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To: Professor Yinon Rudich
Editor, Journal of Atmospheric and Chemistry

RE: Revised manuscript

February 26, 2015

Dear Professor Rudich:

Please attached find the revised manuscript entitled "Arctic Microbial and Next Generation Sequencing Approach for Bacteria in Snow and Frost Flowers: Selected Identification, Abundance and Freezing nucleation " by Mortazavi, Attiya and Ariya.

We would like to first thank you and all three anonymous reviewers and another additional open access reviewer, for time investment, constructive suggestions and inputs. In this revised version, we trust that we have considered all suggestions, have indeed addressed them in the revised manuscript. Our significant modifications based on proposed recommendations includes:

1. Point-by point responses to reviewer's comments, and subsequent changes in the revised manuscript;
2. Improved overall clarity of the presentation of results;
3. Further improvement on clarification and the presentation of the manuscript.

We would like to thank you for your time and timely collaboration. If you have any further questions, please feel free to contact us.

Cordially,

P. A. Ariya
James McGill Professor of Chemistry and Atmospheric and Oceanic Sciences

On behalf of Dr. Mortazavi and Dr. Attiya

Reply

Thanks very much for your detailed comments and suggestions. We have considered all of them, and revised the manuscript accordingly. For your convenience, we put your comments, point-by-point in italic and our replies on regular fonts.

Anonymous Referee #1

“Atmospheric Chemistry and Physics Title: A next generation sequencing of Arctic bacteria in snow and frost flowers: identification, abundance and freezing nucleation This paper provides insights into the microbial composition of frost flowers and other snow sources as well as the ice nucleation capability of culturable bacteria from these samples. This seems to be a novel dataset that is worthy of publication. The paper would greatly benefit from including more details on the methods that were used, particularly with regards to the pyrosequencing and how the samples were prepared and processed in MOTHRU (see specific notes below). The paper would also benefit from being substantially edited and reviewed by a microbial ecologist to improve the flow of language and grammar to more clearly communicate the results and background information (see specific comments below). The paper also mentions more than once the possibility of microbes in snow and ice in contributing to ecological processes in the Arctic but this is not returned to in the discussion of the results with respect to the microbial composition revealed by sequencing. Although this type of discussion requires a bit of speculation, it would be interesting to discuss some hypotheses for future research to build upon.”

Response: Thank you.

Abstract:

“Line 1: Seems like there should not be hyphens between “Ocean, Atmospheric Sea Ice Snowpack”

Response:

Thank you for your comment. Please note that in all their publications, they use hyphens, and thus to assure consistency and respect for the copyrights, we leave it as is.

“Line 2: unclear what is meant by “population”. What about the “population” are you examining?”

Response:

It is changed to “diversity of population”, as suggested.

“Line 3: What is a “snow type”?”

Response:

We used the classification of snow based on guidance provided by the American meteorological society (2009; reference is given below). However for accumulated snow, we use the definition provided by Freiz et al., 2009 (S2.3 and 2.4). We have added a line in the manuscript (page 5) and provided the following additional references.

Additional References:

Glossary of Meteorology (2009). "[Snow](#)". [American Meteorological Society](#). Retrieved 2009-06-28.

Fierz, C., Armstrong, R.L., Durand, Y., Etchevers, P., Greene, E., McClung, D.M., Nishimura, K., Satyawali, P.K. and Sokratov, S.A. 2009. The International Classification for Seasonal Snow on the Ground. IHP-VII Technical Documents in Hydrology N°83, IACS Contribution N°1, UNESCO-IHP, Paris.

Line 5: Awkward to say "conventional culture-based PCR identification approach". Suggested revision: "In addition to culturing and gene sequence-based identification"

Response:

Thank you for your comment. It is incorporated.

Line 6: Suggest replacing "deployed" with "utilized"

Response:

Thank you for your comment. It is incorporated.

Line 7: suggest replacing "diverse" with "diversity of"

Response:

Thank you for your comment. It is incorporated.

Line 7: Do not start a sentence with numbers.

Response:

Thank you for your comment. It is now changed, as suggested.

Line 8: replace "identified" with "detected"

Response:

Thank you for your comment. It is incorporated.

Line 8: eliminate "great"

Response:

Thank you for your comment. It is now eliminated.

Line 9 and 10: Phylum names are not italicized

Response:

Thank you, your comment is incorporated.

Line 10: suggest replacing, “at the genus level, 101-245 different genera” with “The number of genera detected ranged from 101-245.”

Response:

Thank you for your comment. It is modified as suggested.

Line 11: eliminate “in cultured samples”

Response:

Thank you for your comment. It is incorporated.

Line 14: eliminate “Complementary”

Response:

Thank you for your comment. It is incorporated.

Line 16: “isolate” should be “isolates”

Response:

Thank you for your comment. It is incorporated.

Line 18: “An isolate belonging to the Bacillus species” should be “An isolates belonging to the genus Bacillus”.

Response:

Thank you for your comment. It is incorporated.

Line 20: How do you know that the microbes in the Arctic snow originated from distinct ecological environments.

Response:

Next-generation sequencing analysis has identified some bacteria that have been previously detected and reported in literature, to exist within African and Asian dust storms. Hence our reference is based on the previous work, and in the revised

Lines 21 and 22: It is not clear what is meant by “microbial snow”. It seems unlikely that snow would exist that does not have microbes in it. Furthermore, how would the presence of microbes in snow influence the freezing and melting processes of the snowpack in the Arctic.

Response:

Microbial snow is changed to snow microorganisms.

Please see page 12 Line28 to page 13 line 1-14

“The detection of a high number of bacteria with a vast genetic diversity pool, using NGS analysis, further illustrates that the snowpack is a heterogeneous soup of microbial entities. The chemical environment of the snowpack is constantly evolving by novel streams of chemicals

through fresh precipitations, wind transportation and metabolic activity of microbial. With the increased incidence of dust storms, likely due to climate change, the detection of specific bacteria from hot areas into the Arctic environment may suggest a shift in the balance of “native bacterial populations” in the Arctic. One may speculate that it might be conceivable to consider interactions among the heterogeneous population of microbial in Arctic samples, including non-native taxa adaptation in the Arctic snow-ice genome. Though the Arctic does not provide a native habitat for non-native bacteria or biological species originating from elsewhere in the world, their entrance into the Arctic may affect certain bio-chemical reactions, or alter the nutrient pool for the other native microbial entities. In turn, it might affect the ratio and the survival rate of certain populations of microbial with freezing or anti-freezing properties, impacting the melting of ice or snowpack in the Arctic region.”

Introduction:

Page 32095 Lines 4-5: It is not clear what “bio-organic” molecules are and microorganisms (spelled with no hyphen) are not molecules, they are organisms comprised of molecules.

Response:

Thank you for your comment. We removed the hyphen. Instead we are using terms of biomolecule in the revised version which is referred to any molecule that is present in living organisms, including large macromolecules such as proteins, polysaccharides, lipids, and nucleic acids, as well as small molecules such as primary metabolites, secondary metabolites, and natural products.

Line 5: Is there are difference between freezing nucleation and ice nucleation? Both phrases are used and it is not clear what the difference is, if there is one.

Response:

Thank you for your comment. For clarity, freezing nucleation is changed to ice nucleation.

Line 6: there should be a period after “understood” rather than a comma.

Response:

Thank you for your comment. It is incorporated.

Line 14: “microbial” should be “microbes”?

Response:

Thank you for your comment. It is changed to microorganisms.

Line 18: “and survived” should be eliminated.

Response:

Thank you for your comment. It is incorporated.

Page 32096 Line 9: “/z” should be “h” Line 15: “microbial” should be “microbes” or “microorganisms” Line 16: “as an” should be eliminated

Response:

Thank you for your comments. They are incorporated. However, “microbial” is not changed since the intention is to use “microbial”.

Line 20: Not clear what natural and anthropogenic sources are contributing....ice nucleation particles? Please be more specific.

Response:

Thank you for your comment. We included examples of natural ice nuclei including mineral dust, and anthropogenic nucleating agents such as soot in the revised sentence.

Page 32097 Line 3-4: need to cite the studies that indicated diverse bacterial populations exist in snow. Line 10: I think “number density” should be either “number” or “density”

Response:

Thank you for your comment. The following references of bacteria in the snow are now included, and the word “density” was deleted.

References:

Carpenter, E. J., Lin, S., and Capone, D. G.: Bacterial activity in South Pole snow, *Appl Environ Microbiol*, 66, 4514-4517, 2000.

Amato, P., Hennebelle, R., Magand, O., Sancelme, M., Delort, A. M., Barbante, C., Boutron, C., and Ferrari, C.: Bacterial characterization of the snow cover at Spitzberg, Svalbard, *FEMS Microbiol Ecol*, 59, 255-264, 10.1111/j.1574-6941.2006.00198.x, 2007.

Mortazavi, R., Hayes, C. T., and Ariya, P. A.: Ice nucleation activity of bacteria isolated from snow compared with organic and inorganic substrates, *Environmental Chemistry*, 5, 373-381, 2008.

