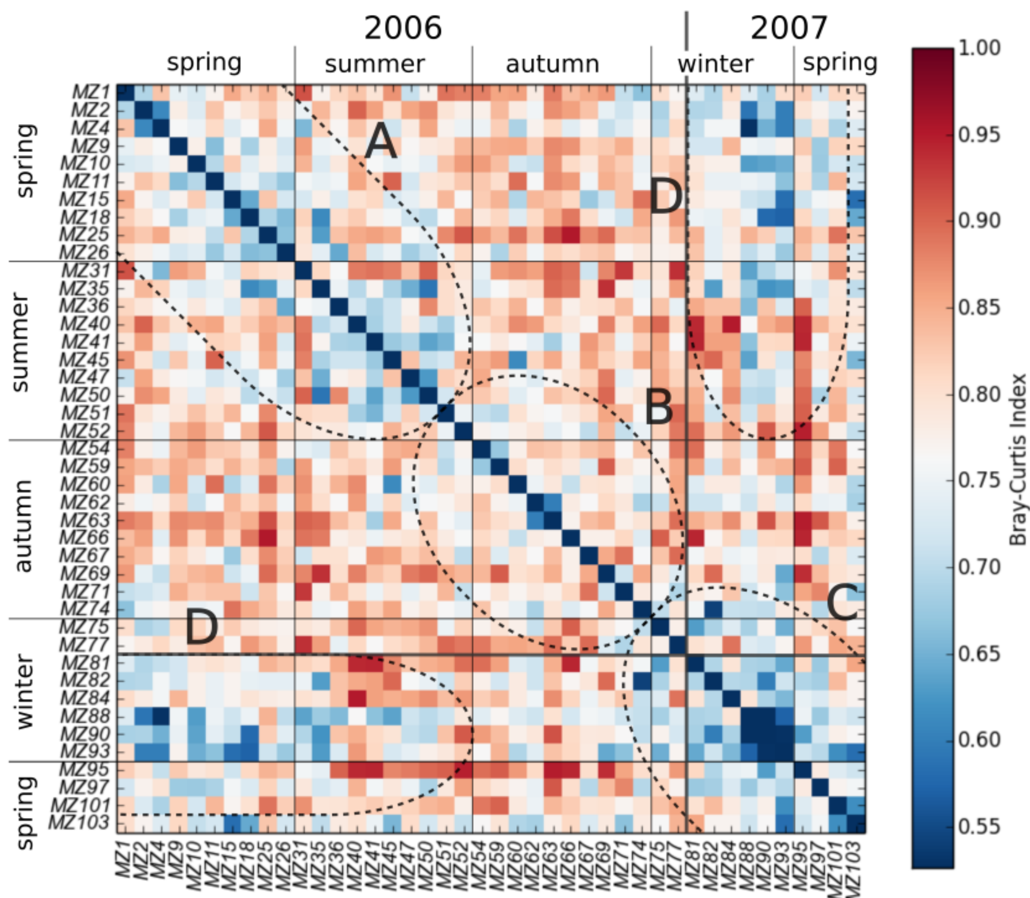

Figure 1: Inter-sample variability of OTU composition (β-diversity) for the entire dataset (TSP) using the Bray-Curtis Dissimilarity index. The consecutively numbered sample IDs of the sampling campaign are shown, which were also grouped into meteorological seasons. The Bray Curtis Index is the ratio of OTUs that differ between two samples, ranging from 1 being a completely different OTU composition to 0 being an identical composition. Note that the used color scale ranges from 1 to 0.52, as this was the lowest index calculated between two samples. A-D: Areas of interest referenced within the text.

Interestingly the end of winter 2006/07 (Figure 1 plot region D) displays high similarities to the samples taken in spring and even summer of 2006. The analyzed period in Germany was rather unique from a climatic perspective. According to the DWD (Deutscher Wetter Dienst), winter 2005/06 was the coldest in over a decade with an average temperature of -0.7°C while the winter of 2006/07 was, up to that time, the warmest in recorded history with an average temperature of +4.4°C (decadal winter time average 2001-10: +1.1°C). The unusually warm winter, is a potential explanation for the observed high similarities, which may have led to a much earlier sporulation onset and could be an indication of the far-reaching influence that a changing climate will have. The winter sample MZ 90 collected in mid-February with an average temperature 4.99 °C even shows similarities to summer samples comparable to the sample-to-sample similarities within the summer season itself. An earlier onset of sporulation might benefit the spread and growth of fungal pathogens, prolong sporulation periods and allow for

additional sporulation events for species with multiple generations per year. These in turn could have unforeseeable effects on, for example, agriculture, local flora and fauna, or the allergic sensitization and exposure towards fungal species (Boddy et al. 2014; Wolf et al. 2010; Gange et al. 2007; Fröhlich-Nowoisky et al. 2016).

To gain a deeper insight into the sample compositions and the hypothesized influence of temperature, in Figure 2a, a PCoA of the Bray-Curtis indices was performed. While the seasonal samples do, very roughly, cluster there is a clearer pattern observed when accounting for the average temperature during sampling. A diagonal increase in average temperature from the top-right to the bottom-left is seen, which also seems to account for the cases where there are larger distances between samples from the same season (temporally close samples). This is an indication that the composition of the airborne fungal community is to a certain extent influenced ambient temperature. This could be due to differing growth optima, an influence on sporulation, but also indirect influences such as a temperature influence on host vegetation. It should also be added that no patterns were observed when accounting for the other available meteorological parameters (seen in Supplementary Figure S1).

In figure 2b a PCoA was performed with separated coarse and fine filter sample OTU compositions. The coarse and fine samples show a clear vertical separation, albeit with some overlap. Furthermore, as shown by the lines connecting corresponding coarse and fine samples there are large differences and no discernable patterns observed between the fractions. Firstly, this indicates that there is a different composition in the coarse and fine fractions above and below the sampler cutoff of 3 µm, which is explainable by differing species-dependent spore sizes. Secondly, the size-dependent communities show little dependency on each other, i.e. clustering of coarse fraction samples does not necessarily lead a clustering of the corresponding fine fraction samples. This may be due to differing species-dependent sporulation strategies, e.g. species producing small spores may be aiming at different substrates than species that produce large spores, which could require different spore release patterns. Also, although it is conceivable that there is different influence of meteorological factors on coarse and fine fraction spore populations, no clear patterning (as seen 2a) was discernable when accounting for any of the meteorological parameters (seen in Supplementary Figure S1) in the separate size fractions.

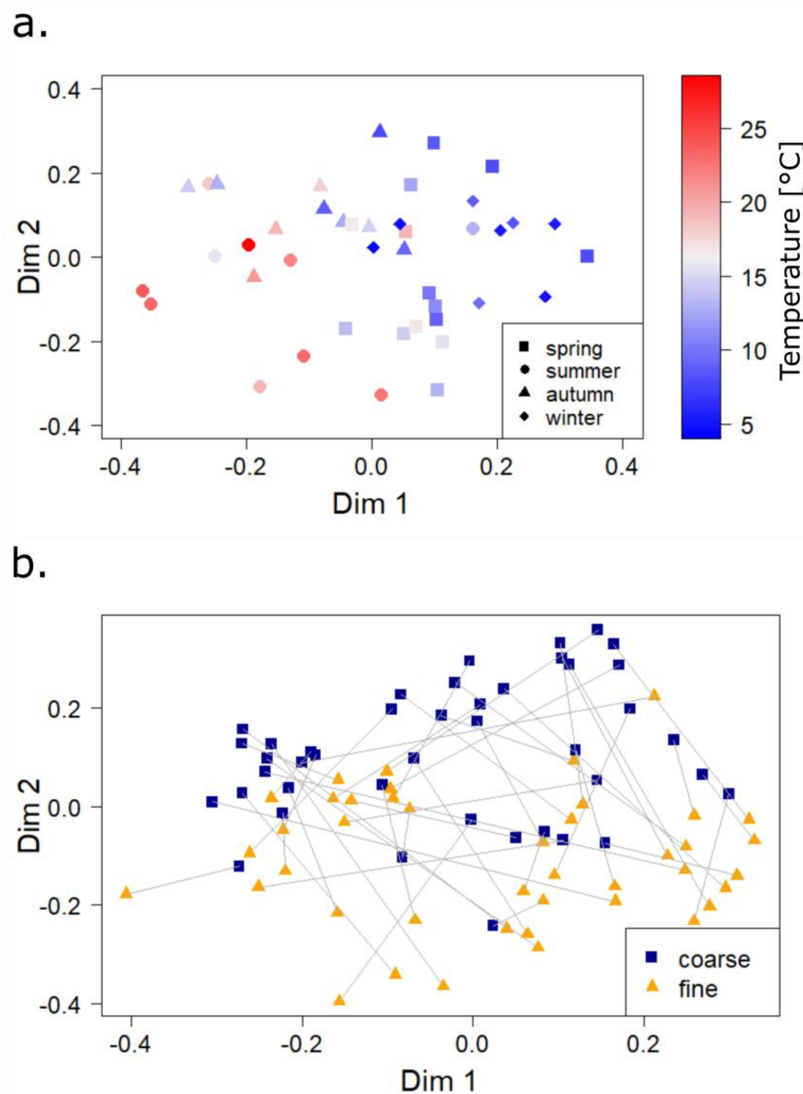

Figure 2: PCoA of Bray-Curtis Dissimilarities. a. PCoA of TSP samples (coarse + fine). The point colors correspond to the average temperature during sampling (color bar). The point shapes correspond to the season the sample was taken. b. PCoA of the separated coarse (blue) and fine (yellow) filter samples. The lines connect corresponding coarse and fine filter samples.

For a deeper insight into the observed seasonalities and the earlier sporulation observed in Figure 2, the RFO (relative frequency of occurrence), timelines of the subset fungi are shown in Figure 3. Boddy et al. (Boddy et al. 2014) investigated the influence of climate change on the development of the fruiting bodies of Basidiomycetes and reported an on average, 18-day earlier onset of springtime fruiting which was correlated with high winter temperatures (Boddy et al. 2014). Our results agree with their findings and point to a similar influence on Ascomycetes (forming the group of non-ligninolytic fungi). April and May of 2006 display a high diversity with nearly all species showing an atmospheric presence within the two-month period. Furthermore, the occurrence shift is especially obvious amongst pathogens and potential pathogens. For example, the non-ligninolytic obligate pathogens (*Ascochyta* sp. to *Itersonilia perplexans*) display mid to high RFO in late spring (May 2006),

which is observed again in mid to late winter (January and February 2007). Although, there is substantial research effort being invested into the influence of climate change on plant pathogens (Chakraborty 2013; Pautasso et al. 2012; West et al. 2012) due to the vast potential influences, there is further need to study and forecast the potentially diverse effects of a changing climate, such as rising temperatures or changing precipitation patterns, on the sporulation of fungi to develop strategies to limit harm to be expected.

In most cases the non-ligninolytic fungi, that is fungi involved primarily in the degradation or infection of non-woody herbaceous plant material, show a clear seasonality with one to two annual peaks that correspond to different stages in the annual life cycle of plants. The mold-like genera *Alternaria* and *Epicoccum* sp. to which many species of opportunistic plant pathogens belong, together with *Botryotinia fuckeliana* (anamorph *Botrytis cinereal)*, with a very large host range, display high RFO values throughout, from spring to late autumn, corresponding to the developing and fully developed vegetative stages of most plants in northern Europe. This reflects a generalist or unspecific sporulation strategy adoptable due to the unspecific nature of the pathogens. The more specialized obligate non-ligninolytic pathogens, *Ascochyta* sp. to *Itersonilia perplexans,* show more temporally confined atmospheric occurrences., in most cases early in the vegetative cycle of plants, aiming at the germinating and early plant developmental stages of plants. Additionally, in the case of *I. perplexans,* the cause of petal blight in its hosts (Gandy 1966), a second RFO peak is observed late in the year, presumably aiming at flowering host plants.

