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).

Cultivable, halotolerant ice nucleating bacteria and fungi in coastal precipitation

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Abstract

Ice nucleating particles (INPs) ~~are~~ represent a rare subset of aerosol particles that initiate cloud droplet freezing at temperatures above the homogenous freezing point of water (-38 °C). Considering that the ocean covers 71~~0~~% of the earth's surface and represent a large potential source of INPs, it is imperative that ~~the uncertainties in~~ the identities, properties ~~and~~ and relative emissions of ocean INP become better understood. However, the specific underlying drivers of marine INP emissions ~~and their identities~~ remain largely unknown due to limited observations and ~~the~~ challenges ~~involved in~~associated with isolating ~~exceptionally~~ rare INPs ~~forming particles~~. By generating isolated nascent sea spray aerosol (SSA) over a range of biological conditions, mesocosm studies have shown that marine microbes can contribute to ~~marine~~ INPs. Here, we identify 14 (30%) cultivable halotolerant ice nucleating microbes and fungi among 47 total isolates recovered from precipitation and aerosol samples collected in coastal air in s~~S~~outhern California. IN isolates collected in coastal air were ~~found to nucleate~~nucleated ice from extremely warm to moderate freezing temperatures (-2.3 to -18 °C). While some Gamma-proteobacteria and fungi are

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known to nucleate ice at temperatures as high as -2 °C, *Brevibacterium* sp. is the first Actinobacteria found to be capable of ice nucleation at a relatively high freezing temperature (-2.3 °C). 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. ~~Air mass trajectory analyses, and cultivability in marine growth media indicate marine origins of these isolates. Further phylogenetic analysis confirmed that at least two of the 14 IN isolates were of marine origin.~~ Moreover, results from cell washing experiments demonstrate that most IN isolates maintained freezing activity in the absence of nutrients and cell growth media. This study ~~provides confirmation of~~supports previous studies~~' findings~~ that implicated microbes as a potential source of marine INPs and additionally demonstrates links between precipitation, marine aerosol and IN microbes.

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1 Introduction

Ice nucleating particles (INPs) are rare aerosols, representing ~~(~~~1 in 10^5 or less of total particles in the free troposphere~~)~~ (Rogers et al., 1998) that induce freezing of cloud droplets at temperatures above the homogenous freezing point of water (-38 °C) and at relative humidities (RH) well below the homogenous freezing RH of aqueous solution droplets. They affect multiple climate-relevant properties of mixed-phase and cold clouds. For example, in-cloud INP distributions can influence the ice-phase partitioning processes that determine ~~a~~ cloud~~'~~s' reflectivity, lifetime and precipitation efficiency (Creamean et al., 2013; DeLeon-Rodriguez et al., 2013; Fröhlich-Nowoisky et al., 2016; Ladino et al., 2016). However, numerical representations of cloud ice processes challenge climate models across all scales (Curry et al., 2000; Furtado and Field, 2017; Kay et al., 2016; Klein et al., 2009; Prenni et al., 2007)~~, and it is believed that the under-characterization of global INP distributions contribute to the relevant uncertainties.~~ 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).

Despite recent evidence showing that sea spray aerosol (SSA) represents a ~~unique~~ source of INPs (DeMott et al., 2016; McCluskey et al., 2016, 2018a, 2018b), that these INPs can contribute significantly to total INP populations (particularly in remote marine regions where terrestrial aerosols are less abundant) (Burrows et al., 2013; Vergara-Temprado et al., 2017; Vergara-Temprado et al., 2018), and that specific parameterization of marine INPs can influence modelled radiative budgets (Wilson et al., 2015), little is known about the actual entities involved in forming marine INPs. ~~(~~Schnell and Vali~~,~~ (1975) were the first to associate phytoplankton blooms with increases in ~~raised~~ ice nucleation activity~~,~~ in seawater sampled shortly after a bloom in Bedford Basin, Nova Scotia. Recent mesocosm studies have linked SSA ice nucleating (IN) activity specifically to the death phase of phytoplankton blooms. ~~(~~McCluskey et al.~~,~~ (2017) showed that increases in INP emissions corresponded to increased emissions of heterotrophic bacteria and the transfer of organic species in SSA, implicating microbes and biomolecules as contributors to marine INP populations. Marine microbes were further linked to INPs in (McCluskey et al., 2018a): subsets of INPs in nascent SSA were found to be heat labile, with sizes greater than 0.2 µm, and INP emissions correlated to increased emissions of cells or cellular material. An IN halotolerant strain of *Pseudomonas fluorescens* was detected in phytoplankton cultures derived from seawater (Fall and Schnell, 1985), and INPs have also been detected in seawater containing marine diatoms, green algae (Alpert et al., 2011; Junge and Swanson, 2007; Ladino et al., 2016; Parker et al., 1985), and sea-ice samples containing marine Antarctic bacteria (Junge and Swanson, 2007; Parker et al., 1985).

While indirect evidence indicates marine microbes and other biogenic entities as ~~possible~~ potential marine INPs, microbial contribution to marine INP populations has not yet been confirmed through direct observations ~~of any marine IN entity in the atmosphere~~ (i.e. through isolation and identification in an atmospheric sample)~~ . were previously nonexistent.~~ Multiple factors make it difficult to determine INP origin, whether terrestrial or marine, including the low abundance of INPs and the diversity of aerosols ~~with IN ability~~that can ice nucleate (e.g. Kanji et al., 2017). ~~Moreover, it is not always possible to differentiate terrestrial and marine air mass influences within the Marine Boundary Layer (MBL).~~ However, cultivable IN microbes have been isolated from clouds and precipitation for decades (e.g. Sands et al., 1982; Failor et al., 2017; Morris et al., 2008), and the origins of IN isolates can be determined by comparing sequences with reference isolates of known origin. There are several caveats to consider when inferring in-cloud INP concentrations or properties from precipitation samples (Petters and Wright, 2015), including "sweep-out" of additional INPs as the hydrometeor traverses the atmosphere below the cloud (Vali, 1974). However, previous studies have derived estimates of in-cloud INP concentrations and origins from the concentrations and identities of IN microbes from ground-level collections (Christner et al., 2008; Failor et al., 2017a; Joyce et al., 2019; Monteil et al., 2014) by assuming that particles in precipitation originate from the cloud rather than the atmospheric column through which the hydrometeor descended. This assumption is~~-~~ supported by ~~(~~Vali~~,~~ (1971), which found that subcloud scavenging of aerosol did not affect INPs observed in precipitation collected at the surface in comparisons of INP spectra from surface samples with samples collected at cloud-base. Furthermore, ~~(~~Wright et al.~~,~~ (2014) estimated that sweep-out contributed between 1.2 and 14% of INPs suspended in a precipitation sample collected at the surface.

While evidence exists for relationships between IN microbes and precipitation in terrestrial systems, studies of the relationship between marine INPs, marine microbes, and precipitation remain quite limited. Here we report the identities and freezing temperatures of 14 cultivable halotolerant IN species derived from marine and coastal precipitation and aerosol samples~~, two of which were marine in origin~~. Over the course of 11 precipitation events during an EI Niño season, 47 cultivable halotolerant bacteria and fungi were recovered from aerosol and precipitation samples collected in a coastal subtropical climate in southern California. Bacterial and fungal species were isolated, identified, and tested for ice nucleation behavior from 0 to -25 °C using an immersion mode droplet freezing assay technique. Precipitating cloud altitudes and isolate source regions were estimated using the High-Resolution Rapid Refresh atmospheric model (HRRR) and the FLEXible PARTicle dispersion model (FLEXPART) (Stohl et al., 1998), respectively. Finally, the effect of media on the observed IN behavior of isolates was investigated through cell washing experiments.

2 Methods

2.1 *Precipitation and Aerosol Sample Collection Methods*

Precipitation and ambient aerosol samples were collected on the Ellen Browning Scripps Memorial Pier at Scripps Institution of Oceanography (SIO) (32.8662 °N, 117.2544 °W) from March 6, 2016 – May 6, 2016. Sampling took place in the surf 8 m above Mean Lower Low Water (MLLW), and samples were only collected during westerly winds. Aerosol samples were collected over 1.5-5 hour periods on polycarbonate filters (45 mm diameter, 0.2 µm pore-size, Whatman® Nucleopore, Chicago, Illinois, USA) placed in open-face Nalgene ® Analytical Filter Units (Waltham, Massachusetts, USA). 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

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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). 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. ~~immersed in 12 mL of ultrapure water. , and particles~~The samples were then hand shaken ~~were shaken off the filter by hand~~ for 20 minutes to resuspend particles from the filter. The precipitation samples were collected using a modified Teledyne Isco© Full-Size Portable Sampler (Lincoln, Nebraska, USA), fitted with 24 1-L polypropylene bottles. Prior to sampling, the bottles were immersed in 10 % hydrogen peroxide for 10 minutes, then rinsed three times with ultrapure water. The automated sampler would engage when triggered by precipitation of at least 0.13 cm h^{-1} and would sample using the first of 24 bottles for 30 minutes, and thereafter switch bottles at hourly intervals. Within one to two hours of sample collection, INP concentrations were measured using the SIO-Automated Ice Spectrometer (SIO-AIS) (Beall et al., 2017), an automated offline freezing assay technique for measurement of immersion mode INPs. To decrease the effect of interstitial particle sweep out by falling raindrops on measured INP concentration, precipitation from the first 30 minutes was discarded. Sweep out effects have been estimated to contribute between 1.2 and 14 % to measured concentrations of INP in a precipitation sample (Wright et al., 2014).

The INP measurement technique is described in detail in (Beall *et al.*, 2017*)*. Briefly, the precipitation samples and aerosol sample suspensions~~samples~~ were distributed in 24-30 50-microliter aliquots into a clean 96-well disposable polypropylene sample tray. An equal number and volume of aliquots of ultrapure water accompany each sample in the disposable tray as control

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for contamination from the loading and/or ultrapure water. The sample trays were then inserted into an aluminum block that is cooled until the samples are frozen. Cumulative INP number concentrations per temperature per volume are calculated using the fraction (*f*) of unfrozen wells per given temperature interval:

$$INP = \frac{-ln(f)}{V_d} \quad \text{Eq. (1)}$$

where V_d is the volume of the sample in each well. For aerosol filter samples, cumulative INP number concentrations are calculated using the ratio of the volume used for resuspension of the particles (V_{re}) to the volume of aerosol sampled (V_A) :

$$INP = \frac{-ln(f) \cdot V_{re}}{V_d \cdot V_A} \quad \text{Eq. (2)}$$

The fraction of unfrozen wells (f) is adjusted for contamination by subtracting the number of frozen ultrapure water wells per temperature interval from both the total number of unfrozen wells and total wells of the sample. For this study, 30 x 50 µL droplets were deposited into the droplet assay, yielding a detection limit of 0.675 INP mL^{-1} liquid.