Amoroso, A., Domine, F., Esposito, G., Morin, S., Savarino, J., Nardino, M., Montagnoli, M., Bonneville, J. M., Clement, J. C., Ianniello, A., and Beine, H. J.: Microorganisms in dry polar snow are involved in the exchanges of reactive nitrogen species with the atmosphere, *Environmental science & technology*, 44, 714-719, 10.1021/es9027309, 2010.

Moller, A. K., Barkay, T., Abu Al-Soud, W., Sorensen, S. J., Skov, H., and Kroer, N.: Diversity and characterization of mercury-resistant bacteria in snow, freshwater and sea-ice brine from the High Arctic, *FEMS Microbiol Ecol*, 75, 390-401, 10.1111/j.1574-6941.2010.01016.x, 2011.

Experimental Methods:

Line 21: Is “hoar” a typographical error?

Response:

Thank you for your comment. Surface hoar snow is not a typographical error, and it is a terminology given in the glossary of meteorology (reference is given below).

Reference:

Glossary of Meteorology: "Snow". American Meteorological Society. , Retrieved 2009-06-28, 2009.

Line 21: It is not clear what frost flowers are. Is there a reference that can be cited here or can a brief description be provided? To some people frost flowers refer to this:

<http://www.kuriositas.com/2012/12/frost-flowers-natures-exquisite-ice.html>

Response:

Thank you for your comment. The following information is added to introduction.

Frost flowers are dendritic shape hypersaline clusters of ice crystals that form at the interface between warm ice surface and sufficiently cold atmospheric temperature and humidity (Obbard et al., 2009).

Additional Reference:

Obbard, R. W., Roscoe, H. K., Wolff, E. W., and Atkinson, H. M.: Frost flower surface area and chemistry as a function of salinity and temperature, *Journal of Geophysical Research: Atmospheres*, 114, D20305, 10.1029/2009JD012481, 2009.

Page 32098 Line 3: Spell out what HDPE means before using the acronym. Line 3: When citing the company where a particular instrument or supply was purchased, put the full company name, city and country in parenthesesâˆAˆ Tnot just "(Fisher)". Do this for the rest of the experimental methods section.

Response:

Your comment is incorporated throughout the text. The sentence is modified to:
"A sterile high-density polyethylene (HDPE) spoon (Fisher Scientific, Montreal, Canada) "

Line 24: a citation or the full recipe needs to be written into this section for "mycological agar"

Response:

Thank you for your comment. As requested, the reference detail is now provided, since it is a well known process in the literature.

Additional Reference:

Rybnikar A. Growth, colonial size and sporulation of lyophilized cultures of the genus trichophyton on agar media. *ACTA VET.BRNO*, 55, 1986: 73-80

Page 32099 Lines 1-13: for the drop-freezing assay, how many 10 uL drops were examined per sample or bacterial culture?

Response:

Thank you for your comment. It is modified. A minimum of 150 drops is used.

Section 2.3: says “used to lyse the cell”. I am pretty sure that more than one cell was lysed. Please specify what material the DNA was extracted from (e.g. liquid culture, filtered environmental sample).

Response:

Thank you for your comment. Page 7 Line 16 is modified to:

Part of each bacterial colony was picked by a sterile disposable inoculating loop (VWR, Mississauga, Canada) and mixed with Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) and proteinase K in 1.5 ml eppendorf tube.

Please cite the original source and names of the PCR primers used, unless these were custom made by the authors. Also please state the region of the 16S rRNA gene that is being amplified and how long the fragment is that is being amplified. Please state the specific PCR mixture components, their manufacturer and the volumes and final reaction concentrations of each that were used. Please state the make a model of the thermalcycler used for PCR. Please state how the PCR products were purified and prepared for DNA sequencing.

Response:

Thank you for the comments. Page 7 Line 17-23 is modified to:

The conserved sequence of extracted DNA was amplified by PCR (Techne Flexigene Thermal Cycler FFG02HSD) in a final volume of 25 µl using 16S universal primers 27F and 149R (Forward primer 5'-AGAGTTTGATCCTGGCTCAG-3' and reverse primer 5'-ACGGCTACCTTGTACGACTT-3', Integrated DNA Technologies, Coralville, USA) yielding a product of about 1465 bp. An Eppendorf™ tube containing all the ingredients but DNA was used as control (blank) for every set of PCR. A typical PCR reaction for one tube contained 2.5 µl of 10X buffer, 1 µl of each primer of 2.5 µM, 0.6 µl of 10mM dNTP, 1 µl of 0.1 µg/ µl DNA, 0.1 µl of 5U/ µl of Taq polymerase, 2.5 µl of 25mM of MgCl₂, 0.8 µl of 1M NaCl, and to 25 µl of nuclease free water (Promega, Madison, USA).

Page 7 Line 27-28 is modified to:

The PCR product of 16S rDNA gene obtained from the cultured bacterial colony were purified using QIAquick PCR Purification Kit (Qiagen, Toronto, Canada), sequenced at McGill University and Génome Québec Innovation Centre, Montreal, Canada.

How many bacterial isolates were selected from each culturing effort for each sample? How many were tested for ice nucleation ability? How many isolates were sequenced?

Response:

For each sample, between 3-8 bacterial isolates were selected and sequenced. For ice nucleation, 5-10 bacterial colonies for each sample were analyzed.

Page 32100 Section 2.4 should be split into separate sections: one for pyrosequencing and one for the electron microscopy.

Response:

Thank you for your comment. It is modified.

How was the DNA extracted from snow? Please describe this in detail. Was the snow filtered and then extracted from a filter? Please explain.

Response:

Thank you for your comment. The following text was added to Page 8 Line6:

Melted snow was passed through 0.22 micron filter (Millipore, Mississauga, Canada). Filter was sonicated in 17 ml of 1X TBE buffer in an ultrasound bath for 10 min. The liquid was collected in sterile 50 ml polycarbonate centrifuge tubes (Nalgen. Rochester, NY, USA). The liquid was centrifuged for 15 min at 18,000 g, and the pellet was resuspended in 200 ul of 1 X TBE buffer. Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) was used to lyse the cell, and DNA was extracted and purified using either a DNAeasy kit (Qiagen, Toronto, Canada) or a Master Pure DNA purification kit (Epicentre Technologies, Madison, USA) according to the manufacturer's instructions.

Please provide the details of how MOTHUR was used to trim and process the libraries.

Response:

Thank you for your comment. The text on Page 8 Line 25 to Page 9 Line 2 was modified with the following text:

The generated sequences from pyrosequencing was analyzed with software MOTHUR formatted version of the RDP classifier using a Bayesian method (Wang et al, 2007) with 1000 bootstrap replicates for pre-processing (quality-adjustment, barcode split), identification of operational taxonomic units (OTUs) defined at ≥ 97 % 16S rRNA sequence identity level, taxonomic assignment, community comparison, and statistical analysis (Schloss et al., 2009). Trimming was done by quality from the 3' end. Sequences containing ambiguous bases, homopolymers longer than eight bases, or an average quality score below 20 over a 50bp long window were excluded (Schloss et al, 2011).

Additional references:

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R.: Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy, Applied and Environmental Microbiology, 73, 5261-5267, 10.1128/AEM.00062-07, 2007.

Schloss, P. D., Gevers, D., and Westcott, S. L.: Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, PloS one, 6, e27310, 10.1371/journal.pone.0027310, 2011.

Results and Discussion

Page 32101

L11-14: What are photobiological reactions? How is this compare with photochemical reactions? L14-15: knowing how microbes interact in snow will not necessarily “reveal the role these play in alterin the Arctic environment and climate”. Is there any reasonable evidence that

suggests bacteria in snow pack are indeed altering the “Arctic environment and climate”? If so, it is necessary to cite those sources here.

Response:

Photobiology is the interactions of light (non-ionizing radiation) and ability of living organisms to absorb by its photoreceptors. The formation of excited molecules is associated with different functions such as: i) an energy source for maintenance and growth, ii) diminishing the effects of damaging radiation (photoprotection), and iii) signal transfer (photosensing).

Photochemistry is the chemical reactions that proceed with the absorption of light by atoms or molecules. Photochemical reactions, i.e. reactions induced by ultraviolet (UV) or visible light, play a major role in the environment. Photochemical activation is the principal driving force of transformations of organic substances in the atmosphere and it plays a significant role in the degradation of compounds which are inefficiently biodegraded in surface waters.

The following text is added to Page 9 Line 13:

Nitrification (Amoroso et al., 2010), transformation of mercury (Møller et al., 2011) and other pollutants within the snowpack have been detected. Ammonia-oxidizing Betaproteobacteria are active nitrifiers in glacial ice microcosms (Miteva et al., 2007), and the presence of nifH genes indicate the potential for nitrogen fixation in supraglacial snow (Boyd et al., 2011).

Additional References:

Miteva, V., Sowers, T., and Brenchley, J.: Production of N₂O by Ammonia Oxidizing Bacteria at Subfreezing Temperatures as a Model for Assessing the N₂O Anomalies in the Vostok Ice Core, *Geomicrobiology Journal*, 24, 451-459, <http://dx.doi.org/10.1080/01490450701437693>, 2007.

