Getting out of the water and into the air: Understanding aerosolization of the bacterium *Pseudomonas syringae* from aquatic environments

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ABSTRACT

Aquatic environments contain a great diversity of microorganisms, some of which may be aerosolized and transported long distances through the atmosphere. The bacterium *Pseudomonas syringae* can be found in aquatic environments and in the atmosphere and may express an ice nucleation protein (bacteria expressing the protein are Ice+ and bacteria not expressing the protein are Ice-). Ice+ bacteria may be involved in cloud formation and precipitation processes due to their ability to freeze water at warmer temperatures. Freshwater aerosolization processes are not well understood, particularly the role the Ice+ phenotype may play. Water samples were collected from Claytor Lake, Virginia, USA and screened for Ice+ *P. syringae*. Results indicated that between 6% and 15% of *Pseudomonas* colonies assayed were Ice+. Preliminary phylogenetic analysis of *cts* (citrate synthase) sequences from strains of *P. syringae* showed a surprising diversity of phylogenetic subgroups present in the lake. A Collison nebulizer was used to aerosolize an Ice+ and an Ice- strain of *P. syringae* under artificial laboratory conditions. The aerosolization of *P. syringae* was not influenced by water temperature between 5° and 30°C. In general, the culturability (viability) of *P. syringae* in aerosols increased with temperature between 5 and 30°C. The Ice+ strain was aerosolized in greater numbers than the Ice- strain at all

temperatures studied, suggesting a possible connection between the Ice+ phenotype and aerosol production. A quantitative empirical assessment of aerosolized droplets was generated using a laboratory flume and high-speed video. Droplet diameter and initial velocity upon leaving the water surface were examined at four wind speeds (3.5, 4.0, 4.5, and 5.0 m/s), and the results showed that droplet diameter and velocity had a gamma distribution and droplet mass flux increased exponentially with wind speed. An estimate of the potential amount of bacteria capable of aerosolizing was made for each wind speed. An interdisciplinary unit for advanced high school students has been developed presenting biological aerosolization and ice nucleation. This interdisciplinary work combines modeling and experimental approaches across biology and engineering interfaces, with the goal of increasing our understanding of microbial aerosols from aquatic environments that may impact our planet's water cycle.

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PUBLIC ABSTRACT

Aquatic environments contain a great diversity of microorganisms, some of which may be aerosolized and transported long distances through the atmosphere. Some microorganisms can function as pathogens and some can function as ice nuclei. Ice nuclei are particles in the atmosphere that that raise the freezing temperature of water. Ice nucleation is an important process in cloud formation and precipitation. The plant pathogenic bacterium *Pseudomonas syringae* can be found in aquatic environments and in the atmosphere and may express an ice nucleation protein (bacteria expressing the protein are referred to as Ice+). Freshwater aerosolization processes are not well understood, particularly the role that Ice+ bacteria may play. First, water was collected from Claytor Lake, Virginia, USA and screened for Ice+ *P. syringae* showing a diversity of different strains of *P. syringae* collected from the lake. Second, a nebulizer was used to aerosolize an Ice+ and an Ice- (not expressing the ice nucleation protein) strain of *P. syringae* under artificial laboratory conditions. The Ice+ strain was aerosolized in greater numbers than the Ice- strain at all temperatures studied, suggesting a possible connection between the Ice+ phenotype and aerosol production. Third, high speed video was used to observe

droplets generated from a laboratory simulated lake surface at four wind speeds. Bacteria can leave the water and enter the air in droplets. An estimate of the potential amount of bacteria capable of aerosolizing was made for each wind speed based on the mass of the droplets produced. Fourth, an interdisciplinary unit for advanced high school students was developed presenting aerosolization of microorganisms and ice nucleation. This interdisciplinary work combines modeling and experimental approaches across biology and engineering. A better understanding of Ice+ bacterial aerosols from aquatic environments may lead us to a better understanding of precipitation processes. This could increase our ability to predict atmospheric processes and perhaps in the future allow manipulation of precipitation processes by distributing Ice+ bacteria in a controlled manner. This work can also lead to a greater understanding of pathogen spread, which can inform more efficient management practices for farmers and growers.

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ATTRIBUTIONS

Cynthia Bohland of the Roanoke Valley Governor's School at Roanoke, Virginia, graciously allowed the use of her biotechnology class to pilot the high school ice nucleation unit and served as a co-author on Chapter 5.

Ray David of the Department of Civil and Environmental Engineering at Virginia Tech, assisted with equipment use and data analysis, as well as serving as a co-author on Chapter 3.

Regina Hanlon of the Department of Plant Pathology, Physiology, and Weed Science at Virginia Tech, gave valuable experimental technique advice and assisted with experiment design for Chapter 3. She also assisted with the lab experiment in the high school curriculum and served as a co-author for Chapter 5.

Sunny Jung of the Department of Biomedical Engineering and Mechanics at Virginia Tech, assisted with experiment design and data analysis and was a co-author on Chapter 4.

Linsey Marr of the Department of Civil and Environmental Engineering at Virginia Tech, provided facilities for aerosolization experiments, analysis of results, and served as a co-author on Chapter 3.

Craig Powers of the Department of Civil and Environmental Engineering at Virginia Tech, assisted with construction of the experiment setup and developed the MATLAB code for data analysis in Chapter 4 as well as serving as a co-author on Chapter 4.

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Chapter 1 – Introduction and Background

Aquatic aerosolization

The ubiquitous presence of bacteria throughout our environment is often cited, however the presence of bacteria in the atmosphere and in clouds is not as commonly known. Bacteria are not often thought of in connection with meteorological processes, but there is evidence suggesting microorganisms may play an active role in precipitation processes as they move between the Earth's surface and the atmosphere. Aerosols are particles dispersed in a gas. Bacteria can become aerosolized in the atmosphere and travel great distances before deposition (Schmale III and Ross, 2015). Aerosolized bacteria can affect the earth's radiation budget (Gabric et al., 2005; Haywood et al., 1999; Park et al., 2014; Slingo, 1990) and pathogen dispersal (Polymenakou, 2012) as well as atmospheric processes (Christner et al., 2008; Morris et al., 2014).

Research has been conducted on aerosolization from marine environments focusing mostly on sea salt and inorganic particles with less emphasis on microorganisms, including bacteria (Cavalli et al., 2004; Facchini et al., 2008; Quinn et al., 2015). Less is known about the aerosolization of bacteria, particularly from freshwater aquatic environments. Aquatic environments contain a great diversity of bacteria with species from many classes found. Comeau et al. (2012) examined the diversity of bacteria in a freshwater lake and found over 10 phyla of bacteria represented. Not only is there considerable diversity of bacteria, but also significant concentrations of bacteria are found in aquatic environments. In freshwater lakes the concentration of bacteria ranges between 7.2×10^4 and 1.3×10^7 cells/mL (Bird and Kalff, 1984; Coveney, 1982; Field et al., 1980). These bacteria have the potential to cross the air-water interface and be aerosolized.

Pseudomonas syringae life cycle

One microorganism found in virtually all parts of the water cycle (Morris et al., 2013; Morris et al., 2008) is the bacterium *Pseudomonas syringae,* a rod shaped gram negative bacterium 0.5 - 1.5 µm wide and 1 - 5 µm long. It has received considerable attention as a plant pathogen in agricultural environments (Hirano and Upper, 1990; Preston, 2000). However, there are many strains of *P. syringae* and not all of them are pathogens (Berge et al., 2014). In Southern France, *P. syringae* was found in 12% of snowfall samples and 65% of rainfall samples (Monteil et al., 2012; Morris et al., 2008). There is considerable diversity in the genotypes of *P. syringae* collected from precipitation. *Pseudomonas syringae* collected in rain above soybean canopies were phenotypically different from *P. syringae* collected on plants indicating the bacteria in rain did not originate from the soybeans (Constantinidou et al., 1990). *Pseudomonas syringae* is also found in lakes, streams, and epilithic biofilms (Morris et al., 2008). Cloud samples in France contained strains of *Pseudomonas syringae* (Amato et al., 2007). Pathogenic strains of the bacterium have been found in lakes and streams in pristine areas at elevations above agricultural habitats. The presence of these strains in these environments may be an indication the bacteria are moving with the water cycle as pathogenic strains are commonly found in agricultural habitats were they can live a pathogenic lifestyle (Morris et al., 2007; Morris et al., 2008).

Ice nucleation

Water is commonly cited to freeze at 0° C. However, pure water molecules can be supercooled to -38° C and remain in a liquid state. Below -38° C pure water molecules freeze by homogenous freezing (Rosenfeld and Woodley, 2000). Above -38° C a particle called an ice

nucleus is necessary to catalyze the formation of ice via heterogenous freezing (Koop et al., 2000; Zachariassen and Kristiansen, 2000). Ice nuclei provide a surface that serves as a scaffold for ice crystal formation (Deininger et al., 1988; Warren et al., 1986; Wolber et al., 1986). Depending on the efficiency of the ice nucleator, the freezing temperature can be raised to anywhere from -38° C to -2° C (Murray et al., 2012; Szyrmer and Zawadzki, 1997). In the atmosphere, water is in a supercooled state and can remain liquid down to -38° C (Rosenfeld and Woodley, 2000).

Cloud formation occurs with either cloud condensation nuclei (CCN), particles which cause water vapor to condense into liquid, or ice nuclei (IN), which cause liquid water to freeze (Harrison, 2000). Ice formation is necessary for certain types of clouds including high altitude clouds and snow producing clouds. Ice clouds are responsible for 60-70% of the world's precipitation (Yakobi-Hancock et al., 2014). There are a variety of known inorganic and organic ice nucleators. Many soot, mineral dust, and metallic particles are present in abundance in the atmosphere (Möhler et al., 2007; Murray et al., 2012). Organic ice nucleators include some bacteria, fungi, pollen, and algae (Felgitsch et al., 2015; Morris et al., 2004; Murray et al., 2012; Pouleur et al., 1992). The best known biological ice nucleator is the bacterium *Pseudomonas syringae* (Fall and Fall, 1998; Morris et al., 2011; Murray et al., 2012). A surface lipoglycoprotein catalyzes freezing by positioning water molecules into the crystal lattice structure of ice (Cochet and Widehem, 2000). Of the known ice nuclei, bacteria such as *P. syringae*, are the highest temperature ice nucleators initiating freezing at temperatures as high as -2°C (Cochet and Widehem, 2000; Després et al., 2012), while inorganic particles only nucleate at temperatures up to -10° C. Expression of the ice nucleation protein is not well understood and appears to be different for different bacteria (Fall and Fall, 1998; Nemecek-Marshall et al., 1993;

Yankofsky et al., 1983). Low nutrient growth media and low temperature seem to enhance IN activity (Nemecek-Marshall et al., 1993). Attard et al. (2012) looked at different conditions, and found that acidic pH reduced ice nucleation activity while UV-A, NO_2 and O_3 levels had little to no effect on ice nucleation activity (Attard et al., 2012).

Bioprecipitation hypothesis

In the early 1980s, the bioprecipitation hypothesis was proposed by David Sands (Morris et al., 2014; Sands et al., 1982). These authors argued that bacteria are not passive "passengers" being transported through the atmosphere, but rather are active players ("pilots") in the precipitation cycle via ice nucleation. The proposed cycle involves ice nucleating bacteria aerosolizing from terrestrial (or aquatic) surfaces. In the atmosphere ice nucleating bacteria can form clouds, which produce precipitation carrying the bacteria back to Earth in a new location (**Figure 1.1**). The production of rain not only brings the bacteria back to Earth but can increase the available habitat for the bacteria by increasing plant growth. This cycle is a positive feedback cycle where the bacteria benefit from the ice nucleation ability using it as a means of dispersal and possibly increasing survival. Ice nucleation may be a method of protecting bacteria from the harsh desiccating atmosphere either by reducing the residence time in the atmosphere or conferring a protection while in the atmosphere (Morris et al., 2014; Polymenakou, 2012). The whole cycle has not been studied, but many pieces of the cycle have been studied individually and seem to support a bioprecipitation hypothesis, though many questions remain unanswered.

Pseudomonas syringae is found in rain and snow collections as well as in freshwater and in clouds (Christner et al., 2008). The proportion of Ice+ (expressing the ice nucleation protein) to Ice- (not expressing the ice nucleation protein) bacteria is much greater in snow compared to

rain, and in rain compared to clouds, with 100% of *P. syringae* collected from snow showing the Ice+ phenotype compared with 60-100% in rain and only 10% in clouds (Joly et al., 2013; Monteil et al., 2014; Morris et al., 2008). The presence of Ice- bacteria in clouds shows that *P. syringae* can definitely be "passengers," but does not answer if they can also serve as "pilots" in meteorological processes. However, the enrichment shows that a selection mechanism of some sort exists for Ice+ bacteria to be in precipitation. If the Ice+ phenotype is involved in precipitation formation that would be an explanation for this enrichment, especially with 100% of the bacteria from snow showing the Ice+ phenotype when snow must come from clouds with ice nuclei. However, *P. syringae* is only found in 12% of the snow collections indicating that some other ice nuclei are capable of making snow (Morris et al., 2013). It cannot be conclusively shown from these data that *P. syringae* are the "pilots" of snow or rain formation.

It would seem that *P. syringae* can influence precipitation, but it is unknown if the bacterium perform this function in natural environments. The concentration of bacteria in the atmosphere is far lower than that of inorganic particles (Morris et al., 2014; Yakobi-Hancock et al., 2014). Within a population of Ice+ phenotype bacteria, only one in 10^{-4} - 10^{-6} bacteria have the entire ice nucleating structure indicating that a small amount of the ice nucleation protein can have a large effect (Cochet and Widehem, 2000; Després et al., 2012; Möhler et al., 2007). Due to the low concentrations, the potential role of *P. syringae* in precipitation formation is expected to be greatest at higher temperatures where other types of ice nuclei are not active.

Even if the indirect evidence suggests that *P. syringae* is involved in precipitation formation, it is not known if this function serves as a means of enhancing the bacteria's growth rate and fitness. The Ice+ ability allows the bacteria to increase frost damage and subsequently gain access to nutrients inside the plant and in this way the Ice+ phenotype is an advantage for the

bacteria. There is some evidence that Ice+ bacteria remain in the atmosphere for shorter periods of time, indicating the Ice+ phenotype may bring them back to earth and limit their time in the harsh desiccating atmospheric environment (Christner, 2010). Ice nucleation may also provide the bacteria with more moisture while in the atmosphere. Bacteria exposed to UV-A in a laboratory setting are no longer viable but retain ice nucleation activity (Attard et al., 2012). Christner (2010) found environmental samples that were Ice+ but *P. syringae* could not be cultured suggesting ice nucleation activity remains either after the bacteria die or when they are viable but nonculturable. This lack of culturability is an interesting consideration for the bioprecipitation cycle, as these bacteria would be able to influence precipitation but not benefit from the bioprecipitation cycle being unable to grow where they are deposited.

Understanding the role of Ice+ *Pseudomonas syringae* in atmospheric processes is important in order to predict meteorological events as well as understanding bacterial dispersal. There is also the potential to manipulate the cycle to alter precipitation events and possibly increase the amount of rainfall. The ice nucleation ability of *Pseudomonas syringae* is already being used for artificially altering precipitation processes. Snomax® is a commercially available product containing *P. syringae* that has been rendered inert, which is successfully used in making artificial snow at temperatures slightly below 0° C (Blondeaux et al., 1999; Möhler et al., 2007). Cloud seeding is a method of altering precipitation by releasing CCN or IN into the atmosphere. The effectiveness of cloud seeding is inconclusive with some cases showing an effect and others not (Bruintjes, 1999). Ice nucleating bacteria have been used in cloud seeding (Ward and DeMott, 1989). In the future, ice nucleating bacteria may perhaps be inoculated on plants or in water with the goal of generating rainfall via aerosolization and ice nucleation of clouds (Moukahel et al., 2015).

The scale of the system makes research challenging, since (1) it is difficult to follow a bacterium through the whole water cycle and (2) it is hard to know if it is playing the role of "pilot" or "passenger" in the precipitation cycle. Aerosolization from aquatic environments and the role of ice nucleation in that process is an understudied area of the cycle. Four objectives were pursued toward the goal of better understanding the process of aquatic aerosolization of *Pseudomonas syringae* and the role of ice nucleation.

Research Objectives

Objective 1. Determine the frequency and diversity of Ice+ *Pseudomonas syringae* in Claytor Lake, Virginia, United States. This objective is based on the hypothesis that Ice+ *P. syringae* from a diversity of phylogroups are found in Claytor Lake.

Objective 2. Determine if temperature and Ice+ phenotype affect the amount and viability of aerosolized *Pseudomonas syringae* in a laboratory setting with a Collison nebulizer. This objective is based on the hypothesis that temperature and Ice+ phenotype increase the production and viability of aerosolized *P. syringae.*

Objective 3. Examine the mechanisms of aerosol production in a laboratory flume and study the relationship between wind speed and droplet parameters of diameter, angle, and speed. This objective is based on the hypothesis that wind speed and the mechanism of droplet production (bubble busting or fragmentation) will affect the diameter, angle, and speed of droplets produced influencing the potential for bacterial aerosolization.

Objective 4. Develop an interdisciplinary curriculum for high school students about ice nucleation and the water cycle. This objective is based on the hypothesis that an interdisciplinary inquiry based unit will increase high school students' interest in STEM fields as well as introduce students to concepts about the water cycle that they are not normally introduced to until college.

References

Amato, P., M. Parazols, M. Sancelme, P. Laj, G. Mailhot, and A.-M. Delort. 2007. Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures. *FEMS Microbiology Ecology*. 59:242-254.

Attard, E., H. Yang, A.-M. Delort, P. Amato, U. Pöschl, C. Glaux, T. Koop, and C. Morris. 2012. Effects of atmospheric conditions on ice nucleation activity of *Pseudomonas. Atmospheric Chemistry and Physics*. 12:10667-10677.

Berge, O., C.L. Monteil, C. Bartoli, C. Chandeysson, C. Guilbaud, D.C. Sands, and C.E. Morris. 2014. A user's guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. *PloS one*. 9:e105547.