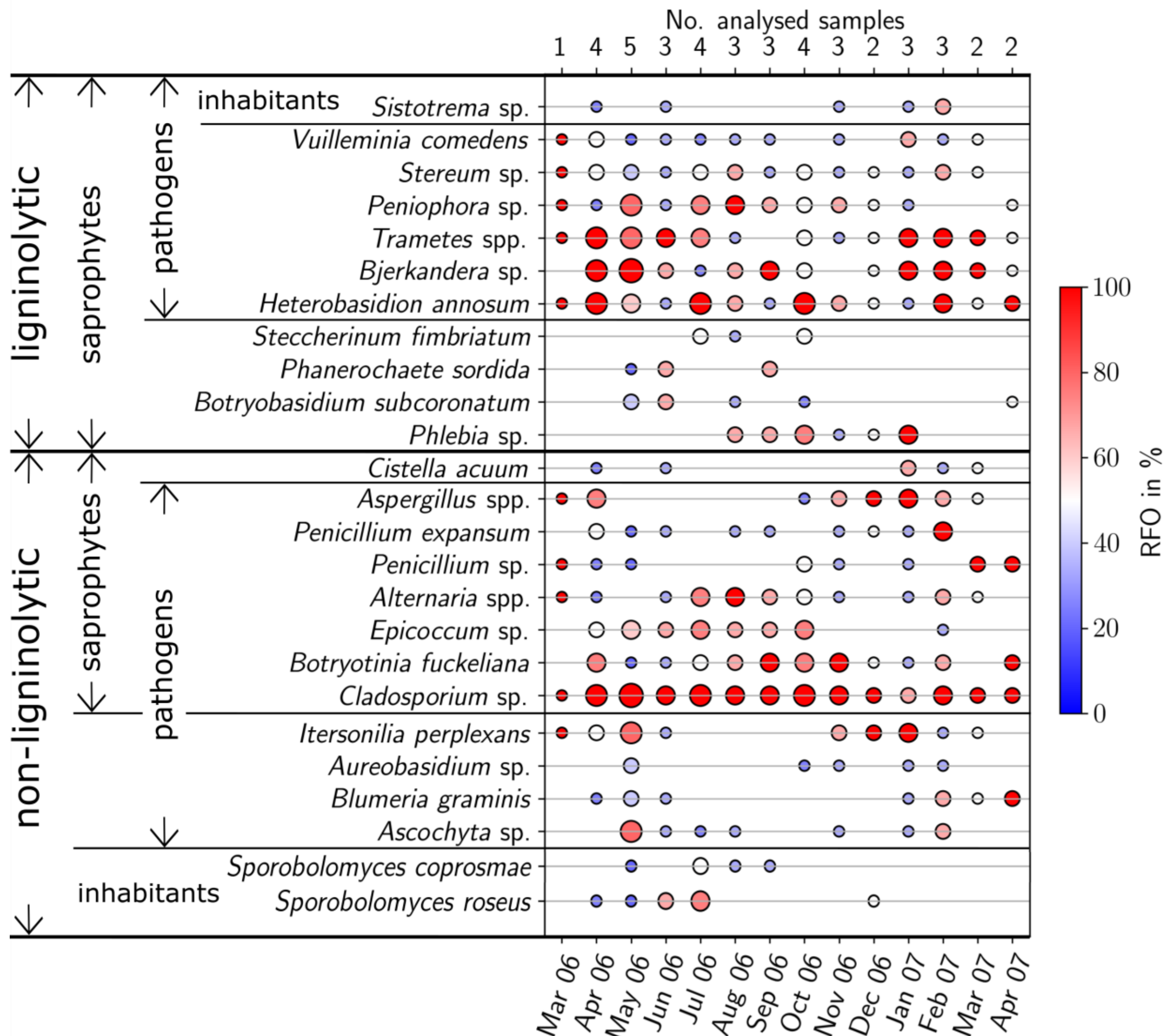

Figure 3: Temporal dynamics of the relative abundance of the subset fungi ordered according to the allocated lifestyle classification. The colors of the points correspond to the relative frequency of occurrence (RFO) for each species or genus and in a given month. The point sizes scale, both with the RFO-value and the number of analyzed samples for each month to additionally weight months with a higher number of analyzed samples (point size = RFO *no. analyzed samples/3).

Three genera of cosmopolitan molds, *Cladosporium Penicillium* and *Aspergillus* which are globally distributed and are mostly unspecific when it comes to habitats or substrates, are also observed. They are frequently amongst the most abundant atmospheric genera in studies (Fröhlich-Nowoisky et al. 2009, 2012; Larsen 1981; Mitakakis et al. 1997; Nayar and Jothish 2013; Shelton et al. 2002). *Cladosporium* sp. is the most abundant taxon in the study and is found in all but one sample, indicating a year-round high abundance. *Penicillium* sp. and *Penicillium expansum* were found with mostly mid to low RFO values spread throughout the year, indicating a continuous atmospheric presence. Furthermore, there were an additional six *Penicillium* OTUs identified in the dataset that didn't meet the criteria of the subset. The continuous presence of the mold genera reflects the unspecific nature

of their sporulation strategies, which can be adopted due to the ubiquity of potential substrate in the environment. *Aspergillus* spp. shows high spring but also winter RFO values, that might reflect the saprophytic nature of the isolated species, aiming at plant litter as a substrate.

In contrast to the non-ligninolytic fungi, the ligninolytic fungi show a distinct difference in occurrence patterns. These fungi to a large part will rely on wood degradation as one of their main energy sources. The saprophytic fungi, apart from *Phlebia* sp., mainly show sporadic occurrences and low RFO values, this might reflect the relatively large distance to larger forested areas (approx. 3.5 km) or even a shielding effect of the forest canopy reducing spore emissions into the free atmosphere. However, the fungi identified as ligninolytic pathogens or tree pathogens are amongst the most abundant identified within the study showing a year-round presence with frequently high RFO values. This may reflect the height of emission above the ground. Saprophytic fungi will mainly release their spores from fruiting bodies near the forest floor, while pathogenic fungi or fungi colonizing trees and dead attached branches will generally release their spores from a greater altitude into more turbulent airflow and therefore travel greater distances. It is also possible that tree pathogenic fungi produce significantly more spores over long periods of time compared to wood saprophytes. Many tree pathogens are known to form long-term fruiting bodies e.g., for *Bjerkandera adusta* which produces fruiting bodies year round (Kuo 2010). Such a sporulation strategy will heighten the infection probability in cases where, for example, a wounded or weakened host is needed, while saprophytes can generally rely on having dead wood in close vicinity to their emission source. So, like the cosmopolitan mold fungi, ligninolytic pathogens could be releasing vast quantities of spores with a less distinct seasonality not due to the ubiquity of substrate in the environment but rather due to its scarcity and therefore lower inoculation success rate.

To further investigate a possible influence of meteorological factors, as seen in Figure 2a. In Figure 4 the Spearman's rank correlation coefficients between the RFO values, seen in Figure 3, and the meteorological factors, seen supplementary Figure S1, are shown. The strongest correlations are observed with temperature and wind speed and only a few significant correlations are observed with relative humidity and precipitation, both known to be important factors for short-term spore release (Jones and Harrison 2004) . However, they primarily have more short-term influence on sporulation and atmospheric residence, which may be masked by the grouping into months.

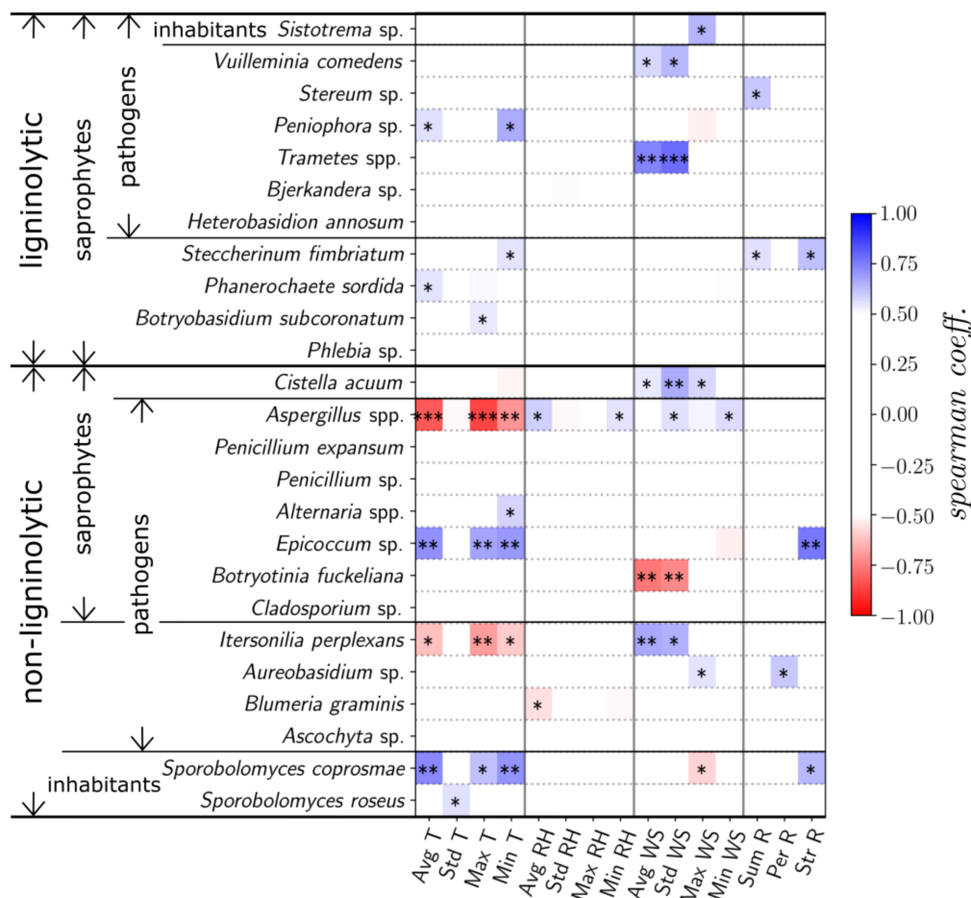

Figure 4: A Spearman's rank correlation matrix between the subset fungi RFOs in TSP with meteorological factors (shown in supplementary Figure S3). The values range from blue (+1) to red (-1), indicating a monotone increase or decrease, respectively, between the compared variables. The color scheme was chosen that begins coloration outside of threshold of -0.5 to 0.5. The p-value is the probability of a random dataset of the same size producing the same results ('*' < 0.05; ''<0.01; '***' < 0.001)**

The non-ligninolytic fungi show the strongest correlations. Temperature especially displays strong positive and negative correlations. Temperature is well known to be a main factor in germination hyphal growth and sporulation (Ayerst 1969; Tommerup 1983) which also makes sense for fungi with seasonal dispersal strategies, aimed at different stages in the vegetative cycle of plants. The ligninolytic fungi on the other hand show fewer sporadic correlations. The more significant correlations are observed with wind speed, seen for the species of *Trametes*. This might reflect the need for higher wind speeds for liberation from the forest canopy.

