Responses to RC1 on "Cultivable, halotolerant ice nucleating bacteria and fungi in coastal precipitation"

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 121 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 estimated using INP concentrations in aerosol sample field blanks assuming the average sampling volume (2270 L). Estimated INP concentrations across the 3 field blanks ranged between between 0 and 0.1 L^{-1} at -20 °C (see Fig. S1).

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 142 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 179: 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 192: The primers were specific to bacterial 16S rRNA gene sequences and were additionally able to capture some 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 441:

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 520 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.

Responses to RC2 on "Cultivable, halotolerant ice nucleating bacteria and fungi in coastal precipitation"

We thank the anonymous referee for their helpful comments and suggestions on which results to emphasize. We include their comments and our responses below. Line numbers in our responses refer to the revised manuscript.

General Comment:

One general comment is that the results from fourteen isolates with IN activity are summarized in the abstract by presenting the temperature range of activities from -2.3 to -18C. I believe the truly interesting find in this work is the very warm IN temperature for the Brevibacterium strain, as I am not aware of another report of this phenotype in this phylum. My suggestion is to specifically emphasize this in the abstract as this will be a strain that will elicit interest from a range of microbiologists interested in novel mechanisms of biological ice nucleation.

Thank you for this suggestion. We have added the following sentence to the abstract, L30:

While some Gamma-proteobacteria and fungi are known to ice nucleate at temperatures as high as -2 °C, *Brevibacterium* sp. is the first Actinobacteria found to be capable of ice nucleation at an exceptionally high freezing temperature (-2.3 °C).

We also added the following to the Sect. 3.3, L468:

Of the known IN bacteria, only Gamma-proteobacteria have been shown to ice nucleate at extremely high temperatures (Morris et al., 2004) and *Brevibacterium* sp. is the first Actinobacteria to be shown capable of IN near 2 °C.

Below are specific points the authors should consider when revising the manuscript:

Lines 32-33; 93-94; 330-332; 462: *"The phylogenetic information available cannot be used to definitively determine the environmental source of these isolates. The study observations collectively support that the principal aerosol source was marine, but the type and amount of sequence data obtained do not allow, for example, saying that Psychrobacter sp. 1b2 is marine and 2a is not. The phylogenetic resolution possible from the V4 region of the 16S/18S rRNA gene, which is about ~1/3 of the gene sequence, is useful for coarse phylogenetic assignments but is not able to resolve evolutionary relationships between closely related taxa. It is safe to conclude that the major source of cultured microbes was from aerosol samples with a marine origin and that many of the isolates are closely related to marine taxa but resolving environmental source from a few hundred nucleotides of small subunit rRNA sequences is not something that can be done with confidence."*

Thank you for this helpful comment. We have edited the text as follows in the indicated sections and any others that referred to marine origin to reflect that we cannot definitively determine origin:

L34: Air mass trajectory analysis demonstrates that marine aerosol sources were dominant during sampling periods, and phylogenetic analysis indicates that at least 2 of the 14 IN isolates are closely related to marine taxa.

L78: While indirect evidence indicates marine microbes and other biogenic entities as possible marine INPs, the microbial contribution to marine INP populations has not yet been confirmed through direct observations (i.e. through isolation and identification in an atmospheric sample).

L102: Here we report the identities and freezing temperatures of 14 cultivable halotolerant IN species derived from marine and coastal precipitation and aerosol samples.

L383: The phylogenetic relationships between isolates and reference sequences (Fig. 3) show that at least two of the 14 IN isolates are closely related to marine taxa, *Idiomarina* sp. and *Psychrobacter* sp. 1c2, both of which were derived from coastal aerosol.

L555: Through isolation and identification of multiple IN microbes in precipitation and aerosol, this study provides identities of multiple halotolerant IN microbes, at least two of which are likely of marine origin.

L570: However, marine origin is highly likely for multiple isolates for the following reasons: aerosol back-trajectories and INP observations during sampling events indicate that marine regions were dominant (Figs. 1-2), multiple isolate sequences show similarity to marine isolation sources in reference sequences (Figs. 3, S8), and isolate freezing temperatures are generally in agreement with previously documented nascent SSA IN freezing temperatures (DeMott et al., 2016; McCluskey et al., 2017, 2018a).

