**Biogeosciences
Discussions**

Interactive comment on "Biogeography in the air: fungal diversity over land and oceans" *by* J. Fröhlich-Nowoisky et al.

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We thank C. Morris for constructive comments and suggestions, which are highly appreciated and have been taken into account upon revision of our manuscript. Detailed responses are given below.

1. General Comments by C. Morris

Morris: The introduction does not present any real compelling reason for this study. This is not a weakness that could make-or-break this manuscript. But the authors have rallied together an incredible work force for sampling. There must have been some great passion that motivated this effort; I was very surprised to not find this passion in the introduction. There is no obvious statement of hypothesis or goal other than

describing the diversity. How did the authors intend to go beyond current knowledge of the abundance and diversity of fungi in the atmosphere?

Response: To clarify the reason for this study we changed the text as follows:

Introduction, paragraph 1: "The biogeographic distribution of microorganisms is a subject of continued discussions in microbial ecology (Bass-Becking, 1934; Finlay, 2002; Papke et al., 2003; Whitaker et al., 2003; Green et al., 2004; Whitfield, 2005; Martiny et al., 2006; Vos, 2008; Womack et al., 2010). One of the major issues debated is, whether only the environment drives biogeography as Baas-Becking postulates (Bass-Becking, 1934) or if other, e.g., historical events like dispersal limitations also can cause biogeographic distribution patterns. Recent studies reported evidence for regional distribution patterns of microorganisms in soil and water (Papke at al., 2003; Whitaker et al., 2003; Green et al., 2004; Martiny et al., 2006; Whitfield, 2005; Vos, 2008), but their global distribution remains largely unknown. The majority of biogeographic studies have focused on terrestrial and marine environments (Womack et al., 2010), but little is known about biogeography in air, although air is the primary medium for the dispersal of microorganisms, connecting all ecosystems at the Earth's surface."

Introduction paragraph 3: "Recent studies using DNA analysis, however, suggest that the species richness of BMC may actually be higher than that of AMC (Hunt et al., 2004; Fröhlich-Nowoisky et al., 2009). The question, however, remains if the species richness of fungi in the atmosphere is generally higher for BMC than for AMC or if there are biogeographic regions in the air as suggested by Womack et al. (2010). Here we inves-tigate. . ."

We added following references:

Bass-Becking, L. G. M.: Geobiologie of Inleiding Tot de Melieukunde, Van Stockkum & Zoon, The Hague, 1934.

Finlay, B. J.: Global Dispersal of Free-Living Microbial Eukaryote Species, Science,

296, 1061-1063, 10.1126/science.1070710, 2002.

Papke, R., Ramsing, NB, Bateson, MM and Ward, DM.: Geographic isolation in hot spring cyanobacteria, Environ. Microbiol. , 5, 650-659, 2003.

Vos, M. V. G.: Isolation by distance in spore-forming soil bacterium Myxococcus xantus, Current Biology, 18, 386-391, 2008.

Whitaker, R. J., Grogan, D. W., and Taylor, J. W.: Geographic Barriers Isolate Endemic Populations of Hyperthermophilic Archaea, Science, 301, 976-978, 10.1126/science.1086909, 2003.

Morris: The major part of the results and conclusions of this manuscript emanate from the assessment of the diversity of fungi via molecular characterization. Data are used for calculating the number of species detected from a range of different fungal phyla and classes and for calculating a range of diversity indices that are based on the abundances of these different species (such as the relative proportion of an individual species, or the number of species detected only once (singletons) or twice (doubletons)). It is debatable if the data can be used for quantifying diversity according to all of the indices presented. Firstly, it is not clear if the sampling at the different sites is comparable. Although the authors present rather detailed information on each sampling site and the associated sampling procedures, some critical comparative information is lacking. Firstly it would have been useful to have comparative information on the sampling efficiency for each of the samplers relative to the size of fungal spores, for the cut-off diameter (cut-off diameter is presented for the sampler used at the German site (P7077 L15) but not for the other samplers), and for the total air volume that the fungal diversity represents at each site. It is important to take into account that differences in sample volume, and hence in the total number of spores collected, can greatly influence the number of species detected (the general principle is that the more individuals analyzed the more species detected).