Aerodynamic properties of spores are also important as they are a determining factor in the potential atmospheric residence time and spore-deposition. In Figure 5 we show the calculated aerodynamic diameter for subset fungi against the ratio of the coarse to fine particle fraction occurrences (cut-off: 3 µm). Factors such as spore density, inhomogeneous density distribution and spore surface composition, will also affect a spore's aerodynamic properties. However, due to lack of information in literature, a uniform density of 1 g cm^{-3} was applied to all species. The few examples of densities

mentioned by Gregory (Gregory 1961) range between 0.56 – 1.44 g cm^{-3}, which would translate to a reduction or increase of the aerodynamic diameters by ~25 % or ~20 %, respectively. It is mentioned that spores are typically slightly denser than water, in the range of 1.1-1.2 g cm^{-2}. Also the hydration of the spores has been observed to have a large effect on the aerodynamic size of spores (Reponen et al. 2001). Despite these uncertainties, an obvious increase in calculated diameters can be observed in Figure 5 over the range from fine to coarse. This is also an indication that the isolated DNA predominantly stemmed from spores rather than hyphal fragments. The yeast-like *Sporobolomyces coprosmae* (19) seemingly contradicts the increasing trend, overweighing in the coarse fraction despite having relatively small spores. However, the larger vegetative cells of the plant surface inhabiting fungus (calculated aerodynamic diameter: 3.5 – 5.3 µm) may predominantly have been sampled as these can be aerosolized from the plant surfaces through splash-dispersal.

When considering the additional information, three groups can be identified (ellipses I-III). All the ligninolytic fungi, with exception of *Vuilleminia comedens*, lie within the central group III that are more or less evenly distributed between the size fractions. Non-ligninolytic fungi on the other hand display two distinct groups: Group I containing species predominant in the fine fraction to species evenly distributed between the size fractions, and Group II with species dominating in the coarse particle fraction. Group I exclusively contains the cosmopolitan mold fungi genera *Cladosporium, Penicillium* and *Aspergillus*, while Group II contains all plant pathogens and plant surface inhabiting fungi.

The three groups could again reflect different distinct dispersal strategies dependent on the primary environment and preferred substrates of the fungi. In general, a large aerodynamic diameter and therefore higher inertia has the benefit of being able to overcome the laminar airflow close to a plant's surface. This will result in a higher impaction probability compared to small spores that will tend to follow the airflow around the plant's surface. This is reflected by the species within Group II, all of which are plant pathogens, live on plant surfaces or in the case of ligninolytic *Vuilleminia comedens* known to inoculate dying, still-attached branches (Boddy and Thompson 1983).

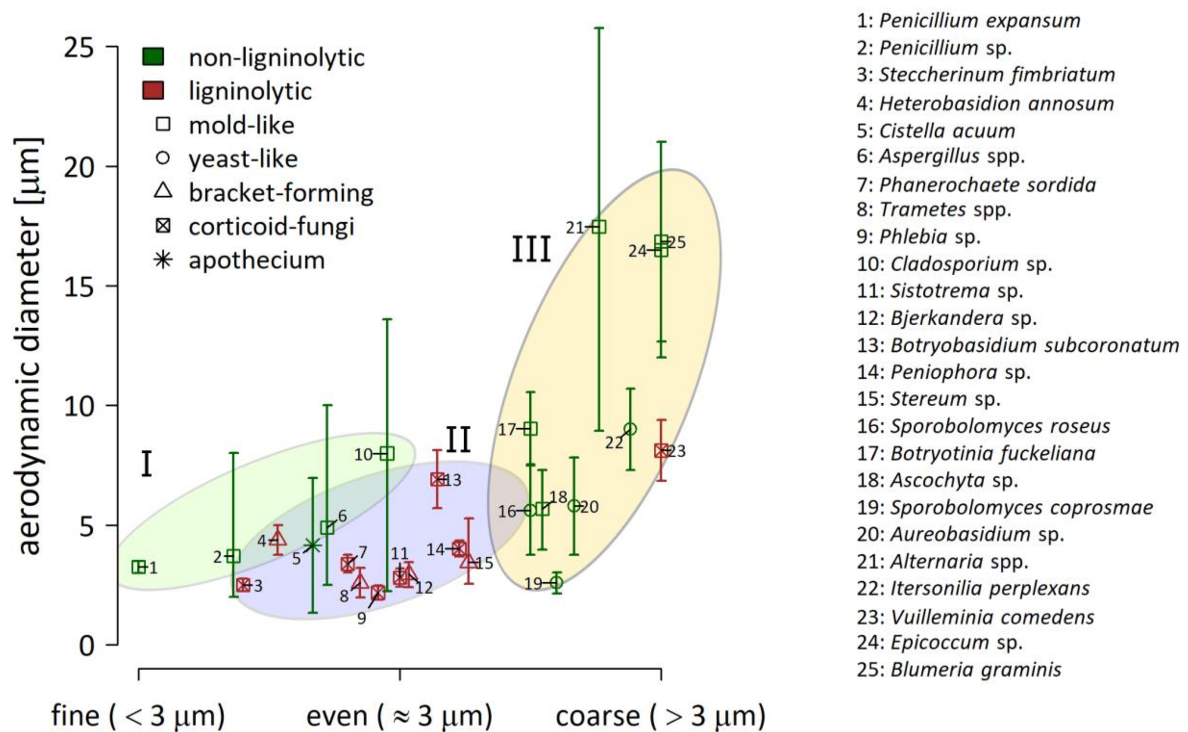

Figure 5: The estimated aerodynamic diameter against the size distribution for the subset fungi. The estimated aerodynamic diameter was calculated using spore dimensions found in literature (see supplementary table S2). The size distribution was calculated by subtracting the number of occurrences in the fine fraction from the occurrences in the coarse fraction and dividing the difference by the TSP occurrences. This shows which size fraction and to what extent the species were predominately found in. The color and point types correspond to the lifestyle categorization displayed in the legend. The shaded ellipses represent three identified clusters. I: hypothesized sedimentation adapted spores; II: hypothesized impaction adapted spores; III: hypothesized forest adapted spores.

Group I is exclusively made up of the cosmopolitan mold fungi (*Cladosporium*, *Penicillium* and *Aspergillus*) which are either evenly distributed between the fractions or overweigh in the fine fraction, i.e., display an aerodynamic diameter around or lower than the 3 µm cut-off. These genera are known to be very unspecific when it comes to substrates and environments. This may be reflected in the small spore sizes. The mold fungi can produce large quantities of small conidia and rely on sedimentation (e.g., on plant litter) for a successful inoculation. Furthermore, the asexual conidia are produced in chains. Dependent on conditions and the species, the mold fungi conidia are known to be liberated in a mixture of single spores and multi-spore chains (Gregory 1961). This leads to a broader aerodynamic size range and thereby does not rule out surface impaction. This may also be a factor playing a role in the even distribution of *Cladosporium* sp. between the fractions.

The ligninolytic fungi and, additionally, Cistella acuum, a degrader of pine needles, form Group III, with aerodynamic diameters around the sampler cut off of 3 µm. These fungi should all be concentrated in woody areas. Dead wood is not as ubiquitous as, for example, decaying plant litter, making a sufficient

spread, key to a successful inoculation. Furthermore, many tree pathogens, such as *Heterobasidion annosum,* can only infect wounded hosts and then further spread across the root systems. Damaged hosts and tree stumps under normal conditions will be relatively scarce, which would make a longer distance spread of a large number of spores essential. Moreover, the small spores which cluster around 3µm could be an adaptation to a forest environment. The forest canopy will act as a wind shield resulting in reduced wind speeds and turbulence. A small aerodynamic diameter will aid the spore spread in calmer conditions due to the longer atmospheric residence times in wind conditions that, dependent on forest type, can be reduced by a factor of four (Moon, Duff, and Tolhurst 2016).

In summary we were able to identify lifestyle-dependent patterns temporal and size fraction occurrences which to large part would have remained hidden by solely viewing the dataset from a taxonomic perspective. This approach opens up new possibilities for the analysis of the atmospheric microbiome. For instance, a similar classification scheme could be used as a simplification for larger-scale atmospheric and forecast modelling of bioaerosol emissions, making the source habitat emission estimates over time of parameters like number concentrations and physical characteristics of the different spore types a far less daunting task. We also presented evidence for a significant shift in early year sporulation due to the unusually warm winter of 2006/2007. This should be of concern for diverse fields, such as food security, agriculture and human health, which will be central challenges for the Anthropocene. Further systematic studies are needed to assess and prepare for potentially far-reaching effects of climate change on the atmospheric microbiome.

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Supplementary material

Figure S1 Meteorological factors

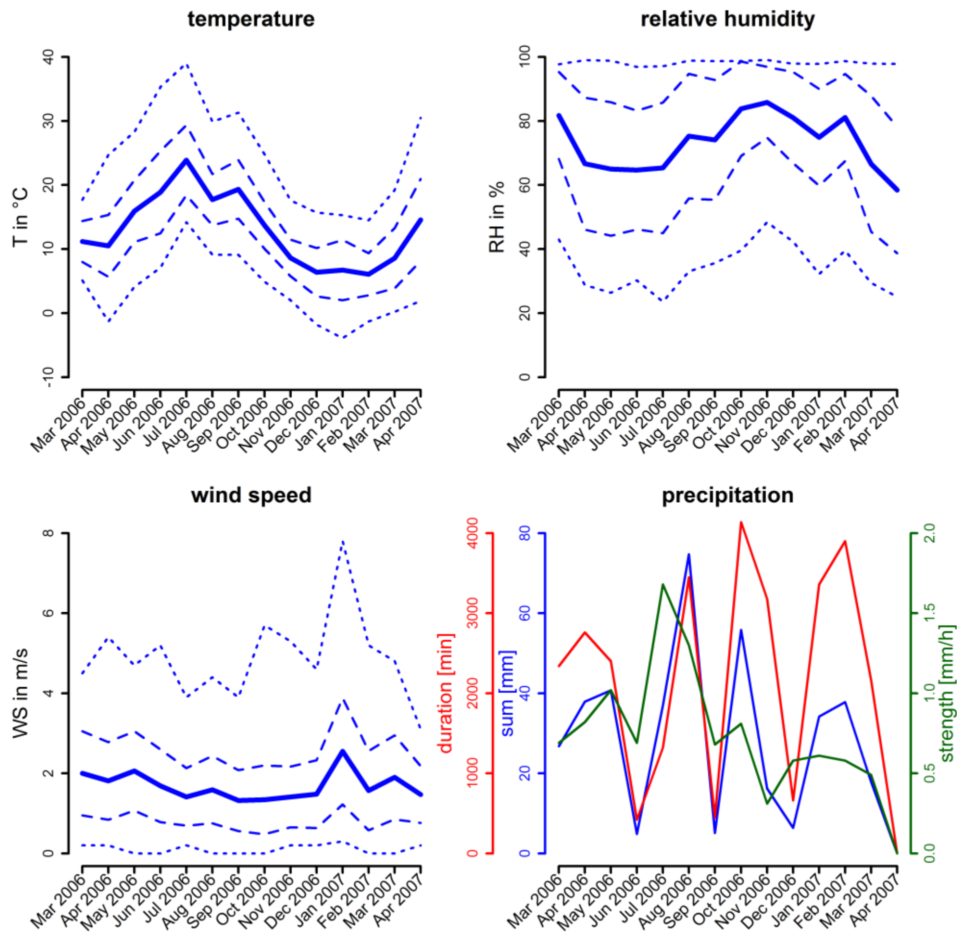

Figure S1: Overview of the meteorological factors used for the correlation analysis seen in figure S2. The values were calculated by grouping the values that lay within the sampling periods of the individual months. For temperature, relative humidity and wind speed the solid lines represent the averages, the dashed lines the standard deviation and the dotted lines the maxima and minima. For precipitation the line colors correspond to the three different y-axes.