Boyd, E. S., Lange, R. K., Mitchell, A. C., Havig, J. R., Hamilton, T. L., Lafreniere, M. J., Shock, E. L., Peters, J. W., and Skidmore, M.: Diversity, abundance, and potential activity of nitrifying and nitrate-reducing microbial assemblages in a subglacial ecosystem, *Appl Environ Microbiol*, 77, 4778-4787, 10.1128/aem.00376-11, 2011.

Amoroso, A., Domine, F., Esposito, G., Morin, S., Savarino, J., Nardino, M., Montagnoli, M., Bonneville, J. M., Clement, J. C., Ianniello, A., and Beine, H. J.: Microorganisms in dry polar snow are involved in the exchanges of reactive nitrogen species with the atmosphere, *Environmental science & technology*, 44, 714-719, 10.1021/es9027309, 2010.

Moller, A. K., Barkay, T., Abu Al-Soud, W., Sorensen, S. J., Skov, H., and Kroer, N.: Diversity and characterization of mercury-resistant bacteria in snow, freshwater and sea-ice brine from the High Arctic, *FEMS Microbiol Ecol*, 75, 390-401, 10.1111/j.1574-6941.2010.01016.x, 2011.

L19-21:

The following should be included in the materials and methods section, not the results and discussion section, “NGS analysis was performed using 1/8 of sequencing 20 plate of GS FLX Titanium (454/Roche) for reading. Individual sequences were based on the V1–V3 primers.”

Response:

Thank you for your comment. It is now classified.

The first paragraph of the results and discussion section needs to be broken up into multiple paragraphs and different topics are in there.

Response:

Thank you for your comment. It is modified, as suggested.

L22: are you referring to the average number of reads per sample? If so state that. Otherwise, it is not clear what you mean by average reads.

Response:

Thank you for your comment. It is modified to:

“average number of total reads”

The following should be eliminated as it is not needed nor usually reported, “The lowest and highest number of reads was obtained for windpack, WP, (13 831) and urban snow, US, (24 968) respectively. The total number of bases was 32 284 312. The 25 lowest and highest number of bases belonged to blowing snow, BS, (4 164 307), and urban snow, US, (7 966 589) respectively. The average raw read length varied among the samples and was obtained as: 542_23 bases (urban snow, US), 422_21 bases (blowing snow, BS), 542_23 bases (snow hoar, SH), 533_23 bases (windpack, WP), and 556_24 bases (frost flower, FF).”

Response:

Thank you for your comment. It is deleted, as suggested.

Page 32102 During the trimming process, all sequences should have been trimmed to the same length. It should not vary from sample to sample as described in the following section, “After trimming and passing through quality control, the final read length was recovered as: 319_18 bases (urban snow, US), 299_17 bases (blowing snow, BS), 401_20 bases (snow hoar, SH), 385_20 bases (windpack, WP), and 419_20 bases (frost flower, FF) (Table A1).”

Response:

Sequence analysis was performed using the MOTHUR platform (Schloss et al, 2009). Sequences containing ambiguous bases, homopolymers longer than eight bases, or an average quality score below 20 over a 50 bp long window were excluded (Schloss et al, 2011). The results of initial step of the trimmed data are represented above. However, SILVA database in MOTHUR pipeline uses equal read lengths during alignment. Hence we opted to proceed with the methodology as written in the manuscript.

Additional reference:

Schloss, P. D., Gevers, D., and Westcott, S. L.: Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, PloS one, 6, e27310, 10.1371/journal.pone.0027310, 2011.

L6-8: Rarefaction Metric is not a way to measure bacterial diversity—it is a way to measure bacterial richness.

Response:

Thank you for your comment. It is modified to:

Simpson Diversity Index; species richness using Rarefaction Metric, and the nonparametric Chao index (Chao, 1984).

Page 32103 L8: “most abundance” should be “greatest abundance”

Response:

Thank you for your comment. It is modified.

L11: “genus” should be genera

Response:

Thank you for your comment. It is modified.

L22: “microbial” should be “microbes” or “microorganisms”

Response:

Thank you for your comment. It is modified.

Page 32106 L6: it is not accurate to say that most aerobic bacteria can grow on TSA. Considering only 0.01% of bacteria can be cultured at this point, you cannot say this about any medium type.

Response:

In 1955, Leavitt et al. (1955) discovered Tryptic Soy Agar (TSA). TSA is a general purpose medium that has enough nutrients to allow for a wide variety of microorganisms to grow; it facilitated vigorous growth of aerobic and anaerobic microorganisms. It contains digests of soybean meal and casein, making it suitable for the growth of a wide variety of fastidious and nonfastidious microorganisms (Murray et al., 2011; Forbes et al., 2007; American Public Health Association., 2012; Isenberg, 1992; MacFaddin, 1985; APHA Technical Committee on Microbiological Methods for Foods., 2001; The Official Compendia of Standards). It is recommended in multiple water & wastewater applications (Greenberg et al., 1995), numerous standard methods for food testing (U.S. Food and Drug Administration, 1998) and bacterial contaminants in cosmetics (Curry et al., 1993).

References:

Leavitt, J. M., I. J. Naidorf and P. Shugaevsky. 1955. The undetected anaerobe in endodontics: a sensitive medium for detection of both aerobes and anaerobes. The NY J. Dentist. 25:377-382.

Murray, P.R., et al. 2011. Manual of Clinical Microbiology, 10th ed. American Society for Microbiology, Washington, D.C.

Forbes, B.A., et al. 2007. Bailey and Scott's Diagnostic Microbiology, 12th ed. C.V. Mosby Company, St. Louis, MO.

American Public Health Association. 2012. Standard Methods for the Examination of Water and Wastewater, 22nd ed. APHA, Washington, D.C.

Isenberg, H.D. 1992. Clinical Microbiology Procedures Handbook, Vol. I, II & III. American Society for Microbiology, Washington, D.C.

MacFaddin, J.F. 1985. Media for Isolation, Cultivation, Identification, Maintenance of Bacteria, Vol. I. Williams & Wilkins, Baltimore, MD.

APHA Technical Committee on Microbiological Methods for Foods. 2001. Compendium of Methods for the Microbiological Examination of Foods, 4th ed. APHA, Washington, D.C.

The Official Compendia of Standards. USP-NF. United States Pharmacopeial Convention, Rockville, MD.

Greenberg, A. E., L. S. Clesceri, and A. D. Eaton (eds.). 1995. Standard methods for the examination of water and wastewater, 19th ed. American Public Health Association, Washington, D.C.

U.S. Food and Drug Administration. 1998. Bacteriological analytical manual, 8th ed., AOAC International, Gaithersburg, MD.

Curry, A. S., G. G. Joyce, and G. N. McEwen, Jr. 1993. CTFA Microbiology guidelines. The Cosmetic, Toiletry, and Fragrance Association, Inc. Washington, D.C.

Conclusions: *This section is an exact repeat of the abstract. Are there any deeper conclusions or implications of the work that can be discussed here?*

Response:

Thank you for your comment. The conclusion is modified to:

We herein examined the identity, population and ice nucleation ability of the microbial communities of five different snow types and frost flowers during the spring 2009 campaign of the Ocean-Atmosphere-Sea Ice-Snowpack (OASIS) program in Barrow, Alaska, USA. We used the next-generation sequencing (NGS) technique to examine the true bacterial communities in snow and frost flowers, in addition to conventional culture techniques. We gained more insight into the wide range of taxa available in different types of snow and frost flowers. Arctic samples and reference urban snow represented 11-18 known phyla or candidate divisions. The majority of sequences (12.3 - 83.1%) belonged to one of the five major phyla: Proteobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Cyanobacteria. At the genus level, 101-245 different genera were detected. A largely diverse community of bacteria exists in the Arctic with many originating from remote ecological environments such as dust storms. This study revealed that snow and frost flowers are rich media for the existence of microbial compounds. Biological materials are shown to act as reactive sites for (photochemical) reactions and thus more studies

are required to decipher the complexity of the snow and frost flowers as a zone of chemical pool. It is conceivable that changes on the ratio of antifreeze bacteria to ice nucleation bacteria may have an impact on the melting and freezing processes of snowpack or frost flowers. It is thus feasible that this shift in bacterial population could ultimately affect the snow melting-freezing processes. Further studies are required to evaluate whether change of nucleation patterns due to biological entities are indeed linked to climate change.

Table 1 is not clear in the information that it contains. What do the percentages refer to?

Response:

The percentages in Table 1 refers to the percentage of bacteria from the total bacteria pool detected by the next-generation sequencing (NGS) that have been previously detected in dust storms (Asian/African), cold oceanic water, or have exhibited antifreeze or ice nucleation properties. Table 1 is divided into five different categories: i) dust, ii) cold oceanic water, iii) antifreeze (AF), iv) ice nucleation (IN), and v) ice nucleation and antifreeze properties (IN & IF). Section (B) is the subtotal of bacteria with AF or IN properties seen only in Asian/African dust. We explained this point in the revised manuscript.

It seems that abundance should be reported in actual numbers of colony forming units. The caption is not clear; it says that “ice nucleation/melting properties as detected by Roche 454 GS-FLX Titanium”. One cannot determine ice nucleating properties using next generation sequencing tools.