Bird, D., and J. Kalff. 1984. Empirical relationships between bacterial abundance and chlorophyll concentration in fresh and marine waters. *Canadian Journal of Fisheries and Aquatic Sciences*. 41:1015-1023.

Blondeaux, A., J. Hamel, P. Widehem, and N. Cochet. 1999. Influence of water activity on the ice-nucleating activity of *Pseudomonas syringae. Journal of industrial microbiology and biotechnology*. 23:514-519.

Bruintjes, R.T. 1999. A review of cloud seeding experiments to enhance precipitation and some new prospects. *Bulletin of the American Meteorological Society*. 80:805-820.

Cavalli, F., M. Facchini, S. Decesari, M. Mircea, L. Emblico, S. Fuzzi, D. Ceburnis, Y. Yoon, C. O'Dowd, and J.P. Putaud. 2004. Advances in characterization of size-resolved organic matter in marine aerosol over the North Atlantic. *Journal of Geophysical Research: Atmospheres*. 109.

Christner, B.C. 2010. Cloudy with a chance of microbes. *Issues*.

Christner, B.C., R. Cai, C.E. Morris, K.S. McCarter, C.M. Foreman, M.L. Skidmore, S.N. Montross, and D.C. Sands. 2008. Geographic, seasonal, and precipitation chemistry influence on the abundance and activity of biological ice nucleators in rain and snow. *Proceedings of the National Academy of Sciences*. 105:18854-18859.

Cochet, N., and P. Widehem. 2000. Ice crystallization by *Pseudomonas syringae*. *Applied microbiology and biotechnology*. 54:153-161.

Comeau, A.M., T. Harding, P.E. Galand, W.F. Vincent, and C. Lovejoy. 2012. Vertical distribution of microbial communities in a perennially stratified Arctic lake with saline, anoxic bottom waters. *Scientific reports*. 2.

Constantinidou, H., S. Hirano, L. Baker, and C. Upper. 1990. Atmospheric dispersal of ice nucleation-active bacteria: The role of rain. *Phytopathology*. 80:934-937.

Coveney, M.F. 1982. Bacterial uptake of photosynthetic carbon from freshwater phytoplankton. *Oikos*:8-20.

Deininger, C., G. Mueller, and P. Wolber. 1988. Immunological characterization of ice nucleation proteins from *Pseudomonas syringae, Pseudomonas fluorescens,* and *Erwinia herbicola. Journal of bacteriology*. 170:669-675.

Després, V.R., J.A. Huffman, S.M. Burrows, C. Hoose, A.S. Safatov, G. Buryak, J. Fröhlich-Nowoisky, W. Elbert, M.O. Andreae, and U. Pöschl. 2012. Primary biological aerosol particles in the atmosphere: a review. *Tellus B*. 64.

Facchini, M.C., M. Rinaldi, S. Decesari, C. Carbone, E. Finessi, M. Mircea, S. Fuzzi, D. Ceburnis, R. Flanagan, and E.D. Nilsson. 2008. Primary submicron marine aerosol

dominated by insoluble organic colloids and aggregates. *Geophysical Research Letters*. 35.

Fall, A.L., and R. Fall. 1998. High-level expression of ice nuclei in *Erwinia herbicola* is induced by phosphate starvation and low temperature. *Current Microbiology*. 36:370-376.

Felgitsch, L., M. Bichler, T. Häusler, V.U. Weiss, M. Marchetti-Deschmann, G. Allmaier, and H. Grothe. 2015. Molecular Ice Nucleation Activity of Birch Pollen. *In* EGU General Assembly Conference Abstracts. Vol. 17. 1560.

Field, J., C. Griffiths, E. Linley, R. Carter, and P. Zoutendyk. 1980. Upwelling in a nearshore marine ecosystem and its biological implications. *Estuarine and Coastal Marine Science*. 11:133-150.

Gabric, A.J., B. Qu, P. Matrai, and A.C. Hirst. 2005. The simulated response of dimethylsulfide production in the Arctic Ocean to global warming. *Tellus B*. 57:391-403.

Harrison, R.G. 2000. Cloud formation and the possible significance of charge for atmospheric condensation and ice nuclei. *Space Science Reviews*. 94:381-396.

Haywood, J., V. Ramaswamy, and B. Soden. 1999. Tropospheric aerosol climate forcing in clear-sky satellite observations over the oceans. *Science*. 283:1299-1303.

Hirano, S.S., and C.D. Upper. 1990. Population biology and epidemiology of *Pseudomonas syringae*. *Annual review of phytopathology*. 28:155-177.

Joly, M., E. Attard, M. Sancelme, L. Deguillaume, C. Guilbaud, C.E. Morris, P. Amato, and A.-M. Delort. 2013. Ice nucleation activity of bacteria isolated from cloud water. *Atmospheric Environment*. 70:392-400.

Koop, T., B. Luo, A. Tsias, and T. Peter. 2000. Water activity as the determinant for homogeneous ice nucleation in aqueous solutions. *Nature*. 406:611-614.

Möhler, O., P. DeMott, G. Vali, and Z. Levin. 2007. Microbiology and atmospheric processes: the role of biological particles in cloud physics. *Biogeosciences Discussions*. 4:2559-2591.

Monteil, C.L., M. Bardin, and C.E. Morris. 2014. Features of air masses associated with the deposition of *Pseudomonas syringae* and *Botrytis cinerea* by rain and snowfall. *The ISME journal*.

Monteil, C.L., C. Guilbaud, C. Glaux, F. Lafolie, S. Soubeyrand, and C.E. Morris. 2012. Emigration of the plant pathogen *Pseudomonas syringae* from leaf litter contributes to its population dynamics in alpine snowpack. *Environmental Microbiology*. 14:2099-2112.

Morris, C., D. Georgakopoulos, and D. Sands. 2004. Ice nucleation active bacteria and their potential role in precipitation. *In* Journal de Physique IV (Proceedings). Vol. 121. EDP sciences. 87-103.

Morris, C., D. Sands, M. Bardin, R. Jaenicke, B. Vogel, C. Leyronas, P. Ariya, and R. Psenner. 2011. Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate. *Biogeosciences*. 8:17-25.

Morris, C.E., F. Conen, J. Alex Huffman, V. Phillips, U. Pöschl, and D.C. Sands. 2014. Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology*. 20:341-351.

Morris, C.E., L.L. Kinkel, K. Xiao, P. Prior, and D.C. Sands. 2007. Surprising niche for the plant pathogen *Pseudomonas syringae*. *Infection, Genetics and Evolution*. 7:84-92.

Morris, C.E., C.L. Monteil, and O. Berge. 2013. The life history of *Pseudomonas syringae:* linking agriculture to earth system processes. *Annual review of phytopathology*. 51:85-104.

Morris, C.E., D.C. Sands, B.A. Vinatzer, C. Glaux, C. Guilbaud, A. Buffiere, S. Yan, H. Dominguez, and B.M. Thompson. 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME journal*. 2:321-334.

Moukahel, A.-A., S. Asaad, B. Debbes, C.E. Morris, and D.C. Sands. 2015. Characterization of wheat genotypes as sources of ice nucleation active bacteria for bioprecipitation aerosols. *International Journal of Phytopathology*. 4:63-72.

Murray, B., D. O'Sullivan, J. Atkinson, and M. Webb. 2012. Ice nucleation by particles immersed in supercooled cloud droplets. *Chemical Society Reviews*. 41:6519-6554.

Nemecek-Marshall, M., R. Laduca, and R. Fall. 1993. High-level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. *Journal of bacteriology*. 175:4062-4070.

Park, J.Y., M. Kim, S. Han, S. Lim, G. Kim, and K. Park. 2014. Measurement of insoluble submicrometer particles and biological materials in seawater to investigate marine aerosol production. *Journal of aerosol science*. 75:22-34.

Polymenakou, P.N. 2012. Atmosphere: a source of pathogenic or beneficial microbes? *Atmosphere*. 3:87-102.

Pouleur, S., C. Richard, J.-G. Martin, and H. Antoun. 1992. Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Applied and environmental microbiology*. 58:2960-2964.

Preston, G.M. 2000. *Pseudomonas syringae pv. tomato*: the right pathogen, of the right plant, at the right time. *Molecular Plant Pathology*. 1:263-275.

Quinn, P.K., D.B. Collins, V.H. Grassian, K.A. Prather, and T.S. Bates. 2015. Chemistry and related properties of freshly emitted sea spray aerosol. *Chemical reviews*. 115:4383-4399.

Rosenfeld, D., and W.L. Woodley. 2000. Deep convective clouds with sustained supercooled liquid water down to-37.5 C. *Nature*. 405:440-442.

Sands, D., V. Langhans, A. Scharen, and G. De Smet. 1982. The association between bacteria and rain and possible resultant meteorological implications. *Idojaras*.

Schmale III, D.G., and S.D. Ross. 2015. Highways in the sky: Scales of atmospheric transport of plant pathogens. *Annual review of phytopathology*. 53:591-611.

Slingo, A. 1990. Sensitivity of the Earth's radiation budget to changes in low clouds.

Szyrmer, W., and I. Zawadzki. 1997. Biogenic and anthropogenic sources of ice-forming nuclei: A review. *Bulletin of the American Meteorological Society*. 78:209-228.

Ward, P., and P. DeMott. 1989. Preliminary experimental evaluation of Snomax, *Pseudomonas syringae,* as an artificial ice nucleus for wheather modification. *J. Wea. Mod*. 21:9-13.

Warren, G., L. Corotto, and P. Wolber. 1986. Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas*. *Nucleic acids research*. 14:8047-8060.

Wolber, P.K., C.A. Deininger, M.W. Southworth, J. Vandekerckhove, M. Van Montagu, and G.J. Warren. 1986. Identification and purification of a bacterial ice-nucleation protein. *Proceedings of the National Academy of Sciences*. 83:7256-7260.

Yakobi-Hancock, J.D., L.A. Ladino, and J.P. Abbatt. 2014. Review of Recent Developments and Shortcomings in the Characterization of Potential Atmospheric Ice Nuclei: Focus on the Tropics. *Revista de Ciencias*. 17:15-34.

Yankofsky, S.A., T.N. Bertold, and Z. Levin. 1983. Induction of latent freezing nucleus capability in an ice nucleation-active bacterium. *Current Microbiology*. 9:263-267.

Zachariassen, K.E., and E. Kristiansen. 2000. Ice nucleation and antinucleation in nature. *Cryobiology*. 41:257-279.

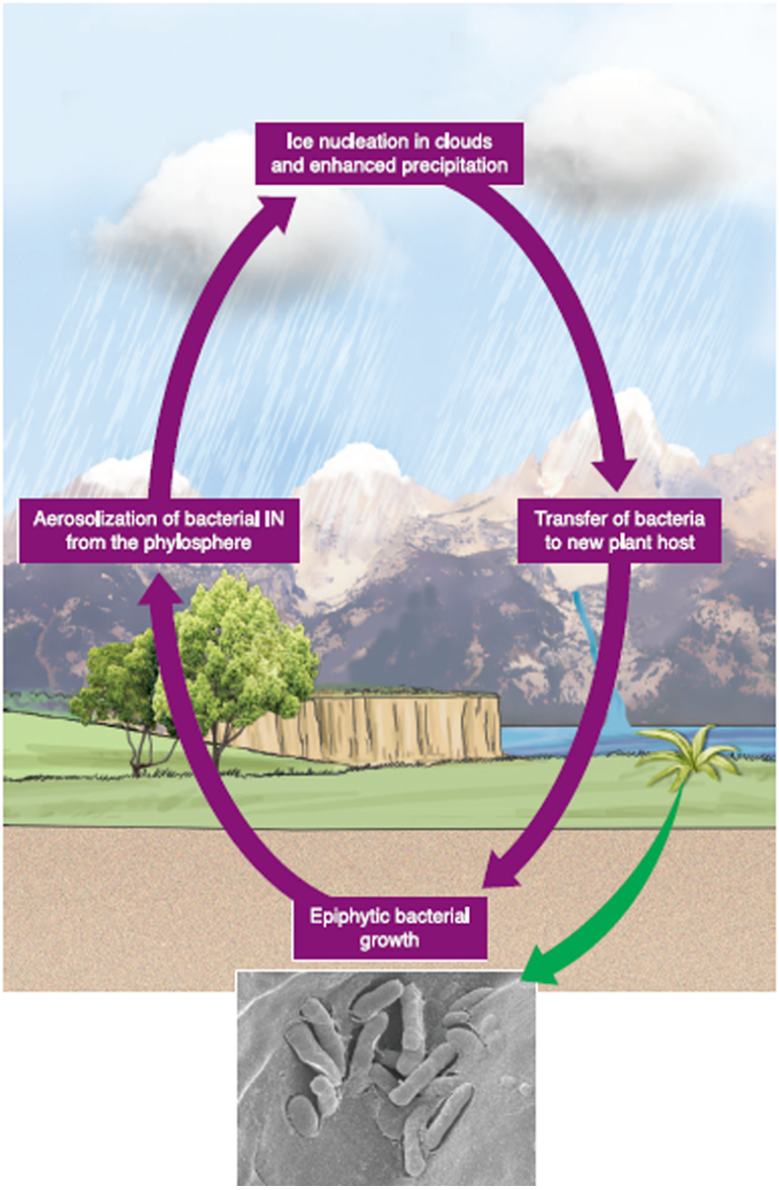

Figure 1.1. Bioprecipitation cycle where ice nucleating bacteria aerosolize, are involved in precipitation formation via ice nucleation in the atmosphere, and return to earth in precipitation depositing on new plant surfaces (Brent Christner, Microbe, 2012, Volume 7 P. 70-75, Figure 3, reproduced with permission from American Society for Microbiology under a Creative Commons Attribution license).

Chapter 2 – Ice nucleating strains of Pseudomonas syringae from a large freshwater lake in Virginia, USA

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Abstract

The bacterium *Pseudomonas syringae* can be found in a variety of terrestrial and aquatic environments. Some strains of the bacterium are plant pathogens, and some express an ice nucleation protein (hereafter referred to as Ice+) allowing them to catalyze the heterogeneous freezing of water at warmer temperatures. Little is known about natural aquatic sources of *P. syringae*. We collected samples of freshwater from four different locations in Claytor Lake, Virginia, USA over five days between November 2015 and February 2016. Samples were concentrated and plated on TSA and KBC to estimate the number of culturable bacteria and *Pseudomonas* in freshwater, respectively. A droplet freezing ice nucleation assay was used to screen colonies for Ice+ phenotype. Colonies with the Ice+ phenotype were identified based on the *cts* (citrate synthase) gene and 16S sequences. Colonies of *Pseudomonas* were recovered from all of the lake samples, and the concentration varied across sampling days. Between 6% and 15% of the *Pseudomonas* colonies were Ice+. Most of the colonies cultured on KBC were identified as *P. syringae.* Phylogenetic analysis of *cts* sequences from strains of *P. syringae* showed a surprising diversity of phylogenetic subgroups present in the lake. Our work shows that freshwater lakes can be a significant source of Ice+ *P. syringae*. Future work is needed to understand the mechanics of aerosolization of *P. syringae* and other microbial ice nucleators from aquatic environments.

Introduction

Pseudomonas syringae has been studied as a plant pathogen since the early 20th century (Elliott, 1951). It is ubiquitous in most terrestrial environments, including agricultural and uncultivated environments, and aquatic environments (Morris et al., 2013). The bacterium has been found in many aquatic environments including rain, snow, clouds, groundwater, streams, and lakes (Amato et al., 2007; Morris et al., 2007; Morris et al., 2008). Some strains are pathogens of plants (Preston, 2000). *Pseudomonas syringae* can also have ice nucleating (IN) properties allowing the bacterium to catalyze the freezing of water at warmer temperatures due to expression of a lipoglycoprotein on the membrane surface (Cochet and Widehem, 2000). Ice nuclei can be a variety of inorganic materials including dust and minerals as well as organic materials such as bacteria, fungi, and pollen (Murray et al., 2012). Ice-nucleating (referred to as Ice+) bacteria have properties which may allow them to be involved in cloud formation and precipitation processes in the atmosphere (Morris et al., 2011). Ice nucleation may allow *P. syringae* to be an active player in atmospheric processes (Moukahel et al., 2015). In *P. syringae*, the ice nucleation protein assists in assembling water molecules into the crystal lattice structure of ice. Not every strain of *P. syringae* produces the ice nucleation protein (Lindow, 1983) and natural environmental conditions that favor the expression of the protein are poorly understood (Nemecek-Marshall et al., 1993). Cold temperatures and low nutrient media tend to favor expression of the protein, but not all strains are induced in the same manner (Nemecek-Marshall et al., 1993).

There are many strains of *Pseudomonas syringae* encompassing a diversity of phenotypes and habitats. Recently, nomenclature was proposed for *P. syringae* with at least 13 different phylogroups (Berge et al., 2014). Though certain strains may favor a particular habitat, a variety

of strains can be found in any given habitat indicating the bacteria move broadly. Strains of *P. syringae* are present throughout the water cycle suggesting they may be moving with the water cycle, providing the bacteria an opportunity to disperse widely (Morris et al., 2013). How the bacteria move with and through the water cycle is not well understood as well as the active and passive roles the bacteria play in this movement. There is evidence that Ice+ bacteria are preferentially found in certain parts of the water cycle such as in rain and snow, and a laboratory experiment showed they may aerosolize preferentially from aquatic environments (Monteil et al., 2012; Morris et al., 2008; Pietsch et al., 2015). This finding may be an indication that Ice+ strains of *P. syringae* are benefiting from, and playing an active role in precipitation processes, a positive feedback bioprecipitation cycle (Morris et al., 2014; Sands et al., 1982). DNA sequencing along with phylogenetic grouping to determine which strains are represented in a population can be used as a tool to look at movement and dispersion. Comparing the diversity of strains present in different environments can help with understanding movement of the bacteria between the environments, and contribute to knowledge about the role of Ice+ bacteria in atmospheric processes.