Table S1 subset fungi

Table S1: Overview of the subset fungi. Dispersal is main means reproduction. The categories ligninolytic, saprophytic, pathogenic, inhabitant (symbionts or known non-pathogenic plant surface inhabitants) are marked with X if the trait is a significant lifestyle or nutritional type. The taxonomic ranks are from the Catalogue of Life database (Bisby et al. 2010). TSP, coarse and fine fractions are the numbers of samples (coarse and fine fractions combined), coarse and fine fraction occurrences. The OTUs refer to the IDs used in Fröhlich-Nowoisky et al. (Fröhlich-Nowoisky et al. 2009). In many cases taxon spore sizes and information were found in the Mycobank database (Robert et al. 2013) from which the citations were extracted.

Name	fruiting-form	ligninolytic	saprophytic	pathogenic	inhabitant	taxonomy	spore dimensions (l x w) in µm	OTU ID	TSP	coarse	fine	literature spore dimensions
Cladosporium sp.	mold-like		X	X		Davidiellaceae, Capnodiales, Dothideomycetes, Ascomycota	(3 - 35)x(2 - 10)	AMC1	41	35	37	(Al-Doory and Domson 1984)
Botryotinia fuckeliana	mold-like		X	X		Sclerotiniaceae, Helotiales, Leotiomycetes, Ascomycota	(9 - 12)x(7 - 10)	AMC2	24	20	8	(Gilman 1957)
Epicoccum sp.	mold-like		X	X		Pleosporaceae, Pleosporales, Dothideomycetes, Ascomycota	(12 - 21)	AMC3	18	18		(Schol-Schwarz 1959)
Alternaria spp.	mold-like		X	X		Pleosporaceae, Pleosporales, Dothideomycetes, Ascomycota	(18 - 83)x(7 - 18)	AMC5 AMC10	12 9	11 8	1 2	(Al-Doory and Domson 1984)
Penicillium sp.	mold-like		X	X		Trichocomaceae, Eurotiales, Eurotiomycetes, Ascomycota	(2 - 8)	AMC8	11	3	10	(Martinez, Calvo, and Ramirez 1982)

Penicillium expansum	mold-like		X	X	Trichocomaceae, Eurotiales, Eurotiomycetes, Ascomycota	(3 - 3.5)	AMC6	12		12	(De Hoog 2000)
Ascochyta sp.	mold-like			X	Not assigned, Pleosporales, Dothideomycetes, Ascomycota	(6 - 16)x(3.4 - 5.6)	AMC7	11	9	3	(Kovachevski 1936)
Blumeria graminis	mold-like			X	Erysiphaceae, Erysiphales, Leotiomycetes, Ascomycota	(25 - 38)x(10 - 17)	AMC9	10	10		(Sperr 1973)
Aureobasidium sp.	yeast-like			X	Dothioraceae, Dothideales, Dothideomycetes, Ascomycota	(7 - 13)x(3 - 6.5)	AMC12	6	5	1	(Kockova-Kratochvilova, Cernakova, and Slavikova 1980)
Cistella acuum	apothecium		X		Hyaloscyphaceae, Helotiales, Leotiomycetes, Ascomycota	(3 - 20)x(1 - 5)	AMC13	6	2	4	(Petersen and Læssøe, n.d.)
Aspergillus spp.	mold-like		X	X	Trichocomaceae, Eurotiales, Eurotiomycetes, Ascomycota	(2.5 - 10)	AMC4	13	7	7	(Noble, Lidwell, and Kingston 1963)
							AMC15	5		5	
Heterobasidion annosum	bracket	X	X	X	Bondarzewiaceae, Russulales, Agaricomycetes, Basidiomycota	(4.5 - 6.5)x(3.5 - 4.5)	BMC1	30	12	26	(Petersen and Læssøe, n.d.)
Bjerkandera sp.	bracket	X	X	X	Meruliaceae, Polyporales, Agaricomycetes, Basidiomycota	(4 - 5)x(2 - 3)	BMC2	29	22	21	(Shaw and Forest 1988)

Trametes spp.	bracket	X	X	X		Polyporaceae, Polyporales, Agaricomycetes, Basidiomycota	(4.5 - 6.5)x(1.5 - 2.5)	BMC3	25	16	19	(Ryvarden and Johansen 1980)
								BMC10	8	3	5	
Peniophora sp.	corticioid fungi	X	X	X		Peniophoraceae, Russulales, Agaricomycetes, Basidiomycota	(6.5 - 8)x(3 - 3.5)	BMC4	22	14	9	(Whelden 1936)
Stereum sp.	bracket	X	X	X		Stereaceae, Russulales, Agaricomycetes, Basidiomycota	(5 - 12)x(2 - 4)	BMC5	19	14	9	(Eriksson, Hjortstam, and Ryvarden 1978)
Itersonilia perplexans	yeast-like			X		Cystofilobasidiaceae, Cystofilobasidiales, Tremellomycetes, Basidiomycota	(12.5 - 17)x(6 - 9)	BMC6	17	16	1	(Ingold 1983)
Vuilleminia comedens	corticioid fungi	X	X	X		Corticiaceae, Corticiales, Agaricomycetes, Basidiomycota	(18 - 23)x(5 - 7)	BMC7	13	13		(Bernicchia and Gorjón 2010)
Phlebia sp.	corticioid fungi	X	X			Meruliaceae, Polyporales, Agaricomycetes, Basidiomycota	(3.5 - 4.5)x(1.5 - 2)	BMC8	12	6	7	(Bridge Cooke 1956)
Sporobolomyces roseus	yeast-like				X	Not assigned, Sporidiobolales, Microbotryomycetes, Basidiomycota	(7 - 14)x(3 - 6)	BMC9	8	7	3	(Ramírez Gómez 1957)
Botryobasidium subcoronatum	corticioid fungi	X	X			Botryobasidiaceae, Cantharellales, Agaricomycetes, Basidiomycota	(8 - 12)x(5 - 7)	BMC11	7	5	4	(Donk 1931)

Sistotrema sp.	corticioid fungi	X	X	X	X	Hydnaceae, Cantharellales, Agaricomycetes, Basidiomycota	(2.7 - 3.7)x(2.3 - 3)	BMC12	6	3	3	(Münzenberger et al. 2012)
Sporobolomyces coprosmae	yeast-like				X	Not assigned, Sporidiobolales, Microbotryomycetes, Basidiomycota	(2.5 - 5)x(2 - 2.5)	BMC14	5	4	1	(Hamamoto and Nakase 1995)
Phanerochaete sordida	corticioid fungi	X	X			Phanerochaetaceae, Polyporales, Agaricomycetes, Basidiomycota	(5 - 7)x(2.5 - 3)	BMC15	5	2	3	(Eriksson, Hjortstam, and Ryvarden 1978)
Steccherinum fimbriatum	corticioid fungi	X	X			Meruliaceae, Polyporales, Agaricomycetes, Basidiomycota	(3 - 3.5)x(2 - 2.5)	BMC16	5	1	4	(Bernicchia and Gorjón 2010)

1

1 **Table S2 Statistical abbreviations and formula**

Symbol	Definition
BC_{ij}	Bray Curtis Dissimilarity Index between sample i and j
C_{ij}	common OTUs found on sample i and j
$S_{i/j}$	number of OTUs found on sample i or j
	$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j}$
$\bar{S}$	coarse-fine ratio
$S_{tot/c/f}$	number of occurrences tot: In total; c: in the coarse fraction; f*: in the fine fraction*
	$\bar{S} = \frac{S_c - S_f}{S_{tot}}$
d_{vol}	volumetric equivalent diameter
d_a	aerodynamic diameter
w, l	spore width and length
Κ	dynamic shape correction factor
q	spore length to width ratio
ρ_0	unit density (1 g cm^{-3})
ρ_{spore}	spore density
	$d_{vol} = (w^2 \times l)^{\frac{1}{3}}$ $\kappa = \frac{8}{3} \frac{q^{-1/3}}{\left\{\frac{q}{(q^2-1)} + \frac{1}{\sqrt{q^2-1}}\left[1 - \frac{1}{2(q^2-1)}\right] \ln\left(\frac{q+\sqrt{q^2-1}}{q-\sqrt{q^2-1}}\right)\right\}}$ $d_a = \sqrt{\frac{\rho_{spore}}{\rho_0 \kappa}} d_{vol}$
$RFO_{x,y}$	Relative Frequency of Occurrence for taxon x in time period y
$N_{x,y}$	Number of samples tested positive for taxon x in time period y
$N_{tot,y}$	Number of total samples taken in time period y
	$RFO_{x,y} = \frac{N_{x,y}}{N_{tot,y}}$

2

C.4 Diversity, abundance, and seasonal dynamics of plant-pathogen Oomycetes in continental air

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Species richness, rRNA gene abundance, and seasonal dynamics of airborne plant-pathogenic Oomycetes

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Author contribution statement

J. F.-N., N.L.-Y., and D.A.P. wrote the paper. J.F.-N. U.P., V.R.D., N.L.-Y., D.A.P., D.T., and J.W. designed the research. J.F.-N. collected air samples and performed DNA extractions, cloning, and Sanger sequencing analysis. N.L.-Y. and I.M. performed qPCR analyses. D.A.P, D.T. and J.W. performed statistical correlations and analysis with meteorological data. J.F.-N, U.P., V.R.D., N.L.-Y, D.A.P., I.M., D.T., and J.W. discussed the results. All co-authors read and contributed to the manuscript.

Keywords

Airborne Oomycetes, Peronosporomycetes, plant pathogens, seasonal distribution, Sanger sequencing, qPCR analysis, Meteorological parameter

In review

Abstract

Word count: 234

Oomycetes, also named Peronosporomycetes, are one of the most important and widespread groups of plant pathogens, leading to significant losses in the global agricultural productivity. They have been studied extensively in ground water, soil, and host plants, but their atmospheric transport vector is not well characterized. In this study, the occurrence of airborne Oomycetes was investigated by Sanger sequencing and quantitative PCR of coarse and fine aerosol particle samples (57 filter pairs) collected over a one-year period and full seasonal cycle in Mainz, Germany. In coarse particulate matter, we found 55 different hypothetical species (OTUs), of which 54 were plant pathogens and 29 belonged to the genus Peronospora (downy mildews). In fine particulate matter (< 3 μm), only one species of Hyaloperonospora was found in one sample. Principal coordinate analysis of the species composition revealed three community clusters with a dependence on ambient temperature. The abundance of Oomycetes rRNA genes was low in winter and enhanced during spring, summer, and fall, with a dominance of Phytophthora, reaching a maximum concentration of ~1.6 X 10^6 rRNA genes per cubic meter of sampled air in summer. The presence and high concentration of rRNA genes in air suggests that atmospheric transport, which can lead to secondary infection, may be more important than currently estimated. Thus, further investigations combining DNA sequencing and quantification of airborne Oomycetes may be useful for improved forecasting and management of related plant diseases.