Response:

Please let us clarify: ice nucleating bacteria in Table 1 were detected by Roche 454 GS-FLX Titanium method. These bacteria were previously shown to have ice nucleation properties.

Does Table 2 contain all of the bacteria that were isolated in this study?

Response:

Table 2 contains those bacteria that their ice nucleation properties were analyzed.

The information in table A1 is not typically presented. When NGS data are trimmed, all sequences, regardless of what sample they are from, should be trimmed to the same length and aligned together to ensure the ability to compare the microbial composition across the samples as was desired in this study.

Response:

Thank you for your comment. Table A1 is removed.

Table A2 would be more useful to the reader if it were converted into a bar chart where the reader could quickly identify the differences in microbial composition among the different sample types.

Response:

Thank you for your comment. There is a wide variation among the detected phylum in each snow category (ex: 49% to 0.03%) which makes it difficult for its true presentation in a graphing format and it does not add any further clarification. Hence we opted to proceed with the Table format as given in the manuscript.

Figure A1. The inverse Simpson index should not be graphed vs the number of sequences sampled. The Simpson index is a dominance index and continued sampling of “rare” types or singletons, is not going to significantly change this index as more sequences are sampled. Hence the rather flat “curves”. The inverse Simpson index should be presented as an endpoint number at the same number of sequences for each of the samples that one wishes to compare. For instance, if the greatest common number of sequences for all samples is 10,000 sequences, then the inverse Simpson index should be reported for 10,000 sequences for all of the samples.

Response:

Thank you for your comment. Inverse Simpson index takes abundance of community into account to estimate diversity. Hence the communities with equal abundances among different species will have more diversity assuming they have equal “richness”. Although, inverse Simpson index were plotted at different sequencing sampling, but it clearly shows, for example: blowing snow is more diverse than surface hoar snow. We agree that the addition of rare species to a sample causes only small changes in the value of index.

Figure 2. The legends are too complicated and are not readable even when the figure is blown up on my computer screen. It is not clear if the bars in the bar graphs each represent a different genus or something different.

Response:

Thank you for your comment. The legends in Figure 2 are modified and changed to bigger font. Plus, additional table (Appendix (A1) Table A.2) is added to Appendix to clarify the legends.

Figure 3. Do the bars represent averages? If yes, please state in the legend how many replicates were averaged. Are the error bars, standard error or standard error? Please state which one.

Response:

Yes, indeed the bar represent the average of three experiments. The error bars represent the standard deviation.

Reviewer 2

Received and published: 24 December 2014

The General Notes In this study, the authors applied a combination of culture dependent and independent methods in order to characterize the microbial community structure of five different

snow types and frost flowers and discuss their potential role in freezing and melting processes. Climate change is frequently linked to the increasing number of dust storms, which can lead to the transport of microbes to unrelated climate zones. Within those climate zones microbes may survive, propagate, and lead to an overall shift in microbial metabolic potential and ecosystem multi-functioning. Thus, this manuscript potentially represents a significant contribution to atmospheric science in general and particularly to the field of microbial ecology.

However, the lack of biological replicates (each of five snow types represented by a single observation) are a significant weakness of the study. Due to the lack of biological replicates, my suggestion is to focus mainly on community structure comparisons based on diversity parameters (alpha and beta diversity parameters) between the five snow types.

Response:

Thanks for your comment. Each of five snow categories and snow flowers were sampled on different days in multiple replications (at least 3). Respectfully, the results are indeed the representative of three experiments. Hence our sampling and analysis included multiple replicates. This sentence has been added in the revised manuscript.

Moreover, I suggest to remove frost flowers from this study based on the following reason: bacterial community structure mainly represents a phyllosphere bacterial community that is heavily influenced by a host (plant species) and that is normally exhibits a significant reduction in bacteria diversity (Fig A1).

Response:

Thanks for your comment. Bacterial community structure are not mainly represents phyllosphere bacterial community. Frost flowers are dendritic shape hypersaline clusters of ice crystals that form at the interface between warm ice surface and sufficiently cold atmospheric temperature and humidity (Obbard et al., 2009). In our study, frost flower sampling was collected from a frost flower “field” on a flat area of thick sea ice, in a single location about 5 km northwest of Barrow

Additional Refernce:

Obbard, R. W., Roscoe, H. K., Wolff, E. W., and Atkinson, H. M.: Frost flower surface area and chemistry as a function of salinity and temperature, *Journal of Geophysical Research: Atmospheres*, 114, D20305, 10.1029/2009JD012481, 2009.

The author should provide a list of accession numbers for both the isolates and the pyrosequencing data.

Response:

Thank for your comment. Please kindly note that the accession number is given in Table 2 & 3. A genus does not have accession number since it is presented by x sequences in a given database.

The authors present their results in a very confusing way: (i) Figure 1. In the diagrams, the axes and their values are unreadable; (ii) Figure 2. The legends are also unreadable; (iii) Figure 4 A. The bars are merged and unreadable.

Response:

Thank you for your comments. Figure 1 is modified and divided by two sections containing pictures obtained from electron microscopy and EDS results. Figure 2: the legends are now modified and changed to bigger font. Plus, additional table (Appendix (A1) Table A.2) is added to Appendix to clarify the legends. Figure 4A: the bars are changed to wider widths and more spaces are given for each group of snow.

The Specific Notes Page 1 Line 8: "the great majority of sequences (12.3–83.1 %) belonging to one of the five major phyla". Please rephrase to "a majority of sequences (12.3-83.1%)"

Response:

Thank you for your correction. It is incorporated.

Page 4 Line 9: "Xant/zomonas" should be Xanthomonas Page 7 Line

Response:

Thank you for your correction. It is incorporated.

20: Please indicate names of 16S universal primers and their concentration in the PCR reactions. Please also provide nucleotide and enzyme concentrations in the PCR reactions

Response:

Thank you for your correction. It is now incorporated.

universal primers 27F and 149R.

A typical PCR reaction for one tube contained 2.5 µl of 10X buffer, 1 µl of each primer of 2.5 µM, 0.6 µl of 10mM dNTP, 1 µl of 0.1 µg/ µl DNA, 0.1 µl of 5U/ µl of Taq polymerase, 2.5 µl of 25mM of MgCl₂, 0.8 µl of 1M NaCl, and to 25 µl of nuclease free water (Promega, Madison, USA).

Page 8 Line 4: Please provide the accession numbers for characterized isolates

Response:

Thank you for your correction. The accession numbers are indicated in Table 2 & 3.

Page 8 Line 5: Please separate the "454 Pyrosequencing, and Electron microscopy analysis" section into two sections: (i) 454 Pyrosequencing and sequence analysis (ii) Electron microscopy analysis

Response:

Thank you for your correction. It is now divided in two sections.

Page 8 Line 6: Please describe the DNA extraction protocol

Response:

Thank you for your comment. The following section has been added to the revised manuscript.

Melted snow was passed through 0.22 micron filter (Millipore, Mississauga, Canada). Filter was sonicated in 17 ml of 1X TBE buffer in an ultrasound bath for 10 min. The liquid was collected in sterile 50 ml polycarbonate centrifuge tubes (Nalgen. Rochester, NY, USA). The liquid was centrifuged for 15 min at 18,000 g, and the pellet was resuspended in 200 ul of 1X TBE buffer. Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) was used to lyse the cell, and DNA was extracted and purified using either a DNAeasy kit (Qiagen, Toronto, Canada) or a Master Pure DNA purification kit (Epicentre Technologies, Madison, USA) according to the manufacturer's instructions.

Page 8 Line 25: Please provide quality filtering parameters (Q values, sequences minimal and maximal lengths); taxonomic assignment algorithm and its parameters.

Response:

Thank you for the comments. The information is inserted on page 8 Line 26:

software MOTHUR formatted version of the RDP classifier using a Bayesian method (Wang et al., 2007) with 1000 bootstrap replicates for pre-processing (quality-adjustment, barcode split), identification of operational taxonomic units (OTUs) defined at ≥ 97 % 16S rRNA sequence identity level, taxonomic assignment, community comparison, and statistical analysis (Schloss et al., 2009). Trimming was done by quality from the 3' end. Sequences containing ambiguous bases, homopolymers longer than eight bases, or an average quality score below 20 over a 50bp long window were excluded (Schloss et al., 2011).

Moreover, the following was added to Page 9 Line22:

The average read length for all the reading was 373 base with average read quality of 34.

Additional References:

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R.: Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy, Applied and Environmental Microbiology, 73, 5261-5267, 10.1128/AEM.00062-07, 2007.

Schloss, P. D., Gevers, D., and Westcott, S. L.: Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, PloS one, 6, e27310, 10.1371/journal.pone.0027310, 2011.

Page 10 Line 7: Please justify the choice of Chao1 diversity index. The Chao 1 index is based upon the number of rare classes (i.e. OTUs) found in a sample, if your samples were filtered to eliminate singletons, then this estimation is not appropriate. Moreover, in order to conduct an inter-sample analysis using Chao1, the comparisons in your library must be equal in size. My suggestion is to use a Shannon diversity index that is much more robust.