Response: Sample descriptions in detail are given in the material and method section

and Tables S1-S9. Even if samples were collected in separate modes (coarse and fine) we were interested in the overall species richness. The results of coarse and fine particle filters were merged together as they represent the totally sampled air mass which is comparable to TSP samples collected at other locations. From each sampling location we analysed several sam-ples (Table S1-S9) and performed several PCR reactions (Table S11, material and method section) to explore the overall species richness. To our knowledge this is the first DNA-based study on a global scale. However, the availability of samples collected on a global scale with identical sampling methods is limited. Nevertheless, like discussed in the manuscript we think that the consistency of major trends and similarities observed over all types of samples sug-gests that the main findings and conclusions of this study are not significantly affected by the sampling conditions.

Tables S1-S9 provide the sample volumes and indicate continental, coastal, and marine sam-ples. As suggested by the reviewer we added the information about sampler type, the type of sample (size range), and the total volume of air in Table S1.

Morris: Secondly, the method of PCR amplification and cloning does not necessarily lead to representation of sequences in the colonies of the cloning vector at the same frequency that they are in the initial sample. This is because there can be competitive interference among sequences for amplification during PCR and during cloning. One consequence is that some sequences might be missed or very poorly amplified, hence the number of species detected is not reliable. But this error can be considered to be equivalent to a detection threshold limit (and these are the type of limits of techniques that we learn to live with). However, the ampli-tude of the error in quantification of the frequency of sequences is unknown. If it depends on the density of the different sequences and their competitors during amplification, then it is not a constant that can be considered to be comparable among samples.

Response: In an earlier study (Fröhlich-Nowoisky et al., 2009) we have tested and applied multiple PCR primer pairs; these and other experimental details were identified

as key ele-ments for efficient amplification of DNA from AMC, BMC, and other fungi. Consequently, we have been able to detect a wide range of species that are well-known from cultivation-based studies (e.g., Cladosporium spp., Penicillium spp., Alternaria spp., and Candida spp.) as well as non cultivable species (e.g., Blumeria graminis and Puccinia spp.) and previously unknown species.

The numbers of sequences per sample are given in Tables S2-S9. As described in the material and method section we used restriction fragment length polymorphism to select as many dif-ferent clones as possible for sequencing reactions which results in different numbers in se-quences per sample.

However, the quantification of the frequency of sequences was not the aim of our study. The principal aim of our study was to explore the overall species richness of airborne fungi. Each species was counted as one in each location, independent if we obtained 1 or more sequences of a certain species (e.g., due to competitive interferences, due to fungal blooms, due to the multicopy nature of the amplified region, or due to the presence on multiple filters).

We agree that the method we used may dismiss some rare species. In this study, we did not compare single species but as discussed in the supporting text we think that the consistency of major trends of BMC and AMC ratios and similarities observed over all types of samples suggests that the main findings and conclusions of this study are not significantly affected by the sampling conditions and method.

Morris: Most of the important conclusions of this manuscript are based on measures of num-ber of species of the different phyla and classes. The diversity indices that involve quantifica-tion of relative abundance of the different species are not used in this manuscript for any of the major conclusions, but simply to state that the level of diversity is comparable to systems studied by other authors (P7085 L4-11). In a supplementary table (S1) the authors have tabulated all the different diversity indices calculated for the different sites. Furthermore, they present means of the indices for continental,

marine and coastal sites. In spite of all these calculations, the authors do not use these indices to formally test the hypothesis, for example, that there is less diversity in marine air compared to the continental and coastal sites – making me wonder if they felt confident in the use of these indices. I would suggest that if they present the values of these indices, then they need to bring to the reader's attention the associated limits. These and other considerations for the quantification of microbial biodiversity can be found in more detail in: Morris C.E., et al. 2002. Microbial biodiversity: approaches to experimental design and hypothesis testing in the primary scientific literature from 1975 to 1999. Microbiol. Molec. Biol. Rev. 66 :592-616.