Within one to two hours of collection, precipitation and aerosol samples were also inoculated in 5 mL ZoBell growth media (ZoBell, 1947) (5 g peptone, 1 g yeast extract per 1 L of filtered (0.22 µm) autoclaved seawater) and grown under ambient conditions (21 - 24 ˚C). Seawater was collected at the Ellen Browning Scripps Memorial Pier and was filtered prior to autoclaving. -INP concentrations in ZoBell enrichments were measured 1-day post inoculation and for several days thereafter to monitor for sustained IN activity.

2.2 *Bacterial and fungal isolation and characterization*

Precipitation and SSA microorganisms were cultivated using the ZoBell enrichment described above (ZoBell, 1947) (Fisher Scientific, Houston, Texas, USA). Isolation was performed by successive plating on ZoBell agar (BD Bacto™ Agar, Sparks, MD, USA). Liquid cultures were inoculated from single colonies and grown to late exponential phase. DNA was extracted from liquid cultures of isolates after an overnight lysis with proteinaseK (100 µg mL^{-1}) and lysozyme (5 mg mL^{-1}) (MilliporeSigma, Burlington, Massachusetts, USA) (Boström et al., 2004) using a QIAamp® kit (QIAGEN, Hilden, Germany). The~~16S~~ V4 region of the 16S rRNA gene was ~~ribosomal DNA fragments were~~ amplified using the primers 515F (5´ GTGYCAGCMGCCGCGGTAA 3´) and 926R (5´ CCGYCAATTCMTTTRAGT 3´) (Walters et al., 2015). The PCR reaction contained 0.5 ng μl^{-1} genomic DNA, 0.2 µM 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.~~-~~ PCR products were purified using GenElute™ PCR Clean-up kit (MilliporeSigma). The sequences of the amplified 16S ~~fragment DNA~~rRNA gene fragments ~~sequences were resolved by~~were determined by Sanger sequencing (Retrogen, San Diego, CA). Taxonomic assignments~~Operational Taxonomic Units (OTUs)~~ 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/). SINA aligns sequences to the SILVA database of rRNA genes using a combination of k-mer searching and partial order matching. Additionally, ~~and ji~~ndividual sequences were inspected using ~~the Basic Local Alignment Search Tool (~~BLAST~~) (https://www.ncbi.nlm.nih.gov/) for further characterization~~and species identities~~.~~–~~ were determined by >97% sequence identity to reference rRNA sequences. The ~~16S~~ 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.

To assess for duplicate isolates within the sampling period, 16S rDNA sequences were compared. Sequences within the same genus~~OTU~~ were adjusted and aligned in DECIPHER(Alignseqs(), AdjustAlignment() with default settings) (Wright, 2015). 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. These sequence alignments were used to generate phylogenetic trees using ClustalW2 (UPGMA) (McWilliam et al., 2013) and visualized with iTOL (Letunic and Bork, 2011). 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 taxonomic assignment where alignment of one sequence to a reference sequence in the database was more successful. To facilitate comparisons between organisms assigned to the same genus~~OTU~~, identity assignments including divisions at distances > 0.1 (e.g. 1, 2, 3…) were further subdivided by distances > 0.01 (e.g. 1a, 1b, 1c…). Nonzero distances < 0.01 were given sub labels (e.g. 1a1, 1a2…). Zero distances were given identical labels. In consideration of overestimations ~~present in~~of tree distances and ~~attempting to avoid~~ the risk of over-reporting numbers of isolates found, we ~~wanted to be more rigorous in our~~applied more conservative ~~threshold~~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

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pigmentation. Each duplicate was tested for its IN ability, and the results are reported in Table S1 ~~and discussed in the main text~~. If the duplicates had the same IN properties only one representative isolate was retained, and the rest were discarded. Maximum likelihood phylogenetic trees were computed in MEGA7~~-~~ (Tamura et al., 2013) after ClustalW alignment with reference sequences (https://www.ncbi.nlm.nih.gov/)~~-~~.

2.3 *Storm and aerosol source characterization methods*

Cloud altitudes at the time of precipitation sample collection were estimated using the High-Resolution Rapid Refresh model (HRRR). The altitudes and pressure levels of clouds were assumed to be located where RH was > 95-100 % in the model. The specific RH criteria applied to each sampling period are provided in Table S2. HRRR model output was compared with surface RH measurements from the SIO pier weather station during sampling periods, and predicted RH was found to agree with observations with an RMSE of < 10 – 15%, which aligns closely with previously reported RH accuracies over the continental US (Benjamin et al., 2016). Three altitudes of the estimated cloud top, middle and bottom were used as release points of FLEXPART 10-day Lag~~G~~rangian backward trajectories. Back-trajectories were used to identify potential sources of INPs in the precipitation samples, and to indicate potential sources of land-based contamination in aerosol and precipitation samples due to local wind patterns or land-sea breezes. Satellite composites from the National Weather Service Weather Prediction Center's North American Surface Analysis Products were used for synoptic weather analysis to generally characterize meteorology during each rain event (see Table S3).

2.4 *Isolate IN activity measurement and controls*

To measure the IN activity of each isolate, liquid cultures were grown to late exponential phase. Growth was monitored by optical density (OD) (590 nm). INP concentrations were measured as described in Sect. 2.1 in liquid cultures and compared to a ZoBell blank as a control. Isolate biomass was estimated from OD measurements using the distribution of OD to biomass conversion factors from (Myers et al., 2013). As Myers et al. (2013) found, in a study of 17 diverse organisms, OD to biomass conversion factors ranged between 0.35 and 0.65 gDW OD^{-1} L^{-1}; we assume that INP g^{-1} biomass may be estimated from OD within a factor of 2. Thus, isolate INP concentrations, and upper and lower limits of 95% confidence intervals were scaled by $\frac{1}{m}$, where m is the mean, minimum or maximum value of the (Myers et al., 2013) biomass conversion factor distribution, respectively (i.e. 0.5, 0.65 and 0.35 gDW OD^{-1} L^{-1}).

To investigate the effect of growth media on IN isolates, a subset of late exponential cultures were washed three times with filtered (0.22 µm) autoclaved seawater (FASW) by successive centrifugation and resuspension. The washing procedure removes everything that is water soluble and whole cells and insoluble molecules pellet upon centrifugation. INP measurements were taken as described within 2-4 hours after the washing procedure and compared to sterile seawater controls (see Fig. S21b and Fig. S21c).

As ZoBell growth media contained INPs at moderate to cold freezing temperatures (-13 to -25 °C, see Fig. S21a), only isolates exhibiting INPs at significantly higher freezing temperatures (-2.3 to -15 °C) or at significantly higher concentrations than their respective ZoBell growth media sample were considered to be IN. The criterion for significance was chosen to be conservative: a data point along an isolate's measured IN spectrum was considered significant if there was no overlap between the 95 % binomial sampling confidence interval of the given data point (Agresti and Coull, 1998) and any ZoBell confidence interval within ± 2.2 °C, the maximum uncertainty in

freezing temperature measurement due to heterogeneity in heat transfer rates across the instrument's droplet assay (Beall et al., 2017). This equates to a significance threshold of $p < 0.005$ (Krzywinski and Altman, 2013). The choice of ± 2.2 °C is likely conservative given that in a study of 11 cooling cycles, the average and maximum ΔT observed across the droplet assay when cooling from 0 to -25 °C was 0.38 and 0.98 °C, respectively (and following this study, the addition of a second thermistor under the second sample tray decreased the observed ΔT to within thermistor uncertainty, ± 0.2 °C). The same criterion was applied to isolates washed and suspended in FASW as described above (Figs. S2~~1~~ b-c). Many isolates were diluted with their respective media (ZoBell or FASW) to decrease opacity such that freezing events could successfully be detected by the camera, so their respective dilution factors were applied to both the INP concentrations measured in the isolate suspension and the INP concentrations measured in the FASW or ZoBell samples for the significance analysis (see Figs. S2~~1~~ b-c and S3~~2~~).

3 Results and discussion

3.1 Subtropical coastal storm properties and origins

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). Observations of INPs in precipitation generally fall within bounds of previously reported INP concentrations from precipitation and cloud water samples (Fig. 1, grey shaded region, adapted from Petters and Wright, 2015). AIS measurement uncertainties are represented with 95% binomial sampling intervals (Agresti and Coull, 1998). Observed freezing temperatures ranged from -6.5 to -22.0 ºC, with concentrations up to the limit of testing at 10^5 INP L^{-1} precipitation. Following the assumptions in (Petters and Wright, 2015) to

estimate in-cloud INP concentrations from precipitation samples (i.e. condensed water content of 0.4 g m^{-3} air), observations of INP concentrations in fresh precipitation samples are additionally compared to studies of field measurements conducted in marine and coastal environments.

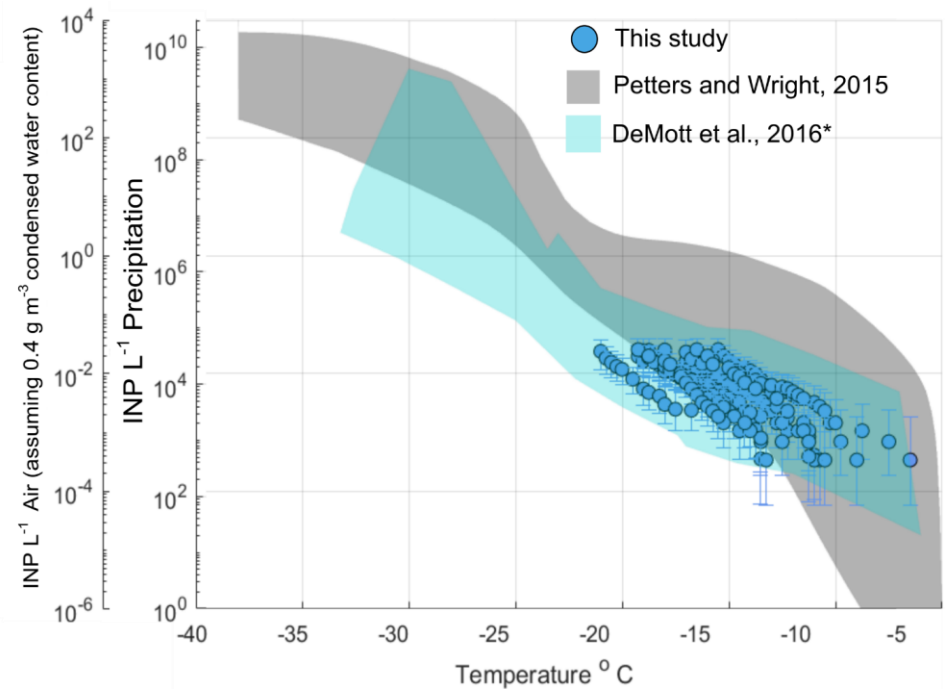

Figure 1. **INP concentrations per liter precipitation and estimated in-cloud INP concentrations per volume of air in 11 precipitation samples collected at Scripps Institution of Oceanography Ellen Browning Scripps Memorial Pier (32.8662 °N, 117.2544 °W, La Jolla, California, USA) between March and May 2016.** Grey shaded region indicates the spectrum of INP concentrations reported in nine previous studies of precipitation and cloud water samples collected from various seasons and locations worldwide, adapted from Fig. 1 in (Petters and Wright, 2015). The blue shaded region represents the composite spectrum of INP concentrations observed in a range of marine and coastal environments including the Caribbean, East Pacific and Bering Sea as well as laboratory-generated nascent sea spray (DeMott et al., 2016).