Lines 110-111: *Just to confirm that the aerosol samples used for culturing in media with ~35ppt salinity were initially placed in deionized water. It is important to note that this process would represent a significant osmotic shock to the cells. Also, assuming that a "hand" was not literally used for this aseptic procedure, so please clarify how the particles were removed from the filters.*

This is a good point, and yes, aerosols were resuspended in deionized water. We have added some text to explain how this could have affected results on aerosol-derived isolates:

L342: The taxonomy of the aerosol and rain isolates show higher diversity in the precipitation samples (Fig. S7 and Table S1), which may be due to artificial biases from low aerosol isolate recovery or sweep out of interstitial particles during raindrop descent. For example, sample handling may have decreased the isolate recovery rate from aerosol samples as cells were osmotically shocked during resuspension in ultrapure water (see Sect. 2.1).

We have also clarified the line about resuspension of aerosols:

L126: After collection, aerosol filters were placed in 50 mL sterile plastic Falcon® tubes (Corning Life Sciences, Corning, NY, USA) and immersed in 12 mL of ultrapure water using sterile polypropylene forceps that were pretreated using the 10 % H_2O_2 process described above.

Line 144: "*Just confirming if it was filtered and then autoclaved. Speculating it could be the reverse because a precipitate typically forms when autoclaving full strength seawater. Please also indicate the source of the seawater.*"

Clarification added. The lat/lon of the pier is provided in the paragraph above L161 (same section).

L165: Seawater was collected at the Ellen Browning Scripps Memorial Pier and was filtered prior to autoclaving.

Line 155 and throughout manuscript: *"16S V4 ribosomal DNA fragments were…" This is common lab slang that I suggest rewording here and throughout as "The V4 region of the 16S rRNA gene".*

Thanks for pointing this out. Edited as suggested.

L176: The V4 region of the 16S rRNA gene was amplified using the primers 515F (5´ GTGYCAGCMGCCGCGGTAA 3´) and 926R (5´ CCGYCAATTCMTTTRAGT 3´) (Walters et al., 2015).

L183: The sequences of the amplified 16S rRNA gene fragments were determined by Sanger sequencing (Retrogen, San Diego, CA)

L185: Taxonomic assignments were determined from 16S rRNA gene sequences using the SILVA Incremental Aligner (SINA) (Pruesse et al., 2012) and the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/).

L192: The primers were specific to bacterial 16S rRNA gene sequences and were additionally able to capture some 18S fungal sequences. Primers specific to 18S rRNA were not used.

L195: To assess for duplicate isolates within the sampling period, 16S rDNA sequences were compared. Sequences within the same genus were adjusted and aligned in DECIPHER(Alignseqs(), AdjustAlignment() with default settings) (Wright, 2015)

L333: This resulted in 34 isolates from rain samples, and 13 isolates from aerosol samples with 29 unique genera as determined by > 97 % sequence identity of 16S rDNA sequences to reference sequences using BLAST (Table S1).

L457: To examine the IN properties of unique strains within samples, multiple sequence alignment of the 16S rDNA sequences was used to identity and remove duplicates. The relationship between 16S rDNA sequences of isolates within their genus is shown in Fig. S11.

Lines 158-159: "*Suggest editing to something like "The sequences of the amplified 16S rRNA gene fragments were determined by...""*

Edited as suggested.

Lines 159 to 161: "*Please define acronyms on first use and describe the criteria used for OTU designation."*

Corrected.

L185: Taxonomic assignments were determined from 16S sequences using the SILVA Incremental Aligner (SINA) (Pruesse et al., 2012) and the Basic Local Alignment Search Tool (BLAST) (https://www.ncbi.nlm.nih.gov/). SINA aligns sequences to the SILVA database of rRNA genes using a combination of k-mer searching and partial order matching. Additionally, individual sequences were inspected using BLAST and species identities were determined by >97% sequence identity to reference rRNA sequences.

Line 163: *Does this mean that the 16S rRNA gene sequences from different isolates were used to create some type of consensus sequence for each OTU? Please explain this process in more detail."*

Great question. A consensus sequence was not used and only sequence identity was used to assign taxonomy during the original SINA and BLAST taxonomic assignments. We've added more detail to describe DECIPHER as more detail of the assignment process in general (see below). We've also greatly removed reference to OTU throughout the document and replaced with clearer taxonomic descriptors.