Response: The diversity indices are meant to characterize the overall dataset. For clar-ification and to avoid any confusion we deleted Table S1 b and we added the following statement and reference.

"Due to well understood limitations of these parameters mentioned by Morris et al. (2002), we focus on the relative proportions of the species richness of different groups of fungi in the investigated samples and the resulting biogeographic patterns."

Morris, C.E., Bardin, M., Berge, O., Frey-Klett, P., Fromin, N., Girardin H., Guine-bretière, M.-H., Lebaron, P., Thiéry, J.M., Troussellier, M.: Microbial biodiversity: ap-proaches to ex-perimental design and hypothesis testing in primary scientific literature from 1975 to 1999, Microbiol. Mol. Biol. Rev. 66: 592-616, 2002.

Morris: To explain the different biogeographies of Ascomycota and Basidiomycota fungi, the authors conduct simulations of residence times and atmospheric transport using existing at-mospheric simulation models. Estimations of residence times and transport are based on spore size. They use the term "aerodynamic diameter" but also "spore size", and it seems as if the spore property used in these calculations is effectively the physical diameter in m of the spore. The spore dimensions used are those corresponding to the predominant fungal spores reported for the species they identified. It is well-known that theoretical estimations of particle trajectories in the

atmosphere predict that larger particles have shorter trajectories than smaller particles. Hence, their results on the increasing relative abundance of Ascomycota with increasing distance is not a surprise given the spore dimensions used in their models. However, these theoretical models ignore an important biological property of fungal spores – their buoyancy. For example, spores of the Ascomycota fungus Erysiphe spp (powdery mildews) and the Basidiomycota fungus Puccinia spp (the rust fungi) are known for their capacity to be disseminated in the air across hundreds of km in spite of their rather large diameter (ca. 20 m in both cases). In fact, the urediospores of rusts have nearly the same residence times in the air as bacteria and generally need rainfall to be washed out, otherwise they can remain in the atmosphere almost indefinitely. Their buoyancy is due in part to wing-like structures on the spores. This is one of numerous examples. If the calculations made by the authors were based on the effective aerodynamic diameter of the spores that accounted for the propensity of the spores for flight, then the calculations would be interesting. In most cases of modeling, the effective aerodynamic diameter has not been reported in the literature for fungal spores of different species and the real physical diameter is used as a proxy (as is the case for all previous efforts to model this phenomenon). This leads to classical predictions that one can expect. The effect of size on simulated transport of microorganisms in the atmosphere is treated in detail in a recent paper. (Wilkinson D.M. et al 2011. Modelling the effect of size on the aerial dispersal of microorganisms. Journal of Biogeography, doi:10.1111/j.1365-2699.2011.02569.x). If the authors choose to maintain the results of their simulations in their revised manuscript, they should point out the assumptions of their analyses and make reference to how their analysis is complementary to the information contained in Wilkinson et al as well as in other previous works.

Response: The result included in the manuscript serves to illustrate the magnitude of differ-ences in relative abundance that can arise from seemingly small differences in particles' (aerodynamic) diameter, after a sufficiently long transport time away from the source. While the predicted increase in the relative abundance of smaller particles is not surprising, the magnitude of the effect is larger than many readers would expect.

In fact, we included these calculations only after discussions with reviewers of earlier versions of this manuscript re-vealed that this result is not obvious to readers less well-versed in atmospheric transport is-sues. For a rigorous test of our hypothesis, more detailed analysis would clearly be required, including a more careful accounting of the physical and aerodynamic sizes of the observed fungal spore species, possible sampling biases, and other technical issues pointed out in the review, many of which likely cannot be resolved for our data set. Here we intend and claim only to present a case that even for small differences in spore size, size differences in atmos-pheric removal rates may be large enough to explain the patterns in biodiversity seen in our data. The suggested reference Wilkinson et al., 2011 was published after we submitted our manuscript. We included the reference in the revised manuscript.