*DeMott et al., 2016 data haves been updated with a completed dataset fromor the ICE-T study, as shown in (Yang et al., 2020).

Figure 1 shows that atmospheric INP concentration estimates compare with INP concentrations observed in a range of marine and coastal environments, including the Caribbean, East Pacific, and Bering Sea, as well as laboratory-generated nascent sea spray aerosol (DeMott et al., 2016). Observations of INPs in aerosol samples are shown in Fig. S4~~3~~ and are also comparable with those of DeMott et al. (2016).

The source regions of aerosols present in precipitating clouds were estimated using 10-day FLEXPART back trajectories (Fig. 2). For each of the 11 sampling periods, back trajectories show that the Pacific Ocean from mid to high latitudes was the primary source region to precipitating cloud layers. Periods 5 – 11 may have been additionally influenced by west coast continental sources (particularly periods 6 and 7). 10-day back trajectory simulations for aerosol samples similarly indicated that marine sources dominated (see Fig. S5~~4~~). Marine aerosols likely originated from regions near the coast (Periods 2, 4-11, A1, A2, A5) or in the mid Pacific Ocean (Periods 1 and 3), where trajectories descended below the marine boundary layer.

Cloud bottom and top altitudes were estimated using the High-Resolution Rapid Refresh model (HRRR), defined by the RH criteria in Table S2. Over the 11 precipitation sampling periods, cloud altitude ranged from 950 – 600 mb, bottom to top, or 500 – 4000 m, with temperatures ranging from 265 – 288 K.

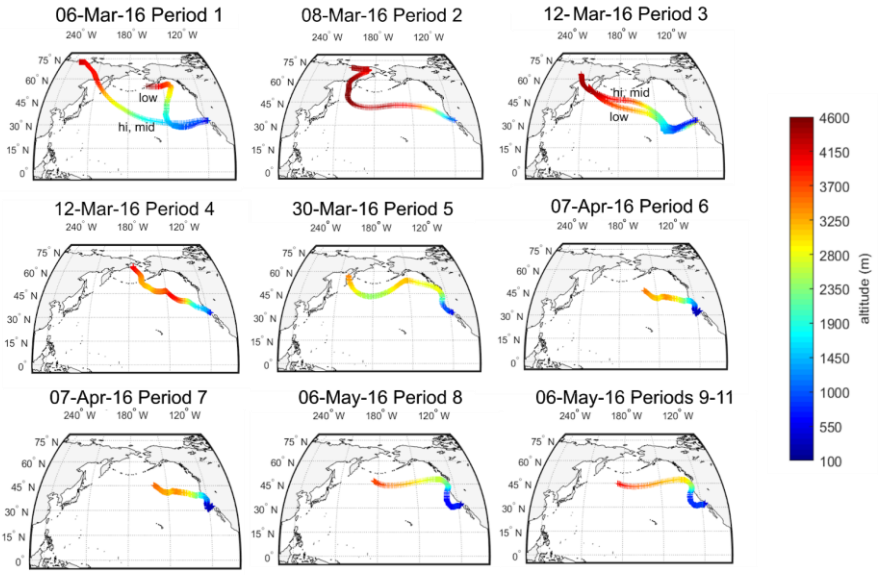

Figure 2. 10-day back-trajectories from cloud base, mid-cloud, and cloud-top during 11 precipitation sampling periods at the SIO Pier (32.8662 °N, 117.2544 °W). FLEXPART back-trajectories were used to estimate potential source regions of INPs to the clouds during precipitation events. Shown are the particle centroids of back-trajectories from three release altitudes within each cloud (see Table S2 for details on altitude selection criteria). If trajectories across the three selected release altitudes differentiated, they are labeled "hi" for cloud top, "mid" for halfway between base and top, and "low" for cloud bottom. Origins of particles in the 10-day simulation are shown to range from 4000 m over Russia to 2500 – 3500 m over the Sea of Okhostk, the Bering Sea, and the north Pacific. FLEXPART results suggest a dominance of marine particle sources to clouds for sampling periods 1-11.

3.2 Bacterial and fungal taxonomy

Cultivable bacteria and fungi were enriched from rain and aerosol samples in marine bacterial growth media, and strains were further isolated on marine agar. This resulted in 34 isolates from rain samples, and 13 isolates from aerosol samples with 29 unique ~~operational taxonomic units (OTUs)~~ 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 S~~4~~5). Many of the isolates derived from rain and aerosol were highly pigmented, as observed in other studies (Delort et al., 2017; Fahlgren et al., 2010, 2015; Hwang and Cho, 2011; Tong and Lighthart, 1997), presumably aiding their survival under high *uv* exposure (Fig. S~~6~~5). This pigmentation was especially prevalent in rain samples.

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 ~~The higher number of precipitation-derived isolates compared to aerosol~~ osmotically shocked during resuspension in ultrapure water (see Sect. 2.1). ~~is likely the result of lower~~ Decreased aerosol bacterial and fungal loads during rain events may have also contributed to lower isolate yield. INP concentration decreases in aerosol during precipitation events support this conclusion. For 3 of the 11 precipitation events featured in this study (see Fig. S~~1~~6), INP concentrations in aerosol were measured immediately before, during, and after precipitation events. In each of the three events, INP concentrations in aerosol decreased below detection levels during precipitation and increased again soon after the end of the precipitation event (in under 24 hours), though not beyond concentrations observed prior to the precipitation event. Interestingly, these features (i.e. the observed decreased INP concentrations during precipitation events and absence of increased INP concentrations within 24 hours of precipitation events) are in opposition to multiple studies of INP concentrations during and after rainfall events in terrestrial systems (Bigg, 1958; Conen et al., 2017; Huffman et al., 2013; Prenni et al., 2013). Additionally, ~~(~~Levin et al.~~,~~ (2019)

observed an increase in INP concentrations after precipitation events in a coastal environment, though this increase may have been related to a shift from marine to terrestrial aerosol sources as indicated by the back trajectories. Thus, results in this study indicate that the positive feedbacks between rainfall and surface INP emissions observed in terrestrial systems (Bigg et al., 2015; Morris et al., 2017) may not always apply to marine environments.

~~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~~. ~~The rain samples had a high proportion of Actinobacteria, whereas in aerosol, Firmicutes and Proteobacteria were more dominant.~~

~~The microbes isolated in our study are closely related to microbial communities described in other studies, despite being isolated from a warmer climate (Bowers et al., 2009; Fröhlich-Nowoisky et al., 2016; Santl-Temkiv et al., 2015; Vaïtilingom et al., 2012).~~ The rain samples had a high proportion of Actinobacteria, whereas in aerosol, Firmicutes and Proteobacteria were more dominant. As ~~(~~Michaud *et al.*~~,~~ (2018) showed, Actinobacteria, as well as select Proteobacteria and Firmicutes, have an increased ability to be aerosolized from seawater~~, and so~~ in SSA emissions ~~may also explain their presence here~~. Two isolates (one from rain and one from aerosol, 3.5% of total isolates) are related to *Pantoea* sp., strains of which are known to possess IN proteins (e.g., Hill et al., 2014). *Pantoea* sp. and *Psychrobacter* sp. were the only bacterial taxa identified previously known to possess ice nucleation activity (Hill et al., 2014; Ponder et al., 2005). However, both *Psychrobacter sp.* and *Idiomarina* sp. have been shown to be capable of inhibiting ice recrystallization, possibly through the production of antifreeze

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proteins (AFPs) which can both inhibit freezing at moderate temperatures and serve as INPs at colder temperatures (Wilson and Walker, 2010).

The phylogenetic relationships between isolates and reference sequences (Fig. 3) ~~indicate~~ show that at least ~~marine origin is highly likely for~~ 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. Additionally, considering the aerosol transport simulation data (Fig. 2), the evidence of marine influence in precipitation INP spectra (Fig. 1), and the use of marine growth media, multiple other IN isolates derived from the precipitation samples are also possibly marine. Furthermore, other IN isolates from precipitation samples cluster closely with marine reference sequences. For example, *Pantoea* sp.1a and *Brevibacterium* sp. show high similarity to reference sequences derived from marine environments (Fig. 3 and S8). However, several of the species identified in this study are likely more ubiquitous, and closely related to reference isolates found in terrestrial and freshwater systems (Bowers et al., 2009; Fröhlich-Nowoisky et al., 2016; Santl-Temkiv et al., 2015; Vaïtilingom et al., 2012), including two of the IN isolates, *Psychrobacter* sp. 1b2 and *Paenibacillus* sp. 1.