L197: DECIPHER uses an iterative process for multiple sequence alignment where two sequences are aligned and merged, and each successive sequence is added until all sequences are aligned.

Lines 167-169: "*The description of this analysis is confusing to me. Distances >0.1 or 10% in the 16S rRNA gene would represent very large phylogenetic distances and not differences that would be confused at being the same "OTU". Please also indicate the length of DNA sequences used in this comparison."*

Thanks for the great observation. This definitely warrants some clarification. The sequences within the same genus were aligned and because the alignments were based upon sequences that often had non-equal spans and unequal length the results possessed numerous gaps that were not manually trimmed prior to tree construction. This resulted in trees with overestimations in mutations per base pair. These errors were not seen in the original assignment phase because alignment of one sequence to a full-length reference in the database.

To address a question below because of the errors in alignment instead of deeming sequences that had identical sequences as clonal, we broadened this to include isolates within a threshold of <0.01distance as we wanted to be more rigorous to avoid overreporting the number.

L201: Alignments were not manually trimmed or adjusted prior to tree construction. Branch distances were used to evaluate sequence similarity. As the sequences resulting from rRNA amplification often covered unequal spans and had unequal lengths, their alignments often resulted in overestimates of tree distances (average mutations per base pair). This error was not seen in original OTU assignment where alignment of one sequence to a reference sequence in the database was more successful.

L210: In consideration of overestimations of tree distances and the risk of overreporting numbers of isolates found, we applied more conservative criteria for removal of potential duplication of the same isolate instead of only considering 100% identical sequences. Distances < 0.01 were determined to be possible duplicates if they were collected during the same sampling period unless the organisms had a different phenotype generally indicated by different pigmentation. Each duplicate was tested for its IN ability, and the results are reported in Table S1. If the duplicates had the same IN properties only one representative isolate was retained, and the rest were discarded.

Lines 206-209: "*It appears that the cells were washed and then tested immediately after nutrient removal. This would provide no opportunity for the microbes to respond to the experimental conditions, so it is difficult to interpret these results as being relevant to the effect of IN activity on the presence of nutrients.*"

We have added clarification here. There were 2-4 hours between the washing process and the INP measurement.

Line 252: INP measurements were taken as described within 2-4 hours after the washing procedure and compared to sterile seawater controls (see Fig. S1b and Fig. S1c).

Line 262: *"Is each period a separate rainstorm? There is more than one period for some days and with different trajectories in Figure 2, so maybe they are just different sampling periods."*

Correct, the 11 sampling periods are just different sampling periods rather than e.g. distinct fronts. Sampling Period 8, for example, is from pre-frontal precipitation, whereas 9-11 are post-frontal, although these were all the same rainstorm. These details are provided in Table S3, so I added a reference to this line.

Line 276: Aerosol and rain samples were collected from a pier on the coast of La Jolla, CA (32°52'01.4"N 117°15'26.5"W) during an El Niño event spanning 11 precipitation sampling periods March 6 to May 7, 2016 (Table S3).

Line 291: *"Please clarify if the sequence identities in Table S1 are BLAST outputs or based on distance matrices from multiple sequence alignments. Also, a general comment is that a >97% OTU estimate is highly conservative (e.g., Stackebrandt and Ebers 2006, Microbiology Today, 33:152-155).*

We added clarification to make it clear that the table is BLAST outputs and added detail and an additional table to explain the role of the SINA outputs.

Thank you for the additional information. We will certainly apply these more stringent thresholds in future work.

Line 333: This resulted in 34 isolates from rain samples, and 13 isolates from aerosol samples with 29 unique genera as determined by > 97 % sequence identity of 16S rDNA sequences to reference sequences using BLAST (Table S1). The assignments by SINA agreed with the assignments by BLAST though their sequence identities were lower in some cases (Table S4).

Line 318-319: "*The isolated bacterial and fungal taxa cannot easily be compared to microbial communities, which are associations of many many different types of interacting microbes. And if they are Pacific-sourced aerosols, I'm not sure to consider it a "a warmer climate" even though that is the case where they were deposited in SoCal. Can the isolates grow at cold temperatures or is there any other evidence for cold tolerance, if that is in fact what is being implied here? Please revise this sentence for clarity.*"

Thank you for pointing out this issue. We have removed this line from the text.