Response:

We used Chao1 over the Shannon in our NGS data since the weight of contribution of rare species to the diversity of overall bacterial population wanted to be considered. Chao1 index is a good estimator for obtaining true species richness based on the observed species accumulation pattern. The number of singletons and doubletons were used to measure Chao1 index. Moreover, SILVA database in MOTHUR pipeline uses equal read lengths during alignment.

Page 17 Line 12: "the great majority of sequences (12.3–83.1 %) belonging to one of the five major phyla" Please rephrase to "a majority of sequences (12.3-83.1%)".

Response:

Thank you for your correction. It is incorporated.

Interactive comment on Atmos. Chem. Phys. Discuss., 14, 32093, 2014.

C10470

Reviewer 3

Received and published: 5 January 2015

The study by R. Mortazavi et al. on arctic bacteria in snow and frost flowers, describe genomically-based characteristics as well as freezing nucleation properties. The investigation presented is valuable and interesting in many aspects; however, there are some important points that should be considered or further explained. in addition, some more information is required, mainly in the method section. My specific comments are below:

1- From the title it is understood that only bacteria were investigated. If the authors chooses to include fungi analysis as well, the title should rephrased to generalize the case for microbes instead of bacteria accordingly.

Response:

Thanks for your comment, we have now changed it to:

Arctic microbial and next-generation sequencing approach for bacteria in snow and frost flowers:
Selected identification, abundance and freezing nucleation

2- P. 32097, lines 20-21: *An explanation of the different snow type as well as the frost flowers is required in the introduction.*

Response:

Thank you for your comment. The following text has been added to the introduction of the revised manuscript (page 5, line 8).

Five different types of Arctic snow: (i) accumulated snow, (ii) windpack, (iii) blowing snow, (iv) surface hoar snow, (v) fresh snow and frost flowers were used for this study (Fierz et al., 2009; Glossary of Meteorology., 2009). Frost flowers are dendritic shape hypersaline clusters of ice crystals that form at the interface between warm ice surface and sufficiently cold atmospheric temperature and humidity (Obbard et al., 2009). The chemistry of frost flowers has garnered increased interest, because these salty ice crystals were shown to act as a source for: (i) sea-salt aerosol (Perovich and Richter-Menge, 1994), and (ii) BrO, which contributes to ozone depletion events (Kaleschke et al., 2004). Increased bacterial abundance was also found in frost flowers (Bowman and Deming, 2010).

Additional references which are now included in the revised version:

Fierz, C., Armstrong, R.L., Durd, Y., Etchevers, P., Greene, E., McClung, D.M., Nishimura, K., Satyawali, P.K. and Sokratov, S.A. 2009. The International Classification for Seasonal Snow on the Ground. IHP-VII Technical Documents in Hydrology N°83, IACS Contribution N°1, UNESCO-IHP, Paris.

Glossary of Meteorology: "Snow". American Meteorological Society. , Retrieved 2009-06-28, 2009.

Obbard, R. W., Roscoe, H. K., Wolff, E. W., and Atkinson, H. M.: Frost flower surface area and chemistry as a function of salinity and temperature, *Journal of Geophysical Research: Atmospheres*, 114, D20305, 10.1029/2009JD012481, 2009.

Perovich D., J.A. Richter-Menge (1994), Surface characteristics of lead ice, *J Geophys Res*, 99, 16341-16350.

Kaleschke L., A. Richter, J. Burrows, O. Afe, G. Heygster, J. Notholt, A. Rankin, H. Roscoe, J. Hollwedel, T. Wagner, H. Jacobi (2004), Frost flowers on sea ice as a source of sea salt and their influence on tropospheric halogen chemistry, *Geophys Res Lett*, 31, L16114.

Bowman J., J.W. Deming (2010), Elevated bacterial abundance and exopolymers in saline frost flowers and implications for atmospheric chemistry and microbial dispersal, *Geophys Res Lett*, 37, L13501-L13505.

3- *Could the authors please provide some information on the poor media used for the fungi (SDA)? How did you make sure that bacteria will not grow in the mycological agar?*

Response:

Thanks for your comment and this is indeed a good question. Please note that we have used a very established and examined technique to optimize fungal growth. As such, fungal Agar is prepared according to the formulation suggested in 1958 by Huppert and Walker (Hupper and Walker., 1958). This media with a neutral or slightly alkaline pH provide for early and luxuriant growth of fungi and the formula is adjusted, standardized to suit performance parameters (Hughes et al., 1954).

Based on the final volume needed for the x number of petri dishes, Sabouraud dextrose agar (SDA) was dissolved in ultrapure Milli-Q water (18 ohms resistance) according to manufacture recommendation. Magnetic stir bar was added to the flask and was heated at the same time. Once it was boiled for one minute or till the medium was completely dissolved, the flask was removed from heater. It was then autoclaved at 121 °C for 15 minutes. Immediate optical microscopy survey was shown the predominance of fungal colonies, yet, we will not be able to preclude of any minute bacterial presence, which is a daunting task with almost all existing fungal growth techniques. We have now added this information in the text.

Additional references:

Hughes, D. E., Moss, E. S., Hood, M., and Henson, M.: Virulence of Mycobacterium tuberculosis: evaluation of a test, using neutral red indicator, American journal of clinical pathology, 24, 621-625, 1954.

Huppert, M., and Walker, L. J.: The selective and differential effects of cycloheximide on many strains of Coccidioides immitis, American journal of clinical pathology, 29, 291-295, 1958.

4- P. 32099, lines 16-18:

i) For DNA isolation - please specify where the bacterial cells were taken from (plates or field sample).

Response:

Bacterial cells were taken from colonies in plates. We have added this sentence to enhance clarity.

ii) In general, the description of the extraction procedure as well as the NGS analysis lacks some information (volume of bacteria sample to be extracted, cell concentration/ mass, the amount of DNA used for NGS analysis etc.).

Response:

Thanks for your comment. The extra information is added to the text. Please note that Part of each bacterial colony was picked by a sterile disposable inoculating loop (VWR, Mississauga, Canada) and mixed with Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) and proteinase K in 1.5 ml eppendorf tube.

0.1 µg/µl, and 5 ng/µl of DNA was used for amplification using Sanger, and NGS method respectively.

A typical PCR reaction for one tube contained 2.5 µl of 10X buffer, 1 µl of each primer of 2.5 µM, 0.6 µl of 10mM dNTP, 1 µl of 0.1 µg/ µl DNA, 0.1 µl of 5U/ µl of Taq polymerase, 2.5 µl of 25mM of MgCl₂, 0.8 µl of 1M NaCl, and to 25 µl of nuclease free water (Promega, Madison, USA). This information is now added in the revised version of manuscript.

For NGS:

Melted snow was passed through 0.22 micron filter (Millipore, Mississauga, Canada). Filter was sonicated in 17 ml of 1X TBE buffer in an ultrasound bath for 10 min. The liquid was collected in sterile 50 ml polycarbonate centrifuge tubes (Nalgen. Rochester, NY, USA). The liquid was centrifuged for 15 min at 18,000 g, and the pellet was resuspended in 200 µl of 1X TBE buffer. Ready-LyseTM Lysozyme (Epicentre Technologies, Madison, USA) was used to lyse the cell, and DNA was extracted and purified using either a DNAeasy kit (Qiagen, Toronto, Canada) or a Master Pure DNA purification kit (Epicentre Technologies, Madison, USA) according to the manufacturer's instructions.

Amplification was done in triplicate and performed in a 20 µl reaction volume containing 13.85 µl of RNase and DNase free water, 2 µl of 5 ng/µl of DNA template, 2 µl of 10X AccuPrime PCR buffer (Invitrogen, Burlington, Canada), 1 µl of 2 µM of each primer, and 0.15 µl of AccuPrime Taq DNA polymerase Hifi (Invitrogen, Burlington, Canada).

iii) Please add some information on the extraction efficiency differences between the two DNA extraction kits.

Response:

Both DNAeasy kit (Qiagen, Toronto, Canada) or Master Pure DNA purification kit (Epicentre Technologies, Madison, USA) gave comparable results. Both were found to function equally well. We have added this section to the revised manuscript.

5- P. 32100, Lines 2-3: abbreviation for BLASTN should be “Basic Local Alignment Search Tool for DNA/nucleic acid”.

Response:

Thank you for the comment. It has been changed.

6- P. 32101, Lines 19-21: (“NGS analysis. . . VI-V3 primers”): this part should be in the method section.

Response:

Thank you for the comment. It has been changed.

7- A statistics paragraph should also be added in the method section where lines 6-8 in P. 32102 should be placed in.

Response:

Thank you for the comment. It has been now incorporated.

The generated sequences from pyrosequencing was analyzed with software MOTHUR formatted version of the RDP classifier using a Bayesian method (Wang et al., 2007) with 1000 bootstrap replicates for pre-processing (quality-adjustment, barcode split), identification of operational taxonomic units (OTUs) defined at ≥ 97 % 16S rRNA sequence identity level, taxonomic assignment, community comparison, and statistical analysis (Schloss et al., 2009). Trimming was done by quality from the 3' end. Sequences containing ambiguous bases, homopolymers longer than eight bases, or an average quality score below 20 over a 50bp long window were excluded (Schloss et al., 2011).