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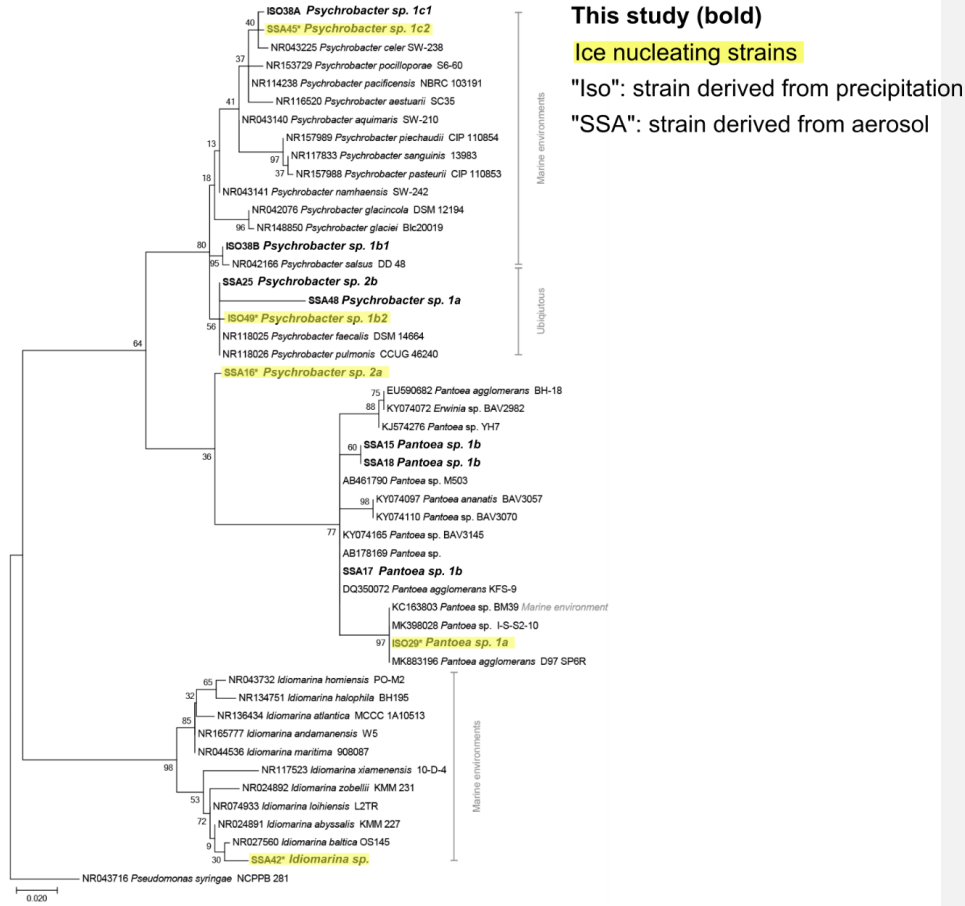

Figure 3. Maximum likelihood phylogenetic tree based on 420 nucleotides of the 16S rRNA gene sequences showing the p~~P~~hylogenetic relationships of isolates (in bold) related to Gamma-proteobacteria reference sequences. The environmental source of the reference sequences (based on NCBI metadata) is 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.

3.3 Ice Nucleating Properties of Rain and SSA isolates

Of the 47 total isolates derived from precipitation and aerosol samples, 14 were found to be significantly ice nucleating according to the selection criterion described in Methods Sect. 2.4. Within the technique's temperature and detection limit of 0.675 INP mL^{-1} liquid between 0 and -25 °C, 11 precipitation isolates exhibited freezing temperatures between -2.3 and -24.3 °C, and 3 aerosol isolates exhibited freezing temperatures between -14.0 and -24.5 °C (Table 1). Prior to this study, *Lysinibacillus* sp. was the only known gram-positive species found to be capable of ice nucleation (Failor et al., 2017a). Yet several IN isolates identified in this study are also gram-positive, including isolates of *Brevibacterium* sp., *Paenibacillus* sp., *Planococcus* sp., *Bacillus* sp., *Arthrobacter* sp., and *Cellulosimicrobium* sp.

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Table 1. Identities of 14 cultivable, halotolerant IN bacteria and fungi derived from aerosol and~~or~~ precipitation samples (see Table S2 for precipitation and aerosol sample details).

IsoID	Isolate	IN Onset Temperature °C	Precipitation or Aerosol Sample Number
Iso2	*Cryptococcus* sp. 1	-9.3	1
Iso10B	*Paenibacillus* sp. 1	-14.8	2
Iso8	*Brevibacterium* sp. 1b	-2.3	4
Iso32B	*Planococcus* sp. 1	-12.3	7
Iso29	*Pantoea* sp. 1a	-17	8
Iso31	*Bacillus* sp.1a1	-14.5	8
Iso21	*Cellulosimicrobium* sp. 1a1	-14	9
Iso23	Unknown ~~*Arthrobacter*~~	-13.3	9
Iso24A	*Metschikowia* sp.	-16.5	9
Iso27	*Cellulosimicrobium* sp. 1a3	-14.8	10
Iso49	*Psychrobacter* sp. 1b2	-13.8	11
SSA42	*Idiomarina* sp.	-14.3	A1
SSA16	*Psychrobacter* sp. 1c2	-17.5	A2
SSA45	*Psychrobacter* sp. 2b	-14	A5

Isolate INP spectra are shown in Fig. 4, normalized to biomass, $n_m\,g^{-1}$ (see Sect. 2.4 for details on biomass estimates). Also plotted in Fig. 4 are observations of a variety of marine and terrestrial bioaerosols from prior studies, including pollens, fungi, lichens, plankton, leaf litter and soil dusts (Conen et al., 2011; Conen and Yakutin, 2018; Després et al., 2012; Fröhlich-Nowoisky et al., 2015; Kunert et al., 2019; O'Sullivan et al., 2015; Wex et al., 2015). Results show that with the exception of *Brevibacterium* sp., isolates from this study are generally less efficient than most terrestrial IN biological particles, with lower concentrations and activation temperatures. Concentrations of INP per mL in ZoBell suspension are additionally shown in Fig. S10.

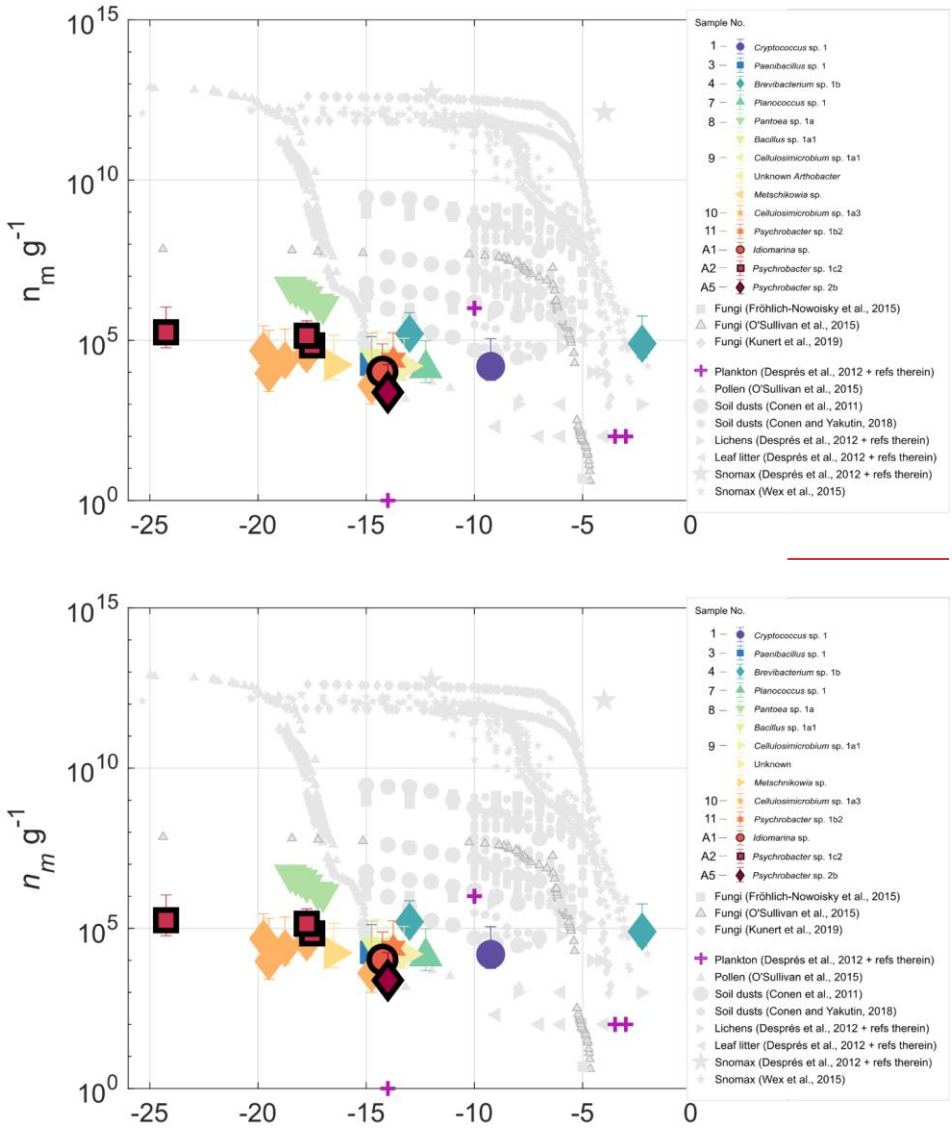

Figure 4. INP concentrations (g^{-1} biomass) for 14 halotolerant isolates derived from precipitation and aerosol samples. Also shown are INP observations of various biological particles from published studies. Sample numbers in the legend indicate the precipitation or aerosol sample from which the isolate was derived (see Table S3). Datapoints corresponding to isolates from aerosol

are outlined in black. Error bars indicate 95% confidence intervals and uncertainty associated with biomass estimate (see Sect. 3.3 for ▪ details). Only freezing activity that was significantly enhanced ($p < 0.005$) above ZoBell growth media is shown. Results show that with the exception of *Brevibacterium* sp., isolates are generally less efficient ice nucleators than most biological INPs of terrestrial origin.

Fungal isolates *Cryptococcus* sp. and *Metschikowia* sp. represent two new asomycotic 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 multiple 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).

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 ~~OTUs~~ genus is shown in Fig. S11. Ice nucleating precipitation and aerosol isolates exhibit moderate IN freezing temperatures

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(< -10 °C) (Fig. 4), with the exception of two warm freezing isolates: a fungal isolate from sampling period 1, *Cryptococcus* sp., which triggered freezing at -9.3 °C, and a bacterial isolate from sampling period 4, *Brevibacterium* sp., at ~~an exceptionally~~a relatively ~~warm~~ high freezing temperature of -2.3 °C. The freezing temperatures of all but *Brevibacterium* sp. 1b overlap with previously reported freezing temperatures of INPs produced ~~from~~ in fresh SSA (-7 to -33 °C), and, in particular, with the freezing temperatures shown to be likely associated with microbes or cellular material in SSA (-8 to -22 °C). (DeMott et al., 2016; McCluskey et al., 2017). Isolate freezing temperatures also overlap with INP freezing temperatures in samples of Arctic marine sea surface microlayer (Irish et al., 2017; Wilson et al., 2015). 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.