Line 334: "*Is it known if the isolates have optimal growth at salt concentrations in seawater, not just tolerance to the concentrations in the seawater-based media? That would provide physiological support for a marine lifestyle.*"

Thank you very much for this suggestion! We did not attempt to grow isolates at different salt concentrations for this study but will certainly consider trying this in future experiments.

Lines 384-385: "*Does this means than isolates having identical sequences in the portion of the 16S rRNA gene examined were deemed clonal and that one isolate was selected as a representative?*"

If isolates had identical sequences or were within 0.01 distance of each other and had the same phenotype and came from the same sampling period and had similar IN freezing temperatures they were deemed clonal and only one was kept. We have added clarification to our process of removing isolate duplicates to the methods section (see above).

Lines 388-390: "*Were any of the observations in Figure S11 replicated to confirm that the patterns of IN at these temperatures and isolates were not more affected by the age of cultures or other potential variations in the way the cultures were handled between experiments?*"

This is a good point. We did not measure INP concentrations in isolates at different points of growth or perform replicate experiments on different suspensions of the isolates, and we agree that it is possible that the observed IN behavior may be affected by the growth phase of the culture.

A line has been added to this paragraph for clarification:

L469: However, INP measurements were not performed repeatedly on isolate suspensions throughout the isolate's growth cycle, so the extent to which the observed freezing behavior was affected by the isolate's growth phase remains unknown.

Lines 411-420; 468: "*Ample time after removal of nutrients may not have been provided in these experiments and the authors should consider limiting this discussion. The one conclusion that can be made is that the activity observed does appear to be associated with the cells and not removed by washing, suggesting the nucleating material is membrane bound or associated with the cell envelope.*"

Thank you. We added some clarification to the section on the washing experiment to say that the washing experiment was performed 2-4 hours prior to the INP measurement. We also shortened this paragraph so that nutrient limitation is only offered as one of multiple possibilities for the observed changes in INP freezing temperatures.

L493: Some of the observed differences in ice nucleation above background between isolates suspended in ZoBell and those suspended in FASW could be a result of the differences in the

background INP concentrations present in the suspension media (i.e. concentrations of INPs in FASW are less than in ZoBell, thus increasing the temperature range in which IN activity could be detected). Another possibility is that the isolates' IN behavior varied depending on multiple factors, including their age, viability, environment, stress, and nutrient availability. As washing cells removes soluble molecules, the apparent IN activity of washed suspensions could indicate that the source of IN activity is membrane-associated, or alternatively, that expression of IN activity is sensitive to environmental factors.

Lines 429-432: "*Please note that the percentage of cells that serve as INPs is temperature dependent, and the "active" fraction values cited from the literature are likely referring to very warm subzero temperatures, whereas for P. syringae populations at temps below -10C, values approaching 100% could be expected. This leads me to suspect the caveats stated are valid for INPs that activate in the -2C range (i.e., Brevibacterium) but may be overly conservative for the colder temperatures of ice nucleation observed in their experiments.*"

This is a good point, thank you. This paragraph has been removed.

Lines 436-440: "*The connection being made with the Failor et al. study is ambiguous. Please clarify if the suggestion is that the taxa in the Failor study were of a marine source, that in the isolates in this study were not marine in origin, or something else entirely.*"

Thank you for bringing this to our attention. I have added some clarification to this section to make the connection clearer, that Failor et al., 2017 also identified multiple halotolerant IN species derived from precipitation samples using marine growth media. I also focused the discussion on comparing their IN halotolerant species and freezing temperatures, since Failor et al., 2017 did not include any discussion or analysis of aerosol source or isolate origins.