Little is known about the relative abundance of *P. syringae* in aquatic environments (Morris et al., 2008). Though studies have been done on distributions of microorganisms in lakes, these have focused on bacterial byproducts in the context of water quality or changes in bacterial communities in response to changes in lake nutrients (McDonough et al., 1986). Lake sediments have also been analyzed for vertical distributions of bacteria (Haglund et al., 2003; Ye et al., 2009). Several studies examined the vertical distribution of bacteria classifying them into broad taxonomic groups (Comeau et al., 2012; Glöckner et al., 2000). Comeau et al. (2012) found λ-Proteobacteria accounted for approximately <2% of the bacteria collected in a lake. The

analysis did not classify λ-Proteobacteria further to determine if any *Pseudomonas* were present. Concentrations of bacteria were not determined, but the relative proportion of different types of bacteria changed with depth from 2m to 60 m as well as differing by sampling season (Comeau et al., 2012). To our knowledge, concentration gradients of Ice+ *Pseudomonas syringae* across the water column have not been examined in detail.

The overall goal of this study was to examine the relative abundance of Ice+ strains of *P. syringae* in a large freshwater lake in Virginia, USA. The specific objectives of this study were to: (1) examine the frequency of *P. syringae*, (2) examine concentrations of *P. syringae* at different locations and at different depths within the lake, (3) determine the relative proportion of Ice+ colonies of *Pseudomonas* in the lake, and (4) examine the phylogenetic relationship within and among strains of *P. syringae* collected from the lake and those from rain collections. This research has applications in understanding how *P. syringae* is distributed in natural bodies of water, and may work towards greater understanding of the potential for a lake to release aerosolized Ice+ bacteria into the atmosphere.

Methods and Materials

Collection of freshwater samples

Samples were collected from Claytor Lake, VA, USA on five different calendar dates (5 November 2015, 18 November 2015, 16 December 2015, 21 January 2015, and 4 February 2016). A 3.2 L Van Dorn (Wildco, Yulee, Florida, USA) water sampler was used to collect samples of lake water off the side of a kayak. For each sample, the time, GPS coordinates, and water temperature were recorded **(Table 2.1)**. The samples were taken from four different locations selected to provide a variety a location types **(Figure 2.1)**; 1) deep water in the middle

of the lake, 2) shallow water near the mouth of an inlet, 3) shallow water in the inlet, 4) the mouth of the boat launch inlet. Samples were collected at the surface of the water at locations 2-4. At location 1, samples were collected at the water surface, at 4.6 m, and at 9.1 m. Two Van Dorn samplers were used to collect the surface and 4.6 m samples simultaneously in location 1. **Table 2.2** shows the number of samples taken at each location on each sampling day.

Processing of samples for culturable bacteria

The lake water samples were place on ice in a cooler immediately following collection, and transported back to the laboratory for analysis. In the laboratory, one liter of each sample was filtered with 0.2 µm nitrocellulose filters. The filters were placed in 5 mL of the filtrate for 10 min with a stir bar to resuspend the bacteria in a 200X concentration. King's medium B modified with cephalexin (80 mg/L), cyclohexamide (200 mg/L), and boric acid (1500 mg/L) (KBC) (Mohan and Schaad, 1987), selective for *Pseudomonas*, were plated with 200 µL of the suspension, with three replicated per suspension. The filtrate was also used to make a 2X concentration, which was plated onto 10% tryptic soy agar (TSA) media to obtain counts of all culturable bacteria. The plates were incubated for 48-72 hours at ambient room temperature (~ 20° C), and the colonies were counted.

Ice nucleation assays

Colonies that grew on the KBC plates were selected at random (at least 12 colonies per plate) and transferred to 140µL of water with a sterile toothpick. Two droplets of 12 µL of each sample were loaded onto a Parafilm® M boat floating on an Alpha 12 cooling bath (Lauda, New Jersey, USA). Sterile 0.2 µm filtered water was used as a negative control. The temperature of the bath

was set at -5° C during loading of the samples, and was then lowered to -12°. The temperature that each of the droplets froze was recorded in one degree increments. Strains for which both droplets froze were selected for further analysis.

Sequence-assisted identification of bacteria

DNA was extracted from cultures using a Puregene Yeast/Bacteria Kit B (Qiagen #1042607) and a BioSprint15 DNA Plant Kit (Qiagen #941517) following manufacturer's protocols. PCR was conducted using a Mastercycler ep Gradient S thermocycler (Eppendorf, New York, USA) with the extracted DNA using primers (forward: 5' CCC GTC GAG CTG CCA ATW CTG A 3', reverse: 5' ATC TCG CAC GGS GTR TTG AAC ATC 3') for the citrate synthase (*cts*) housekeeping gene to identify *P. syringae* colonies. A 1% TBE Ethidium Bromide gel was used to visualize products from the PCR reaction. Samples that did not produce a product specific for *P. syringae* were subjected to PCR with 16S primers (518 forward: 5' CCA GCA GCC GCG GTA ATA CG 3', 1491 reverse: 5' ATC GGY TAC CTT GTT ACG ACT TC 3'). PCR was performed with GoTaq® Green Master Mix (Promega M712) under the following parameters: 1x cycle denaturation 95° C for 10 min, 5x cycle denaturation 95 for 1 min, annealing 53° C for 30 sec, extension 72° C 1min 40 sec, 25x cycle 94° C 30 sec, 53° C 20 sec, 72° C 1 min, 1x cycle 72° C 10 min, hold 4° C). The samples were purified using ExoI / rSAP prior to sequencing (AB1 3730x1 DNA Sequencer, Eton Biosciences, 104 T.W. Alexander Drive, Bldg 4A, RTP, NC 27709, USA). The sequences from 16S primers were blasted against GenBank (http://www.ncbi.nlm.nih.gov/blast) to identify the sequence in the GenBank database with the closest match. The sequences from *cts* primers along with 38 reference strains of *P. syringae* (Berge et al., 2014) were aligned using the Clustal W method and a phylogenetic tree was

constructed using neighbor-joining method using MEGA7 with 1000 bootstrap replicates. Samples from a previous study with rain and stream water collections (Streett, 2015) were also compared to the samples collected in this study using MEGA7.

Results

Concentration of bacteria

The concentration of bacteria (CFU Liter^{-1}) and *Pseudomonas* in lake water was determined from the counts of colonies on TSA and KBC media, respectively **(Table 2.1)**. Between 0.16% and 1.7 % of the total bacteria present were *Pseudomonas.* The concentration of colonies that grew on KBC media (*Pseudomonas)* in CFU/Liter showed a weak trend of increasing concentration with depth of sampling from surface to 9.1 m (**Figure 2.2a**). The concentration of colonies that grew on KBC media (*Pseudomonas)* for each sampling location for each day of collection showed considerable variation across all days and across all sampling locations (**Figure 2.2b**). Although the concentration varied across days, **Figure 2.2b** showed 5 November 2015 and 16 December 2015 location 1 had a higher concentration of *Pseudomonas* than location 2 while on 18 November 2015 the opposite was seen with location 1 having a higher concentration than location 2. **Figure 2.3** shows the average concentration (CFU Liter^{-1}) of *Pseudomonas* from all locations that grew on KBC media for all days of sampling indicating *Pseudomonas* is consistently found throughout Claytor Lake. There is considerable diversity seen across the days from a low of 110 CFU/Liter on 5 November 15 to 8055 CFU/Liter on 4 February 16.

Ice+ *Pseudomonas* strains

The colonies from KBC media were tested for Ice+ phenotype with an ice nucleation assay. The % of the colonies tested which froze on both replicates (**Table 2.1** and **Figure 2.4)** was between10 and 15% of the colonies on all sampling days, with the exception of 4 February 2016 when only 6.5% of the tested colonies were Ice+. The following numbers of Ice+ colonies were obtained: 5 November 2015 n=24, 18 November 2015 n=33, 16 December 2015 n=51, 21 January 2015 n=37, and 4 February 2016 n=26. A majority of the Ice+ samples produced a product with the *cts* primer indicating they were *P. syringae.* The % of Ice+ *Pseudomonas* colonies that did not produce a *cts* PCR product, and were therefore likely not *P. syringae*, was between 15 and 20% on each day of sampling with the exception of 4 February 2016 where 47% of the Ice+ colonies were not *P. syringae* (**Figure 2.5)**. The 16S sequence results confirmed that these colonies were not *P. syringae.*

Phylogenetic analysis of *Pseudomonas syringae* strains

A phylogenetic tree was constructed with all of the samples producing a product with *cts* primers as well as 38 reference strains representing 13 phylogroups of *P. syringae* (Berge et al., 2014) **(Figure 2.6)**. The strains from Claytor Lake were compared to the 13 phylogroups with all groupings having bootstrap values of 84 or higher. The samples from Claytor Lake show diversity representing samples from multiple phylogroups including group 2, 3, 4, 7, 9, and 13. The 5 November 2015 collection showed less diversity with all of the samples coming from phylogroup 2 and one sample from phylogroup 3. This is the only sample collection to include samples from location 3 and 4. Thus all of the samples from these two locations are in phylogroup 2 and 3. Another phylogenetic tree was made adding samples collected from rain collections in Blacksburg, VA and a stream collection in Pembroke, VA (Streett, 2015) along

with the Claytor Lake samples and 38 reference strains **(Figure 2.7)**. The rain and stream samples also span multiple phylogroups, although overall most of the samples are clumped in relatively few phylogroups. Some of these phylogroups overlap with phylogroups seen with the Claytor Lake samples while others do not. The samples that did not produce a *cts* product were sequences with 16S primer and BLAST results showed they were all *Pseudomonas* with the exception of three colonies which were *Xanthamonas.* The *Pseudomonas* colonies showed 100% match with *P. fragi, P. fluorescens, P. viridiflava, and P. plecoglossicida.*

Discussion

Little is known about the abundance of *Pseudomonas* in aquatic environments. Here, we show the distribution of *Pseudomonas* in a freshwater lake, examining the concentration across sampling dates, locations, and depths. *Pseudomonas* colonies were found in every sample collected on every day of collection, indicating they are ubiquitous throughout Claytor Lake. Variation was observed in the concentration of *Pseudomonas* at different sampling locations and across different sampling days. The concentrations of *Pseudomonas* collected ranged widely, between 25 and 9250 CFU/Liter indicating a non-uniform distribution. Within each day of sampling there was variation between sampling locations and the location with the highest concentration varied from day to day, suggesting that one location within the lake does not consistently have the highest concentrations; thus, the bacteria appear to be continually moving and mixing. Thus the amount of bacteria with the potential to enter the environment is not constant. Comparisons to previous concentrations are limited due to a lack of reported concentrations of *Pseudomonas*. Morris et al. (2008) sampled four freshwater lakes and found concentrations of *Pseudomonas syringae* between 130 and $1x10^4$ CFU/Liter. The relative

abundance we found of *Pseudomonas* ranged between 0.1 and 1.7% of the total bacterial concentrations indicating they were a small minority of the bacteria present. Previous studies examining the relative abundances of bacteria in freshwater lakes found between 5 and 10% of the bacteria sampled were λ-Proteobacteria (which includes *Pseudomonas*) (Briée et al., 2007; Hiorns et al., 1997; Tamaki et al., 2005; Wang et al., 2012).

A trend of increasing *Pseudomonas* concentration with depth was observed. The Claytor Lake water temperature did not change with depth indicating temperature is not a cause for this change in concentration. Concentration of nutrients and pH were not measured but may contribute to the increase in concentration with depth. De Wever et al. (2005) observed increases in total bacterial concentrations over a similar depth profile to this study, however some locations in the lake did not show an increase. The proportion of total bacteria that were *Pseudmonas* did not change with depth. Comeau et al. (2012) looked at the relative proportion of total bacteria that was λ-Proteobacteria and did not see any change over a similar depth profile to our Claytor Lake samples.

Between 6% and 15% of the *Pseudomonas* colonies were Ice+ on each sampling day indicating Ice+ strains are consistently present throughout Claytor Lake. The Ice+ colonies were not evenly distributed across sampling locations. Some collections did not have any Ice+ colonies and others had up to 50%. This finding might indicate a preference for Ice+ strains to associate with each other. Joly et al. (2013) sampled cloud water for Ice+ *Pseudomonas* and found 12% of the strains tested were Ice+. Based on ice nucleation activity the population of *Pseudomonas* in Claytor Lake and in the clouds are similar. These bacteria may be moving between the clouds and freshwater lake via rain and aerosolization (Morris et al., 2014).

Strains of Ice+ *Pseudomonas syringae* were identified from diverse phylogroups. Berge et al. (2014) conducted phylogenetic analysis using 4 housekeeping genes from 763 strains of *P. syringae* and found that using only the *cts* gene sequence was a reliable and efficient method of classifying *P. syringae.* Berge et al. (2014) examined the phenotypic and genetic characteristics of strains of *P. syringae* within each of 13 phylogroups. Many of the strains from Claytor Lake were in phylogroup 2, which is the most widespread phylogroup found in all habitats studied with around 85% of the strains previously sampled being Ice+ (Morris et al., 2010). Two samples were in phylogroup 3, which includes many pathovars although few strains have been found in the environment perhaps due to competition from faster growing strains (Monteil et al., 2014). About 20% of previously sampled phylogroup 3 strains were Ice+ (Berge et al., 2014). Five strains may be in phylogroup 4, although there was weak bootstrap support for this grouping at only 60. All strains previously reported in phylogroup 4 have been Ice+ although these strains are rarely detected in the environment (Berge et al., 2014). Many of the strains from Claytor Lake were in phylogroup 7, which includes strains previously called *P. viridiflava* (Berge et al., 2014). These strains have been commonly found in environmental reservoirs with about 45% being Ice+ (Berge et al., 2014). We found 7 strains in phylogroup 9. All previous strains in phylogroup 9 have been found in aquatic habitats, but only 4% have been Ice+ (Berge et al., 2014). Berge et al. (2014) reported none of the samples in phylogroups 8, 11, 12, and 13 showed Ice+ activity. We were selecting only for Ice+ strains so it is not surprising that none of the samples we collected were in these phylogroups, with the exception of phylogroup 13. We found 4 samples in phylogroup 13. There does not appear to be any differences in phylogroups represented based upon sampling depth or sampling location in the lake. The diversity of

phylogroups represented may be an indication that the *P. syringae* in the lake are moving through the lake from a variety of habitats.

The diversity of *P. syringae* strains in Claytor Lake appears to be greater than in rain. The samples were also compared to 185 strains of *P. sryingae* collected from rain in Blacksburg, VA and 13 strains collected from a stream in Pembroke, VA. The rain samples were not screened for Ice+ phenotype before phylogenetic analysis limiting the comparison that can be made with the samples from Claytor Lake. The rain samples also showed diversity across the phylogroups, however not as many phylogroups were represented by the rain samples compared to the lake samples. A majority of the strains from rain were in phylogroups 2 and 6. Phylogroup 4 was represented with 9 strains, phylogroup 9 with 8 strains, and phylogroup 13 with 2 strains. All of the stream samples, with the exception of two were in phylogroup 2 however this sample size was small making assessments of diversity more limited. With the rain samples being a broader group encompassing both Ice- (bacteria not expressing the ice nucleation protein) and Ice+ strains compared to only Ice+ strains for the Claytor Lake samples it appears less diversity was found in the rain samples compared to the Claytor Lake samples. This difference could be due to certain phylogroups being preferentially incorporated into rain either by scavenging or in the process of rain formation. Perhaps certain phylogroups are comparatively more viable in the atmosphere leading to a higher representation in rain. A majority of the *Pseudomonas* Ice+ strains were *P. syringae*, which may support previous studies indicating *P. syringae* is the most wide spread biological ice nucleator (Fall and Fall, 1998; Morris et al., 2011; Murray et al., 2012). Many of the non- *P. syringae* colonies showed the highest match with *P. fluorescens* when blasted against the NCBI database. *Pseudomonas fluorescens* has previously been reported to have the Ice+ phenotype (Warren and Corotto, 1989).

This study indicates that freshwater lakes may be significant sources of Ice+ *P. syringae.* These bacteria have the potential to move to other parts of the water cycle and into the atmosphere (Morris et al., 2008). Future work could include examining connections between bacterial populations in the lake and weather, particularly rain events. The samples collected on 4 February 2016 had significantly higher concentrations of bacteria, as well as more non-*P. syringae* Ice+ colonies, and fewer overall Ice+ colonies compared to all previous collection days. A rain event with heavy precipitation totaling approximately 2.5 cm took place within 24 hours of sample collection, which could have affected the bacterial composition within the lake. Future work should also include further characterization of the Ice- strains to investigate the diversity of *Pseudomonas* species. Morris et al. (2008) used fluorescence and oxidase tests to isolate *P. syringae* from other *Pseudomonas* species on KBC media. This method could be implemented with the Ice- strains from Claytor Lake. The Ice- *P. syringae* strains could then be sequenced with the *cts* primers to examine differences in the phylogroups of Ice- strains and Ice+ strains. Comparing the concentrations and diversity of Ice- and Ice+ strains may give information towards understanding possible effects the Ice+ phenotype has on the movement and lifestyle of *P. syringae.* Ice+ bacteria may be actively involved in atmospheric processes including formation of rain and subsequently using rain to return to earth thus functioning as a dispersal strategy for the bacteria (Morris et al., 2014). Understanding movement of bacteria can also be helpful in managing pathogens as well as learning how strains are adapted to local habitats (Constantinidou et al., 1990; Morris et al., 2013).

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References

Amato, P., M. Parazols, M. Sancelme, P. Laj, G. Mailhot, and A.-M. Delort. 2007. Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures. *FEMS Microbiology Ecology*. 59:242-254.

Berge, O., C.L. Monteil, C. Bartoli, C. Chandeysson, C. Guilbaud, D.C. Sands, and C.E. Morris. 2014. A user's guide to a data base of the diversity of *Pseudomonas syringae* and its application to classifying strains in this phylogenetic complex. *PloS one*. 9:e105547.

Briée, C., D. Moreira, and P. López-García. 2007. Archaeal and bacterial community composition of sediment and plankton from a suboxic freshwater pond. *Research in Microbiology*. 158:213-227.

Cochet, N., and P. Widehem. 2000. Ice crystallization by *Pseudomonas syringae*. *Applied microbiology and biotechnology*. 54:153-161.