Of the known IN bacteria, only Gamma-proteobacteria have been shown to nucleate ice at high temperatures (Morris et al., 2004). *Brevibacterium* sp. was the first Actinobacteria to be shown capable of IN near 2 °C. Considering that only IN microbes of continental origins, such as *Pseudomonas syringae*, have been reported with freezing temperatures as high as -2 or -3 °C (e.g. Fröhlich-Nowoisky *et al.*, 2016 and references therein), and that SSA is associated with 1000 times fewer ice nucleating active sites per unit surface area compared to mineral dust (McCluskey et al., 2018b), ~~it would be unexpected~~one would not expect to find a marine IN isolate with an extremely warm freezing onset temperature. However, the presence of bacteria closely related to the *Brevibacterium* sp. in marine environments suggests that a marine origin is possible (Fig. S8, see also discussion in Sect. 3.2). Furthermore, the back-trajectory analysis for the sample from which *Brevibacterium* sp. was isolated indicates that the North Pacific

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region~~sources~~ dominated the sampling period. Actinobacteria are common in marine environments (e.g. Bull et al., 2005) and have been identified in nascent SSA (Michaud et al., 2018).

~~-~~To explore the role of the growth media on ~~isolate~~ IN properties of isolates, controls were run on nine washed isolates (Fig. S2~~1~~ and Table S5~~4~~, see Methods Sect. 2.4). Five of the selected isolates were found to not be significantly IN above sterile ZoBell background, while four were chosen from the subset of significantly IN isolates. Interestingly, the observed INP concentrations of washed isolates above that of the FASW were inconsistently related to activity when grown in ZoBell media, and were generally enhanced. Seven of the nine media-free isolates exhibited significant IN behavior, including 4 isolates that were not IN in ZoBell. 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 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. ~~For example, limited nutrient availability has been shown to enhance IN behavior of both *Lysinibacillus* sp. and *P. syringae* sp. (Failor et al., 2017a; Nemecek-Marshall et al., 1993).~~ The difference in IN activity between ZoBell and FASW suspensions indicates that *in situ* measurements of IN bacteria will be necessary to determine the abundance of active IN microbes in the atmosphere.

~~Another limitation of the cultivation approach is that the concentrations of the IN species in the precipitation samples from which we derived them are unknown. Considering that typically, only a fraction of an IN isolate's cells are actively ice nucleating, even for highly efficient IN microbes such as *P.syringae* (2-4%, Amato et al., 2015), concentrations of active IN microbes in the precipitation samples were likely below the limit of detection (0.675 mL^{-1}, see Sect. 2.1). For example, assuming a high active fraction of 2% and the maximum concentration of cultivable microbes in precipitation observed in (Failor et al., 2017b, ~22,000 L^{-1}), the concentration of actively ice nucleating microbes equal 0.44 mL^{-1}, which is below the limit of detection.~~

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).

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~~One study of note (Failor *et al.*, 2017) used similar cultivation and INP measurement techniques on precipitation samples.~~ ~~While Failor et al. (2017) did not report estimates of source regions or claim marine origin of the IN microbes that were cultivable in marine growth media, they report the presence and IN freezing temperatures of *Pseudomonas* sp. and *Pantoea* sp., both of which were also found here (see also Fall and Schnell, 1985). Additionally, whereas (Failor *et al.*, 2017) reports warm freezing temperatures between -4 and -12 °C for multiple *Pseudomonas* sp. samples, none of the *Pseudomonas* sp. isolated in our study exhibited detectable IN activity. Similarly, pseudomonads were common, but all lacked ice nucleation activity in rain and cloud water samples collected on the coast of Scotland (Ahern et al., 2007).~~ ~~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.~~

Finally, where the~~Interestingly,~~ results from 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, w~~s~~e also find that ~~, supporting our findings that~~ IN behavior can vary between different types of isolate suspensions (i.e. ZoBell vs. FASW).~~between different isolate suspensions.~~ (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~~ may generally be produced in higher amounts in oligotrophic conditions, ~~like~~ such as those found in the atmosphere. ~~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).~~ ~~Finally, whereas (Failor *et al.*, 2017) report only IN Gammaproteobateria that were cultivable in marine growth~~

~~media, we find greater diversity among the IN isolate taxonomies, including Actinobacteria, Bacilli, Saccharomycetes, and Tremellomycetes~~.

4 Conclusions

Through isolation and identification of multiple IN microbes in precipitation and aerosol, this study provides identities of multiple halotolerant IN microbes that are likely of marine origin. ~~Through isolation and identification of multiple IN microbes in precipitation and aerosol, this study reveals two specific marine INP identities, *Idiomarina* sp. and *Psychrobacter* sp. 1c2, confirming previous mesocosm studies' implication of marine microbes as INP candidates (McCluskey et al., 2017, 2018a). Furthermore, we~~Furthermore, we isolated six new IN gram-positive bacteria capable of ice-nucleation.~~,~~ ~~as p~~Prior to this study, *Lysinibacillus* sp. was the only gram-~~negative~~ positive species capable of ice nucleation (Failor et al., 2017). Additionally, through cell washing experiments in which soluble molecules and growth media are eliminated from isolate suspensions, we find that the IN activities of most isolates~~' IN activities~~ are dependent on growth conditions.

Due to the challenge of distinguishing between marine ~~from~~ and terrestrial INPs in environmental samples, it is impossible to definitively claim marine or terrestrial origins for ~~10 of~~ the 14 IN isolates measured~~featured~~ in this study. In order to survive atmospheric transport and deposition ~~through~~ in rainwater, cultivable isolates derived from precipitation must be tolerant of near-freshwater conditions. However, marine origins is highly likely for multiple isolates ~~are possible~~ for the following reasons: aerosol back-trajectories and INP observations during sampling events indicate that marine regions~~sources~~ were dominant (Figs. 1-2), multiple isolate sequences show similarity to marine isolation sources in reference sequences (Figs. 3, S8~~), and~~), and isolate

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freezing temperatures are generally in agreement with previously documented nascent SSA IN freezing temperatures (DeMott et al., 2016; McCluskey et al., 2017, 2018a).

While cultivation methods preclude quantification of atmospheric abundance and exclude a large fraction of uncultivable microorganisms, we captured several possible contributors to precipitation IN populations and through isolation maintained the ability to assess their IN activity and other characteristics. Considering the general rarity of atmospheric INPs (1 in 10^5 at -20 °C) (Rogers et al., 1998), the relatively lower concentrations of INPs in marine air masses (DeMott et al., 2016; McCluskey et al., 2018c), and the rarity of cultivable microbes, it is quite surprising that a substantial fraction of the cultivable microbial isolates from precipitation samples were found to be IN at temperatures above -17 °C (11 out of 34 total, or 32%), and suggests that there are ~~more~~ a significant fraction ~~– i.e., a significant fraction –~~ of IN species in aerosols among the substantially larger uncultivable community.

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. ~~better identify the putative IN microbes that may not be recovered by cultivation techniques.~~ 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 ~~emission~~ from the ocean surface.

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Data Availability: The 16S rRNA gene sequences from this study were deposited to GenBank under the accession numbers MW704027- MW704080. The data set supporting this manuscript is hosted by the UCSD Library Digital Collections (https://doi.org/10.6075/J0GQ6W2Z).

Supplement Link: The supplement related to this article is available here:

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Author Contributions: CMB and JMM wrote the manuscript, prepared figures, and conducted the field campaign and laboratory measurements. MAF contributed HRRR analysis and characterized meteorology during sampling periods. JD provided phylogenetic analyses and prepared figures. GCC supported FLEXPART simulations. MDS, MDB, TCH, PJD and KAP provided feedback on the study design, analyses and manuscript. KAP and PJD are principal

investigators on awards CHE-1801971 and AGS-1451347, and MDB is the principal investigator on award IOS-1516156.

References

Agresti, A. and Coull, B. A.: Approximate Is Better than "Exact" for Interval Estimation of Binomial Proportions, Am. Stat., 52(2), 119–126, doi:10.2307/2685469, 1998.

Ahern, H. E., Walsh, K. A., Hill, T. C. J. and Moffett, B. F.: Fluorescent pseudomonads isolated from Hebridean cloud and rain water produce biosurfactants but do not cause ice nucleation, Biogeosciences, 4(1), 115–124, doi:10.5194/bg-4-115-2007, 2007.

Alpert, P. A., Aller, J. Y. and Knopf, D. A.: Initiation of the ice phase by marine biogenic surfaces in supersaturated gas and supercooled aqueous phases, Phys. Chem. Chem. Phys., 13(44), 19882, doi:10.1039/c1cp21844a, 2011.

Amato, P., Joly, M., Schaupp, C., Attard, E., Möhler, O., Morris, C. E., Brunet, Y. and Delort, A.-M.: Survival and ice nucleation activity of bacteria as aerosols in a cloud simulation chamber, Atmos. Chem. Phys., 15(11), 6455–6465, doi:10.5194/acp-15-6455-2015, 2015.

Amend, A. S., Barshis, D. J. and Oliver, T. A.: Coral-associated marine fungi form novel lineages and heterogeneous assemblages, ISME J., 6(7), 1291–1301, doi:10.1038/ismej.2011.193, 2012.

Amend, A., Burgaud, G., Cunliffe, M., Edgcomb, V. P., Ettinger, C. L., Gutiérrez, M. H., Heitman, J., Hom, E. F. Y., Ianiri, G., Jones, A. C., Kagami, M., Picard, K. T., Quandt, C. A., Raghukumar, S., Riquelme, M., Stajich, J., Vargas-Muñiz, J., Walker, A. K., Yarden, O. and Gladfelter, A. S.: Fungi in the Marine Environment: Open Questions and Unsolved Problems, edited by D. A. Garsin, MBio, 10(2), e01189-18, doi:10.1128/mBio.01189-18, 2019.

Ansmann, A., Tesche, M., Seifert, P., Althausen, D., Engelmann, R., Fruntke, J., Wandinger, U., Mattis, I. and Müller, D.: Evolution of the ice phase in tropical altocumulus: SAMUM lidar observations over Cape Verde, J. Geophys. Res. Atmos., 114(17), 1–20, doi:10.1029/2008JD011659, 2009.

Beall, C. M., Stokes, M. D., Hill, T. C., DeMott, P. J., DeWald, J. T. and Prather, K. A.: Automation and Heat Transfer Characterization of Immersion Mode Spectroscopy for Analysis of Ice Nucleating Particles, Atmos. Meas. Tech., (February), 1–25, doi:10.5194/amt-2016-412, 2017.