Wilkinson, D. M., Koumoutsaris, S., Mitchell, E. A. D., and Bey, I.: Modelling the effect of size on the aerial dispersal of microorganisms. Journal of Biogeography. doi: 10.1111/j.1365-2699.2011.02569.x, 2011.

Morris: In the discussion, the authors suggest the possible interactions of air borne fungi with atmospheric processes: ice nucleation and cloud condensation. They cite Diel et al to state that large particle size makes for effective ice nucleators. Although size above several microns has an overriding role on the capacity of a particle to act as a CCN (overriding the impact of the chemical composition on cloud condensation activity), it is surprising that they claim the effect of size on INA. Size of the water binding site has a positive effect on INA, but ice nucleation sites are generally specific sites and the whole particle is not necessarily concerned. Secondly, the authors cite Bowers et al (2009) indicating that their data suggest that there are more INA fungal species than currently described. Bowers et al (2009) report ice nucleation activity of their samples at levels below 100 ice nuclei / m3. They sequenced nearly 5000 microbial ribosomal RNAs from their samples, but their samples represented populations of 10e5 and 10e6 individuals/m3. Hence, they were not able to detect microorganisms whose abundance was on the same order as ice nucleation activity. The organisms responsible for the

ice nucleation activity in their samples are most likely not among the taxonomic groups constituting the dominant part of the populations that they described and therefore they have not eliminated the usual suspects as candidates.

Response: The ice nucleation activity of particles is related to the chemical-physical charac-teristics of the surface, and for chemically-physically similar particles it is strongly related to their surface area. This has been documented extensively for dust particles, e.g., Archuleta et al. (2005), Kanji et al. (2008), Welti et al. (2009), as well as for silver iodide particles, e.g., Vonnegut (1947). The likely explanation for this phenomenon is that ice nucleation sites are stochastically distributed across particle surfaces, such that larger particles of the same type will, on average, have a larger number of such sites (e.g., Fletcher, 1969; Lüönd et al., 2010; Niedermeier et al., 2010). Based on this understanding of IN activity, large biological parti-cles such as fungal spores and pollen would be expected to be good IN (activity comparable to or better than mineral dust) unless their surface site density were far lower than that of mineral dust, which does not seem to be the case for pollen, and we assume in the ab-sence of better information that it is also not the case for fungal spores (ice nucleation activity of fun-gal spores is less well studied). This is what our comment was intended to express.

We are not sure what the reviewer means concerning the citation of Bowers et al., 2009. We did not claim that fungi are within the dominant part of ice active microbial populations.

Original text from Bowers et al., 2009: "Alternatively, fungal spores and/or pollen grains may be responsible for the observed increase in IN abundance during periods of cloud cover, as they have also been shown to possess high-temperature ice-nucleating ca-pabilities."

However, we deleted the word "many" and would like to point out that ongoing investi-gations in our lab showed that there are more ice active fungal species than currently

known. To avoid misinterpretation we deleted the citation of Bowers et al., 2009.

For clarification the section about ice nucleation activity has been rewritten. We added a par-agraph describing the relation between surface area and ice nucleation activity and a statement about ongoing investigations.