Ethics statements

(Authors are required to state the ethical considerations of their study in the manuscript, including for cases where the study was exempt from ethical approval procedures)

Does the study presented in the manuscript involve human or animal subjects: No

Species richness, rRNA gene abundance, and seasonal dynamics of airborne plant-pathogenic Oomycetes

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12 **Number of word:** 4243
13 **Number of figures:** 3

14 **Keywords: Airborne Oomycetes, Peronosporomycetes, plant pathogens, seasonal distribution,**
15 **Sanger sequencing, qPCR analysis, meteorological parameter**

In review

16 **Abstract**

17 Oomycetes, also named Peronosporomycetes, are one of the most important and widespread groups
18 of plant pathogens, leading to significant losses in the global agricultural productivity. They have
19 been studied extensively in ground water, soil, and host plants, but their atmospheric transport vector
20 is not well characterized. In this study, the occurrence of airborne Oomycetes was investigated by
21 Sanger sequencing and quantitative PCR of coarse and fine aerosol particle samples (57 filter pairs)
22 collected over a one-year period and full seasonal cycle in Mainz, Germany. In coarse particulate
23 matter, we found 55 different hypothetical species (OTUs), of which 54 were plant pathogens and 29
24 belonged to the genus *Peronospora* (downy mildews). In fine particulate matter (< 3 µm), only one
25 species of *Hyaloperonospora* was found in one sample. Principal coordinate analysis of the species
26 composition revealed three community clusters with a dependence on ambient temperature. The
27 abundance of Oomycetes rRNA genes was low in winter and enhanced during spring, summer, and
28 fall, with a dominance of *Phytophthora*, reaching a maximum concentration of ~1.6×10^6 rRNA
29 genes per cubic meter of sampled air in summer. The presence and high concentration of rRNA genes
30 in air suggests that atmospheric transport, which can lead to secondary infection, may be more
31 important than currently estimated. Thus, further investigations combining DNA sequencing and
32 quantification of airborne Oomycetes may be useful for improved forecasting and management of
33 related plant diseases.

34 ## 1 Introduction

35 The dispersal of pathogenic microorganisms through the atmosphere has major implications for
36 agriculture and public health. Some pathogens can travel over long distances, spreading diseases
37 across and even between continents (Brown and Hovmoller, 2002;Burrows et al., 2009a;Burrows et
38 al., 2009b;Womack et al., 2010;Després et al., 2012;Fisher et al., 2012;Fröhlich-Nowoisky et al.,
39 2016).

40 Oomycetes are one of the most economically important and widespread group of plant pathogens.
41 They are a diverse group of 'fungus-like` eukaryotic organisms distributed globally in diverse
42 environments and spreading through water, seeds, soil, and air (Göker et al., 2007;Dick, 2013;Beakes
43 et al., 2015). Historically, Oomycetes were classified as fungi due to their similarities in hyphal
44 organization and in nutrition (osmotrophy). However, since their molecular analysis in recent years,
45 they have been redefined as stramenopiles (or heterokonts), to which brown algae also belong
46 (Baldauf et al., 2000;Latijnhouwers et al., 2003;Andersen, 2004;Spring and Thines, 2004;Garcia-
47 Blazquez et al., 2008;Riisberg et al., 2009;Dick, 2013;Beakes et al., 2015).

48 Oomycetes mainly spread as zoospores, wall-less free-swimming cells, which are released from
49 sporangia during wet conditions (such as water splash, ground or underground water) and at
50 temperatures below 12°C (Walker and van West, 2007;Dick, 2013). They can generate survival
51 structures (formed for over-wintering, hot summer temperatures, or drought survival), i.e., thick-
52 walled chlamydospores (asexual) and oospores (sexual) that are temperature-resistant (Dick,
53 1995;Fay and Fry, 1997;Vercesi et al., 1999;Fry and Grünwald, 2010;Crone et al., 2013). Oospores
54 and sporangia can be dispersed through the air or attached to soil and plant particles (Kakde et al.,
55 2001;Docampo et al., 2011;Mallo et al., 2011;Delmas et al., 2014;Manzano et al., 2015). Moreover,
56 the spores are known to stay viable in soil up to 10 years (Judelson, 2008;Spencer-Phillips and Jeger,
57 2012). Under optimal growth conditions, these spores can germinate and infect host plants.

58 More than 60% of known Oomycetes species are plant pathogens, such as species from the families
59 Albuginaceae (white blister rusts), Peronosporaceae (downy mildews), and Pythiaceae (Aylor et al.,
60 1982;Göker et al., 2007;Walker and van West, 2007;Garcia-Blazquez et al., 2008;Voglmayr,
61 2008;Thines and Kamoun, 2010;Beakes et al., 2015). An understanding of their diversity, dynamics,
62 and spreading behavior in the atmosphere on local and larger scales is important to improve infection
63 risk prediction and disease management strategies (West et al., 2008). Improving monitoring and
64 forecast of infection risk would also be advantageous for both economic and environmental reasons:
65 curative treatment of already infected plants usually is more expensive and more stressful for the
66 environment than treatments which are applied before the disease actually infects the plants
67 (Scholthof, 2006;Bebber and Gurr, 2015). However, for an improved disease forecasting and
68 management a more precise knowledge of the time of arrival, diversity, and abundance of infectious
69 spores is necessary. Moreover, understanding the dynamics of plant pathogens and influences
70 thereupon is important in food security and climate change (Pautasso et al., 2012).

71 Because of the parasitic nature of many Oomycetes species they are often not detected with standard
72 culture-based methods (Arcate et al., 2006;Spring and Thines, 2010). Therefore, spore traps and
73 microscopy combined with meteorological data are used to forecast sporulation and infection risk
74 (West et al., 2008;Delmas et al., 2014). The development and application of DNA-based detection
75 and quantification methods for airborne Oomycetes could provide a faster monitoring and more
76 accurate disease forecast (Lévesque, 2011;Judelson, 2012). Furthermore, DNA-based methods enable
77 the detection of target organisms or genetic changes in pathogen populations by choice of primers,

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78 which dependent on specificity allow the detection of all, some, or selected organisms in a sample
79 (West et al., 2008).

80 Here, we combined DNA Sanger sequencing of the internal transcribed spacer (ITS) region with
81 qPCR analysis of ribosomal RNA genes to investigate the species richness, rRNA gene abundance,
82 and seasonal dynamics of airborne Oomycetes as well as their relationships with meteorological
83 factors in continental air over a one-year period.

84 2 Material and Methods

85 2.1 Aerosol sampling

86 Aerosol samples (57 pairs of fine and coarse particle samples) were collected on glass fiber filters
87 (Pall Corporation, Dreieich, Germany, Type A/E, 102 diameter) over one year in Mainz, Germany
88 (March 2006 - April 2007) as described previously (Fröhlich-Nowoisky et al., 2009) and detailed in
89 Table S1.

90 Briefly, a self-built high-volume-dichotomous sampler (Solomon et al. 1983), was operated with a
91 rotary vane pump (Becker, Wuppertal, Germany, Type VT 4.25) at a total flow rate of ~0.3 $m^3 min^{-1}$,
92 corresponding to a nominal cut-off diameter of ~3 µm. Thus, coarse particles with an aerodynamic
93 diameter larger than ~3 µm were collected on one glass fiber filter (~0.03 $m^3 min^{-1}$), while the fine
94 particles from the same air sample were collected on a second glass fiber filter (~0.27 $m^3 min^{-1}$). The
95 sampling period was generally ~7 days, corresponding to a sampled air volume of 3000 m^3. A few
96 samples were collected over shorter periods (volumes of ~400-2000 m^3). The sampling station was
97 positioned on a mast about 5 m above the flat roof of the three-story high Max Planck Institute for
98 Chemistry building located in the campus of the University of Mainz (49°59'31.36''N,
99 8°14'15.22''E). The sampled air masses represent a mix of urban and rural continental boundary
100 layer air in central Europe. To ensure that all filters were DNA free prior to sampling, all glass fiber
101 filters were baked overnight at 500°C prior to sampling and the loaded filters were packed in
102 aluminum foil (baked at 500°C) and stored at -80°C until DNA extraction.

103 To detect possible contaminants from the sample handling or the sampler, blank samples were taken
104 at 4-week intervals as previously described (Fröhlich-Nowoisky et al., 2009). Prebaked filters were
105 mounted in the sampler in a similar manner for regular sampling, but the pump was either not
106 activated ("mounting blanks") or activated only for 5 s ("start-up blank"), respectively.

107 2.2 DNA extraction and amplification

108 Filter aliquots (about ⅛ – ¼ of the filter) were extracted with a soil DNA extraction kit
109 (LysingMatrixE, FastDNASpin Kit for Soil, MP Biomedicals, Eschwege, Germany) according to the
110 supplier's instructions with the following modifications: After lysis the mixtures were centrifuged for
111 10 - 15 min, followed by an addition of 900 µL buffer (kit-supplied) and a second repeat of bead-
112 beating and centrifugation step. Both supernatants were combined for the further extraction process.
113 Finally, the DNA was dissolved in 100 µL elution buffer. Extraction kit blanks containing no filter
114 and baked filter blanks were included as extraction blanks.

115 For each DNA extract up to two PCR reactions were performed with the primer pair ITS4Oo/ITS5
116 and nested primer pairs ITS4Oo/ITS1 or ITS4/ITS5 (White T J, 1990;Nikolcheva and Bärlocher,
117 2004). The 50 µL reaction mixture contained 1 - 2 µL template DNA, 0.33 µM of each primer
118 (Sigma-Aldrich, Munich, Germany), 1× JumpStart™ PCR buffer (Sigma-Aldrich), 0.2 mM of each

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119 dNTP (Sigma-Aldrich) and 2.5 units of JumpStart^{TM} REDTaq DNA polymerase (Sigma-Aldrich). A
120 negative control containing no template DNA was included in all PCR runs.

121 The thermal cycling conditions (DNA Engine, Bio-Rad Laboratories, Munich, Germany) consisted of
122 an initial 3 min denaturation at 94°C, followed by 35 cycles of 30 s denaturation at 94°C, and 60 s
123 annealing at 49°C (for ITS4Oo/ITS5 and ITS4Oo/ITS1) or 30 s at 54°C (ITS4/ITS5), proceeded with
124 90 or 45 s, respectively of elongation at 72°C and 3 min of final extension at 72°C.

125 While PCR products were obtained for all coarse particle filter extracts, PCR amplicons were only
126 detected on six fine particle filter extracts. No DNA could be amplified from any of the six mounting,
127 six start-up, 12 extractions, and 36 PCR-blanks, indicating that no contamination occurred during
128 sample handling and analysis in the laboratory.

129 Amplification products for sequencing were cloned using the TOPO TA Cloning® Kit (Thermo
130 Fisher Scientific, Darmstadt, Germany) following the supplier's instructions. Colonies containing
131 inserts were identified by blue-white selection and lysed in 20 µL H_2O for 10 min at 95°C. The
132 inserts of 12-24 randomly picked colonies of each cloning reaction were amplified using 3 µL cell
133 lysate in a 40 µL reaction. The PCR reaction mixture contained 1× PCR buffer (New England
134 BioLabs, Frankfurt, Germany), 0.25 mM of each dNTP (New England BioLabs), 0.25 µM of each
135 primer (Sigma-Aldrich) and 1.25 units of Taq DNA Polymerase (New England BioLabs). The PCR
136 reactions were performed with the primer pair M13F-40 and M13R, and the thermal cycling
137 conditions consisted of an initial 5 min denaturation at 94°C, followed by 40 cycles of 30 s
138 denaturation at 94°C, 60 s annealing at 55°C, 60 s elongation at 72°C, and 15 min of final extension
139 at 72°C. Up to 12 colony PCR products per original PCR product were sequenced.