Comeau, A.M., T. Harding, P.E. Galand, W.F. Vincent, and C. Lovejoy. 2012. Vertical distribution of microbial communities in a perennially stratified Arctic lake with saline, anoxic bottom waters. *Scientific reports*. 2.

Constantinidou, H., S. Hirano, L. Baker, and C. Upper. 1990. Atmospheric dispersal of ice nucleation-active bacteria: The role of rain. *Phytopathology*. 80:934-937.

De Wever, A., K. Muylaert, K. Van der Gucht, S. Pirlot, C. Cocquyt, J.-P. Descy, P.-D. Plisnier, and W. Vyverman. 2005. Bacterial community composition in Lake Tanganyika: vertical and horizontal heterogeneity. *Applied and environmental microbiology*. 71:5029-5037.

Elliott, C. 1951. Manual of bacterial plant pathogens. *Manual of bacterial plant pathogens.*

Fall, A.L., and R. Fall. 1998. High-level expression of ice nuclei in *Erwinia herbicola* is induced by phosphate starvation and low temperature. *Current Microbiology*. 36:370-376.

Glöckner, F.O., E. Zaichikov, N. Belkova, L. Denissova, J. Pernthaler, A. Pernthaler, and R. Amann. 2000. Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. *Applied and environmental microbiology*. 66:5053-5065.

Haglund, A.-L., P. Lantz, E. Törnblom, and L. Tranvik. 2003. Depth distribution of active bacteria and bacterial activity in lake sediment. *FEMS Microbiology Ecology*. 46:31-38.

Hiorns, W.D., B.A. Methe, S.A. Nierzwicki-Bauer, and J.P. Zehr. 1997. Bacterial diversity in Adirondack mountain lakes as revealed by 16S rRNA gene sequences. *Applied and environmental microbiology*. 63:2957-2960.

Joly, M., E. Attard, M. Sancelme, L. Deguillaume, C. Guilbaud, C.E. Morris, P. Amato, and A.-M. Delort. 2013. Ice nucleation activity of bacteria isolated from cloud water. *Atmospheric Environment*. 70:392-400.

Lindow, S. 1983. The role of bacterial ice nucleation in frost injury to plants. *Annual review of phytopathology*. 21:363-384.

McDonough, R.J., R.W. Sanders, K.G. Porter, and D.L. Kirchman. 1986. Depth distribution of bacterial production in a stratified lake with an anoxic hypolimnion. *Applied and environmental microbiology*. 52:992-1000.

Mohan, S., and N. Schaad. 1987. An improved agar plating assay for detecting *Pseudomonas syringae pv. syringae* and *P. s. pv. phaseolicola* in contaminated bean seed. *Phytopathology*. 77:1390-1395.

Monteil, C.L., M. Bardin, and C.E. Morris. 2014. Features of air masses associated with the deposition of *Pseudomonas syringae* and *Botrytis cinerea* by rain and snowfall. *The ISME journal*. 8:2290-2304.

Monteil, C.L., C. Guilbaud, C. Glaux, F. Lafolie, S. Soubeyrand, and C.E. Morris. 2012. Emigration of the plant pathogen *Pseudomonas syringae* from leaf litter contributes to its population dynamics in alpine snowpack. *Environmental Microbiology*. 14:2099-2112.

Morris, C., D. Sands, M. Bardin, R. Jaenicke, B. Vogel, C. Leyronas, P. Ariya, and R. Psenner. 2011. Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate. *Biogeosciences*. 8:17-25.

Morris, C., D. Sands, J. Vanneste, J. Montarry, B. Oakley, C. Guilbaud, and C. Glaux. 2010. Inferring the evolutionary history of the plant pathogen *Pseudomonas syringae* from its biogeography in headwaters of rivers in North America, Europe, and New Zealand. *MBio*. 1:e00107-00110.

Morris, C.E., F. Conen, J. Alex Huffman, V. Phillips, U. Pöschl, and D.C. Sands. 2014. Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology*. 20:341-351.

Morris, C.E., L.L. Kinkel, K. Xiao, P. Prior, and D.C. Sands. 2007. Surprising niche for the plant pathogen *Pseudomonas syringae. Infection, Genetics and Evolution*. 7:84-92.

Morris, C.E., C.L. Monteil, and O. Berge. 2013. The life history of *Pseudomonas syringae:* linking agriculture to earth system processes. *Annual review of phytopathology*. 51:85-104.

Morris, C.E., D.C. Sands, B.A. Vinatzer, C. Glaux, C. Guilbaud, A. Buffiere, S. Yan, H. Dominguez, and B.M. Thompson. 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME journal*. 2:321-334.

Moukahel, A.-A., S. Asaad, B. Debbes, C.E. Morris, and D.C. Sands. 2015. Characterization of wheat genotypes as sources of ice nucleation active bacteria for bioprecipitation aerosols. *International Journal of Phytopathology*. 4:63-72.

Murray, B., D. O'Sullivan, J. Atkinson, and M. Webb. 2012. Ice nucleation by particles immersed in supercooled cloud droplets. *Chemical Society Reviews*. 41:6519-6554.

Nemecek-Marshall, M., R. Laduca, and R. Fall. 1993. High-level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. *Journal of bacteriology*. 175:4062-4070.

Pietsch, R.B., R.F. David, L.C. Marr, B. Vinatzer, and D.G. Schmale III. 2015. Aerosolization of Two Strains (Ice+ and Ice–) of *Pseudomonas syringae* in a Collison Nebulizer at Different Temperatures. *Aerosol Science and Technology*. 49:159-166.

Preston, G.M. 2000. *Pseudomonas syringae pv. tomato*: the right pathogen, of the right plant, at the right time. *Molecular Plant Pathology*. 1:263-275.

Sands, D., V. Langhans, A. Scharen, and G. De Smet. 1982. The association between bacteria and rain and possible resultant meteorological implications. *Idojaras*.

Streett, H.E., Failor, Kevin C., Llontop, Marco E., Vinatzer, Boris A. 2015. Isolation of *Pseudomonas syringae* from rain and other water sources in southwest Virginia.

Tamaki, H., Y. Sekiguchi, S. Hanada, K. Nakamura, N. Nomura, M. Matsumura, and Y. Kamagata. 2005. Comparative analysis of bacterial diversity in freshwater sediment of a

shallow eutrophic lake by molecular and improved cultivation-based techniques. *Applied and environmental microbiology*. 71:2162-2169.

Wang, Y., H.-F. Sheng, Y. He, J.-Y. Wu, Y.-X. Jiang, N.F.-Y. Tam, and H.-W. Zhou. 2012. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Applied and environmental microbiology*. 78:8264-8271.

Warren, G., and L. Corotto. 1989. The consensus sequence of ice nucleation proteins from *Erwinia herbicola*, *Pseudomonas fluorescens* and *Pseudomonas syringae. Gene*. 85:239-242.

Ye, W., X. Liu, S. Lin, J. Tan, J. Pan, D. Li, and H. Yang. 2009. The vertical distribution of bacterial and archaeal communities in the water and sediment of Lake Taihu. *FEMS Microbiology Ecology*. 70:263-276.

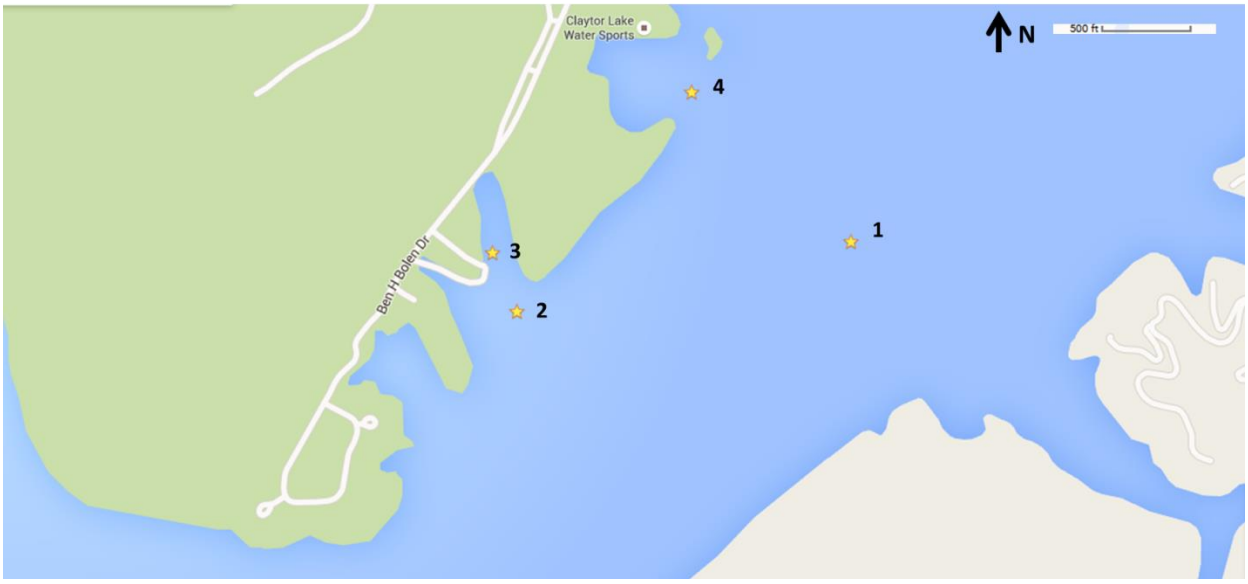

Figure 2.1. Map of the four sampling locations in Claytor Lake, VA. 1) deep water in the middle of the lake, 2) shallow water near the mouth of an inlet, 3) shallow water in the inlet, 4) the mouth of the boat launch inlet. GPS coordinates of the sampling location are shown in **Table 2.1.**

Table 2.1. Data for each 3.2 Liter water sample collected from Claytor Lake, VA with a Van Dorn sampler including collection date, ambient air temperature (° F), water temperature at collection location (° F), collection depth, GPS location, CFU/Liter of Pseudomonas grown on KBC media, CFU/Liter of total bacteria on TSA media, percent of Ice+ strains from colonies grown on KBC media.

Date	Air temp (° F)	Water temp (° F)	Location area	Sample depth	GPS coordinates	CFU/Liter on KBC media	CFU/Liter on TSA media	%Ice+ colonies
5-Nov-15	66	n.d.	4	surface	37.054566,-80.620337	192	n.d.	10.34%
5-Nov-15	66	n.d.	2	surface	37.051167,-80.623722	100	n.d.	43.75%
5-Nov-15	66	n.d.	3	surface	37.05208, -80.624204	108	n.d.	26.32%
5-Nov-15	66	n.d.	1	surface	37.052242, -80.617239	42	n.d.	0.00%
5-Nov-15	66	n.d.	1	3.0 m	37.052242, -80.619078	25	n.d.	4.17%
18-Nov-15	52	n.d.	2	surface	37.050904,-80.623690	367	9.42E+04	0.00%
18-Nov-15	52	n.d.	2	surface	37.051063,-80.623709	208	8.50E+04	5.56%
18-Nov-15	52	n.d.	2	surface	37.051246,-80.623684	50	2.75E+04	0.00%
18-Nov-15	52	n.d.	1	surface	37.051911,-80.619642	283	8.42E+04	5.88%
18-Nov-15	52	n.d.	1	surface	37.052095,-80.619428	442	7.92E+04	20.69%
18-Nov-15	52	n.d.	1	surface	37.052487,-80.619154	525	1.25E+05	32.00%
18-Nov-15	52	n.d.	4	surface	37.054582,-80.620274	267	1.70E+05	7.69%
16-Dec-15	48	48.9	2	surface	37.051218,-80.623385	1950	1.93E+05	7.35%
16-Dec-15	48	49.1	2	surface	37.050978,-80.623684	3275	6.60E+05	17.86%
16-Dec-15	48	48.9	2	surface	37.051368,-80.623841	3600	5.35E+05	10.29%
16-Dec-15	48	48.4	1	surface	37.052293,-80.619069	1300	7.50E+04	14.71%
16-Dec-15	48	48.2	1	surface	37.052179,-80.619059	1100	1.85E+05	16.67%
16-Dec-15	48	48.9	1	surface	37.052249,-80.619043	500	8.50E+04	13.46%
21-Jan-16	30	40.5	1	4.2 m	37.0515635,-80.6169486	900	9.50E+04	10.87%
21-Jan-16	30	40.2	1	surface	37.0509283,-80.6169794	600	1.08E+05	4.84%
21-Jan-16	30	40.3	1	4.2 m	37.0522448,-80.6170897	950	2.78E+05	10.29%
21-Jan-16	30	40.3	1	surface	37.0522448,-80.6170897	600	1.05E+05	8.33%
21-Jan-16	30	39.5	1	4.2 m	37.0520847,-80.6169546	550	2.33E+05	12.50%
21-Jan-16	30	40.3	1	surface	37.0517267,-80.6169077	575	6.75E+04	9.62%
21-Jan-16	30	40.1	1	9.1 m	37.052242, -80.617239	1025	1.83E+05	11.76%
4-Feb-16	42	35.6	1	4.2 m	37.0522073,-80.6180414	7475	1.47E+06	1.79%
4-Feb-16	42	35.8	1	surface	37.0520805,-80.6184315	5025	1.54E+06	7.69%
4-Feb-16	42	35.4	1	4.2 m	37.0520144,-80.6188472	8175	1.58E+06	13.33%
4-Feb-16	42	35.8	1	surface	37.0519812,-80.6188476	5800	2.21E+06	8.33%
4-Feb-16	42	35.6	1	4.2 m	37.0521998,-80.6190779	9175	2.09E+06	3.85%
4-Feb-16	42	35.8	1	surface	37.0521254,-80.6189773	9250	1.72E+06	12.50%
4-Feb-16	42	35.6	1	9.1 m	37.0522846,-80.6189418	9200	2.95E+06	0.00%

Table 2.2. Five sampling dates with the number of 3.2 Liter water samples collected from Claytor Lake, VA with a Van Dorn sampler at each location. All locations were collected at the water surface, except location 1 where samples were collected at the surface, 4.6 m depth, and 9.1 m depth. Each sample was collected with a 3.2 Liter Van Dorn sampler. See **Figure 2.1** for map of locations and **Table 2.1** for GPS coordinates.

Date	Location 1 surface	Location 1 4.2 m	Location 1 9.1 m	Location 2	Location 3	Location 4
5-Nov-15	1	0	0	1	1	1
18-Nov-15	3	0	0	3	0	1
16-Dec-15	3	0	0	3	0	0
21-Jan-16	3	3	1	0	0	0
4-Feb-16	3	3	1	0	0	0
Total	13	6	2	7	1	2

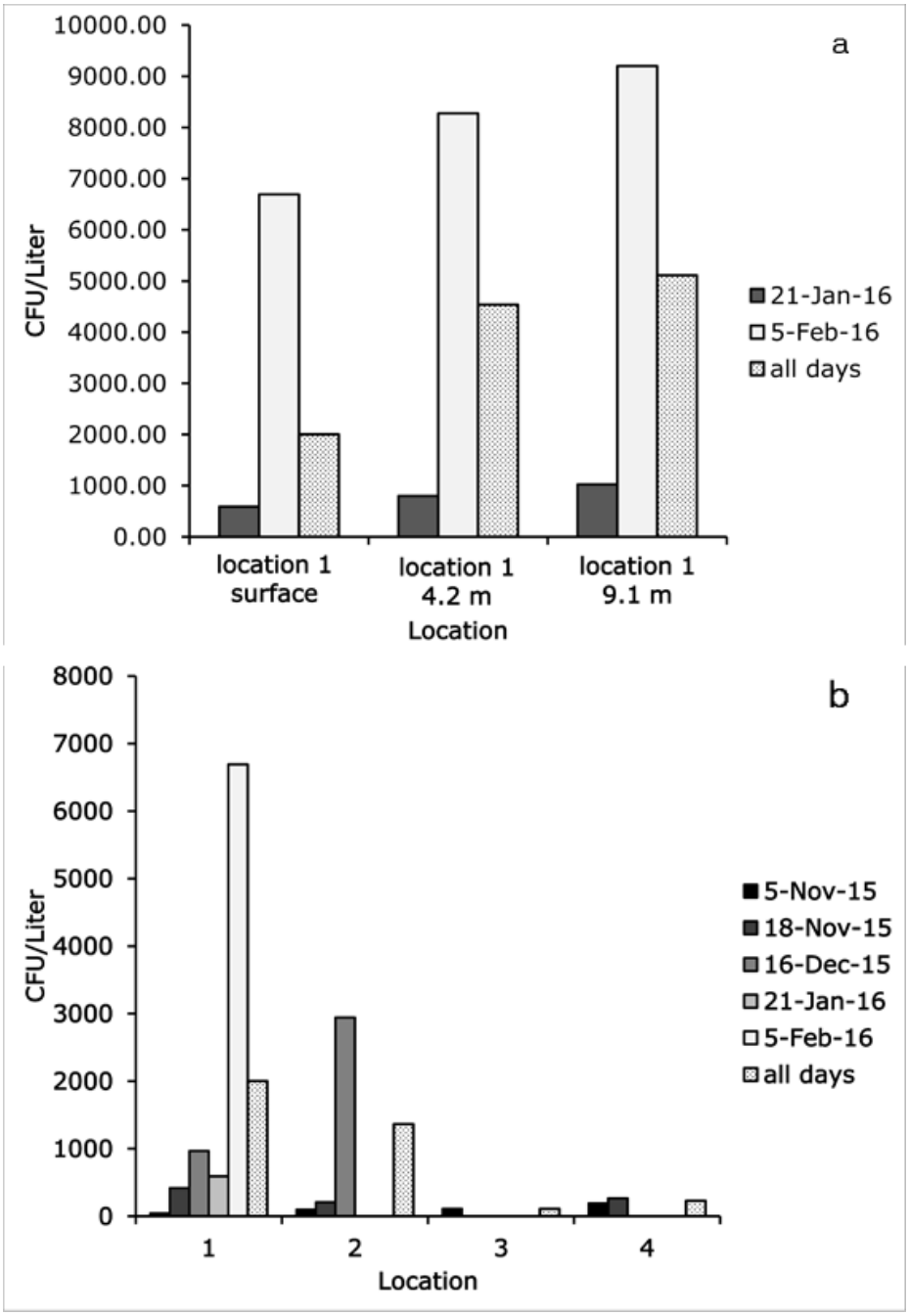

Figure 2.2. Concentration in CFU/Liter of *Pseudomonas* grown on KBC media at each three different depths (surface, 4.6 m, and 9.1 m) for two sampling days and an average over both days (a), and at four different locations for each day of collection and an average for all days of collection (b). Note different y axis values. See **Figure 2.1** for map of locations.