Benjamin, S. G., Weygandt, S. S., Brown, J. M., Hu, M., Alexander, C. R., Smirnova, T. G., Olson, J. B., James, E. P., Dowell, D. C., Grell, G. A., Lin, H., Peckham, S. E., Smith, T. L., Moninger, W. R., Kenyon, J. S. and Manikin, G. S.: A North American Hourly Assimilation and Model Forecast Cycle: The Rapid Refresh, Mon. Weather Rev., 144(4), 1669–1694, doi:10.1175/MWR-D-15-0242.1, 2016.

Bigg, E. K.: A LONG PERIOD FLUCTUATION IN FREEZING NUCLEUS CONCENTRATIONS, J. Meteorol., doi:10.1175/1520-0469(1958)015<0561:alpfif>2.0.co;2,

659 1958.

660 Bigg, E. K., Soubeyrand, S. and Morris, C. E.: Persistent after-effects of heavy rain on
661 concentrations of ice nuclei and rainfall suggest a biological cause, Atmos. Chem. Phys., 15(5),
662 2313–2326, doi:10.5194/acp-15-2313-2015, 2015.

663 Boström, K. H., Simu, K., Hagström, Å. and Riemann, L.: Optimization of DNA extraction for
664 quantitative marine bacterioplankton community analysis, Limnol. Oceanogr. Methods, 2, 365–
665 373, 2004.

666 Bowers, R. M., Lauber, C. L., Wiedinmyer, C., Hamady, M., Hallar, A. G., Fall, R., Knight, R.
667 and Fierer, N.: Characterization of airborne microbial communities at a high-elevation site and
668 their potential to act as atmospheric ice nuclei, Appl. Environ. Microbiol., 75(15), 5121–5130,
669 doi:10.1128/AEM.00447-09, 2009.

670 Bull, A. T., Stach, J. E. M., Ward, A. C. and Goodfellow, M.: Marine actinobacteria:
671 Perspectives, challenges, future directions, Antonie van Leeuwenhoek, Int. J. Gen. Mol.
672 Microbiol., doi:10.1007/s10482-004-6562-8, 2005.

673 Burrows, S. M., Hoose, C., Pöschl, U. and Lawrence, M. G.: Ice nuclei in marine air: Biogenic
674 particles or dust?, Atmos. Chem. Phys., 13(1), 245–267, doi:10.5194/acp-13-245-2013, 2013.

675 Carro-Calvo, L., Hoose, C., Stengel, M. and Salcedo-Sanz, S.: Cloud glaciation temperature
676 estimation from passive remote sensing data with evolutionary computing, J. Geophys. Res.,
677 121(22), 13,591-13,608, doi:10.1002/2016JD025552, 2016.

678 Christner, B. C., Cai, R., Morris, C. E., McCarter, K. S., Foreman, C. M., Skidmore, M. L.,
679 Montross, S. N. and Sands, D. C.: Geographic, seasonal, and precipitation chemistry influence
680 on the abundance and activity of biological ice nucleators in rain and snow., Proc. Natl. Acad.
681 Sci. U. S. A., 105(48), 18854–18859, doi:10.1073/pnas.0809816105, 2008.

682 Conen, F. and Yakutin, M. V: Soils rich in biological ice-nucleating particles abound in ice-
683 nucleating macromolecules likely produced by fungi, Biogeosciences, 15(14), 4381–4385,
684 doi:10.5194/bg-15-4381-2018, 2018.

685 Conen, F., Morris, C. E., Leifeld, J., Yakutin, M. V and Alewell, C.: Biological residues define
686 the ice nucleation properties of soil dust, Atmos. Chem. Phys., 11(18), 9643–9648,
687 doi:10.5194/acp-11-9643-2011, 2011.

688 Conen, F., Eckhardt, S., Gundersen, H., Stohl, A. and Yttri, K. E.: Rainfall drives atmospheric
689 ice-nucleating particles in the coastal climate of southern Norway, , (2013), 11065–11073, 2017.

690 Creamean, J. M., Suski, K. J., Rosenfeld, D., Cazorla, A., DeMott, P. J., Sullivan, R. C., White,
691 A. B., Ralph, F. M., Minnis, P., Comstock, J. M., Tomlinson, J. M. and Prather, K. A.: Dust and
692 biological aerosols from the Sahara and Asia influence precipitation in the Western U.S, Science
693 (80-.)., 340(6127), 1572–1578, doi:10.1126/science.1227279, 2013.

694 Curry, J. A., Hobbs, P. V., King, M. D., Randall, D. A., Minnis, P., Isaac, G. A., Pinto, J. O.,
695 Uttal, T., Bucholtz, A., Cripe, D. G., Gerber, H., Fairall, C. W., Garrett, T. J., Hudson, J., Intrieri,
696 J. M., Jakob, C., Jensen, T., Lawson, P., Marcotte, D., Nguyen, L., Pilewskie, P., Rangno, A.,
697 Rogers, D. C., Strawbridge, K. B., Valero, F. P. J., Williams, A. G. and Wylie, D.: FIRE arctic
698 clouds experiment, Bull. Am. Meteorol. Soc., 81(1), 5–29, doi:10.1175/1520-
699 0477(2000)081<0005:FACE>2.3.CO;2, 2000.

700 D.C., S., V.E., L., A.L., S. and G., D. S.: The association between bacteria and rain and possible

resultant meteorological implications., edited by F. A. O. of the UN, , 86, v.148-152(2-4), 1982.

DeLeon-Rodriguez, N., Lathem, T. L., Rodriguez-R, L. M., Barazesh, J. M., Anderson, B. E., Beyersdorf, A. J., Ziemba, L. D., Bergin, M., Nenes, A. and Konstantinidis, K. T.: Microbiome of the upper troposphere: species composition and prevalence, effects of tropical storms, and atmospheric implications., Proc. Natl. Acad. Sci. U. S. A., 110(7), 2575–80, doi:10.1073/pnas.1212089110, 2013.

Delort, A. M., Vaïtilingom, M., Joly, M., Amato, P., Wirgot, N., Lallement, A., Sancelme, M., Matulova, M. and Deguillaume, L.: Clouds: A Transient and Stressing Habitat for Microorganisms BT - Microbial Ecology of Extreme Environments, edited by C. Chénard and F. M. Lauro, pp. 215–245, Springer International Publishing, Cham., 2017.

DeMott, P. J., Hill, T. C. J., McCluskey, C. S., Prather, K. A., Collins, D. B., Sullivan, R. C., Ruppel, M. J., Mason, R. H., Irish, V. E., Lee, T., Hwang, C. Y., Rhee, T. S., Snider, J. R., McMeeking, G. R., Dhaniyala, S., Lewis, E. R., Wentzell, J. J. B., Abbatt, J., Lee, C., Sultana, C. M., Ault, A. P., Axson, J. L., Diaz Martinez, M., Venero, I., Santos-Figueroa, G., Stokes, M. D., Deane, G. B., Mayol-Bracero, O. L., Grassian, V. H., Bertram, T. H., Bertram, A. K., Moffett, B. F. and Franc, G. D.: Sea spray aerosol as a unique source of ice nucleating particles, Proc. Natl. Acad. Sci., 113(21), 5797–5803, doi:10.1073/pnas.1514034112, 2016.

Després, V. R., Alex Huffman, J., Burrows, S. M., Hoose, C., Safatov, A. S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M. O., Pöschl, U. and Jaenicke, R.: Primary biological aerosol particles in the atmosphere: A review, Tellus, Ser. B Chem. Phys. Meteorol., 64(1), doi:10.3402/tellusb.v64i0.15598, 2012.

Fahlgren, C., Hagström, Å., Nilsson, D. and Zweifel, U. L.: Annual Variations in the Diversity, Viability, and Origin of Airborne Bacteria, Appl. Environ. Microbiol., 76(9), 3015 LP – 3025, doi:10.1128/AEM.02092-09, 2010.

Fahlgren, C., Gómez-Consarnau, L., Zábori, J., Lindh, M. V, Krejci, R., Mårtensson, E. M., Nilsson, D. and Pinhassi, J.: Seawater mesocosm experiments in the Arctic uncover differential transfer of marine bacteria to aerosols, Environ. Microbiol. Rep., 7(3), 460–470, doi:10.1111/1758-2229.12273, 2015.

Failor, K. C., Schmale, D. G., Vinatzer, B. A. and Monteil, C. L.: Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms, ISME J., 11(12), 2740–2753, doi:10.1038/ismej.2017.124, 2017a.

Failor, K. C., Iii, D. G. S., Vinatzer, B. A. and Monteil, C. L.: Ice nucleation active bacteria in precipitation are genetically diverse and nucleate ice by employing different mechanisms, , 1–14, doi:10.1038/ismej.2017.124, 2017b.

Fall, R. and Schnell, R.: Association of an ice-nucleating pseudomonad with cultures of the marine dinoflagellate, Heterocapsa niei., 1985.

Fröhlich-Nowoisky, J., Hill, T. C. J., Pummer, B. G., Yordanova, P., Franc, G. D. and Pöschl, U.: Ice nucleation activity in the widespread soil fungus Mortierella alpina, Biogeosciences, 12(4), 1057–1071, doi:10.5194/bg-12-1057-2015, 2015.

Fröhlich-Nowoisky, J., Kampf, C. J., Weber, B., Huffman, J. A., Pöhlker, C., Andreae, M. O., Lang-Yona, N., Burrows, S. M., Gunthe, S. S., Elbert, W., Su, H., Hoor, P., Thines, E., Hoffmann, T., Després, V. R. and P?öschl, U.: Bioaerosols in the Earth system: Climate, health,

and ecosystem interactions, Atmos. Res., 182, 346–376, doi:10.1016/j.atmosres.2016.07.018, 2016.

Furtado, K. and Field, P.: The Role of Ice Microphysics Parametrizations in Determining the Prevalence of Supercooled Liquid Water in High-Resolution Simulations of a Southern Ocean Midlatitude Cyclone, J. Atmos. Sci., 74(6), 2001–2021, doi:10.1175/JAS-D-16-0165.1, 2017.

Hill, T. C. J., Moffett, B. F., DeMott, P. J., Georgakopoulos, D. G., Stump, W. L. and Franc, G. D.: Measurement of ice nucleation-active bacteria on plants and in precipitation by quantitative PCR, Appl. Environ. Microbiol., 80(4), 1256–1267, doi:10.1128/AEM.02967-13, 2014.