In review

140 The DNA sequences were determined with ABI Prism 377, 3100, and 3730 sequencers (Thermo
141 Fisher Scientific) using BigDye-terminator v3.1 chemistry the Max Planck-Genome-centre Cologne,
142 Germany (http://mpgc.mpipz.mpg.de/home/). The quality of all sequences was manually checked and
143 the vector sequences were cut. Out of 499 sequenced clones 30 sequencing reactions failed.

144 For comparison with known sequences, database queries using the Basic Local Alignment Search
145 Tool (BLAST) were performed via National Center for Biotechnology Information (NCBI,
146 http://www.ncbi.nlm.nih.gov/). Each of the remaining 469 sequences was identified to the lowest
147 taxonomic rank common to the top BLAST hits. Sixty sequences produced non-Oomycetes results
148 and 6 sequences were assumed to be chimeric results of PCR recombination of the ITS1 and ITS2
149 regions and were excluded from further analysis. The Oomycetes DNA sequences were grouped into
150 55 OTUs (similarity scores ≥ 97%; Table S2). Fifty-two OTUs, obtained by direct PCR
151 amplification, were used for the species richness analysis, whereas three (OTU 31, 32 and 55, Table
152 S2) were excluded, as they were obtained by co-amplification of the 16S region (in an Acidiobacteria
153 PCR with the primer pair Acid31/Eub518; Fierer et al. (2005)). For each filter, sequences that
154 produced the same BLAST results were pairwise aligned using the BioEdit program (BioEdit
155 Sequence Alignment Editor 7.2.5, http://www.mbio.ncsu.edu/bioedit/bioedit.html). Sequences with
156 similarity scores ≥ 97% were clustered into an operational taxonomic unit (OTU) and can be seen as
157 hypothetical species as the inner-species variability is lower than 3% (Robideau et al., 2011), with a
158 mean intra-species variability of 0.5% and a mean inter species variability of 30%. The sequences
159 from the obtained OTUs of the present study have been deposited in the GenBank database with the
160 accession numbers MF095126 - MF095180, detailed in Table S3.

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161 **2.3 Quantitative PCR**

162 The rRNA genes of four selected Oomycete taxa and total Oomycetes were quantified from coarse
163 particle filter extracts using the CFX96 quantitative PCR (qPCR) instrument (Bio-Rad Laboratories).
164 Selection was based on taxa identified by Sanger-sequencing. We used the SYBR green method for
165 *Pythium*, Albuginaceae, and *Peronospora* and for the total Oomycetes (Lang-Yona et al.,
166 2012;Müller-Germann et al., 2015), and the TaqMan probe method for the *Phytophthora* (Kox et al.,
167 2007) as specified in Table 1. All qPCR reactions were performed in triplicates of 10 µL mixtures.
168 The SYBR green reactions contained 5 µL SsoAdvanced universal SYBR green supermix (Bio-Rad
169 Laboratories), 1 µL extracted DNA, 500 nM of each primer (500 nM, Sigma-Aldrich), and 3 µL
170 sterile, filtered water (Sigma-Aldrich). TaqMan probe reactions contained 5 µL SsoAdvanced
171 universal probes supermix (Bio-Rad Laboratories), 1 µL extracted DNA, 500 nM of each primer (500
172 nM, Sigma-Aldrich) 200 nM probe (200 nM, Eurofins Genomics, Ebersberg, Germany), and 2.6 µL
173 sterile, filtered water (Sigma-Aldrich).

174 The thermal cycling conditions consisted of an initial 30 s or 2 min denaturation for SYBR green or
175 TaqMan method respectively, enzyme activation at 98°C, followed by 40 cycles of 10 s denaturation
176 at 98°C, and 25 s annealing for both reaction types, and extension at primer pair specific
177 temperatures as detailed in Table 1. The examination of the melt peaks confirmed amplification of
178 the single desired product.

179 The qPCR results are reported in gene copy number (GCN) per cubic meter of air. Oomycete genes
180 were not detected in mounting, start-up, extraction, and qPCR blanks, verifying that no
181 contamination occurred during sample handling and analysis.

In review

182 **2.4 gBlock DNA fragments and qPCR calibration**

183 Calibration curves were derived using two self-designed gBlock DNA fragments (IDT, Iowa, USA).
184 Fragment 'gBlock A' contains two binding sites and 'gBlock B' contains three binding sites for
185 primer pairs detailed in Table 1 and illustrated Figure S1. Cross amplification of primer pairs was
186 excluded using NCBI BLAST against Nucleotide collection (nr/nt) database. The designed amplicon
187 size is based on the theoretical amplicon sizes of the selected taxa (Table 1).

188 Amplification efficiencies, tested on the gBlock fragments were higher than 90% in all qPCR assays,
189 calculated from standard curves of 10-fold dilutions (10^9-10^1 rRNA gene copies). Limits of
190 quantification, as calculated from the standard curves were 2.5 ± 0.6, 1.8 ± 1.1, 1.4 ± 0.6, 1.3 ± 0.5,
191 and 1.0 ± 0.1 gene copies for total Oomycetes, Albuginaceae, *Phytophthora*, *Peronospora*, and
192 *Pythium*, respectively (Forootan et al., 2017).

193 **2.5 Meteorological data**

194 Local meteorological data (temperature, relative humidity (RH), atmospheric pressure, wind speed,
195 and precipitation) provided by the ZIMEN Luftmessnetz, Rheinland-Pfalz, Station Mainz-Mombach
196 were provided in half hour values. The values were averaged for each sample period and are detailed
197 in Table S1.

198 ## 2.6 Statistical analysis

199 The Pearson correlation coefficient was calculated between the quantitative PCR values and
200 meteorological factors, using OriginPro 9, to assess if there are potential significant linear
201 correlations (p-value < 0.05).

202 Bray-Curtis (BC) dissimilarity in OTU composition was calculated for all aerosol filter pairs.

203 $$BC_{ij} = 1 - \frac{2C_{ij}}{S_i + S_j} \quad (1)$$

204 Here C_{ij} is the number of OTUs per filter pair, i and j have in common and S is the number of total
205 OTUs (species richness) found on a filter pair i or j. A principle coordinate analysis (PCoA) was then
206 performed on the resulting BC distances. Diversity clusters were identified using EM algorithm,
207 using the R package mclust (vers.: 5.3) (Fraley and Raftery, 2002;Fraley et al., 2012).

208 # 3 Results and Discussion

209 The species richness and rRNA gene abundance of airborne Oomycetes were investigated over a one-
210 year period and full seasonal cycle in Mainz, Germany, and correlated with meteorological factors to
211 gain a better understanding of their seasonal dynamics in the atmosphere.

In review

212 ## 3.1 Species richness of airborne Oomycetes

213 Oomycetes were identified by PCR amplicon Sanger sequencing on all 57 coarse particle filters, but
214 only on one fine particle filter (Table S2). This is consistent with previous observations of an
215 enrichment of plant pathogenic fungi in the coarse particle fraction (Fröhlich-Nowoisky et al., 2009).
216 A larger aerodynamic diameter is beneficial for a plant pathogen as a higher inertia will aid the
217 impaction on a plants surface, thereby heightening the infection probability (Herries, 1961).
218 Moreover, plant pathogens associated with aerosolized plant tissue fragments or soil particles will be
219 concentrated in the coarse particle fraction (Després et al. (2012), and reference therein).

220 The 55 identified operational taxonomic units (OTUs) comprise up to 3.6-6.1% of the estimated 900-
221 1500 existing Oomycetes species (Arcate et al., 2006;Walker and van West, 2007;Dotzler et al.,
222 2008;Voglmayr, 2008;Sandle, 2014). Of all identified OTUs, 54 were identified as pathogens (Table
223 S2). No information on the pathogenicity was found for the single OTU identified as *Pythium*
224 *apiculatum.*

225 The detected OTUs were distributed over three families (Figure 1A). About 90% of the OTUs
226 detected in coarse particle filters belonged to widespread obligate pathogenic family of
227 Peronosporaceae to which all species of downy mildew belong. Albuginaceae (*Albugo* and
228 *Wilsoniana*), to which many of white rusts belong, and Pythiaceae (*Pythium*), containing species
229 known to cause root rot or damping off, represent 8% and 2% of the total identified OTUs,
230 respectively. The Peronosporaceae OTUs were assigned to three (out of eight to seventeen) reported
231 wind-dispersed genera (Riethmuller et al., 2002;Göker et al., 2004;Göker et al., 2007;Spencer-
232 Phillips and Jeger, 2012) i.e., *Peronospora* (57%), *Hyaloperonospora* (29%), and
233 *Pseudoperonospora* (4%). For just one OTU, discernment between *Peronospora* and
234 *Hyaloperonospora* was not possible. From the three families described above, 24% of the OTUs
235 were identified down to the species level (Table S2). The most abundant OTU, *Peronospora*

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236 *conglomerata*, a pathogen for geraniums (Farr and Rossman, 2017), was found on 30% of the coarse
237 particle filters of all seasons.

238 Although the genus *Phytophthora* was expected in the airborne fraction (Fall et al., 2015;Manzano et
239 al., 2015), it was not detected by Sanger sequencing using the ITS primer pairs. However, it was the
240 most abundant genus amongst the taxa quantified with qPCR. This is most probably due to a PCR
241 selectivity of the general Oomycetes primers (albeit that the primers were previously reported to
242 amplify *Phytophthora* spp.; Nikolcheva and Bärlocher (2004)). Further evidence for selectivity was a
243 successful co-amplification of sequences that best matched *P. ramorum* (sequence similarity of 97%)
244 using primers aiming at 16S rRNA of prokaryotes. This pathogen has a wide host range of 75 plant
245 genera (e.g., oak, larch, rhododendron). *Phytophthora ramorum* was found in samples that were
246 mostly taken in periods of higher humidity and rain (apart from MZ 15) during fall (Table S1). This
247 corresponds to the spreading mechanism of *P. ramorum*, by airborne spores carried by wind-blown
248 rain (Grunwald et al., 2008). The co-amplification in turn implies significant concentrations of
249 airborne *P. ramorum* as it was the single oomycete identified amongst the abundant atmospheric
250 bacterial population.

251 Seasonal dynamics of OTU composition of all analyzed samples is shown in Figure 1B. The BC
252 dissimilarity index between two samples can vary between 1, indicating a completely different OTU
253 composition, and 0, indicating an identical composition. Samples with half year differences, in
254 general, show higher dissimilarities (values between 0.8 to 1) than samples in close temporal vicinity
255 (along the diagonal) which show higher consistencies in OTU composition. This is not surprising in
256 the seasonally changing climate of northern Europe as the plant pathogenic Oomycetes community
257 structure will change with the annual vegetation cycle and will also change due to favorable
258 meteorological conditions.