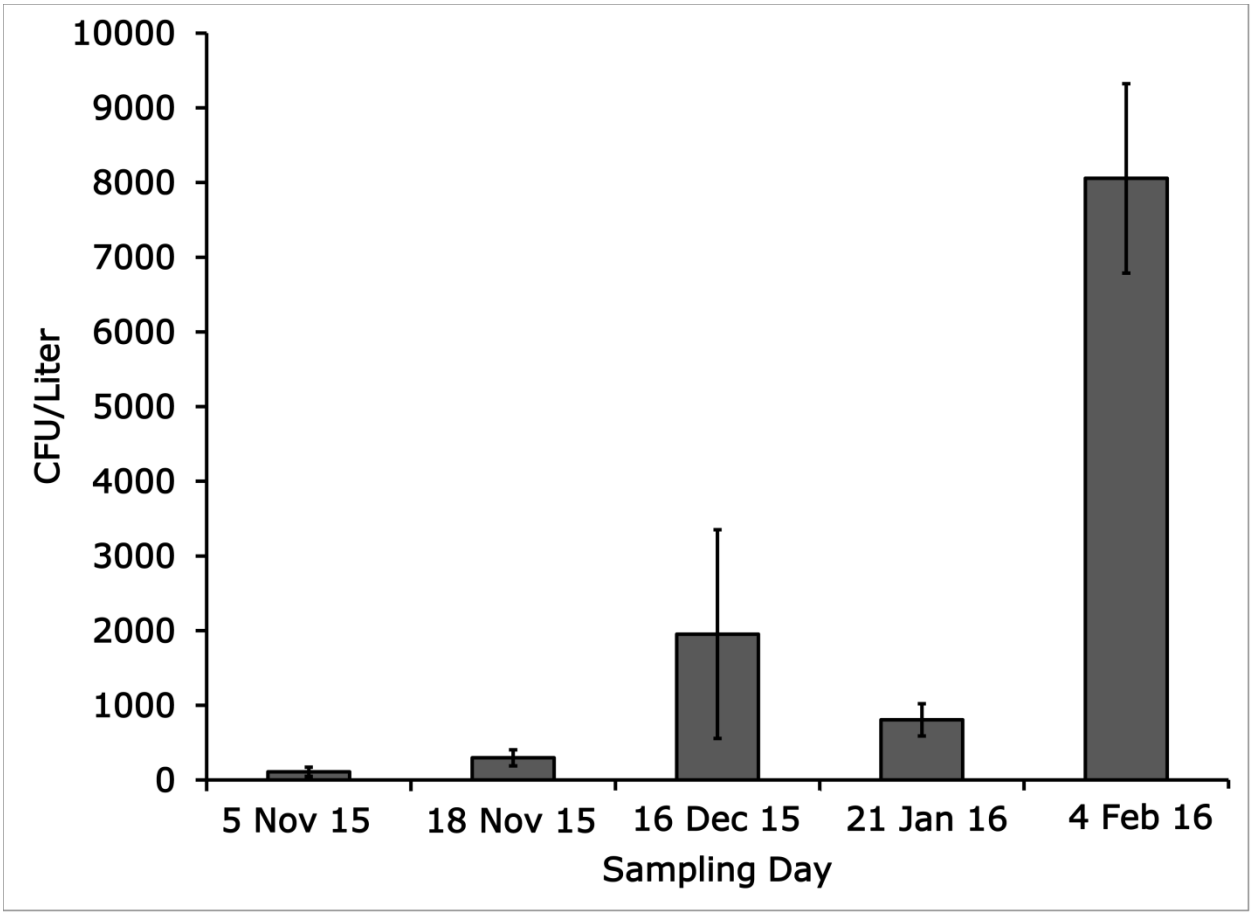

Figure 2.3. Concentration in CFU/Liter of *Pseudomonas* grown on KBC media across all locations on each day of sampling.

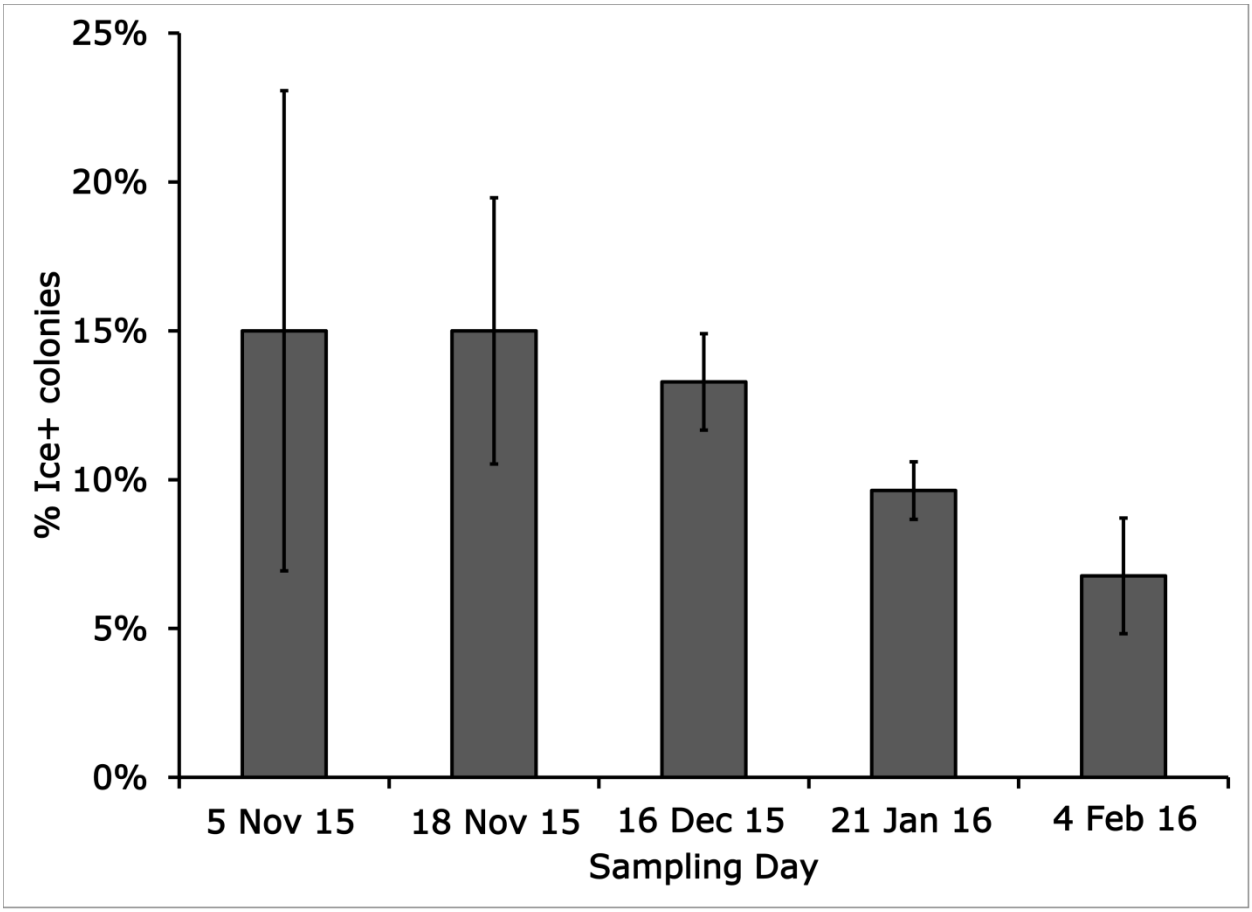

Figure 2.4. Percent of Ice+ *Pseudomonas* colonies grown on KBC media for each day of sampling.

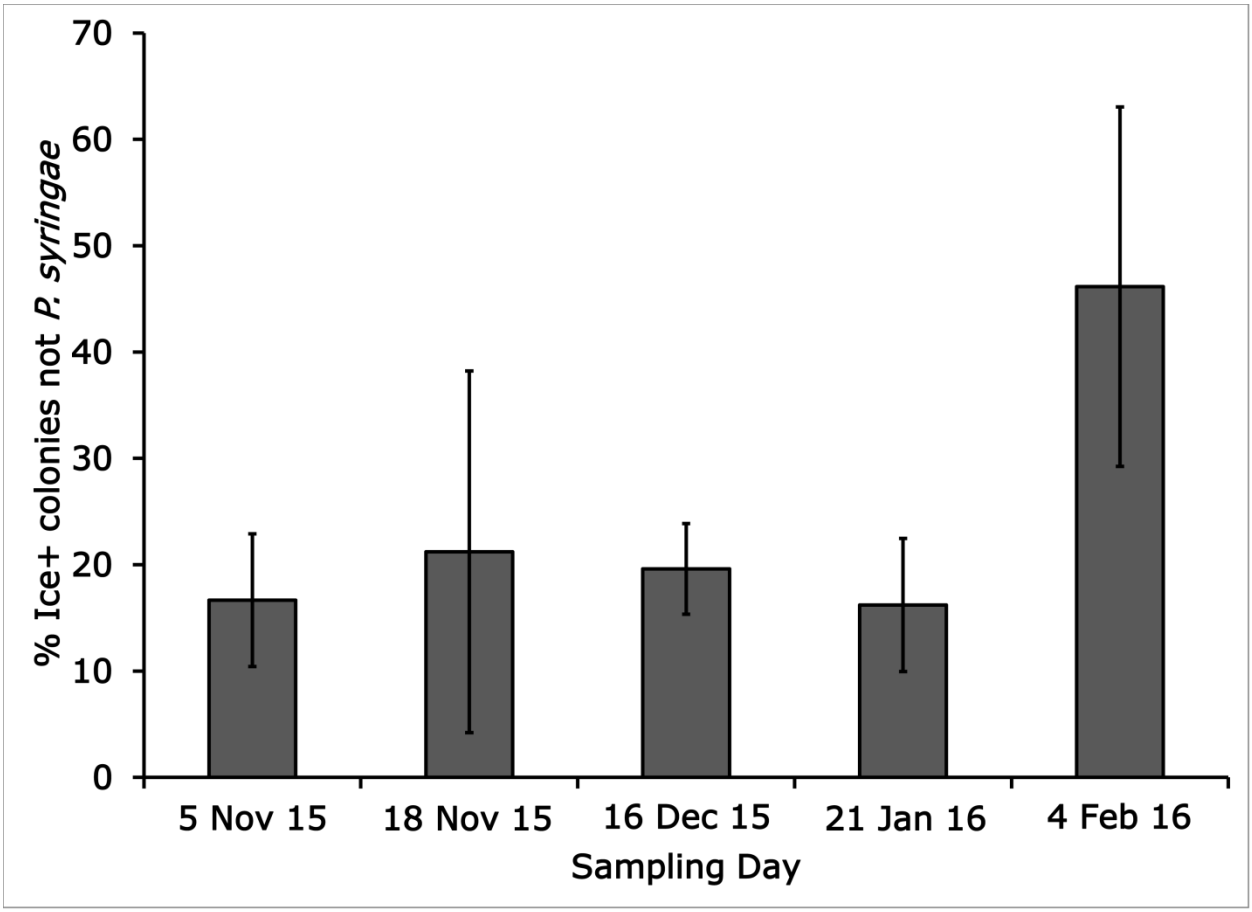

Figure 2.5. The % of Ice+ *Pseudomonas* colonies not producing a PCR product with *cts* primers and therefore not *P. syringae* for each sampling day.

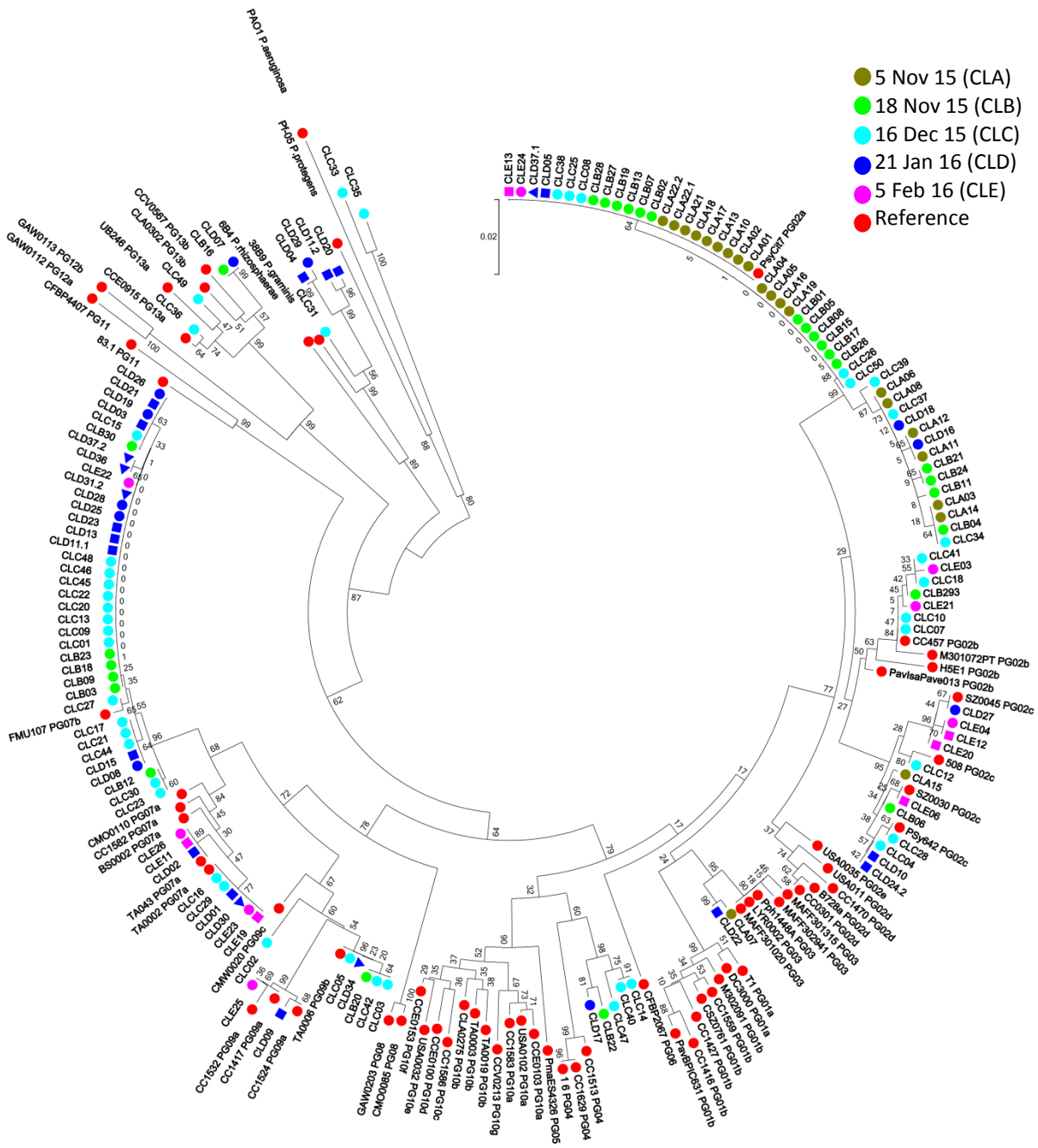

Figure 2.6. Phylogenetic tree with samples from all days of collection along with 38 reference strains representing the diversity of *Pseudomonas syringae* strains. Bootstrap values are listed. 5 November 2015 = CLA (yellow), 18 November 2015 = CLB (green), 16 December 2015 = CLC (light blue), 21 January 2015 = CLD (blue), and 4 February 2016 (pink) = CLE. Reference strains = red. Circle = surface, square = 4.6m depth, triangle = 9.1m depth.

