

Interactive comment on “Spores of most common airborne fungi reveal no ice nucleation activity” by B. G. Pummer et al.

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Referee#1: There is currently a very high interest in the field of atmospheric sciences biological ice nuclei and their impacts on clouds and precipitations. A number of studies reported that bacteria were the most active IN in the atmosphere. To my opinion, such emphasis on biological and microbial IN may have biased the vision of scientists of the atmosphere other than biologists that in reality most of the microorganisms are NOT IN active. This paper reports the ice nucleation activity of a number of fungi relevant in aerobiology. Only 1 species out of 26 investigated here demonstrated significant IN activity, and it is probably the main result of this work. However, the knowledge that is gained from it are rather limited, and one can wonder about the relevance of the fungal strains investigated since they were actually not isolated from atmospheric samples.

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Answer: Our approach was the application of fungal strains that are known to be very common in the atmosphere, since they make up a large amount of spores and therefore would be very significant, if found to be IN-positive. Surely, under the condition that the composition of fungal spores in the atmosphere assumed with today's knowledge is indeed correct. Our tests show furthermore that absolutely no IN-activity originates from the the fungal spore bodies. Although many of our samples were acquired from databases, the spores of the food moulds came directly from the air. The cultures received from the TU database were taken from the mother cultures at -80°C, which were once taken directly from the environment – so they have not been exposed to cultivation over many generations, as it is the case in older databases. Since the natural spores originated from a source on the ground, too, our samples can be considered as rather close to outdoor samples.

Referee#1: One of my main concerns about this work is the quantity of spores used in the assays. It was apparently normalized to 20 mg/mL, but there is neither mention about how this concentration was achieved (by weighting dried spores after collection??), nor to the approximate number of spores it corresponds to. This is crucial since heterogeneous freezing of supercooled water is a matter of probability and it strongly depends on the number of freezing sites available. In the most efficient IN active bacteria, about only 1% of the cells are actually IN. It would have been necessary here to quantify the number of spores or the number of IN sites in the suspension for normalizing the results and be able to compare strains with each others.

Answer: The 20 mg/ml (in respect to water) is achieved by adding the proper amount of spores to the emulsion and shake it, until the spores are homogeneously distributed. The spore number this corresponds to depends on the spore size, which varies with the species. As Fig. 2 (new fig. 2, see manuscript) shows, every droplet contains several spores. We made sure that the loss of spores in the oil phase is compensated and that there are enough spores in the droplets, so that any IN activity should be detectable. As it was shown in other studies (Kieft and Ruscetti 1990; Pouleur et al. 1992), fungal IN

can be separated from the spores, therefore it is not correct to parameterize IN activity with the total spore surface. It is analogous to the pollen, where the IN activity remains after removal of the pollen grain (Pummer et al. 2012), meaning that the total pollen grain surface per droplet is zero. However, the number of IN-active molecules per spore was not quantified up to now. To compare different samples with each other, we normalized our spore amount to the mass – as stated in the paper to 20 mg/ml – since it is the most reliable way to determine an amount. Alternatively, one could normalize to spore numbers, however, as different species vary in their spore size, but this way we would artificially create a size dependence, since larger particles are expected to carry more active sites, if IN-active. Further attempts of IN quantification would only make sense, if there were IN that could be quantified. The total inactivity of most samples suggests that the number of IN is indeed zero. The possibility of an activity below the detection limit might be of concern. As mentioned, only a fraction of bacteria in a culture express IN. Out tests with Snomax showed that our method can detect also IN in an excess of IN-inactive material, since Snomax contains such bacterial cultures plus the nutrient substrate. As our data showed, there are sufficient IN present in the Snomax droplets to initiate freezing-through even at high temperatures (Pummer et al., 2012). The IN amount of the fungal spores, which were pure and not diluted with nutrient substrate, should have several orders of magnitude less IN, if they show no IN activity at all under the very same conditions. In this case, they are of no relevance.

Referee#1: Using the T50 values might not be relevant since the spore concentrations might have been quite variable from a suspension to another. It could even have not been sufficient to detect any significant IN activity since some droplets may have remained free of any IN spore in the case were concentrations too low were used, i.e. if a weak (which is different from absence) IN activity exists. Hence, I agree with Reviewer 1 that the possibility that IN activity remained not detected due to experimental issues should be discussed in more details.