"Members of fungal species that can act as ice nuclei (IN) (Jayaweera and Flanagan, 1982; Kieft and Ahmadjian, 1989; Pouleur et al., 1992; Iannone et al., 2011) were found in all re-gions: Cladosporium spp., Fusarium spp., Microdochium spp., Penicil-lium spp. (Tab. S10). While Cladosporium is the genus with the highest frequency of occurrence in continental air samples (98%) (Fröhlich-Nowoisky et al., 2009), Peni-cillium is the genus most frequently detected in marine samples (60%). So far, all reported IN-active fungi belong to the AMC (Jayaweera and Flanagan, 1982; Kieft and Ahmadjian, 1989; Pouleur et al., 1992; Henderson-Begg et al., 2009; Iannone et al., 2011), but recent findings indicate that there are also IN-active fungal species from other phyla. As described for pollen (Diehl et al., 2000), the IN activity of biological particles may in-crease with size. For mineral dust, it is well-known that rates of ice nucleation increase with particle surface area, i.e. larger dust particles are on aver-age more efficient ice nuclei than smaller particles with similar chemical composition (Archuleta et al., 2005, Kanji et al., 2008, Welti et al., 2009). It seems plausible that a similar relationship would hold for fungal spores, with larger spores tending to be more effective IN than small spores. Ongoing investigations (Haga et al., in preparation) suggest that there is indeed some correlation between spore size and median freez-ing temperature, and that spores of prominent BMC species may be more effective IN than spores of prominent AMC species. Particles that are more effective IN can be expected to be scavenged at higher rates in mixed-phase and ice clouds. Simulations of global atmospheric transport suggest that the effectiveness of particles acting as IN would affect their concentration in surface air primarily in polar regions (Bourgeios and Bey, 2011). Thus, if BMC are better IN than AMC, this could contribute to explaining the very low frac-tion of BMC species observed in the filter samples collected near the

coast of Antarctica."

Archuleta, C. M., DeMott, P. J., and Kreidenweis, S. M.: Ice nucleation by surrogates for at-mospheric mineral dust and mineral dust/sulfate particles at cirrus temperatures, Atmos. Chem. Phys., 5, 2617-2634, doi:10.5194/acp-5-2617-2005, 2005.

Fletcher, N. H.: Active sites and ice crystal nucleation. J. Atmos.Sci., 26, 1266-1271, 1969.

Kanji, Zamin A.; Florea, Octavian; Abbatt, Jonathan P. D..: Ice formation via deposition nu-cleation on mineral dust and organics: dependence of onset relative humidity on total particu-late surface area, Environ. Res. Lett., 3, 025004, doi:10.1088/1748-9326/3/2/025004, 2008.

Lüönd, F., Stetzer, O., Welti, A. and Lohmann, U.: Experimental study on the ice nu-cleation ability of size-selected kaolinite particles in the immersion mode, J. Geophys. Res., 115, D14201, doi:10.1029/2009JD012959, 2010.

Niedermeier, D., Shaw, R. A., Hartmann, S., Wex, H., Clauss, T., Voigtländer, J., and Strat-mann, F.: Heterogeneous ice nucleation: exploring the transition from stochastic to singular freezing behavior, Atmos. Chem. Phys., 11, 8767-8775, doi:10.5194/acp-11-8767-2011, 2011.

Welti, A., Lüönd, F., Stetzer, O., and Lohmann, U.: Influence of particle size on the ice nu-cleating ability of mineral dusts, Atmos. Chem. Phys., 9, 6705-6715, doi:10.5194/acp-9-6705-2009, 2009.

Morris: The concluding statement of the discussion - "we suggest that air flow patterns in the global atmospheric circulation, as well as spore size-driven selection, may be important for the evolution and spread of fungi" – is the most troubling part of this manuscript. It gives the impression that this work is detached from the fundamental and founding literature on aerobiology. Yet the authors have cited some of this work in a supplementary section on emission and transport of fungal spores, and in par-

ticular: Gregory, P.H. (1961; 1973) The Microbiology of the Atmosphere. New York: Interscience Publishers, Inc. This work is a several hundred-page tour de force of most of the major concepts in aerobiology richly illustrated with abundant data. Several of its pages are dedicated to the microbiology of oceanic air, and can serve as an important basis of comparison. In conjunction with the work of E. Stakman (for example: Stakman, E., and Christensen, C.M. (1946) Aerobiology in relation to plant disease. Botanical Review 12: 205-253.) and the subsequent research that was inspired up to about the early 1990's, this body of research has clearly establish that dissemination capacity is linked to the evolution of pathogens. Dissemination is also a fundamental principle of population genetics (gene flow), a body of concepts that describe how organisms evolve. I encourage the authors to clearly anchor their conclusions in this body of knowledge and to specify how their findings are complementary and go beyond it.