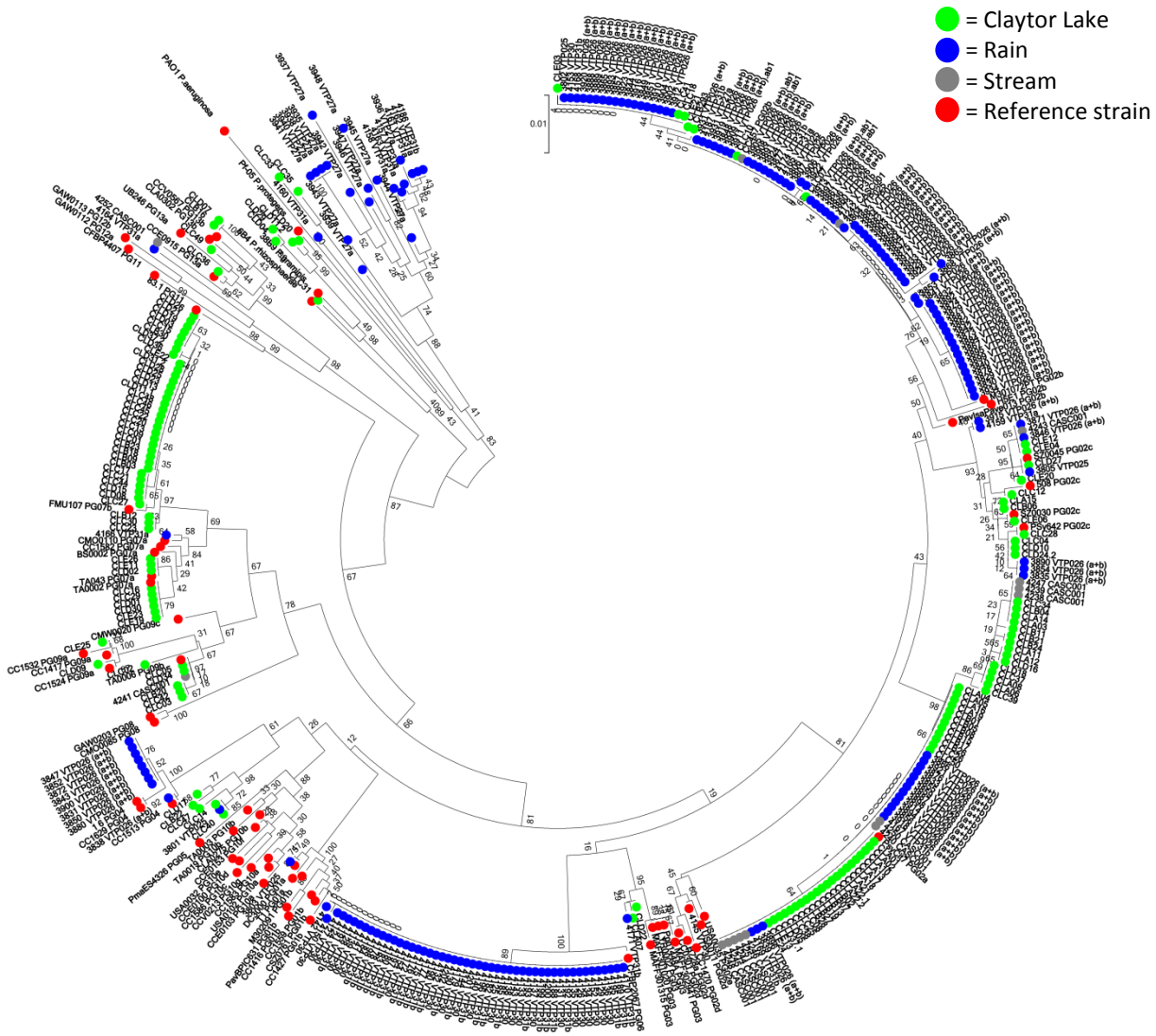

Figure 2.7. Phylogenetic tree with samples from all days of collection along with 38 reference strains representing 13 phylogenetic subgroups of *Pseudomonas syringae* along with samples from rain and stream collections in Blacksburg, VA and Pembrook, VA, respectively. Bootstrap values are listed. 5 November 2015 = CLA, 18 November 2015 = CLB, 16 December 2015 = CLC, 21 January 2015 = CLD, and 4 February 2016 = CLE, VTP = rain sample from Blacksburg, VA, CASC = stream sample from Pembrook, VA. Green = Claytor Lake, blue = rain, grey = stream, red = reference strains.

Chapter 3 – Aerosolization of two strains of Pseudomonas syringae in a Collison nebulizer at different temperatures

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Abstract

The aerosolization of microorganisms from aquatic environments is understudied. In this study, an ice nucleation active (Ice+) strain and a non-ice nucleation active (Ice-) strain of the bacterium *Pseudomonas syringae* were aerosolized from aqueous suspensions under artificial laboratory conditions using a Collison nebulizer. The aerosolization of *P. syringae* was not influenced by water temperatures between 5° and 30°C. In general, the culturability (viability) of *P. syringae* in aerosols increased with temperature between 5 and 30°C. The ice+ strain was aerosolized in greater numbers than the Ice- strain at all temperatures studied, suggesting a possible connection between the ice nucleation phenotype and aerosol production. Together, our results suggest that *P. syringae* has the potential to be aerosolized from natural aquatic environments, such as streams, rivers, ponds, and lakes; known reservoirs of *P. syringae*. Future work is needed to elucidate the mechanisms of aerosolization of *P. syringae* from natural aquatic systems.

Introduction

Aerosols (inorganic ions, organic material, algae, fungal spores, viruses, and bacteria) are formed from aquatic environments by several methods, such as the bursting of bubbles brought to the surface by waves (Baylor et al., 1977; Bigg, 2007; Blanchard, 1989). Particles are released as the bubbles burst, and are subsequently carried into the air by wind. Studies in marine environments have examined the content of aerosols (Blot et al., 2013; Cavalli et al., 2004; Claeys et al., 2010; Després et al., 2012; Fu et al., 2011; Marks et al., 2001), and have showed that they contain a large amount of inorganic materials, such as salt (Blot et al., 2013; Pósfai et al., 2003) with a lesser contribution from biological aerosols.

The aerosolization of microorganisms from aquatic environments is understudied. Fahlgren et al., (2011) investigated bacteria in natural aerosols and found agreement between three sampling methods in regards to distribution of species among phylogenetic groups. Seaver, (1999) released and collected bacterial aerosol to develop an aerosol collection method. Bacteria have also been aerosolized and collected in laboratory settings (Brosseau et al., 2000; Fergenson et al., 2004; Heidelberg et al., 1997; Jensen et al., 1992; Seaver, 1999; Zhao et al., 2011).

The bacterium *Pseudomonas syringae* is present in a variety of different natural and managed ecosystems (Morris et al., 2007). Some strains of *P. syringae* are important crop pathogens (Hirano and Upper, 2000), but the majority of the genetic diversity within *P. syringae* is found in non-agricultural environments. Previous research on the life history of *P. syringae* has focused mostly on agricultural environments. *Pseudomonas syringae* has been found on vegetation and in the atmosphere (Clarke et al., 2010; Kozloff et al., 1983; Morris et al., 2011). The bacterium is known to aerosolize from plants such as corn and wheat, and to a lesser degree from soil (Lindemann et al., 1982). It is found in clouds (Amato et al., 2007; Sands et al., 1982),

and in rain (Constantinidou et al., 1990; Monteil et al., 2014; Morris et al., 2008), and deposition from rain is recognized as a means of pathogen dissemination (Morris et al., 2007; Morris et al., 2013). The bacterium has also been found in a variety of aquatic environments such as lakes, streams, and snow pack, including locations in pristine habitats at altitudes above cultivated zones (Morris et al., 2013; Morris et al., 2008; Riffaud and Morris, 2002). Though *P. syringae* is known to move through the water table to agricultural environments, the potential aerosolization of the bacterium directly from aquatic sources has not yet been characterized in detail. Other species of bacteria are known to aerosolize from aquatic environments (Aller et al., 2005; Després et al., 2012; Lighthart, 1997), and this pathway has the potential to be an important component of the life history of *P. syringae.*

Some strains of *P. syringae* express ice nucleation active (INA) proteins (Lindow, 1983), which may be important for its pathogenicity (Hirano and Upper, 2000), contributions to the water cycle (Maki et al., 1974), and its reproductive success (Weidner, 2013). Expression of the Ice+ phenotype leads to frost damage in plants at higher temperatures (Lindow, 1983). The bacteria may take advantage of the frost damage to gain access to the plant as a pathogen (Hirano and Upper, 2000). Cloud formation involves water condensing around a small particle, either a cloud condensation nucleus or ice nucleus to form liquid cloud droplets or ice particles, respectively (Howell, 1949; Mason and Ludlam, 1951; Murray et al., 2012; Schaefer, 1952) . Water droplets can stay in a supercooled liquid state at temperatures as cold as -40°C (Lundheim, 2002; Mason, 1952; Mossop, 1955). An ice nucleus catalyzes the freezing of water at higher temperatures and is important in cloud formation (Edwards and Evans, 1960; Hudson, 1993; Vonnegut, 1947) and the initiation of precipitation (Bigg and Miles, 1964; Hoose et al., 2010). A variety of particulates including dust, pollen, fungal spores, and bacteria can cause ice nucleation

(DeMott et al., 2003; Diehl et al., 2002; Jayaweera and Flanagan, 1982; Lindow et al., 1978; Lohmann and Diehl, 2006; Pouleur et al., 1992; Pratt et al., 2009; Vali et al., 1976). An outer membrane lipoglycoprotein on some *P. syringae* strains enables the bacteria to catalyze ice formation at temperatures between -2° and -8°C (Cochet and Widehem, 2000; Kozloff et al., 1991). The exact mechanism involved has not yet been determined, but a recent study suggests the ice nucleation protein may remove thermal energy from the water and organize the water molecules into an ice lattice initiating crystal growth (Weidner, 2013). *Pseudomonas syringae* is currently used in making artificial snow due to its ability to freeze water at higher than normal temperatures (Rixen et al., 2003).

In this study, aqueous suspensions of *P. syringae* were aerosolized with a Collison nebulizer under a variety of different temperatures and monitored using a particle counter. The specific objectives of this study were to: (1) determine the association of temperature with the aerosolization of *P. syringae*, (2) examine the effect of temperature on culturability (viability) of aerosolized *P. syringae*, and (3) investigate the potential role of ice nucleation on the aerosolization of *P. syringae*. This work will increase our understanding of the potential transmission of *P. syringae* from aquatic environments to the atmosphere.

Materials and Methods

Preparation of bacteria for aerosolization experiments

Two strains of *P. syringae* (642 and 892) from the 2c phylogenetic subgroup were used in the experiments. These strains were collected locally in Blacksburg, VA in 2007 and 2008 (Clarke et al., 2010). Based on the genome sequence of strain 642, and based on a molecular test for strain

892, both strains contain at least a partial ice nucleation activity (INA) gene (Clarke et al., 2010). However, based on multiple ice nucleation assays in the laboratory (see section below on ice nucleation), strain 642 was found to be Ice- (not ice nucleating) while strain 892 was found to be Ice+ (ice nucleating). Bacteria were taken from -80°C glycerol stocks and inoculated on King's medium B modified with cephalexin (80 mg/L), cyclohexamide (200 mg/L), and boric acid (1500 mg/L) (KBC) media for 48 hours at 23°C (Mohan and Schaad, 1987). Bacteria were prepared in two different aqueous suspensions for the two different experiments, the direct particle counting method (to determine the number of aerosolized bacteria) and the indirect culture method (to determine the number of viable aerosolized bacteria). The bacteria were suspended in sterile nanopure water or 10 mM $MgSO_4$. Suspension in water was necessary when using the direct particle counting method due to the salt being counted as particles masking the count of the bacteria. For the indirect culture method, the bacteria were suspended in $MgSO_4$ because viability of the bacteria is greatly reduced when suspended in water due to the hypotonic condition. A spectrophotometer (Beckman Coulter DU 800, Brea, CA) was used to obtain OD_{600} for each suspension. Each suspension was diluted to 0.1 OD. For both methods, a tenfold dilution was prepared to obtain samples of 0.01 OD. The concentration of the bacteria was determined to be 1.5×10^9 CFU/mL at 0.01 OD.

Ice nucleation assays

Droplet freezing assays were conducted using a Lauda model Alpha RA 24 (Delran, NJ) freezing bath to determine expression of the ice nucleation phenotype under specific conditions. Bacteria were suspended in sterile nanopure water and incubated for 30 min at 5°, 15°, 20°, 30° C, and at ambient room temperature (~ 25° C). Droplets of 12 µL were used in the assay, with two

droplets each from two different suspensions for each strain. The temperature was lowered from -4° C to -12° C over 30 minutes, and the temperature at which the droplets froze was recorded.

Nebulizer

A 1-jet Collison Nebulizer (BGI, Waltham, MA) was used for the aerosolization experiments. This equipment has been used to aerosolize bacteria previously (Brosseau et al., 2000; Fergenson et al., 2004; Heidelberg et al., 1997; Jensen et al., 1992; Seaver, 1999). Jensen et al. 1992 used a 6-jet version of the Collison nebulizer, but the number of jets used in the other publications (references) was not stated. The top of the nebulizer had a tee connection; ambient air flowed into one side of the tee at 2 lpm, and the other side of the tee was equipped with a female nut and an adjustable plug to obtain a pressure of 20 psi (**Figure 3.1**). This air flow (2 lpm) and pressure setting (20 psi) was selected based on manufacturer's protocols to minimize damage to the bacteria. An aliquot of 30 mL of 0.01 OD bacterial suspension was placed in the nebulizer.

Direct method to assess the number of particles in aerosols collected at four different temperatures

The direct method was based on particle counting of aerosols collected at four different temperatures (5°, 15°, 20°, and 30° C). For this method, the aerosol passed through a diffusion dryer model 3062 (TSI, INC, Shoreview, MN) prior to entering the particle counter to remove water droplets (which otherwise could be counted as particles). Water was aerosolized with the nebulizer as a negative control. Particles were counted with the Aerodynamic Particle Sizer (APS) Model 3321 (TSI, INC, Shoreview, MN). Because the flow rate of the APS was greater than that of the nebulizer, particle-free air was introduced as make-up flow to the APS through

an uncontrolled (open) tee connection to the ambient air with a HEPA filter (1602051, TSI, INC, Shoreview, MN) (**Figure 3.1**).

Indirect culture method to assess the number of colony forming units (viable cells) from aerosols collected at four different temperatures

The indirect method was based on plating of aerosols collected at four different temperatures (5°, 15°, 20°, and 30° C). In this method, the aerosol was collected into 1 mL of 10 mM $MgSO_4$ in a sterile 50 mL tube at ambient room temperature by bubbling the aerosol into the liquid in the tube. Each collection was plated on KBC media. Three plates were used for each collection. For each collection, tenfold dilutions were made for plates of aerosol collected at 15°, 20°, and 30° C. Dilutions were not used for the samples collected at 5° C due to lower aerosol production. All of the plates were incubated at ambient room temperature and CFUs were counted after 72 to 96 hours, depending on the growth of the colonies. At time less than 72 hours the colonies were not large enough to count, and at timelonger than 96 hours the colonies were beginning to grow on top of each other making counting distinct CFUs difficult.

Test schedule

Collection periods for both methods were three minutes, and three replicates were collected sequentially at each temperature (**Figure 3.2**). The nebulizer jar was submerged in a circulating water bath. The jar was submerged for 30 minutes with the bath set at 5°C, then collection took place for a total of 9 minutes (**Figure 3.2**). The bath temperature was then changed, and 21 minutes passed for the temperature of the sample to stabilize before the next collection period (**Figure 3.2**). The jar remained submerged for the entire experiment. It took approximately 3

minutes for the water bath to change temperature allowing approximately 18 minutes for the aerosol to stabilize at each temperature. Temperatures of 5°, 15°, 20°, and 30° C were used. Both strains were tested in alternating order each day. After data were collected for the first strain, the bath temperature was set back to 5°C to begin testing the second strain. It took about 45 minutes for the bath temperature to return to 5°C. Each sample was in the nebulizer for approximately 2 hours. The test schedule was developed based on a series of preliminary laboratory experiments designed to determine (1) the amount of time needed for a sample to reach equilibrium following changing temperature regimes and (2) that fluctuations in aerosolization rates did not vary over total sampling time.

Test to monitor potential changes in the CFUs of the suspensions

To test if the CFUs changed from the beginning to the end of the experiment, the Ice- 642 and Ice+ 892 strains of *P. syringae* were prepared at concentrations of 0.01 OD in sterile nanopure water or 10 mM $MgSO_4$. A 30 mL sample was placed in the nebulizer. Aliquots of 10 µL were removed from the bench sample and nebulizer sample at 0, 3, 6, 9, and 12 hours, and were plated on KBC. CFUs were counted 3 days later. The rate of change for concentration of bacteria in the suspension was calculated by subtracting the final concentration of CFUs from the initial concentration divided by the time.

Statistical analyses

Statistical analyses were conducted with JMP software. Tukey's post-hoc comparison ($P < 0.05$) was used to test for significant differences between aerosolized samples at different temperatures.

Results

Ice nucleation assays to confirm ice+ and ice- phenotypes

Strain 892 exhibited the Ice+ phenotype at all temperatures of incubation, and strain 642 exhibited the ice- phenotype at all temperatures of incubation. Four 12 µL droplets were used for each temperature of incubation (ambient room temp, 5°, 15°, 20°, and 30°C). For strain 892, the droplets incubated at 20°C froze at -6°C, and the droplets incubated at ambient room temp, 5°, and 15°C froze between -7° and -8°C. The 30°C incubated droplets did not freeze until the temperature reached -10°C. None of the drops of strain 642 froze at any of the temperatures studied.

Direct method to assess the number of particles in the aerosol

The amount of aerosolized particles was not influenced by temperature between 5° and 30° C (**Figure 3.3**). There was no significant difference between any of the temperatures for either strain. In general, the Ice+ strain produced a higher number of particles than the Ice- strain at each temperature (**Figure 3.3**). There was a significant difference between the strains at 5°, 15°, and 20° C ($P < 0.05$), but not at 30° C ($P = 0.11$).

The mean diameter reported by the APS ranged from 0.71 – 0.84 µm, depending on the day of testing (**Figure 3.4**). Cells of *P. syringae* are rod shaped with a length of 1-5 µm and a width of 0.5 – 1.5 µm, so submicron aerodynamic diameters are reasonable, especially since the rods are likely to be oriented with their long axis parallel to the direction of flow and they may have been subject to shrinkage in the dryer. Nanopure water was aerosolized as a negative control and the particle counts were very low (around 1 particle per cm^3), indicating the dryer was removing a sufficient amount of water. The size distribution of particles differed slightly

between the two strains of bacteria (**Figure 3.4**). While more particles were generated by the Ice+ strain than by the Ice- strain at aerodynamic diameters of 0.8 μm and less, the opposite was generally true for particles >0.8 μm (**Figure 3.4**). There was a pronounced rightward shift of the size distribution, especially at the higher temperatures (**Figure 3.4**).

Indirect method to assess the number of colony forming units (culturable cells) from the aerosol

The culturability of aerosolized *P. syringae* increased with temperature between 5° and 30° C (**Figure 3.5**). In the Ice- strain, the temperature pairs 5° and 15° C and 15° and 20° C and 5° and 20° C were not significantly different ($P = 0.4724$, P = 0.9427, and $P = 0.1891$ respectively). The temperature of 30° C was significantly different from all of the other temperatures studied ($P < 0.001$). For the Ice+ strain, the temperature pair of 5° and 15° was not significantly different ($P = 0.999$), while all other pairs were significantly different ($P < 0.001$). Comparing the two strains, the Ice+ strain showed greater culturability of the aerosol at all temperatures, with the exception of 30°C (**Figure 3.5**). There was a significant difference between the culturability of the aerosols between the strains at 20°C ($P = 0.0362$). The trend of increasing culturability of aerosol with temperature fits an exponential curve with an r-squared value of 0.8783 and 0.8297 for the Ice- and Ice+ strains, respectively.