Answer: In air-samplings, the active fraction is low even for the known IN-positive

species, however, there we sample a mixture of different strains, of which some are totally IN-negative, while others show appreciable IN activity. Since the active spores can release their active sites (and an IN-active spore will carry more than one IN-active molecule), and since we had an appreciable amount of spores in the sample, the fraction of active droplets should be higher than 1%. If there was an activity in the sample, which was simply below our detection limit, it would be of low significance for the atmosphere as well, since there it competes with other ice nuclei (like mineral dusts), which have shown far higher IN activity in the same setup. We furthermore chose the T50, because it is statistically most reliable. The onset temperature depends on the behavior of a single droplet – namely the one that freezes first. We consider it quite unserious to make our evaluation based on a single event, which could have been influenced by many different parameters. The onset freezing has far larger statistical error bars than that of the median freezing temperature, and should not be used to compare data. Furthermore, a single IN-active contaminant can lead to an overestimation of the onset freezing temperature, while the median freezing temperature is only affected by larger amounts of contamination. The interpretation whether an early onset temperature is caused by contamination or by the sample itself remains therefore speculative. To rule out any wrong-negative results caused by the oil matrix, we did some further measurements with a classical droplet array, where microliter droplets are arranged on a sample carrier and cooled down. The method is described in a new chapter (2.4.), and the results are shown in Table 4. “To confirm the data from the oil immersion method, some of the samples were further investigated with a setup where the droplets are not surrounded by oil, which might influence the results. The sample carrier is in this case a PCR plate with 96 wells. Each one of them was filled with 50 μ l of fluid, which was either pure water, or a suspension with a concentration of 1 mg/ml. Only for birch pollen, a concentration of 2.5 mg/ml was applied (see Pummer et al. (2012) for further explanation). Higher dilutions than in the oil immersion setup were necessary, since the droplet volume is much bigger and therefore contains more spores at a given concentration. Droplets with 20 mg/ml spores were so loaded that

it became intransparent and were therefore not applicable. The PCR plate was then sealed with adhesive foil to prevent external contamination. The PCR-plate was then placed in a cooling bath, which was thermostated to 273 K, and then cooled down in 3-K-steps each 12 minutes, which was ample time for the system to equilibrate at the demanded temperature. Via optical inspection, the aggregation state of each droplet was determined and counted. In case of liquid droplets, the reflection of the ambient light formed a bright ring on the well floor. When a droplet froze, this reflection ring vanished, or was at least severely reduced. The fraction of frozen droplets for each sample was then plotted against temperature the same way it was done for the oil immersion measurements. The applied samples and their T50 are presented in Table 4.”

Referee#1: In addition, more details concerning the determination of IN activity should be given: how many droplets counted? What was the method of counting? Was cooling stopped and temperature steady during counting? What was the reproducibility of the results?

Answer: We added more information about the handling of the setup (see p.5/L.19 – p.6/L.11) and some statistical information about the measurements (see Table 2): We now show the number of counted droplets and the values for T10 (the threshold where 10% of droplets are frozen), which is, when compared with the corresponding T50, also a measure for the spreading of freezing events across the temperature axis.

Referee#1: Quantification of spore material was attempted by determining total protein content in spore suspensions. This was not correlated with IN activity, and Figure 2 appears quite useless. From the pictures showed in Figure 1, the size of the spores varies quite widely from a species to another, and the protein content itself also varies from a species to another. Furthermore, the spores were harvested from 2 independent plates, in ethanol for IN assays, and in water for protein concentration measurements. The authors seem to assume that those 2 methods lead in similar collection efficiencies of spores from colonies, but this has apparently not been verified? This also has to be discussed, and a few additional experiments for verifying this could be a real plus.

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Answer: The current Fig. 2 was removed. The harvesting techniques described in our paper are standardized approaches, what does not make them unquestionable, but at least in agreement with comparative data. The absolute amount of harvested spores might vary between the two approaches; however, since we were mainly interested in a relative comparison between data, the absolute amount is not of interest. The samples are normalized in this approach via the dilution to a given transmission (if we had a more concentrated suspension, it would have to be diluted more). The fact that all samples showed about the same median freezing temperature proves that the differences that do exist are not responsible for potential IN activity.

Referee#1: Finally, while the problematic of the presence of biological IN in the atmosphere was clearly exposed in the introduction, this is not even mentioned in the conclusion. It would be interesting to indicate what those results imply for ice nucleation in the atmosphere.

Answer: The atmospheric impacts were presented as a motivation for this study, which led to the key question of this paper, how common ice nucleation is among the common species in the atmosphere. Our conclusions are, as we already stated, that only a fraction of the fungal spores in the environment possess the ability to nucleate ice. Further interpretation (e.g. if it needs a high fraction of ice nuclei to be relevant, or if a small fraction is sufficient for major global impacts) is beyond the scope of this study.

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