Huffman, J. A., Prenni, A. J., Demott, P. J., Pöhlker, C., Mason, R. H., Robinson, N. H., Fröhlich-Nowoisky, J., Tobo, Y., Després, V. R., Garcia, E., Gochis, D. J., Harris, E., Müller-Germann, I., Ruzene, C., Schmer, B., Sinha, B., Day, D. A., Andreae, M. O., Jimenez, J. L., Gallagher, M., Kreidenweis, S. M., Bertram, A. K. and Pöschl, U.: High concentrations of biological aerosol particles and ice nuclei during and after rain, Atmos. Chem. Phys., 13(13), 6151–6164, doi:10.5194/acp-13-6151-2013, 2013.

Hwang, C. Y. and Cho, B. C.: Prokaryotic abundance and 16S rRNA gene sequences detected in marine aerosols on the East Sea (Korea), FEMS Microbiol. Ecol., 76(2), 327–341, doi:10.1111/j.1574-6941.2011.01053.x, 2011.

Irish, V. E., Elizondo, P., Chen, J., Chou, C., Charette, J., Lizotte, M., Ladino, L. A., Wilson, T. W., Gosselin, M., Murray, B. J., Polishchuk, E., Abbatt, J. P. D., Miller, L. A. and Bertram, A. K.: Ice-nucleating particles in Canadian Arctic sea-surface microlayer and bulk seawater, , 10583–10595, 2017.

Jayaweera, K. and Flanagan, P.: Investigations on biogenic ice nuclei in the Arctic atmosphere, Geophys. Res. Lett., 9(1), 94–97, doi:https://doi.org/10.1029/GL009i001p00094, 1982.

Joyce, R. E., Lavender, H., Farrar, J., Werth, J. T., Weber, C. F., D'Andrilli, J., Vaitilingom, M. and Christner, B. C.: Biological Ice-Nucleating Particles Deposited Year-Round in Subtropical Precipitation, edited by A. J. M. Stams, Appl. Environ. Microbiol., 85(23), e01567-19, doi:10.1128/AEM.01567-19, 2019.

Junge, K. and Swanson, B. D.: High-resolution ice nucleation spectra of sea-ice bacteria: implications for cloud formation and life in frozen environments, Biogeosciences Discuss., 4, 4261–4282, doi:10.5194/bgd-4-4261-2007, 2007.

Kanji, Z. A., Ladino, L. A., Wex, H., Boose, Y., Burkert-Kohn, M., Cziczo, D. J. and Krämer, M.: Overview of Ice Nucleating Particles, Meteorol. Monogr., 58, 1.1-1.33, doi:10.1175/AMSMONOGRAPHS-D-16-0006.1, 2017.

Kay, J. E., Wall, C., Yettella, V., Medeiros, B., Hannay, C., Caldwell, P. and Bitz, C.: Global climate impacts of fixing the Southern Ocean shortwave radiation bias in the Community Earth System Model (CESM), J. Clim., 29(12), 4617–4636, doi:10.1175/JCLI-D-15-0358.1, 2016.

Kieft, T. L.: Ice Nucleation Activity in Lichens, Appl. Environ. Microbiol., 54(7), 1678 LP – 1681, 1988.

Kjer, J., Debbab, A., Aly, A. H. and Proksch, P.: Methods for isolation of marine-derived endophytic fungi and their bioactive secondary products, Nat. Protoc., 5(3), 479–490, doi:10.1038/nprot.2009.233, 2010.

Klein, S. A., McCoy, R. B., Morrison, H., Ackerman, A. S., Avramov, A., Boer, G. de, Chen,

M., Cole, J. N. S., Del Genio, A. D., Falk, M., Foster, M. J., Fridlind, A., Golaz, J.-C., Hashino, T., Harrington, J. Y., Hoose, C., Khairoutdinov, M. F., Larson, V. E., Liu, X., Luo, Y., McFarquhar, G. M., Menon, S., Neggers, R. A. J., Park, S., Poellot, M. R., Schmidt, J. M., Sednev, I., Shipway, B. J., Shupe, M. D., Spangenberg, D. A., Sud, Y. C., Turner, D. D., Veron, D. E., Salzen, K. von, Walker, G. K., Wang, Z., Wolf, A. B., Xie, S., Xu, K.-M., Yang, F. and Zhang, G.: Intercomparison of model simulations of mixed-phase clouds observed during the ARM Mixed-Phase Arctic Cloud Experiment. I: single-layer cloud, Q. J. R. Meteorol. Soc., 135(641), 979–1002, doi:10.1002/qj.416, 2009.

Knopf, D. A., Alpert, P. A., Wang, B. and Aller, J. Y.: Stimulation of ice nucleation by marine diatoms, Nat. Geosci., 4(2), 88–90, doi:10.1038/ngeo1037, 2011.

Krzywinski, M. and Altman, N.: Error bars, Nat. Methods, 10, 921, 2013.

Kunert, A. T., Pöhlker, M. L., Tang, K., Krevert, C. S., Wieder, C., Speth, K. R., Hanson, L. E., Morris, C. E., Schmale III, D. G., Pöschl, U. and Fröhlich-Nowoisky, J.: Macromolecular fungal ice nuclei in ~~\textit{~~*Fusarium*~~}~~: effects of physical and chemical processing, Biogeosciences, 16(23), 4647–4659, doi:10.5194/bg-16-4647-2019, 2019.

Ladino, L. A., Yakobi-Hancock, J. D., Kilthau, W. P., Mason, R. H., Si, M., Li, J., Miller, L. A., Schiller, C. L., Huffman, J. A., Aller, J. Y., Knopf, D. A., Bertram, A. K. and Abbatt, J. P. D.: Addressing the ice nucleating abilities of marine aerosol: A combination of deposition mode laboratory and field measurements, Atmos. Environ., 132, 1–10, doi:10.1016/j.atmosenv.2016.02.028, 2016.

Letunic, I. and Bork, P.: Interactive Tree of Life v2: Online annotation and display of phylogenetic trees made easy, Nucleic Acids Res., 39(SUPPL. 2), 475–478, doi:10.1093/nar/gkr201, 2011.

McCluskey, C. S., Hill, T. C. J., Malfatti, F., Sultana, C. M., Lee, C., Santander, M. V., Beall, C. M., Moore, K. A., Cornwell, G. C., Collins, D. B., Prather, K. A., Jayarathne, T., Stone, E. A., Azam, F., Kreidenweis, S. M. and DeMott, P. J.: A dynamic link between ice nucleating particles released in nascent sea spray aerosol and oceanic biological activity during two mesocosm experiments, J. Atmos. Sci., (2015), JAS-D-16-0087.1, doi:10.1175/JAS-D-16-0087.1, 2016.

McCluskey, C. S., Hill, T. C. J., Malfatti, F., Sultana, C. M., Lee, C., Santander, M. V., Beall, C. M., Moore, K. A., Cornwell, G. C., Collins, D. B., Prather, K. A., Jayarathne, T., Stone, E. A., Azam, F., Kreidenweis, S. M. and DeMott, P. J.: A dynamic link between ice nucleating particles released in nascent sea spray aerosol and oceanic biological activity during two mesocosm experiments, J. Atmos. Sci., 74(1), 151–166, doi:10.1175/JAS-D-16-0087.1, 2017.

McCluskey, C. S., Hill, T. C. J., Sultana, C. M., Laskina, O., Trueblood, J., Santander, M. V., Beall, C. M., Michaud, J. M., Kreidenweis, S. M., Prather, K. A., Grassian, V., DeMott, P. J., McCluskey, C. S., Hill, T. C. J., Sultana, C. M., Laskina, O., Trueblood, J., Santander, M. V., Beall, C. M., Michaud, J. M., Kreidenweis, S. M., Prather, K. A., Grassian, V. and DeMott, P. J.: A mesocosm double feature: Insights into the chemical make-up of marine ice nucleating particles, J. Atmos. Sci., JAS-D-17-0155.1, doi:10.1175/JAS-D-17-0155.1, 2018a.

McCluskey, C. S., Ovadnevaite, J., Rinaldi, M., Atkinson, J., Belosi, F., Ceburnis, D., Marullo, S., Hill, T. C. J., Lohmann, U., Kanji, Z. A., O'Dowd, C., Kreidenweis, S. M. and DeMott, P. J.: Marine and Terrestrial Organic Ice-Nucleating Particles in Pristine Marine to Continentally Influenced Northeast Atlantic Air Masses, J. Geophys. Res. Atmos., 123(11), 6196–6212,

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doi:10.1029/2017JD028033, 2018b.

McCluskey, C. S., Hill, T. C. J., Humphries, R. S., Rauker, A. M., Moreau, S., Strutton, P. G., Chambers, S. D., Williams, A. G., McRobert, I., Ward, J., Keywood, M. D., Harnwell, J., Ponsonby, W., Loh, Z. M., Krummel, P. B., Protat, A., Kreidenweis, S. M. and DeMott, P. J.: Observations of Ice Nucleating Particles Over Southern Ocean Waters, Geophys. Res. Lett., 45(21), 11,911-989,997, doi:10.1029/2018GL079981, 2018c.

McWilliam, H., Li, W., Uludag, M., Squizzato, S., Park, Y. M., Buso, N., Cowley, A. P. and Lopez, R.: Analysis Tool Web Services from the EMBL-EBI., Nucleic Acids Res., 41, 597–600, doi:10.1093/nar/gkt376, 2013.

Michaud, J. M., Thompson, L. R., Kaul, D., Espinoza, J. L., Richter, R. A., Xu, Z. Z., Lee, C., Pham, K. M., Beall, C. M., Malfatti, F., Azam, F., Knight, R., Burkart, M. D., Dupont, C. L. and Prather, K. A.: Taxon-specific aerosolization of bacteria and viruses in an experimental ocean-atmosphere mesocosm, Nat. Commun., 9(1), doi:10.1038/s41467-018-04409-z, 2018.

Monteil, C. L., Bardin, M. and Morris, C. E.: Features of air masses associated with the deposition of Pseudomonas syringae and Botrytis cinerea by rain and snowfall, , 8(11), 2290–2304, doi:10.1038/ismej.2014.55, 2014.

Morris, C. E., Georgakopoulos, D. G. and Sands, D. C.: Ice nucleation active bacteria and their potential role in precipitation, in Journal De Physique. IV : JP., 2004.

Morris, C. E., Sands, D. C., Vinatzer, B. A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H. and Thompson, B. M.: The life history of the plant pathogen Pseudomonas syringae is linked to the water cycle, Isme J., 2, 321, 2008.

Morris, C. E., Soubeyrand, S., Bigg, E. K., Creamean, J. M. and Sands, D. C.: Mapping rainfall feedback to reveal the potential sensitivity of precipitation to biological aerosols, Bull. Am. Meteorol. Soc., 98(6), 1109–1118, doi:10.1175/BAMS-D-15-00293.1, 2017.