Confirming CFU of suspensions in the nebulizer over test duration

The concentrations of the bacterial $MgSO_4$ suspensions showed an increase at 3, 6, and 12 hours, but this increase was not associated with increased time in the nebulizer (i.e., longer incubation times did not result in higher concentrations of bacteria) (**Figure 3.6**). In general, concentrations

decreased for the suspensions in H_2O over all times tested (**Figure 3.6**). The volume of the suspensions in the nebulizer were measured in a graduated cylinder before and after the 2 hour aerosolization period, and the amount lost was less than the resolution of the graduated cylinder (data not shown). After 12 hours of aerosolization in the nebulizer, the volume of the suspension decreased from 30 mL to ~20 mL.

Discussion

Freshwater aerosols are understudied, and few efforts have tracked the life history of microorganisms in aerosols. Here, we show the culturability of two strains of *P. syringae* aerosolized in a Collison nebulizer is impacted by temperature while particle counts of aerosol are not impacted by temperautre. Our experiments were designed to test the hypotheses that: (1) the production of aerosolized *P. syringae* particles will increase with temperature, (2) the culturability of aerosolized *P. syringae* will increase with temperature, and (3) an ice-nucleating strain (Ice +) of *P. syringae* will aerosolize at a greater rate than a non ice-nucleating strain (Ice-). Results from our experiments suggest that *P. syringae* has the potential to be aerosolized from natural aquatic environments (streams, rivers, ponds, and lakes—known reservoirs of *P. syringae* (Morris et al., 2008)) at a variety of temperatures. This process may be an important part of *P. syringae's* life history. Strains of *P. syringae* have been found in the atmosphere and in aquatic environments (Morris et al., 2013), yet the movement between these two environments and factors affecting the flux has not been studied in detail. Aerosolized bacteria are known to be transported in the atmosphere (Franc, 1988; Morris et al., 2013). The results described here improve our understanding of the aerosolization of *P. syringae* from aquatic environments, and could be applied to a generalized model of bacterial aerosolization flux in the future.

Aerosolization of *P. syringae* was not influenced by water temperatures between 5° and 30°C. Previous studies have suggested that temperature may increase bacterial aerosol concentration above plant canopies (Lighthart and Shaffer, 1994; Lindemann and Upper, 1985). Thus, our observation that aerosolization was not influenced by temperature was unexpected. This observation suggests that aerosolization of *P. syringae* in natural aqueous environments would not change with seasonal temperature changes. Unknown, however, are the contributions of other factors correlated with seasonal changes, such as changes in water velocity and turbulence (Sharma et al., 2007), that may affect aerosolization during different times of the year. Additionally, the temperature at the surface of the water in natural environments may be higher because of direct solar radiation.

In general, the culturability of *P. syringae* in aerosols increased with temperature between 5 and 30°C. This observation is supported by a shorter doubling time for *P. syringae* at higher temperatures (between 0 and 36°C). (Young et al., 1977) reported the doubling rate of *P. syringae* to be 10.45 hours at 5.3° C, and 1.3 hours at 26.0° C. In natural aqueous systems, viable aerosolized particles of *P. syringae* might be expected to increase with temperature and may therefore change seasonally. Viable cells of *P. syringae* are essential for infection and reproductive success, but nonviable bacteria can nucleate ice. (Attard et al., 2012) found that aerosolized *P. syringae* lost viability after UV-A exposure, but retained ice nucleation ability.

The Ice+ strain produced greater total numbers of particles than did the Ice- strain at all temperatures studied (5° to 30° C). This observation suggests a possible connection between ice nucleation phenotype and aerosol production. Provided the same volume of liquid is aerosolized, the same total number of bacteria must be aerosolized in either case, but the strain affects the resulting size of the aerosols. Perhaps the bacteria of the Ice- strain stick together more, such that

two or more cells comprise a single aerosol, while the bacteria of the Ice+ strain tend toward single-bacteria aerosols. In other bacteria an aggregation protein has been shown to cause differing amount of aggregation and expression of different surface antigens appears to change hydrophobicity (Lindahl et al., 1981; Wells et al., 2000). In this scenario, the Ice- strain would produce a smaller total number of aerosols and have a rightward shifted size distribution compared to the Ice+ strain. It is also possible that there are changes in the relative solubility of each of the strains that could be related to Ice+ phenotype differences, but this was not examined in this study.

At 30°C, the Ice+ 892 strain did not freeze until it reached -10°C, which was 2 to 4°C cooler than bacteria incubated at the other temperatures. In the direct particle counting method, 30°C was the only temperature which did not report a significant difference between strains, while in the indirect culture method 30°C was the only temperature which did not show higher aerosol production for the Ice+ strain. Perhaps the ice nucleation activity is reduced at 30°C, and this is reflected in aerosol production. It is possible that the Ice+ strain may be suited for transport and survival in the atmosphere. Harsh atmospheric conditions including lack of water are harmful to bacteria, especially non-spore forming bacteria such as *P. syringae* (Chi and Li, 2007; Polymenakou, 2012). Morris et al. (2011) found all strains of *P. syringae* collected from snow and rain were ice nucleation active, but not all strains collected from other sources including plants and water were ice nucleation active (Morris et al., 2011; Morris et al., 2008). Monteil et al. (2014) found all strains collected from snow were ice nucleation active while some strains collected from rain were not ice nucleation active. This may indicate that the ice nucleation phenotype favors survival in atmospheric conditions either by preferentially allowing the bacteria to enter the atmosphere or increasing survival once bacteria have entered the

atmosphere. Even if the difference between strains is not due to the ice nucleation phenotype, our data indicate that aerosolization rates may not be constant for all strains of the same species. Both of the strains used in this study are closely related and part of the 2c phylogenic subgroup (Clarke et al., 2010). A difference in aerosol production between these two strains is an interesting finding regardless of a connection to ice nucleation phenotype. It should be noted that bacterial suspensions were plated before and after the aerosolization experiments, and there was not a significant increase in concentration for the Ice+ 892 strain compared to the Ice- 642 strain, indicating differences in the concentration of the suspension were not the cause of differences in aerosol production between the strains. The concentrations of the bacterial $MgSO_4$ suspensions showed an increase at 3, 6, and 12 hours, but this increase was not associated with increased time in the nebulizer, and concentrations generally decreased for the suspensions in H_2O over all times tested. Thus, changes in aerosol production are not likely a result of changes in bacterial concentration in the suspension. Use of other nebulizers may increase the robustness of our results but that was beyond the scope of this study. A number of studies have generated bacteria aerosols using only a Collison nebulizer (Brosseau et al., 2000; Fergenson et al., 2004; Heidelberg et al., 1997).

Future work should include knocking out the ice nucleation gene in the Ice+ 892 strain, and performing aerosolization experiments comparing the Ice+ strain with the genetically modified Ice- strain. Since the only difference between the two strains will be the ice nucleation gene, differences between strains can then be interpreted as being directly caused by the ice nucleation gene. Other strains of *P. syringae* with Ice- and Ice+ phenotypes could also be subjected to similar aerosolization experiments. Future work aims to examine the aerosolization of *P. syringae* from aqueous field environments. Such studies may assist in elucidating the

mechanisms of natural aerosol production in the future, a necessary component of a comprehensive understanding of the aerosolization process and the ecology of microbial life in the atmosphere.

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References

Aller, J.Y., Kuznetsova, M.R., Jahns, C.J., Kemp, P.F., 2005. The sea surface microlayer as a source of viral and bacterial enrichment in marine aerosols. *Journal of Aerosol Science.* 36:801-812.

Amato, P., Parazols, M., Sancelme, M., Laj, P., Mailhot, G., Delort, A.M., 2007. Microorganisms isolated from the water phase of tropospheric clouds at the Puy de Dôme: major groups and growth abilities at low temperatures. *FEMS Microbiology Ecology.* 59:242-254.

Attard, E., Yang, H., Delort, A.-M., Amato, P., Pöschl, U., Glaux, C., Koop, T., Morris, C., 2012. Effects of atmospheric conditions on ice nucleation activity of *Pseudomonas*. *Atmospheric Chemistry and Physics.* 12:10667-10677.

Baylor, E., Baylor, M., Blanchard, D.C., Syzdek, L.D., Appel, C., 1977. Virus transfer from surf to wind. *Science.* 198:575-580.

Bigg, E., Miles, G., 1964. The results of large-scale measurements of natural ice nuclei. *Journal of the Atmospheric Sciences.* 21:396-403.

Bigg, E.K., 2007. Sources, nature and influence on climate of marine airborne particles. *Environmental Chemistry.* 4:155-161.

Blanchard, D.C., 1989. The ejection of drops from the sea and their enrichment with bacteria and other materials: a review. *Estuaries.* 12:127-137.

Blot, R., Clarke, A., Freitag, S., Kapustin, V., Howell, S., Jensen, J., Shank, L., McNaughton, C., Brekhovskikh, V., 2013. Ultrafine sea spray aerosol over the south eastern Pacific: open-ocean contributions to marine boundary layer CCN. *Sea.* 13:3279-3322.

Brosseau, L.M., Vesley, D., Rice, N., Goodell, K., Nellis, M., Hairston, P., 2000. Differences in

detected fluorescence among several bacterial species measured with a direct-reading particle sizer and fluorescence detector. *Aerosol Science & Technology.* 32:545-558.

Cavalli, F., Facchini, M., Decesari, S., Mircea, M., Emblico, L., Fuzzi, S., Ceburnis, D., Yoon, Y., O'Dowd, C., Putaud, J.P., 2004. Advances in characterization of size-resolved organic matter in marine aerosol over the North Atlantic. *Journal of Geophysical Research: Atmospheres (1984–2012).* 109.

Chi, M.-C., Li, C.-S., 2007. Fluorochrome in monitoring atmospheric bioaerosols and correlations with meteorological factors and air pollutants. *Aerosol Science and Technology*. 41:672-678.

Claeys, M., Wang, W., Vermeylen, R., Kourtchev, I., Chi, X., Farhat, Y., Surratt, J.D., Gómez-González, Y., Sciare, J., Maenhaut, W., 2010. Chemical characterisation of marine aerosol at Amsterdam Island during the austral summer of 2006–2007. *Journal of Aerosol Science.* 41:13-22.

Clarke, C.R., Cai, R., Studholme, D.J., Guttman, D.S., Vinatzer, B.A., 2010. *Pseudomonas syringae* strains naturally lacking the classical *P. syringae* hrp/hrc locus are common leaf colonizers equipped with an atypical type III secretion system. *Molecular Plant-microbe Interactions.* 23:198-210.

Cochet, N., Widehem, P., 2000. Ice crystallization by *Pseudomonas syringae*. *Applied microbiology and biotechnology.* 54:153-161.

Constantinidou, H., Hirano, S., Baker, L., Upper, C., 1990. Atmospheric dispersal of ice ucleation-active bacteria: the role of rain. *Phytopathology*. 80:934-937.

DeMott, P.J., Sassen, K., Poellot, M.R., Baumgardner, D., Rogers, D.C., Brooks, S.D., Prenni,

A.J., Kreidenweis, S.M., 2003. African dust aerosols as atmospheric ice nuclei. *Geophysical Research Letters.* 30.

Després, V.R., Huffman, J.A., Burrows, S.M., Hoose, C., Safatov, A.S., Buryak, G., Fröhlich-Nowoisky, J., Elbert, W., Andreae, M.O., Pöschl, U., 2012. Primary biological aerosol particles in the atmosphere: a review. *Tellus B.* 64.

Diehl, K., Matthias-Maser, S., Jaenicke, R., Mitra, S., 2002. The ice nucleating ability of pollen:: Part II. Laboratory studies in immersion and contact freezing modes. *Atmospheric Research.* 61:125-133.

Edwards, G., Evans, L., 1960. Ice nucleation by silver iodide: I. Freezing vs sublimation. *Journal of Meteorology.* 17:627-634.

Fahlgren, C., Bratbak, G., Sandaa, R.-A., Thyrhaug, R., Zweifel, U.L., 2011. Diversity of airborne bacteria in samples collected using different devices for aerosol collection. *Aerobiologia.* 27:107-120.

Fergenson, D.P., Pitesky, M.E., Tobias, H.J., Steele, P.T., Czerwieniec, G.A., Russell, S.C., Lebrilla, C.B., Horn, J.M., Coffee, K.R., Srivastava, A., 2004. Reagentless detection and classification of individual bioaerosol particles in seconds. *Analytical Chemistry.* 76:373-378.

Franc, G.D., 1988. Long distance transport of *Erwinia carotovora* in the atmosphere and surface water. Colorado State University.

Fu, P., Kawamura, K., Miura, K., 2011. Molecular characterization of marine organic aerosols collected during a round-the-world cruise. *Journal of Geophysical Research: Atmospheres (1984–2012).* 116.

Heidelberg, J., Shahamat, M., Levin, M., Rahman, I., Stelma, G., Grim, C., Colwell, R., 1997.

Effect of aerosolization on culturability and viability of gram-negative bacteria. *Applied and Environmental Microbiology.* 63:3585-3588.

Hirano, S.S., Upper, C.D., 2000. Bacteria in the Leaf Ecosystem with Emphasis on *Pseudomonas syringae*—a Pathogen, Ice Nucleus, and Epiphyte. *Microbiology and Molecular Biology Reviews.* 64:624-653.

Hoose, C., Kristjánsson, J., Burrows, S., 2010. How important is biological ice nucleation in clouds on a global scale? *Environmental Research Letters.* 5:024009.

Howell, W.E., 1949. The growth of cloud drops in uniformly cooled air. *Journal of Meteorology.* 6:134-149.

Hudson, J.G., 1993. Cloud condensation nuclei. *Journal of Applied Meteorology;(United States).* 32.

Jayaweera, K., Flanagan, P., 1982. Investigations on biogenic ice nuclei in the arctic atmosphere. *Geophysical Research Letters*. 9:94-97.

Jensen, P.A., Todd, W.F., Davis, G.N., Scarpino, P.V., 1992. Evaluation of eight bioaerosol samplers challenged with aerosols of free bacteria. *The American Industrial Hygiene Association Journal.* 53:660-667.

Kozloff, L., Schofield, M., Lute, M., 1983. Ice nucleating activity of *Pseudomonas syringae* and *Erwinia herbicola*. *Journal of bacteriology.* 153:222-231.

Kozloff, L., Turner, M., Arellano, F., 1991. Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes. *Journal of Bacteriology*. 173:6528-6536.

Lighthart, B., 1997. The ecology of bacteria in the alfresco atmosphere. *FEMS Microbiology Ecology.* 23:263-274.

Lighthart, B., Shaffer, B., 1994. Bacterial flux from chaparral into the atmosphere in mid-

summer at a high desert location. *Atmospheric Environment.* 28:1267-1274.

Lindahl, M., Faris, A., Wadström, T., Hjertén, S., 1981. A new test based on 'satling out' to measure relative surface hydrophobicity of bacterial cells. *Biochimica et Biophysica Acta.* 677:471-476.

Lindemann, J., Constantinidou, H.A., Barchet, W.R., Upper, C.D., 1982. Plants as sources of airborne bacteria, including ice nucleation-active bacteria. *Applied and Environmental Microbiology*. 44:1059-1063.

Lindemann, J., Upper, C., 1985. Aerial dispersal of epiphytic bacteria over bean plants. *Applied and Environmental Microbiology.* 50:1229-1232.

Lindow, S., 1983. The role of bacterial ice nucleation in frost injury to plants. *Annual Review of Phytopathology.* 21:363-384.

Lindow, S., Arny, D., Upper, C., 1978. *Erwinia herbicola*: a bacterial ice nucleus active in increasing frost injury to corn. *Phytopathology*. 68:523-527.

Lohmann, U., Diehl, K., 2006. Sensitivity studies of the importance of dust ice nuclei for the indirect aerosol effect on stratiform mixed-phase clouds. *Journal of the Atmospheric Sciences*. 63:968-982.

Lundheim, R., 2002. Physiological and ecological significance of biological ice nucleators. *Philosophical Transactions of the Royal Society of London. Series B: Biological Sciences.* 357:937-943.

Maki, L.R., Galyan, E.L., Chang-Chien, M.-M., Caldwell, D.R., 1974. Ice nucleation induced by *Pseudomonas syringae*. *Applied Microbiology*. 28:456-459.

Marks, R., Kruczalak, K., Jankowska, K., Michalska, M., 2001. Bacteria and fungi in air over the Gulf of Gdańsk and Baltic sea. *Journal of Aerosol Science*. 32:237-250.

Mason, B., 1952. The spontaneous crystallization of supercooled water. *Quarterly Journal of the Royal Meteorological Society.* 78:22-27.

Mason, B., Ludlam, F., 1951. The microphysics of clouds. *Reports on progress in physics*. 14: 147.

Mohan, S., Schaad, N., 1987. An improved agar plating assay for detecting *Pseudomonas syringae* pv. *syringae* and *P. s*. pv. *phaseolicola* in contaminated bean seed. *Phytopathology*. 77:1390-1395.

Monteil, C.L., Bardin, M., Morris, C.E., 2014. Features of air masses associated with the deposition of *Pseudomonas syringae* and *Botrytis cinerea* by rain and snowfall. *The ISME journal.*

Morris, C., Sands, D., Bardin, M., Jaenicke, R., Vogel, B., Leyronas, C., Ariya, P., Psenner, R., 2011. Microbiology and atmospheric processes: research challenges concerning the impact of airborne micro-organisms on the atmosphere and climate. *Biogeosciences*. 8:17-25.

Morris, C.E., Kinkel, L.L., Xiao, K., Prior, P., Sands, D.C., 2007. Surprising niche for the plant pathogen *Pseudomonas syringae*. *Infection, Genetics and Evolution*. 7:84-92.

Morris, C.E., Monteil, C.L., Berge, O., 2013. The Life History of *Pseudomonas syringae*: Linking Agriculture to Earth System Processes. *Annual Review of Phytopathology.*

Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H., Thompson, B.M., 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME Journal.* 2:321-334.

Mossop, S., 1955. The freezing of supercooled water. *Proceedings of the Physical Society. Section B* 68, 193.

