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Reply on RC1

Charlotte M. Beall et al.

Author comment on "Cultivable halotolerant ice-nucleating bacteria and fungi in coastal precipitation" by Charlotte M. Beall et al., Atmos. Chem. Phys. Discuss., https://doi.org/10.5194/acp-2020-1229-AC1, 2021

We thank the anonymous referee for their suggestions and thoughtful comments on how to supplement the discussion of the results. We include their comments and our responses below. Line numbers in our responses refer to the revised manuscript.

Specific comments:

Line 25: *"Better use INP as introduced in line 19 instead of "IN forming particles" as IN is not introduced here and based on IN definition in line 58 it would mean "ice nucleating forming particles"*

This has been corrected as suggested.

Line 108: *"How were the filters pretreated for decontamination before aerosol sampling? Information on blank samples for aerosol sampling and handling should be added."*

Line 110 now reads: "Prior to sampling, filters were pretreated for decontamination by soaking in 10 % H_2O_2 for 10 minutes and rinsing 3X with ultrapure water.

Background levels of INPs from sampling handling processes were simulated using INP concentrations in aerosol sample field blanks assuming the average sampling volume (2270 L). Simulated INP concentrations across the 3 field blanks ranged between between 0 and 0.1 L^{-1} at -20 °C (see Fig. S6)."

Line 124: *"Please add here also the aerosol samples. Moreover, I suggest to add the information given in line 140 about the volume (50 µL) and number of aliquots (30) already here as "microliter aliquots" covers a wide range of possible droplet sizes."*

Corrected as suggested. Line 129 now reads: "Briefly, the precipitation samples and aerosol sample suspensions were distributed in 24-30 50-microliter aliquots into a clean 96-well disposable polypropylene sample tray."

Line 155-157: "*Please add full information (or a reference) for the performed PCRs (PCR components, concentrations, cycling conditions). Note also, that ribosomal DNA in fungi is 18S and not 16S. Which primers were used for amplification of fungal 18S or did the bacterial primers only coamplify fungal 18S? This part needs some clarification on how the*

authors obtained fungal 18S sequences. The authors should also clarify and correct this in other parts of the manuscript., e.g., caption table S1, figure S11."

Thank you for bringing this to our attention. The 16S primers were able to capture the fungal 18S sequences and we did not use additional primers. We have added this clarification as well as the PCR reagents and cycling conditions to the manuscript.

Line 163: The PCR reaction contained 0.5 ng ml^{-1} genomic DNA, 0.2 mM of each primer, and 1x KAPA HiFi HotStart ReadyMix (KAPA Biosystems, KK2601), and the thermocycler was set to the following program: 95°C for 30 seconds; 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds; 72°C for 5 minutes.

Line 170: The 16S primers were able to capture 18S fungal sequences. Primers specific to 18S rRNA were not used.

Caption Table S1 and Fig. S11: 18S fungal sequences were obtained from 16S primers due to coamplification (see Methods Sec. 2.2).

Line 360: *"As Cryptococcus and Metschikowia are not bacteria but fungi, please change caption to "Identities of 14 …IN bacteria and fungi". Overall, both fungal species did not receive much attention in the manuscript although the title and abstract raised some expectations. The authors should add some discussion and comparison with the literature for the fungi they advertise in the title."*

Corrected. Table 1's caption now reads: Identities of 14 cultivable, halotolerant IN bacteria and fungi derived from aerosol or precipitation samples.

We agree that some discussion of fungal INPs is needed given the two fungal IN isolates featured here. Thank you for bringing this to our attention. The following paragraph has been added to Sec. 3.3, Line 391:

Fungal isolates *Cryptococcus* sp. and *Metschikowia* sp. represent two new ascomycotic and basidiomycotic IN fungal species, respectively, with INP concentrations 7-8 orders of magnitude lower than the highest reported values for fungal isolates *F. armeniacum* and *F. acuminatum* (Kunert et al., 2019). While other IN species of the Ascomycota and Basidiomycota phyla have been previously reported (e.g. Jayaweera and Flanagan, 1982; Kieft et al., 1988; Pouleur et al., 1992), very little is known regarding the distribution and source potential of fungal INPs. Moreover, multiple issues pose challenges to the differentiation of marine vs. terrestrial fungal species (Amend et al., 2019). Many fungi found in the sea are also found in terrestrial environments, and strong correlations with abiotic environmental conditions (Orsi et al., 2013; Tisthammer et al., 2016) and gene expression data (Amend et al., 2012) suggest that some fungi are truly amphibious. Issues with amplicon sequencing pose additional challenges due to coamplification of other eukaryotes and large biases toward terrestrial species in ITS rDNA primers, which were designed using sequence alignments from largely terrestrial representatives (Amend et al., 2019). However, future studies could take advantage of established marine fungi isolation and cultivation techniques to probe the INP source potential of various cultivable marine fungal species (e.g. Kjer et al., 2010; Overy et al., 2019).