Myers, J. A., Curtis, B. S. and Curtis, W. R.: Improving accuracy of cell and chromophore concentration measurements using optical density, BMC Biophys., 6(1), 4, doi:10.1186/2046-1682-6-4, 2013.

Nemecek-Marshall, M., LaDuca, R. and Fall, R.: High-level expression of ice nuclei in a Pseudomonas syringae strain is induced by nutrient limitation and low temperature., J. Bacteriol., 175(13), 4062–4070, 1993.

Orsi, W., Biddle, J. F. and Edgcomb, V.: Deep Sequencing of Subseafloor Eukaryotic rRNA Reveals Active Fungi across Marine Subsurface Provinces, PLoS One, 8(2), e56335, 2013.

O'Sullivan, D., Murray, B. J., Ross, J. F., Whale, T. F., Price, H. C., Atkinson, J. D., Umo, N. S. and Webb, M. E.: The relevance of nanoscale biological fragments for ice nucleation in clouds., Sci. Rep., 5, 8082, doi:10.1038/srep08082, 2015.

Overy, D. P., Rämä, T., Oosterhuis, R., Walker, A. K. and Pang, K.-L.: The Neglected Marine Fungi, Sensu stricto, and Their Isolation for Natural Products' Discovery, Mar. Drugs , 17(1), doi:10.3390/md17010042, 2019.

Parker, L., Sullivan, C., Forest, T. and Ackley, S.: Ice nucleation activity of antarctic marine microorganisms, Antarct. J., 20, 126–127, 1985.

Petters, M. D. and Wright, T. P.: Revisiting ice nucleation from precipitation samples, Geophys.

Res. Lett., 42(20), 8758–8766, doi:10.1002/2015GL065733, 2015.

Ponder, M. A., Gilmour, S. J., Bergholz, P. W., Mindock, C. A., Hollingsworth, R., Thomashow, M. F. and Tiedje, J. M.: Characterization of potential stress responses in ancient Siberian permafrost psychroactive bacteria, FEMS Microbiol. Ecol., 53(1), 103–115, doi:10.1016/j.femsec.2004.12.003, 2005.

Pouleur, S., Richard, C., Martin, J. and Antoun, H.: Ice nucleation activity in Fusarium acuminatum and Fusarium avenaceum, Appl. Environ. Microbiol., 58(9), 2960–2964, 1992.

Prenni, A. J., Harrington, J. Y., Tjernström, M., DeMott, P. J., Avramov, A., Long, C. N., Kreidenweis, S. M., Olsson, P. Q. and Verlinde, J.: Can Ice-Nucleating Aerosols Affect Arctic Seasonal Climate?, Bull. Am. Meteorol. Soc., 88(4), 541–550, doi:10.1175/BAMS-88-4-541, 2007.

Prenni, A. J., Tobo, Y., Garcia, E., DeMott, P. J., Huffman, J. A., McCluskey, C. S., Kreidenweis, S. M., Prenni, J. E., Pöhlker, C. and Pöschl, U.: The impact of rain on ice nuclei populations at a forested site in Colorado, Geophys. Res. Lett., 40(1), 227–231, doi:doi:10.1029/2012GL053953, 2013.

Pruesse, E., Peplies, J. and Glöckner, F. O.: SINA: Accurate high-throughput multiple sequence alignment of ribosomal RNA genes, Bioinformatics, 28(14), 1823–1829, doi:10.1093/bioinformatics/bts252, 2012.

Rogers, D. C., DeMott, P. J., Kreidenweis, S. M. and Chen, Y.: Measurements of ice nucleating aerosols during SUCCESS, Geophys. Res. Lett., 25(9), 1383, doi:10.1029/97GL03478, 1998.

Sands, D.C., Langhans, V.E., Scharen, A.L., De Smet, G.: The association between bacteria and rain and possible resultant meteorological implications, J. Hungarian Meteorol. Ser., 86, v.148-152(2-4), 1982.

Santl-Temkiv, T., Sahyoun, M., Finster, K., Hartmann, S., Augustin-Bauditz, S., Stratmann, F., Wex, H., Clauss, T., Nielsen, N. W., Sorensen, J. H., Korsholm, U. S., Wick, L. Y. and Karlson, U. G.: Characterization of airborne ice-nucleation-active bacteria and bacterial fragments, Atmos. Environ., 109, 105–117, doi:10.1016/j.atmosenv.2015.02.060, 2015.

Schnell, R. C. and Vali, G.: Freezing nuclei in marine waters, Tellus, 27(3), 321–323, doi:10.1111/j.2153-3490.1975.tb01682.x, 1975.

Stohl, A., Hittenberger, M. and Wotawa, G.: Validation of the Lagrangian particle dispersion model FLEXPART against large scale tracer experiment data, Atmos. Environ., 32(24), 4245–4264, 1998.

Tamura, K., Stecher, G., Peterson, D., Filipski, A. and Kumar, S.: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0., Mol. Biol. Evol., 30(12), 2725–2729, doi:10.1093/molbev/mst197, 2013.

Tisthammer, K. H., Cobian, G. M. and Amend, A. S.: Global biogeography of marine fungi is shaped by the environment, Fungal Ecol., 19, 39–46, doi:https://doi.org/10.1016/j.funeco.2015.09.003, 2016.

Tong, Y. and Lighthart, B.: Solar Radiation Is Shown to Select for Pigmented Bacteria in the Ambient Outdoor Atmosphere, Photochem. Photobiol., 65(1), 103–106, doi:10.1111/j.1751-1097.1997.tb01884.x, 1997.

910 Vaïtilingom, M., Attard, E., Gaiani, N., Sancelme, M., Deguillaume, L., Flossmann, A. I.,
911 Amato, P. and Delort, A. M.: Long-term features of cloud microbiology at the puy de Dôme
912 (France), Atmos. Environ., 56, 88–100, doi:10.1016/j.atmosenv.2012.03.072, 2012.

913 Vali, G.: Freezing Nucleus Content of Hail and Rain in Alberta, J. Appl. Meteorol., 10(1), 73–
914 78, 1971.

915 Vali, G.: Comments on "Freezing Nuclei Derived from Soil Particles," J. Atmos. Sci., 31(5),
916 1457–1459, doi:10.1175/1520-0469(1974)031<1457:CONDFS>2.0.CO;2, 1974.

917 Vergara-Temprado, J. V., Carslaw, K. S., Murray, B. J., Ardon-dryer, K., Bertram, K., Browse,
918 J., Burrows, S. M., Ceburnis, D., Demott, P. J., Mason, R. H., Dowd, C. D. O., Sullivan, D. O.,
919 Pringle, K., Rinaldi, M. and Wilson, T. W.: Contribution of feldspar and marine organic aerosols
920 to global ice nucleating particle concentrations, 17, 3637-3658, doi:10.5194/acp-17-3637-2017,
921 2017.
922
923 Vergara-Temprado, J., Miltenberger, A. K., Furtado, K., Grosvenor, D. P., Shipway, B. J., Hill,
924 A. A., Wilkinson, J. M., Field, P. R., Murray, B. J. and Carslaw, K. S.: Strong control of
925 Southern Ocean cloud reflectivity by ice-nucleating particles, Proc. Natl. Acad. Sci., 201721627,
926 doi:10.1073/pnas.1721627115, 2018.

927 Walters, W., Hyde, E. R., Berg-lyons, D., Ackermann, G., Humphrey, G., Parada, A., Gilbert, J.
928 a and Jansson, J. K.: Improved bacterial 16S rRNA gene (V4 and V4-5) and fungal internal
929 transcribed spacer marker gene primers for microbial community surveys, mSystems, 1(1),
930 e0009-15, doi:10.1128/mSystems.00009-15.Editor, 2015.

931 Wex, H., Augustin-Bauditz, S., Boose, Y., Budke, C., Curtius, J., Diehl, K., Dreyer, A., Frank,
932 F., Hartmann, S., Hiranuma, N., Jantsch, E., Kanji, Z. A., Kiselev, A., Koop, T., Möhler, O.,
933 Niedermeier, D., Nillius, B., Rösch, M., Rose, D., Schmidt, C., Steinke, I. and Stratmann, F.:
934 Intercomparing different devices for the investigation of ice nucleating particles using Snomax®
935 as test substance, Atmos. Chem. Phys., 15(3), 1463–1485, doi:10.5194/acp-15-1463-2015, 2015.

936 Wilson, S. L. and Walker, V. K.: Selection of low-temperature resistance in bacteria and
937 potential applications, Environ. Technol., 31(8–9), 943–956, doi:10.1080/09593331003782417,
938 2010.

939 Wilson, T. W., Ladino, L. a., Alpert, P. a., Breckels, M. N., Brooks, I. M., Browse, J., Burrows,
940 S. M., Carslaw, K. S., Huffman, J. A., Judd, C., Kilthau, W. P., Mason, R. H., McFiggans, G.,
941 Miller, L. a., Nájera, J. J., Polishchuk, E., Rae, S., Schiller, C. L., Si, M., Temprado, J. V.,
942 Whale, T. F., Wong, J. P. S., Wurl, O., Yakobi-Hancock, J. D., Abbatt, J. P. D., Aller, J. Y.,
943 Bertram, A. K., Knopf, D. a. and Murray, B. J.: A marine biogenic source of atmospheric ice-
944 nucleating particles, Nature, 525(7568), 234–238, doi:10.1038/nature14986, 2015.

945 Wright, E. S.: DECIPHER: harnessing local sequence context to improve protein multiple
946 sequence alignment, BMC Bioinformatics, 16(1), 322, doi:10.1186/s12859-015-0749-z, 2015.

947 Wright, T. P., Hader, J. D., McMeeking, G. R. and Petters, M. D.: High relative humidity as a
948 trigger for widespread release of ice nuclei, Aerosol Sci. Technol., 48(11), i–v,
949 doi:10.1080/02786826.2014.968244, 2014.

950 Yang, J., Wang, Z., Heymsfield, A. J., DeMott, P. J., Twohy, C. H., Suski, K. J. and Toohey, D.
951 W.: High ice concentration observed in tropical maritime stratiform mixed-phase clouds with top
952 temperatures warmer than −8 °C, Atmos. Res., 233, 104719,

953 doi:https://doi.org/10.1016/j.atmosres.2019.104719, 2020.
954 ZoBell, C. E.: Marine Bacteriology, Annu. Rev. Biochem., 16, 565–586, 1947.