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259 Samples taken at the late fall, winter, and spring show comparatively similar compositions. The
260 summer samples, however, show a high sample-to-sample consistency (~0.5 and lower), and a nearly
261 completely different OTU composition than samples from other seasons, apart from a transitional
262 phase in the first half of the fall season. This again can be explained by the vegetative cycle of host
263 plants. Different species of oomycetes are adapted to infect the different annual developmental stages
264 of the hosts, such as leaf- or fruit-development (Latijnhouwers et al., 2003;Thines and Kamoun,
265 2010). The high consistency between winter 2007 and the two spring seasons could be due to the
266 aerosolization of soil particles during winter which contain soil dwelling oomycetes species.

267 Principle coordinate analysis (PCoA) of the BC revealed three OTU composition clusters (Figure 2
268 A). However, the community clusters don't seem to be primarily defined by a distinct seasonality
269 (Figure 1B), but rather through the average ambient temperature during sampling (distinguished by
270 point color) as each cluster contains samples from at least three seasons (distinguished by point
271 shapes). Other meteorological parameter, such as mean relative humidity, wind speed, sum and
272 duration of precipitation, did not show correlations with OTU clusters. Therefore, the clusters were
273 named "warm", "intermediate", and "cold". The mean temperatures for the different clusters are 9,
274 14 and 20°C (Figure 2B). Furthermore, the temperature distributions of the three clusters, analyzed
275 by the Wilcoxon Rank test, were significantly different from another (p-value $< 10^{-16}$). The cold
276 cluster had the lowest number of OTUs (22), which all fell in the two genera *Hyaloperonospora* and
277 *Peronospora* (Figure 2C). The intermediate and warm clusters display higher species richness
278 consisting of 35 and 28 OTUs, respectively. Taxonomically, both clusters are similar, with slight
279 differences. The intermediate cluster contains the non-classified Peronosporaceae, while the warm
280 cluster contains the only non-pathogenic OTU from the genus *Phytium*. A similar temperature

281 dependency has been previously shown to influence the distribution and seasonal abundancies of
282 various *Halophytophthora* species in river water (Nakagiri, 2000). The changing atmospheric
283 community structure can be explained by two possible scenarios: A rapid response of the
284 phyllospheric Oomycetes community composition, shifting the abundance towards other species,
285 with changing temperatures, or an influence of temperature on the emission or sporulation process, of
286 different species. Sporangia are very sensitive to mild changes in temperature (Byrt and Grant,
287 1979;Suzaki et al., 1996), and zoospore formation has been shown to be induced by a drop in
288 ambient temperature (Hardham and Hyde, 1997).

289 **3.2 Ribosomal RNA gene abundance of airborne Oomycetes**

290 To evaluate the abundance and seasonal variation of airborne Oomycetes in coarse particulate matter,
291 rRNA genes of selected taxa and total Oomycetes were quantified using qPCR with the primer pairs
292 detailed in Table 1. The concentration of rRNA genes for total Oomycetes ranges between ~1.4×10^4
293 up to ~5.1×10^6 GCN m^{-3} (Figure 3A and Table S4). Highest concentrations of rRNA genes were
294 observed for *Phytophthora* (ranging between 4.3×10^3 to ~1.8×10^6 GCN m^{-3}), while Albuginaceae
295 and *Peronospora* had lower values (ranging between 0 to ~3.3×10^4, and 0 to ~1.3×10^4 GCN m^{-3},
296 respectively). *Pythium* values were lowest (with maximum of ~33 GCN m^{-3}; MZ 11).

297 The seasonal averages of rRNA gene abundance are shown in Figure 3B. Total Oomycetes,
298 *Phytophthora*, and *Peronospora* did not differ significantly during spring, summer, and fall, with
299 highest levels observed during summer (~$1.6 \times 10^6 \pm 7.0 \times 10^5$, ~$4.9 \times 10^5 \pm 2.4 \times 10^5$, and
300 ~$1.7 \times 10^3 \pm 9.6 \times 10^2$ GCN m^{-3}, respectively). This corresponds to the seasons with the highest host
301 plant availability in Europe, and conditions most beneficial for growth (Ellenberg, 2009). The
302 Albuginaceae exhibited highest values in fall (~$8.1 \times 10^3 \pm 5.0 \times 10^3$ GCN m^{-3}), while no significant
303 seasonal variation and low rRNA gene abundances were observed for *Pythium*. All other taxa had
304 lowest concentrations in winter, with ~$1.7 \times 10^5 \pm 6.2 \times 10^4$, ~ $6.0 \times 10^2 \pm 4.5 \times 10^2$, ~$7.3 \times 10^4 \pm$
305 3.5×10^4, and ~5.8 ± 2.9 GCN m^{-3} for total Oomycetes, Albuginaceae, *Phytophthora,* and
306 *Peronospora*, respectively. Winter in central Europe is characterized by low vegetative yield
307 (Ellenberg, 2009). Thus, a lower abundance of airborne plant pathogens can be expected. This
308 finding is in concordance with previous studies of fungal spore abundance, reporting lower spore
309 counts for lower outdoor temperatures (Tang (2009), and references therein).

310 Disease warning systems for plant pathogenic Oomycetes are based on temperature and leaf wetness,
311 under the assumption that certain leaf moistures and temperatures will allow efficient infection of
312 plants (Abraham et al., 1995;Madden et al., 2000;Gilles, 2004;Henderson et al., 2007;Reis, 2013).
313 Our results show a medium to high positive correlation of total Oomycetes rRNA GCN m^{-3} with RH
314 only during fall (See Table S5). No significant correlations were observed with temperature and
315 precipitation. The inconsistency between the correlations found in our study and the disease
316 forecasting system might be due to different factors, such as the 7-day sampling periods used in this
317 study. Furthermore, while plant infection risk is correlated with RH and temperature (Palmieri et al.,
318 2006;Li et al., 2014;Morales et al., 2018), atmospheric gene abundance of Oomycetes may not. The
319 positive correlation with RH in fall could indicate preferential aerial transport under humid
320 conditions, which is in agreement with previously reviewed dispersal of fungal and Oomycetes
321 pathogens in tropical areas (Drenth and Guest, 2016).

322 As discussed above, temperatures might relate to preferential distribution of Oomycetes in a non-
323 linear manner, i.e., specific temperatures trigger release of certain species (Jones, 2014). The
324 differential influence of meteorological factors on oospore release is outlined in Fry et al. (2008).

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Rain intensity and precipitation type (i.e., rain, drizzle, fog, etc.) could also affect the distribution of aerosolized Oomycetes. For example, prolonged rain will lead to wet deposition and therefore a washing of the atmosphere (Hemond and Fechner, 2015). However, short intensive rain may induce highly efficient release of oospores and Oomycetes fragments through a splash effect (Huffman et al., 2013), whereas a light drizzle could only have a minimal effect on aerosolization or deposition. Therefore, to estimate correlation with rain, further characterization of the precipitation type is required. Zoospores are free swimming in water films and can settle on surfaces and retract their flagella (Hardham and Hyde, 1997;Walker and van West, 2007). A subsequent secretion of a mucilaginous matrix affixes them to the surfaces, e.g., soil particles or leaf fragments which can then be aerosolized.

To further visualize common patterns in atmospheric presence, the rRNA gene abundance of the individual taxa was normalized by calculating the ratio to the highest taxon-specific value (Fig. 3C). Common periodical tendencies with similar sample-to-sample dynamics are observed amongst all taxa. This again is most probably an indication of a passive influence affecting the atmospheric presence of all taxa, such as meteorological factors influencing emission and deposition. Additionally, in Figure 3D the gene abundance of the different taxa in individual air samples display a linear correlation with the gene abundance of total Oomycetes. The linear tendency indicates near constant proportions of single taxa within the total airborne Oomycetes, which might be due to dispersal mechanisms (e.g., attached to soil particles or plant fragments (Sutton et al., 2006)), rather than species-related emissions (e.g., wind-dispersed sporangia etc.).

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Our results demonstrate the presence of plant-pathogenic Oomycetes over a one-year period and full seasonal cycle in Mainz, Germany. Species composition analysis revealed occurrences of three plant-pathogenic families with seasonal dynamics and three community clusters with a dependence on ambient temperature. Higher concentrations of Oomycete rRNA genes in spring, summer, and fall, imply higher atmospheric transport rates in those seasons. The complementary input of the two methods combined in this study underlines the importance of parallel approaches in microbial ecology, where supportive analyses could help answering complex questions in this field. Further investigations and monitoring of airborne Oomycetes, combining high throughput DNA sequencing with quantitative approaches may be useful for improved forecasting and disease management.

4 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5 Author Contributions

J. F.-N., N.L.-Y., and D.A.P. wrote the paper. J.F.-N. U.P., V.R.D., N.L.-Y., D.A.P., D.T., and J.W. designed the research. J.F.-N. collected air samples and performed DNA extractions, cloning, and Sanger sequencing analysis. N.L.-Y. and I.M. performed qPCR analyses. D.A.P, D.T. and J.W. performed statistical correlations and analysis with meteorological data. J.F.-N, U.P., V.R.D., N.L.-Y, D.A.P., I.M., D.T., and J.W. discussed the results. All co-authors read and contributed to the manuscript.

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593 ## 9 Tables

594 **Table 1. Primers used for qPCR.** The amplified region, primer names, sequences, length, and
595 annealing temperatures of the different qPCR primer sets for analysis of the total Oomycetes,
596 Albuginaceae, *Phytophthora*, *Pythium*, and *Peronospora***.**

Target	Amplified region	Primer name	Sequence	Amplicon length (bp)	Annealing temperature (°C)	Reference
Oomycetes	18S rRNA	AFP293-F AFP294-R	TTTCCGTAGGTGAACCTGCG GCGAGCCTAGACATCCAC	220-300	65	(Brouwer et al., 2003)
Albuginaceae	5.8S+18S rRNA	Albug-F Albug-R	GCTTCGGCTTGACACATTAG TCCGTCTCCTTGATGACCTT	93	62	(van Molken et al., 2014)
Phytophtora	18S rRNA	15Ph-F 279Ph-R All-Phy-P	TGCGGAAAGGATCATTACCACACC GCGAGCCTAGACATCCACTG FAM-TTGCTATCTAGTTAAAAGCA- TAMRA	248	60	(Kox et al., 2007)
Pythium	18S rRNA	Pyth664-F Pyth712-R	GCCCTTTCGGGTGTGTTACTAG CTGAATGGCAGAAGAACATCCTC	66	60	(Thomas et al., 2011)
Peronospora	5.8S+18S rRNA	Peron-F Peron-R	CACGTGAACCGTATCAACC GATAGGGCTTGCCCAGTAG	98	62	(Hukkanen et al., 2006)

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597 ## 10 Figure captions

598 **Figure 1. Species richness and seasonal dynamics in composition of airborne Oomycetes.** (A)
599 Relative proportions of different genera (n.c. = not classified), and (B) heatmap of Bray-Curtis
600 dissimilarity in OTU composition between all analyzed samples. The Bray Curtis index can vary
601 between 0, for an identical OTU composition, and 1, for no common OTUs on the samples.

602 **Figure 2. Temperature dependency of OTU composition.** (A) Principle coordinate analysis of
603 Bray-Curtis dissimilarities, revealing three temperature-dependent clusters with high, intermediate,
604 and low average sampling temperatures. Point shape represents the sampling season, and color
605 represents the average temperature for each aerosol filter pair. (B) Temperature distributions of the
606 clusters (middle band: median, box: the 25th to 75th percentile, whiskers: 95% confidence interval.
607 (C) Relative proportions of different genera within the three clusters (n.c. = not classified).