The diversity of the bacterial communities for four different snow types and frost flowers was estimated using the Simpson Diversity Index, species richness using Rarefaction Metric, and the nonparametric Chao index (Chao, 1984). Chao1 index is a good estimator for obtaining true species richness based on the observed species accumulation pattern wherein the number of singletons and doubletons were used.

Additional references:

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R.: Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy, *Applied and Environmental Microbiology*, 73, 5261-5267, 10.1128/AEM.00062-07, 2007.

Schloss, P. D., Gevers, D., and Westcott, S. L.: Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, *PloS one*, 6, e27310, 10.1371/journal.pone.0027310, 2011.

8- Why figure A1-3 and Tables A1-2 are not under numbers? If the authors meant to separate them as supporting information, it should be clearly presented. The current presentation is confusing and unnecessary. The figures are also very hard to understand (axis titles as well as legends in most Figures).

Response:

Figure A1 is divided by Figure A1-A, A1-B, and A1-C. For the clarity the labels are modified to A1-A, A1-B, and A1-C. The title is changed to: “(A1) Figure 1.”

Figure 1 is modified and divided by two sections containing pictures obtained from electron microscopy and EDS results. Figure 2: the legends are now modified and changed to bigger font. Plus, additional table (Appendix (A1) Table A.2) is added to Appendix to clarify the legends. Figure 4A: the bars are changed to wider widths and more spaces are given for each group of snow.

9- P. 32102, Lines 23-24: *the authors explain that pyrosequencing was done only for bacteria. In the present terminology, it is understood that this is not the case for other analyses (IN, electron microscopy etc.), however fungi were analyzed here only for cultivability. Therefore, please rephrase this sentence to be more accurate.*

Response:

Thank you for your comments. This paragraph is changed to:

In the next-generation sequencing part of this study, pyro-sequencing was done only for bacteria and not fungi which was feasible under our existing facilities. However, high resolution electron microscopy (Fig. 1) further revealed the appearance of the existence of several biological materials, remnants of biological activities, and not only biological entities in their entirety.

10- P. 32103, lines 22-26: *The authors should refer the readers to some published data on the different chemical/physical composition or other elements such as RH, microclimate etc., which might affect the growth of the microbes in the different snow forms. Additional discussion on this point is required.*

Response:

Thanks for your comments. Indeed, Bhatia et al. (2006) compared bacterial communities from solid snow and snow melt water from the high Arctic John Evans glacier with basal ice and subglacial communities of the same glacier. Distinct bacterial communities were found in each one of these different environments with very few common profiles. Similar to this study, our NGS analysis clearly showed variation of distinct sets of microbial among different Arctic samples and urban snow. Our observation also suggests the importance of the selective pressure of specific physical and chemical characteristics of each snow type that may serve as a predictor of microbial abundance and composition (Rosa et al., 2008). This may specifically favors the growth conditions for microbial communities that originated from diverse sources. We have added the additional references and discussed previous literature in the revised version of the manuscript.

Additional references:

Bhatia M, Sharp M, Foght J (2006) Distinct bacterial communities exist beneath a high arctic polythermal glacier. *Appl Environ Microbiol* 72:5838–5845

Rosa Margesin, Franz Schinner, Jean-Claude Marx, Charles Gerday (2008). Chapter 3. Bacteria in Snow and Glacier Ice in *Psychrophiles: from Biodiversity to Biotechnology*. ISBN: 978-3-540-74334-7 (Print) 978-3-540-74335-4 (Online)

11- P. 32104, lines 10-14: *please expand some more on the anti-freezing properties and IN abilities in the same microbe. Why this phenomenon is interesting? was this observed in other previously published data? What do the references at the end of the sentence relate to? Is there any significance for the presence of such microbes in this environment? It is understood from the*

text that you describe here your results. However, there are few citations at the end of the line. If it is another published data, please rephrase this sentence.

Response:

Thank you for the comment. The following paragraph is now added to the manuscript.

Some bacteria such as *Pseudomonas fluorescens* KUAF-68 and *Pseudomonas borealis* DL7 have been reported to have both antifreeze and ice nucleation activity (Kawahara et al., 2004; Wilson et al., 2006). Having these two properties may enhance the freeze tolerance survival of bacteria by: i) maintaining small ice crystals with ice recrystallization inhibition protecting against freeze-thaw stress with antifreeze proteins (Xu et al., 1998), ii) minimizing damage from explosive ice crystal growth and stabilizing the outer membrane with the low thermal hysteresis value (Xu et al., 1998), and iii) minimizing the supercooling point with ice nucleation proteins (Kawahara et al., 2004)

References:

Kawahara, H., Nakano, Y., Omiya, K., Muryoi, N., Nishikawa, J., and Obata, H.: Production of two types of ice crystal-controlling proteins in Antarctic bacterium, *Journal of bioscience and bioengineering*, 98, 220-223, 10.1016/s1389-1723(04)00271-3, 2004.

Wilson, S. L., Kelley, D. L., and Walker, V. K.: Ice-active characteristics of soil bacteria selected by ice-affinity, *Environmental microbiology*, 8, 1816-1824, 10.1111/j.1462-2920.2006.01066.x, 2006.

Xu, H., Griffith, M., Patten, C. L., and Glick, B. R.: Isolation and characterization of an antifreeze protein with ice nucleation activity from the plant growth promoting rhizobacterium *Pseudomonas putida* GR12-2, *Canadian Journal of Microbiology*, 44, 64-73, 10.1139/w97-126, 1998.

12- P. 32109, lines 3-5: where was this species shown to oxidize radioactive materials?

Please add relevant citations. additional explanation on this anecdote is required. Is there any information on uranium in this area? Or does it require some future investigation?

Response:

Thank you for your comment. We have added this section and added the following references, as requested.

Some of *Geobacter* species is shown to be able to catalyze anaerobic U(IV) oxidation with nitrate serving as a potential electron acceptor leading to the subsequent mobilization of uranium (Finneran et al., 2002). *Geobacter* species can also reduce soluble U(VI) to the less soluble U(IV) (Lovley et al., 1991). Arctic area does contain radioactive waste due to military activity, oil and gas, and uranium mines exploitation (Thomas et al., 1992; Dowdall et al., 2004, Convey, 2010;

Emmerson and Lahn., 2012). We have added this section and added the following references, as requested.

Additional References:

Finneran, K. T., M. E. Housewright, and D. R. Lovley. 2002. Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. *Environ. Microbiol.* 4:510–516.

Lovley, D.R., E.J.P. Philips, Y.A. Gorby, E. R. Landa. (1991) Microbial Reduction of Uranium. *Nature.* 350: 413-415

Thomas, D. J., Tracey, B., Marshall, H., and Norstrom, R. J.: Arctic terrestrial ecosystem contamination, *The Science of the total environment*, 122, 135-164, 1992.

Dowdall, M., Vicat, K., Frearson, I., Gerland, S., Lind, B., and Shaw, G.: Assessment of the radiological impacts of historical coal mining operations on the environment of Ny-Alesund, Svalbard, *Journal of environmental radioactivity*, 71, 101-114, 10.1016/s0265-931x(03)00144-9, 2004.

Convey, T., (2010). Environmental overnace and the new arctic frontier: when the north opens and security declines, (Carleton University Symposium; 2009-2010)

Emmerson C, and Lahn G. (2012). Arctic opening: Opportunity and Risk in the High North. London: Chatham House-Lloyd's Risk Insight Report. available at www.chathamhouse.org/publications/papers/view/182839

13- It is hard to understand from the presented data how the fungal cultivated results relate or contribute to the main conclusions of this paper. Could the authors clarify this point, and emphasize it more clearly in the discussion? In the present form, this specific data on fungi seems unrelated to the whole paper.

Response:

Thank you for your comment. The following paragraph with additional references is added to discussion.

The objective of this paper was the identification of microbial body in Arctic snow and frost flowers. Indeed, we could not have done next-generation sequencing for fungi due to experimental conditions. Hence, there are more genetic information about bacterial species. Yet, we found the existing presented data which are still very scarce of great value, and this is why we opted to include them. With Arctic regions, currently warming at rapid rates (Hansen et al. 2006; Convey et al. 2009), the interrelationship of ice/snow microbial, and increased water availability is yet to be determined. Though fungi species and their spore are wide spread in the atmosphere, little is known about their role and presence at Arctic. Interestingly, few studies have shown that fungi like bacteria can be effective ice nucleators, capable of initiating ice nucleation at temperatures as high as -2°C (Kieft and Ahamdjian., 1989; Pouleur et al., 1992). Some could also prevent ice crystal expansion by synthesising antifreeze proteins permitting

their growth at subzero temperatures (Tojo and Newsham., 2012; Hoshino et al., 1998). In our study, besides bacteria we thence sought to explore the role of fungi in Arctic environment by quantifying and comparing their population in six different Arctic samples

References:

Convey, P., Bindschadler, R., di Prisco, G., Fahrbach, E., Gutt, J., Hodgson, D. A., Mayewski, P. A., Summerhayes, C. P., and Turner, J.: Antarctic climate change and the environment, *Antarctic Science*, 21, 541-563, <http://dx.doi.org/10.1017/S0954102009990642>, 2009.