L515: One study of note Failor *et al.* (2017) used similar cultivation and INP measurement techniques on precipitation samples and additionally identified multiple halotolerant IN species using marine growth media. However, the IN species identified in Failor et al. (2017) were limited to Gamma-proteobacteria, whereas we find greater diversity among the IN isolate taxonomies, including Actinobacteria, Bacilli, Saccharomycetes, and Tremellomycetes. Two of the halotolerant IN Gamma-proteobacteria identified in Failor et al. (2017) were also found here (see also Fall and Schnell, 1985). Additionally, whereas Failor et al. (2017) reports high freezing temperatures between -4 and -12 °C for multiple halotolerant *Pseudomonas* spp., none of the *Pseudomonas* spp. isolated in our study exhibited detectable IN activity. IN observations for *Pantoea* sp. also differ. The *Pantoea* sp. isolate in our study exhibited a moderate IN freezing temperature of -17 °C, but Failor et al. (2017) reports warm freezing activity between -4 and -10 °C. In addition to environment-dependent changes in isolate IN activity, the differences between the two studies could also be the result of inherent differences in IN activity between different strains of the same species (Morris et al., 2008).

Line 449: "*I am not able to find where this is discussed further. This suggests some of the IN activities reported were difficult to repeat, which would be consistent with other similar attempts, Failor et al. being one good example.*"

I have added some clarification to this section to make clear that this section is discussing how some isolates' IN behavior was found to vary between different types of isolate suspensions. We did not perform repeat measurements on the same isolate suspension at different points of its growth phase.

L534: Finally, where Failor *et al.'s* (2017) results show discrepancies between IN behavior of isolates directly plated from precipitation samples and those from suspensions of purified strains, we also find that IN behavior can vary between different types of isolate suspensions (i.e. ZoBell vs. FASW). (Failor et al., 2017) suggests that changes in an isolate's IN activity may be explained in part by growth conditions not conducive for the expression of INA, and that INA molecules might generally be produced in higher amounts in oligotrophic conditions, like those found in the atmosphere.

Line 484-486: "*General comment to authors: I am most surprised by the fact that out of a group of less than 50, you found one that is active at warmer than -5C and is a member of a phylum where I am not aware of other known examples of this phenotype. In my view, this single isolate may be the most important contribution of this study and will be of interest for additional work to decipher if the mechanism of ice nucleation differs from that of certain Gammaproteobacteria."*

Thank you for pointing this out. We have added the following to emphasize that Brevibacterium sp. is the first Actinobacteria to be shown capable of ice nucleating at -2C.

Abstract, L30: While some Gamma-proteobacteria and fungi are known to ice nucleate at temperatures as high as -2 °C, *Brevibacterium* sp. is the first Actinobacteria found to be capable of ice nucleation at an exceptionally high freezing temperature (-2.3 °C).

L468: Of the known IN bacteria, only Gamma-proteobacteria have been shown to ice nucleate at extremely high temperatures (Morris et al., 2004) and *Brevibacterium* sp. is the first Actinobacteria to be shown capable of IN near 2 °C.

Data availability: "*Please provide database accession information to access the DNA sequence data from this study."*

Thank you for reminding us to make our data accessible. We have added the following to the Data availability section.

The16S rRNA gene sequences from this study were deposited to GenBank under the accession numbers MW704027- MW704080.

Figure 3: "*Please indicate the number of aligned nucleotides and method of phylogenetic analysis used for evolutionary tree construction."*

The Figure 3 legend has been edited as suggested: Maximum likelihood phylogenetic tree based on 420 nucleotides of the 16S rRNA gene sequences showing the phylogenetic relationships of isolates (in bold) related to Gamma-proteobacteria reference sequences. The environmental source of the reference sequences (based on NCBI metadata) is indicated in grey. Isolates with ice nucleating properties are shaded in yellow; bootstrap values (n=500) are indicated at nodes; scale bar represents changes per positions.

In addition to the changes listed above in response to the RCs, we have made minor editorial (e.g. grammar, style) changes throughout the text. We also made the following minor changes**:**

For clarity, we added “all” to L34**:** Air mass trajectory analysis demonstrates that marine aerosol sources were dominant during all sampling periods, and phylogenetic analysis indicates that at least 2 of the 14 IN isolates are closely related to marine taxa.

In-text references written in the style (Author et al., xxxx) have been updated to Author et al., (xxxx) format.

L21, editorial changes and updated statistic: Considering that the ocean covers 71% of the earth’s surface and represent a large potential source of INPs, it is imperative that the identities, properties and relative emissions of ocean INP become better understood.

L54, editorial changes and references added: It has been hypothesized that an enhanced understanding of marine and terrestrial INP populations could contribute to improved representation of ice processes in models (Seinfeld et al., 2016; Storelvmo, 2017; Kanji et al., 2017).