Line 424: "*Remove the "sp." after "syringae" as syringae is the species name."*

Corrected, thank you.

Line 441: "*please use "spp." and not "sp." if multiple species are meant."*

Corrected. Line 469 now reads: Additionally, whereas (Failor *et al.*, 2017) reports high freezing temperatures between -4 and -12 °C for multiple *Pseudomonas* spp., none of the *Pseudomonas* spp. isolated in our study exhibited detectable IN activity.

Line 456: *"Gammaproteobateria" – typo in bacteria, and missing hyphen (see line 345 Gamma-proteobacteria, be consistent).*

Corrected, thank you.

Line 465: *"Lysinibacillus is not gram-negative. Please correct to gram-positive."*

Corrected.

Line 490: "*Can the authors please add some more discussion and more specific suggestions on the "state-of-the art sequencing approaches" they mention here. I wonder how combining INP measurements with state-of the art sequencing should help to identify putative IN microbes that are not recovered by cultivation. The sequencing gives information about composition of the community, which are usually highly diverse, but only a small number of species possesses ice nucleation activity. A diversity analysis, however, does not give information about putative IN abilities of the organisms. Metagenomic (and transcriptomic approaches) are limited by database entries of IN genes, as these genes are not known for the many of the known IN organisms. Also note that some microorganisms (e.g., most known IN fungi) release cell-free IN into the environment. These IN would be covered by the IN measurements but as they do not contain DNA or RNA they would not be covered by the sequencing approaches. Furthermore, without cultivation it seems not feasible to proof the ice nucleation activity of a microorganism, even when (hopefully in future) gene similarities might suggest more candidates."*

We agree that more discussion is needed here to explain how advanced sequencing approaches could help advance understanding of the factors that modulate bio-INP emissions. While it is indeed unlikely that we could identify a single IN species, advanced sequencing methods could illuminate relationships between specific communities and INP freezing activity. For example, high-throughput sequencing techniques for low biomass samples will enable sequencing of individual e.g. 50 µL droplets such that droplet assay measurements of INP concentrations could be related to communities present in low temperature vs high temperature freezing droplets (Minich et al., 2018). The referee also makes a good point about the inability of such methods to identify cell-free INPs. This paragraph has been edited as follows:

Finally, as cultivable populations represent a small fraction of the total microbial community, future studies should combine INP measurements with state-of-the-art sequencing approaches to identify relationships between specific microbial communities and INP freezing activity. Furthermore, a combination of advanced fractionation methods to identify the putative ice nucleating metabolites associated with specific microbial communities and computational networking could illuminate molecular and microbial linkages to ice nucleation and the mechanisms by which the entities work individually or in concert. Further study is also needed to understand the factors, such as atmospheric processing or nutrient limitation, that inhibit or enhance microbe IN behavior, as well as the factors that modulate the emissions of IN bacteria from the ocean surface.

Table S1: "*Be consistent - genus and species names should be italics, "sp." should not; contains several typos in e.g., Bacillaceae, Metschnikowiaceae. Paenibacillus is not a family but a genus, thus it should be Paenibacillaceae in the family column. Column blast*

identity has an extra comma in line Iso39, missing space in line iso3. IN ability column seems not needed, as IN onset temperature gives the "yes" or "no" information."

Thank you for pointing out the typos. These have been corrected. We agree that the column for IN ability is unnecessary and it has been removed.

Table S4: "*Genus and species names should be italics, Iso5 – missing space, SSA18 – 7tewartia?*"

Corrected.

Figure S7: "*Typo in legend: Metschnikowiaceae; what does the line and the Y? at the right side of the legend mean?*"

Corrected, thank you. The symbols are alpha and gamma (to indicate gamma vs alpha-proteobacteria).

Figure 4 and S10: *"It is confusing that the orange and yellow triangle symbols (sample 9) described in the legend point to a different direction in the plot. Caption for figure S10 needs to be checked "Sample numbers in the legend indication the precipitation"?"*

The triangle orientation has been corrected. (I also found another legend typo for *Arthrobacter* and *Metschnikowia* sp. typo and corrected).

The S10 caption typo has been corrected: "Sample numbers in the legend indicate the precipitation or aerosol sample from which the isolate was derived (see Table S3). Datapoints corresponding to isolates from aerosol are outlined in black."

References:

Minich, J. J., Zhu, Q., Janssen, S., Hendrickson, R., Amir, A., Vetter, R., Hyde, J., Doty, M. M., Stillwell, K., Benardini, J., Kim, J. H., Allen, E. E., Venkateswaran, K. and Knight, R.: KatharoSeq Enables High-Throughput Microbiome Analysis from Low-Biomass Samples, mSystems, 3(3), e00218-17, doi:10.1128/mSystems.00218-17, 2018.