608 **Figure 3. Oomycetes rRNA gene abundance retrieved from qPCR analysis.** (A) The abundance
609 (rRNA genes m^{-3} air) of selected taxa and total Oomycetes in coarse particle filter samples. Error bars
610 represent standard deviation of triplicates. (B) Average seasonal rRNA gene abundance for selected
611 taxa and total Oomycetes. Boxes limit 25 and 75% percentile, median presented as line, and mean
612 values as point inside, connected in line. Error bars present 1% and 99% percentile. Outliers are
613 shown (Student´s two sample *t*-test p-value < 0.01). (C) Gene abundance of selected taxa and total
614 Oomycetes scaled to maximal taxon-specific values. (D) Gene abundance of selected taxa scaled to
615 total Oomycetes gene abundance and correlated with gene abundance of the selected taxa. Color
616 codes in all panels: total Oomycetes marked as black squares, Albuginaceae as green inverted
617 triangle, *Phytophthora* as red circles, *Peronospora* as orange triangles, and *Pythium* as blue diamond.

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Figure 1.TIF

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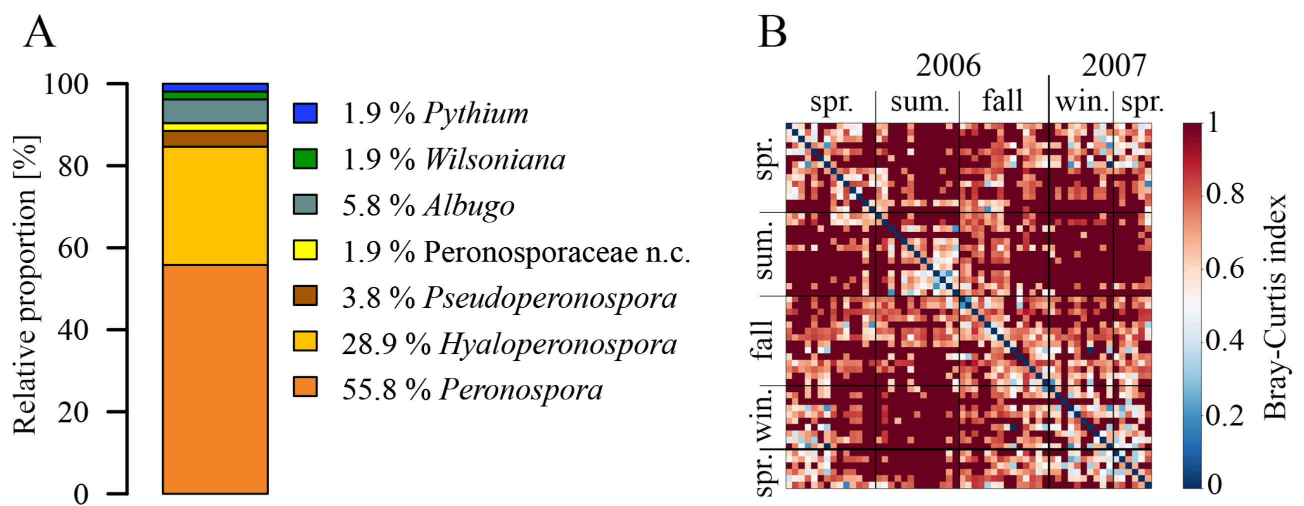

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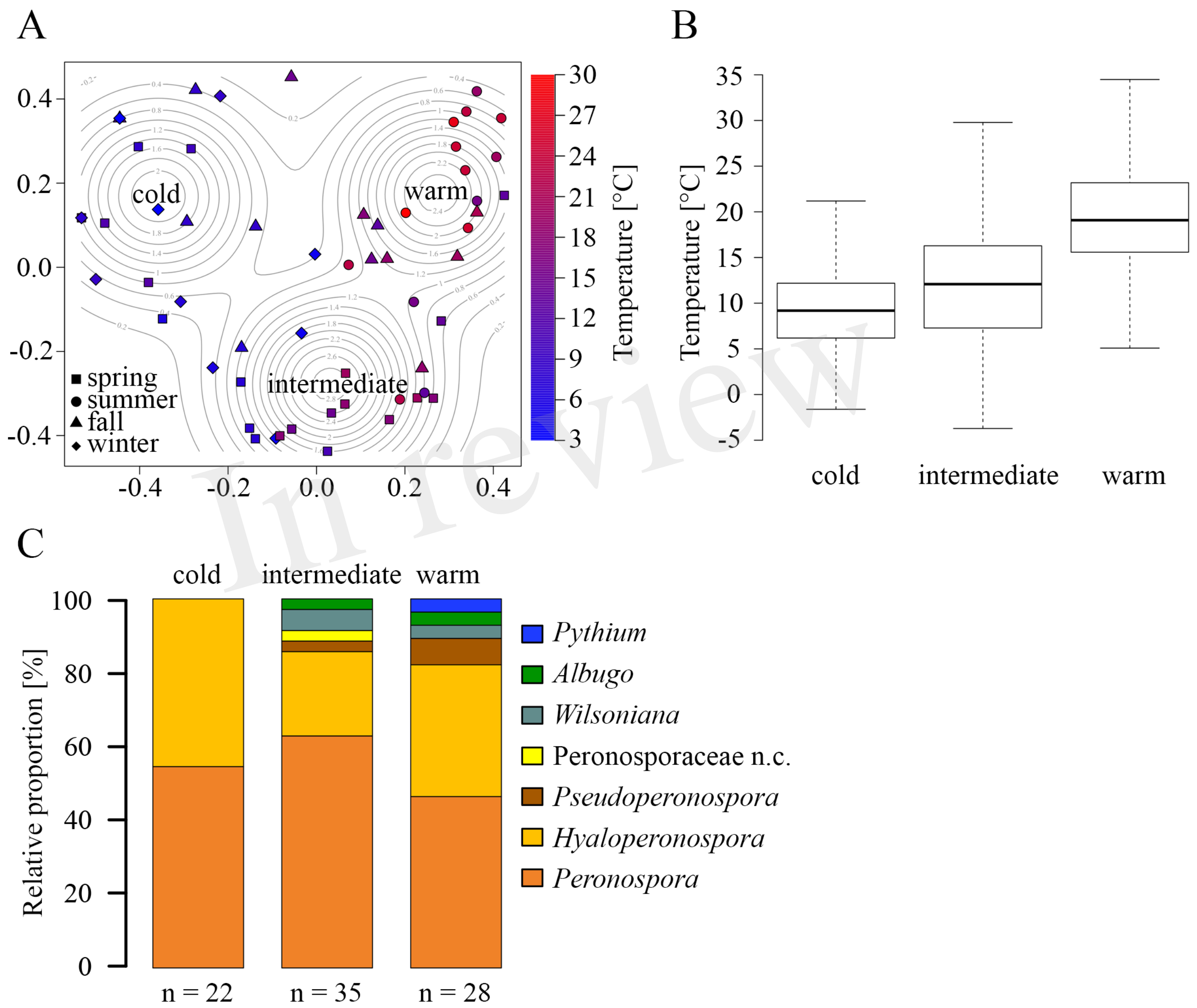

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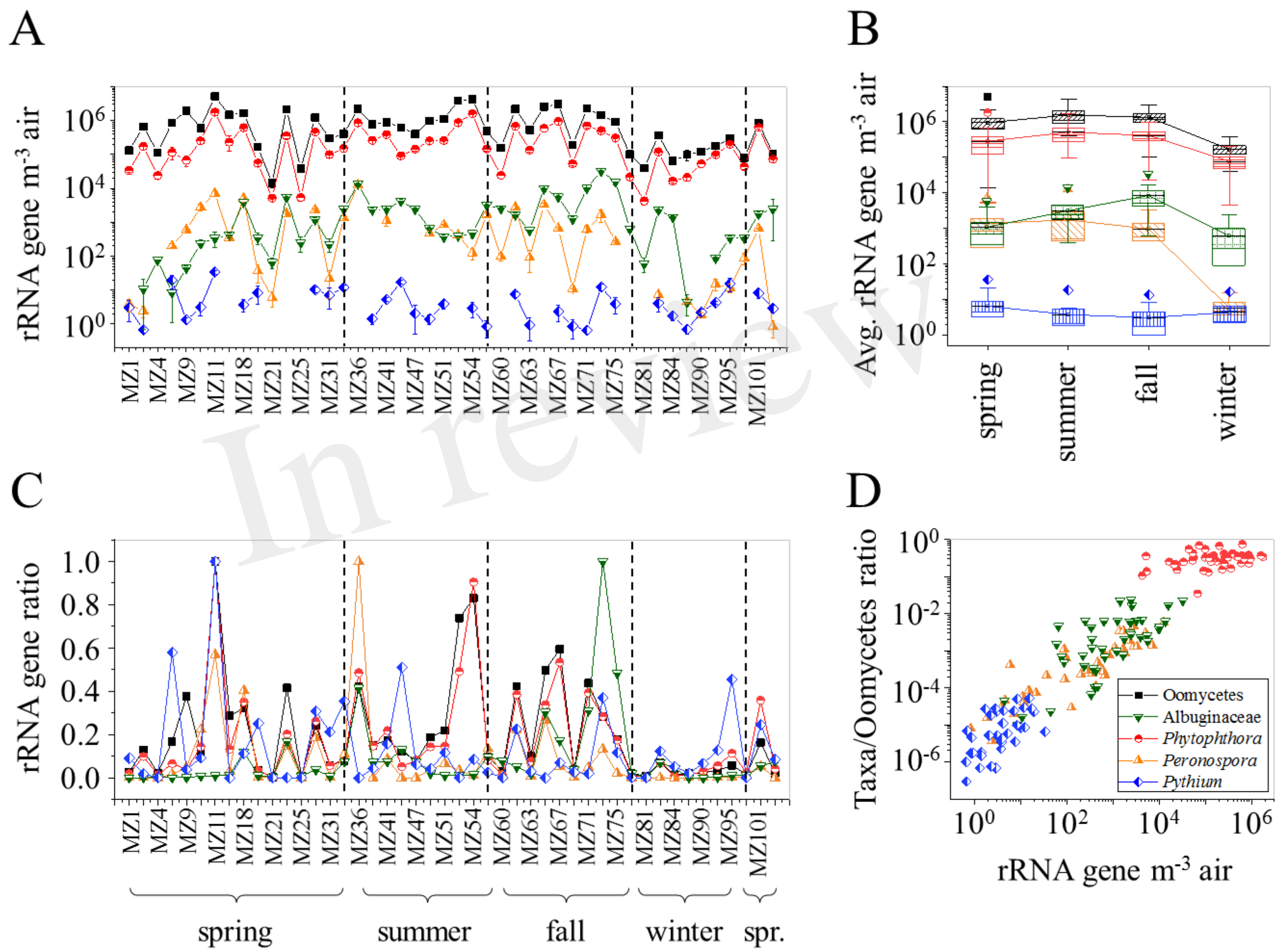
Figure 3.TIF



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Selbstständigkeitserklärung

Sehr geehrte Damen und Herren,

hiermit erkläre ich, dass ich die beigefügte Dissertation selbstständig verfasst und keine anderen als die angegebenen Hilfsmittel genutzt habe. Alle wörtlich oder inhaltlich übernommenen Stellen habe ich als solche gekennzeichnet.

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