Response: With our statement we intended to provide a broader perspective (suggesting the global aspect based on our global data set) rather than claiming new discoveries related to evolution. Neither in the review of Stakman and Christensen, 1946 nor in a recent review (Womack et al., 2010) we could find the link between global atmospheric circulation and bio-geography and evolution of fungi. In fact we included the statement "we suggest that air flow patterns in the global atmospheric circulation, as well as spore size-driven selection, may be important for the evolution and spread of fungi. " after discussions with reviewers of earlier versions of this manuscript. To avoid misinterpretation we delete the statement from the re-vised manuscript.

2. Specific comments by C. Morris:

Comment 1: As mentioned above, it would be very useful to add a summary table comparing the different sampling sites (above ground height; sampler type, efficiency, and total volume of air sampled in particular) to make it easier for the reader to determine the degree to which samples can be compared. This table should also indicate which samples are continental, coastal and oceanic.

Response: We included the suggested information in Table S1.

Comment 2: In section 2.4 on DNA analysis, the authors indicate that they have eliminated possible chimers from the analysis. How frequent was the occurrence of chimers? Likewise they indicate that sequences corresponding to contaminations originating from the filters were eliminated from the analyses. The supplementary information about background DNA on blanks was very useful.

Response: Out of 3360 sequenced clones we obtained 2654 sequences which were included in the analysis. The remaining 706 sequences were excluded from the analysis due to failed sequencing reaction/bad/heterogeneous sequences (247), coamplifications (9), chimeric se-quences (51) and sequences that may origin from contaminations (399). As described in Fröh-lich-Nowoisky et al. (2009), no DNA was detected in the blank samples from Mainz, Ger-many, indicating that no contaminations occurred during sample handling and analysis in the laboratory. However, 180 sequences out of the 399 possible contamination sequences were detected in the samples from Mainz. To avoid any bias in the comparison with other sample sets, we excluded the possible contaminations also from the statistical analysis of the Mainz samples.

We have added the information in the text (material and method section).

"Out of 3360 sequenced clones 247 sequencing reactions failed and nine sequences produced non-fungal results. Each of the 3113 remaining sequences was identified to the lowest taxo-nomic rank common to the top BLAST hits (up to ~100 data base sequences with highest sim-ilarity and total scores). Sequences (51), for which the ITS1 and ITS2 regions matched in dif-ferent genera and thus were assumed to be chimeric results of PCR recombination. These se-quences and were excluded from further analysis. Sequences (399), which were obtained from field, extraction or PCR blanks and identical sequences obtained from the air filter samples and filter blank samples were also excluded from further analysis."

Comment 3: P7085 L14-27, Here the authors present data about proportions of AMC

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and BMC. They should clearly define how they calculate 'proportion'. I assume that it is: [total number of AMC species (independent of the frequency of occurrence of each species)/ total number of species detected in the sample]

Response: We included the description of how to calculate a proportion of AMC and BMC in the text.

"….we focus on the relative proportions of the species richness of different groups of fungi in the investigated samples and the resulting biogeographic patterns. The relative proportion of AMC and BMC discussed below are defined as the ratio of AMC or BMC to the total number of species detected in the samples." . Comment 4: P7088 L14, The statement "diversity and spread of ecosystems" is not clear. What is the spread of an ecosystem?

Response: We changed the text as follows: "spread in ecosystems"

Comment 5: In the supplementary information, the authors discuss the impact of dif-ferent sampling methods and conditions. This type of analysis is critical for putting the results into perspective and the whole of this should be in the main manuscript.

Response: The text and corresponding references were added to the main body of the manu-script (material and method section) as the reviewer suggested

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