Hansen, J., Sato, M., Ruedy, R., Lo, K., Lea, D. W., and Medina-Elizade, M.: Global temperature change, *Proceedings of the National Academy of Sciences of the United States of America*, 103, 14288-14293, 10.1073/pnas.0606291103, 2006.

Hoshino, T., Tronsmo, A. M., Matsumoto, N., Araki, T., Georges, F., Goda, T., Ohgiya, S., and Ishizaki, K.: Freezing resistance among isolates of a psychrophilic fungus, *typhula ishikariensis*, from Norway (19th Symposium on Polar Biology), *Proceedings of the NIPR Symposium on Polar Biology*, 11, 112-118, 1998.

Kieft, T., and, and Ahmadjian, V.: Biological ice nucleation activity in lichen mycobionts and photobionts. , *Lichenologist* 21, 355–362., 1989.

Pouleur, S., Richard, C., Martin, J. G., and Antoun, H.: Ice Nucleation Activity in *Fusarium acuminatum* and *Fusarium avenaceum*, *Appl Environ Microbiol*, 58, 2960-2964, 1992.

Tojo, M., and Newsham, K. K.: Snow moulds in polar environments, *Fungal Ecology*, 5, 395-402, <http://dx.doi.org/10.1016/j.funeco.2012.01.003>, 2012.

14- P. 32109, lines 25-28 and P. 32110 lines 1-3: it seems that there is some mistake in these two sentences. Please check.

Response:

Thanks for your comment. We modified the sentence in the enclosed manuscript.

Biological materials are shown to act as reactive sites for (photochemical) reactions and thus more studies are required to decipher the complexity of the snow as a zone of chemical pool. It is conceivable that changes on the ratio of antifreeze bacteria to ice nucleation bacteria may have an impact on the melting and freezing processes of snowpack or frost flowers. It is thus possible that this shift in bacterial population could ultimately affect the snow melting-freezing processes.

Reviewer 4

Atmos. Chem. Phys. Discuss., 14, C10490–C10493, 2014

www.atmos-chem-phys-discuss.net/14/C10490/2014/

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Atmospheric Chemistry and Physics Open Access Discussions

Interactive comment on “A next generation sequencing of Arctic bacteria in snow and frost flowers: identification, abundance and freezing nucleation” by R. Mortazavi et al.

J. Bowman bowmanjs@gmail.com

Received and published: 25 December 2014

General Comments

Frost flowers are an interesting ice-type both biologically and chemically. The authors collected data on the chemical and biological composition of frost flowers and various other snow/ice environments. This kind of data is much needed and could serve as fodder for an interesting analysis. There are, however, numerous issues with the analysis and the resulting manuscript that should be corrected before publication.

First, the authors are missing an opportunity to place their sequence results in the context of existing work on frost flowers

(e.g. Bowman et al, 2013, EMIR; Bowman et al, 2014, FEMS; Barber et al, 2014, JGR Oceans) and snow

(e.g. Hauptmann et al. 2014, Extremophiles, and Maccario et al. 2014, Frontiers in Microbiology among many others).

In particular the three frost flower studies noted above describe two dramatically different frost flower communities. Where does the community described by this work fit in? As NGS technologies were used in all of these studies the authors should take the opportunity to compare and contrast these results. For example, are some of the same sequences described in more than one study? What might lead to the observed differences?

Response:

Thank you for your comment. We have indeed cited one of your papers which was relevant to the subject. However, for the limited space, we opted to not proceed with the references to your other suggestions, although they were quite interesting, they were not linked directly, as much as other cited references, to the discussions of the paper. For instance you have suggested “First, the authors are missing an opportunity to place their sequence results in the context of existing work on frost flowers”

The suggested articles:

“Hauptmann AL1, Stibal M, Bælum J, Sicheritz-Pontén T, Brunak S, Bowman JS, Hansen LH, Jacobsen CS, Blom N. Bacterial diversity in snow on North Pole ice floes. Extremophiles. 2014 Nov;18(6):945-51. doi: 10.1007/s00792-014-0660-y. Epub 2014 Jun 21.”

Maccario L, Vogel TM, Larose C. Potential drivers of microbial community structure and function in Arctic spring snow. *Front Microbiol.* 2014 Aug 7;5:413. doi: 10.3389/fmicb.2014.00413. eCollection 2014.

Did not cover any study on frost flowers.

Bowman et al, 2013, EMIR; and Bowman et al, 2014, FEMS articles are very informative and interesting. Samples were taken from one young ice and one frost flower sample, from a small lead off shore (Pt. Barrow, Alaska). The papers mainly investigated the comparison of microbial community in frost flowers to underlying young ice, as well as, the hypotheses regarding the source of the observed frost flower Rhizobiales and to explore the potential biogeochemical impact of this community at the critical ice–ocean–atmosphere interface. As author indicated: “A quantitative approach was developed to assess the taxonomic composition of these communities and the presence of key functional genes.” Our study did not investigate the expression of chromosomes, plasmids and metabolic profiles in frost flower metagenome; thus, not a strong link exist within our studies. Barber et al, 2014, JGR did an excellent study on the impact of different factors such as climate, chemicals and salinity on formation of young frost flowers on the manmade open ocean. The frost flowers used in our study were old on a very thick layer of snow near the land (Kos et al., 2014). Thus, these articles are not directly related to the scope of this paper.

Reference:

Kos, G., Kanthasami, V., Adechina, N., and Ariya, P. A.: Volatile organic compounds in Arctic snow: concentrations and implications for atmospheric processes, *Environmental science. Processes & impacts*, 16, 2592-2603, 10.1039/c4em00410h, 2014.

“Where does the community described by this work fit in? As NGS technologies were used in all of these studies the authors should take the opportunity to compare and contrast these results. For example, are some of the same sequences described in more than one study? What might lead to the observed differences? “

Response:

Thanks for your suggestion. The aim of this paper was not to review the existing works using NGS methodology on microbial content of Arctic environment. We have clearly discussed the relevance of the topic, but your proposed suggestion, although interesting, it is beyond the scope of this paper to compare and contrast large numbers of taxonomic categories. This is a different study of this own, that we might pursue in the future. However, it was not amongst the objectives of this collaborative work.

Bowman et al (2014) specifically evaluated a frost flower metagenome for ice structuring genes, finding fewer genes in frost flowers than in the underlying sea ice. Some discussion of this in the context of the ice nucleation work presented here would be useful.

Response:

Thank you for your comment. In the presented work, no sampling was done for underlying sea ice, due to logistical challenges. However, other colleagues have done similar works and we expect to see their results in near future.

The results and discussion are presented somewhat haphazardly, making it difficult to determine what the authors have done and why. Some important data collected is not adequately discussed; why is the EDS data not discussed further, or presented in the results in a more quantified manner? Similarly, beyond some brief discussion of their ice nucleation potential the different microbial genera “identified” (at what confidence?) are not discussed.

A lot of information was collected in this study, what does it tell us about these different environments? I would encourage the authors to read the manuscript carefully for wording and grammatical errors. There are numerous errors which I did not document in detail.

Response:

Thank you for your comments. Please note that we have revised the manuscript. We have now discussed your suggestion for further discussion on EDS in the revised manuscript.

Specific Comments

Suppliers of materials are inconsistently identified. For example the manufacture of the freezer is identified (not necessary), but the manufacture of the Ready-Lyse reagent is not (necessary). This should be corrected throughout.

Response:

Thank you for your comment. It has been clarified throughout of manuscript.

For example: Page 7 Line16 is modified to:

Part of each bacterial colony was picked by a sterile disposable inoculating loop (VWR, Mississauga, Canada) and mixed with Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) and proteinase K in 1.5 ml eppendorf tube.

What were the temperature and salinity of the collected frost flowers? How old do the authors estimate they are? How thick was the ice underneath? These are essential details for interpreting the community composition data.

Response:

Frost flower sampling was collected from a frost flower “field” on a flat area of sea ice, in a single location about 5 km northwest of Barrow on Mar 20, 2009 (also described by Beine et al., 2012; and Douglas et al., 2012). Average snow temperature was at -19 °C and average air temperature was at -21 °C during our sampling (Kos et al., 2014). The Frost flowers were characterized as “old frost flowers” having coatings from increased vapour phase deposition (Douglas et al., 2012). A pre-cleaned single-use HDPE shovel was used to carefully lift Frost flower samples to minimize (but not completely eliminating) brine content.

Additional References:

Beine, H., Anastasio, C., Domine, F., Douglas, T., Barret, M., France, J., King, M., Hall, S., and Ullmann, K.: Soluble chromophores in marine snow, seawater, sea ice and frost flowers near Barrow, Alaska, *Journal of Geophysical Research: Atmospheres*, 117, D00R15, 10.1029/2011JD016650, 2012.

Douglas, T. A., Domine, F., Barret, M., Anastasio, C., Beine, H. J., Bottenheim, J., Grannas, A., Houdier, S., Netcheva, S., Rowland, G., Staebler, R., and Steffen, A.: Frost flowers growing in the Arctic ocean-atmosphere–sea ice–snow interface: 1. Chemical composition, *Journal of Geophysical Research: Atmospheres*, 117, D00R09, 10.1029/2011JD016460, 2012.

Kos, G., Kanthasami, V., Adechina, N., and Ariya, P. A.: Volatile organic compounds in Arctic snow: concentrations and implications for atmospheric processes, *Environmental science. Processes & impacts*, 16, 2592-2603, 10.1039/c4em00410h, 2014.

What pore-size filter was used to collect cells for DNA extraction? Was a pre-filtration step used?

Response:

Thank you for your comment. It was incorporated in text as:

Melted snow was passed through 0.22 micron filter (Millipore, Mississauga, Canada). Filter was sonicated in 17 ml of 1X TBE buffer in an ultrasound bath for 10 min. The liquid was collected in sterile 50 ml polycarbonate centrifuge tubes (Nalgen. Rochester, NY, USA). The liquid was centrifuged for 15 min at 18,000 g, and the pellet was resuspended in 200 ul of 1X TBE buffer. Ready-Lyse™ Lysozyme (Epicentre Technologies, Madison, USA) was used to lyse the cell, and DNA was extracted and purified using either a DNAeasy kit (Qiagen, Toronto, Canada) or a Master Pure DNA purification kit (Epicentre Technologies, Madison, USA) according to the manufacturer's instructions.

Cyanobacterial reads were reported, were these chloroplasts? If so, from what eukaryote?

Response:

No, they were not chloroplasts.

32095 Line 4 – citation needed

Response:

Thank you for your comment. References are added.

(Amoroso et al., 2010; Fujii et al., 2010; Segawa et al., 2005).

Reference:

Segawa, T., et al. (2005). "Seasonal change in bacterial flora and biomass in mountain snow from the Tateyama Mountains, Japan, analyzed by 16S rRNA gene sequencing and real-time PCR." *Appl Environ Microbiol* **71**(1): 123-130.

Amoroso, A., et al. (2010). "Microorganisms in dry polar snow are involved in the exchanges of reactive nitrogen species with the atmosphere." *Environ Sci Technol* **44**(2): 714-719.

Fujii, M., et al. (2010). "Microbial community structure, pigment composition, and nitrogen source of red snow in Antarctica." *Microbial Ecology* **59**(3): 466-475.

32095 Line 28 – there is a growing body of literature on bacterial survival in long distance dust. Some of this work should be cited here (e.g. Smith et al. 2010, Aerobiologia).

Response:

Thank you for your comment. The reference is incorporated.

Smith, D., Griffin, D., and Schuerger, A.: Stratospheric microbiology at 20 km over the Pacific Ocean, *Aerobiologia*, 26, 35-46, 10.1007/s10453-009-9141-7, 2010.

32096 – I think the introduction needs to get more specific regarding the known and potential role of IN in high latitudes. Quite a bit of low latitude work is introduced that is not particularly informative.

Response:

Thank you for your comment. In page 4, line: 15-19 covers the roles of microbial in atmosphere.

32097 Line 4 – citations needed (e.g. Hauptmann et al. 2014, Maccario et al. 2014)

Response:

Thank you for the comment. The following references which they used standard microbiology techniques in showing diverse population of bacteria in snow were added.

(Liu et al., 2011; Harding et al., 2011)

Additional References:

Harding, T., Jungblut, A. D., Lovejoy, C., and Vincent, W. F.: Microbes in high arctic snow and implications for the cold biosphere, *Appl Environ Microbiol*, 77, 3234-3243, 10.1128/aem.02611-10, 2011.

Liu, Y., Yao, T., Jiao, N., Tian, L., Hu, A., Yu, W., and Li, S.: Microbial diversity in the snow, a moraine lake and a stream in Himalayan glacier, *Extremophiles : life under extreme conditions*, 15, 411-421, 10.1007/s00792-011-0372-5, 2011.

32098 Line 11 – how long did samples sit at -20 before analysis? Communities are not stable (particularly against loss) at these temps.

Response:

For one year.

32099 Line 20 – *what region is this amplifying? Important to identify.*

Response:

Thank you for your comment. It was incorporated in the text.

The conserved region was amplified yielding a product of about 1465 bp.

32100 Line 2 – *BLASTN is not an appropriate way to classify 16S sequences. The authors should use the RDP classifier, the classifier built in to Mothur, or a different tool and an appropriate database.*

Response:

Thank you for your comment. BLASTN was used for identification of sequences obtained using Sanger method and not for NGS.

32100 Line 27 – *the authors should more clearly describe the sequence analysis undertaken with Mothur.*

Response:

Thank you for the comments. The information is inserted on page 8 Line 26:

The generated sequences from pyrosequencing was analyzed with software MOTHUR formatted version of the RDP classifier using a Bayesian method (Wang et al, 2007) with 1000 bootstrap replicates for pre-processing (quality-adjustment, barcode split), identification of operational taxonomic units (OTUs) defined at $\geq 97\%$ 16S rRNA sequence identity level, taxonomic assignment, community comparison, and statistical analysis (Schloss et al., 2009). Trimming was done by quality from the 3' end. Sequences containing ambiguous bases, homopolymers longer than eight bases, or an average quality score below 20 over a 50bp long window were excluded (Schloss et al, 2011).

Additonal References:

Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R.: Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy, *Applied and Environmental Microbiology*, 73, 5261-5267, 10.1128/AEM.00062-07, 2007.

Schloss, P. D., Gevers, D., and Westcott, S. L.: Reducing the effects of PCR amplification and sequencing artifacts on 16S rRNA-based studies, *PloS one*, 6, e27310, 10.1371/journal.pone.0027310, 2011.

32101 Line 14 – *true, but I don't think the current study is addressing interactions at all. . .*

Response:

Thank you for your comment. It was a general statement: “A clear understanding of the bacterial population and their interactions will further reveal the role these play in altering the Arctic environment and its climate.”

Definitely, this study is only one study and many more studies are required to analyze Arctic microbial population both spatially and temporally to reveal bacterial population and their interactions. However, this study exhibits the existence of heterogeneous population of bacteria in Arctic in which potentially they interact within each other as many studies such as Fiegna et al., (2014) have previously shown in different environment.

Reference:

Fiegna, F., Moreno-Letelier, A., Bell, T., and Barraclough, T. G.: Evolution of species interactions determines microbial community productivity in new environments, ISME J, 10.1038/ismej.2014.215, 2014.

32101 Line 15 – here and elsewhere, it is a little odd the way the authors refer to the sequencing methodology as the NGS technique. They relied on a sequencing platform (454), which is one of a suite of technologies that differ from Sanger sequencing. These technologies are very different from one another and are, collectively, now the standard technologies for obtaining environmental sequences. The authors should just state the platform used and move on. As currently used the authors run the risk of a reader perceiving the study as about NGS, not about the study of a particular environment.

This further obscures interesting findings in this work.

Response:

Thank you for the comment. This information was moved to Experimental Methods.

Interactive comment on Atmos. Chem. Phys. Discuss., 14, 32093, 2014.

Atmos. Chem. Phys. Discuss., 14, C10494–C10494, 2014

www.atmos-chem-phys-discuss.net/14/C10494/2014/

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Atmospheric Chemistry and Physics

Open Access Discussions

Interactive comment on “A next generation sequencing of Arctic bacteria in snow and frost flowers: identification, abundance and freezing nucleation” by R. Mortazavi et al.

J. Bowman

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I think there is some confusion here that can be cleared up with an improved introduction. Referee #2 refers to a phyllosphere association for the frost flower microbial community, which seems a reference to the botanical frost flower and not the saline ice formation. The authors should take the time to clearly describe the environments they have selected for investigation and their reasons for selecting them.

Response:

Thank you for your comment. A clarification is added to the Experimental Methods.

Frost flower sampling was collected from a frost flower “field” on a flat area of thick sea ice, in a single location about 5 km northwest of Barrow on Mar 20, 2009 (also described by Beine et al., 2012; and Douglas et al., 2012). Average snow temperature was at -19 °C and average air temperature was at -21 °C during our sampling (Kos et al., 2014). The Frost flowers were characterized as “old frost flowers” having coatings from increased vapour phase deposition (Douglas et al., 2012).

Additional References:

Beine, H., Anastasio, C., Domine, F., Douglas, T., Barret, M., France, J., King, M., Hall, S., and Ullmann, K.: Soluble chromophores in marine snow, seawater, sea ice and frost flowers near Barrow, Alaska, *Journal of Geophysical Research: Atmospheres*, 117, D00R15, 10.1029/2011JD016650, 2012.

Douglas, T. A., Domine, F., Barret, M., Anastasio, C., Beine, H. J., Bottenheim, J., Grannas, A., Houdier, S., Netcheva, S., Rowland, G., Staebler, R., and Steffen, A.: Frost flowers growing in the Arctic ocean-atmosphere-sea ice-snow interface: 1. Chemical composition, *Journal of Geophysical Research: Atmospheres*, 117, D00R09, 10.1029/2011JD016460, 2012.

Kos, G., Kanthasami, V., Adechina, N., and Ariya, P. A.: Volatile organic compounds in Arctic snow: concentrations and implications for atmospheric processes, *Environmental science. Processes & impacts*, 16, 2592-2603, 10.1039/c4em00410h, 2014.

Once again we would like thanks again for inputs and suggestions.