Murray, B., O'Sullivan, D., Atkinson, J., Webb, M., 2012. Ice nucleation by particles immersed in supercooled cloud droplets. *Chemical Society Reviews*. 41:6519-6554.

Polymenakou, P.N., 2012. Atmosphere: A source of pathogenic or beneficial microbes? *Atmosphere*. 3:87-102.

Pósfai, M., Li, J., Anderson, J.R., Buseck, P.R., 2003. Aerosol bacteria over the Southern Ocean during ACE-1. *Atmospheric Research*. 66:231-240.

Pouleur, S., Richard, C., Martin, J.-G., Antoun, H., 1992. Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Applied and Environmental Microbiology*. 58:2960-2964.

Pratt, K.A., DeMott, P.J., French, J.R., Wang, Z., Westphal, D.L., Heymsfield, A.J., Twohy, C.H., Prenni, A.J., Prather, K.A., 2009. In situ detection of biological particles in cloud ice-crystals. *Nature Geoscience*. 2:398-401.

Rixen, C., Stoeckli, V., Ammann, W., 2003. Does artificial snow production affect soil and vegetation of ski pistes? A review. *Perspectives in Plant Ecology, Evolution and Systematics*. 5:219-230.

Sands, D., Langhans, V., Scharen, A., De Smet, G., 1982. The association between bacteria and rain and possible resultant meteorological implications. *Idojaras.*

Schaefer, V.J., 1952. Formation of ice crystals in ordinary and nuclei-free air. Industrial & *Engineering Chemistry*. 44:1300-1304.

Seaver, M., 1999. Size and fluorescence measurements for field detection of biological aerosols. *Aerosol Science and Technology*. 30:174-185.

Sharma, N.K., Rai, A.K., Singh, S., Brown, R.M., 2007. Airborne algae: their present status and relevance. *Journal of Phycology*. 43:615-627.

Vali, G., Christensen, M., Fresh, R., Galyan, E., Maki, L., Schnell, R., 1976. Biogenic ice nuclei. Part II: Bacterial sources. *Journal of the Atmospheric Sciences*. 33:1565-1570.

Vonnegut, B., 1947. The nucleation of ice formation by silver iodide. *Journal of Applied Physics*. 18:593-595.

Weidner, T., 2013. Uncovering the tricks of nature's ice-seeding bacteria. Catherine Meyers [cited 1 December 2013]. Available from http://www.eurekalert.org/pub_releases/2013-10/aiop-utt102313.php.

Wells, C.L, Moore, E.A., Hoag, J.A., Hirt, H., Dunny, G.M., Erlandsen, S.L., 2000. Inducible expression of *Enterococcus faecalis* aggregation substance surface protein facilitates bacterial internalization by cultured enterocytes. *Infection and Immunity*. 68:7190-7194.

Young, J., Luketina, R., Marshall, A., 1977. The effects on temperature on growth in vitro of *Pseudomonas syringae* and *Xanthomonas pruni*. *Journal of Applied Bacteriology*. 42:345-354.

Zhao, Y., Aarnink, A.J., Doornenbal, P., Huynh, T.T., Koerkamp, P.W.G., de Jong, M.C., Landman, W.J., 2011. Investigation of the efficiencies of bioaerosol samplers for collecting aerosolized bacteria using a fluorescent tracer. I: effects of non-sampling processes on bacterial culturability. *Aerosol Science and Technology*. 45:423-431.

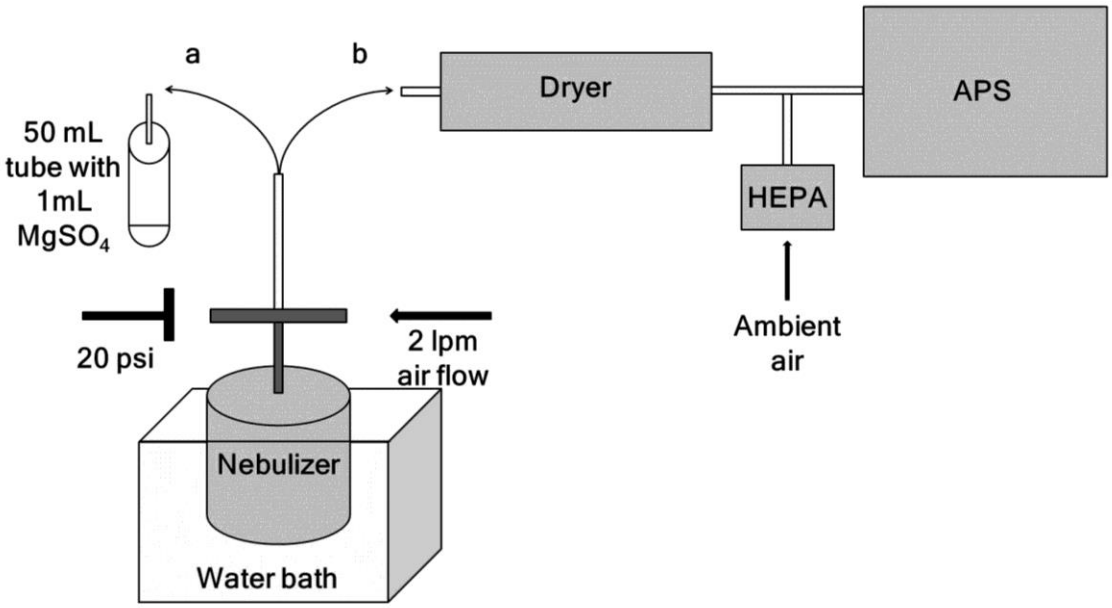

Figure 3.1. Schematic of the aerosolization equipment. Aerosol was created in the Collison nebulizer with a flow rate of 2 lpm and pressure of 20 psi and collected in 50 mL tubes containing 1 mL of 10 mM $MgSO_4$ for the indirect method (a). For the direct method (b), the aerosol passed through a diffusion dryer and into the Aerodynamic Particle Sizer (APS). Free air was allowed to enter the system through a HEPA filter prior to entering the APS.

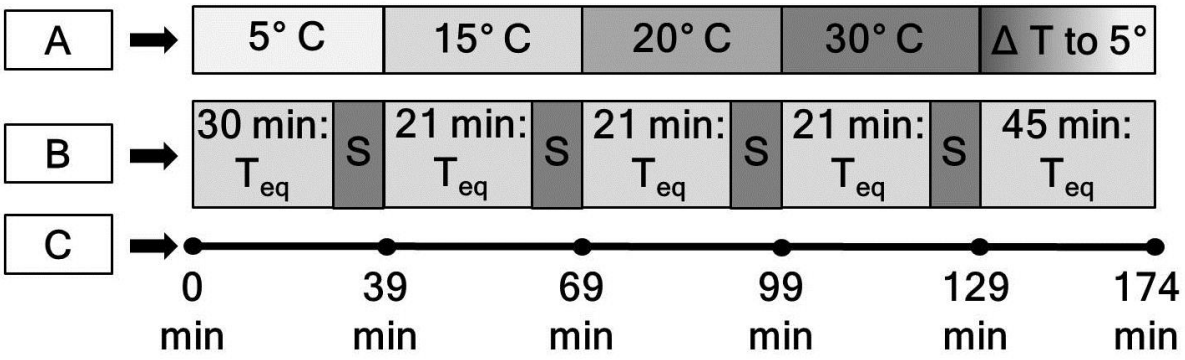

Figure 3.2. Aerosolization test schedule for each strain showing temperature of the water bath (a), testing activity (b), with wait periods in light grey and collection periods in dark grey, and a timeline (c) showing cumulative time. Total test time was 129 minutes for each strain with an additional 45 minutes between the first and second strain for the temperature to return to 5° C. A = temperature, B = activity, C = time line (not to scale), T_{eq} = temperature equilibrium, S = 3, 3 min samples (9 min).

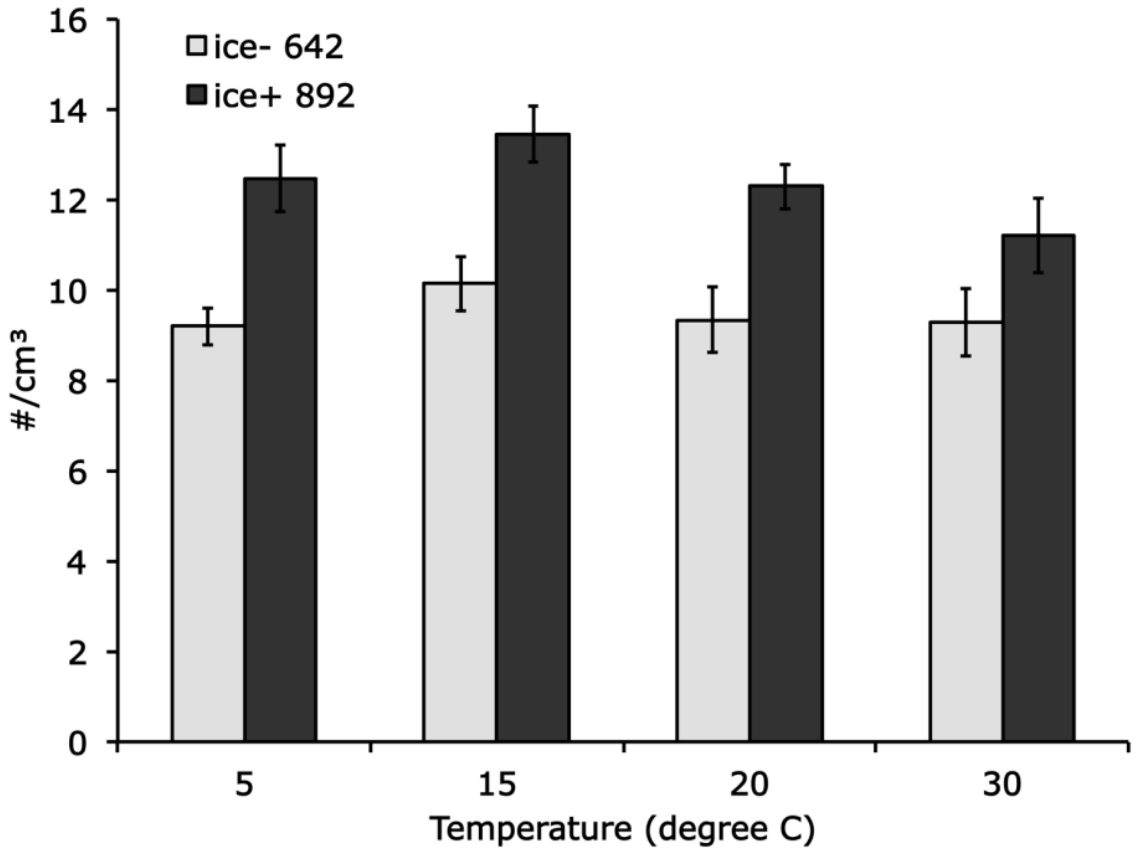

Figure 3.3. Concentration of *Pseudomonas syringae* aerosols produced by a Collison nebulizer measured for a 3 minute period with an Aerodynamic Particle Sizer at four temperatures for Ice- and Ice+ strains with n=9 in the direct particle counting method. There was a significant difference between the strains at 5°, 15°, and 20° C ($P < 0.05$), but not at 30° C ($P = 0.11$).

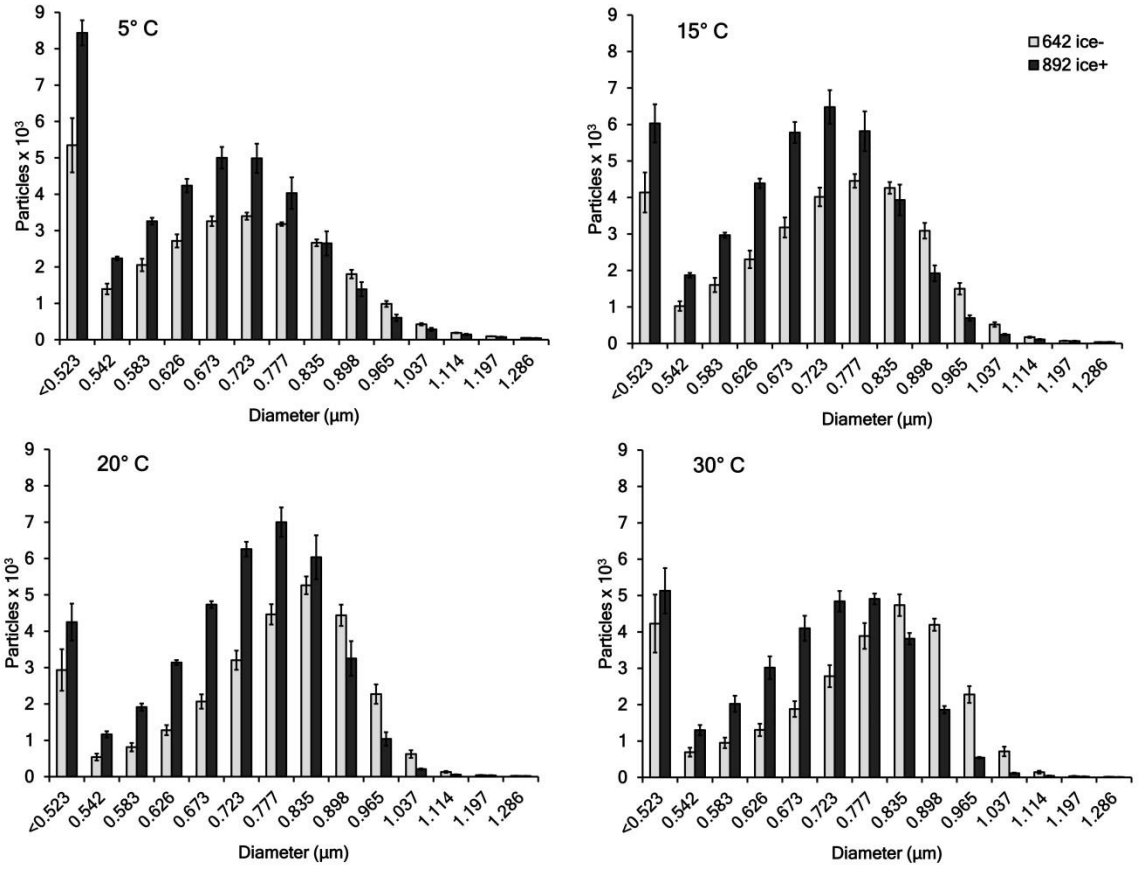

Figure 3.4. Number of aerosol particles/3 min in size bins by diameter size generated from tests with the Aerodynamic Particle Sizer for four temperatures comparing the distribution pattern for Ice- 642 and Ice+ 892 strains, n=6.

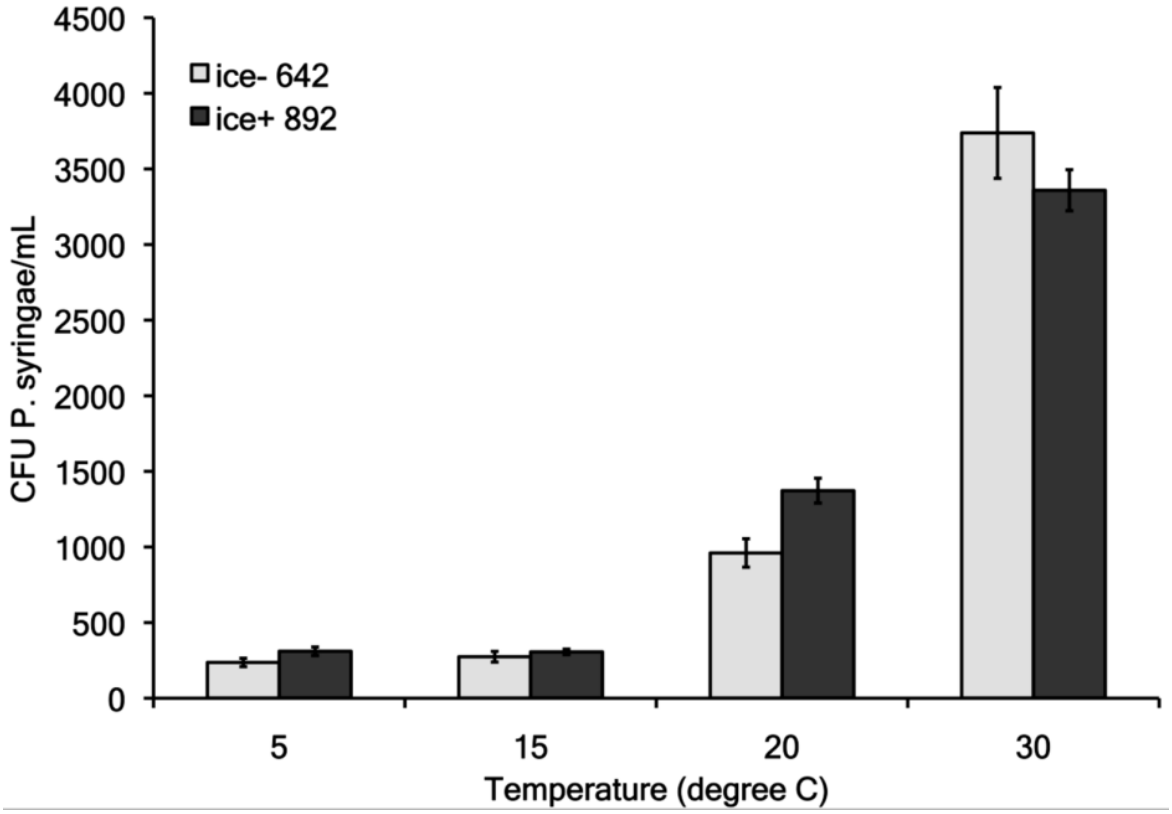

Figure 3.5. CFU of *Pseudomonas syringae* in the indirect culture method collected in 1 mL of 10 mM $MgSO_4$ over 3 minutes. Aerosols were produced by a Collison nebulizer at four temperatures for Ice- and Ice+ strains with n=9, except Ice- at 15° C where n=6. There was a significant difference between the culturability of the aerosols between the strains at 20°C (P = 0.0362).

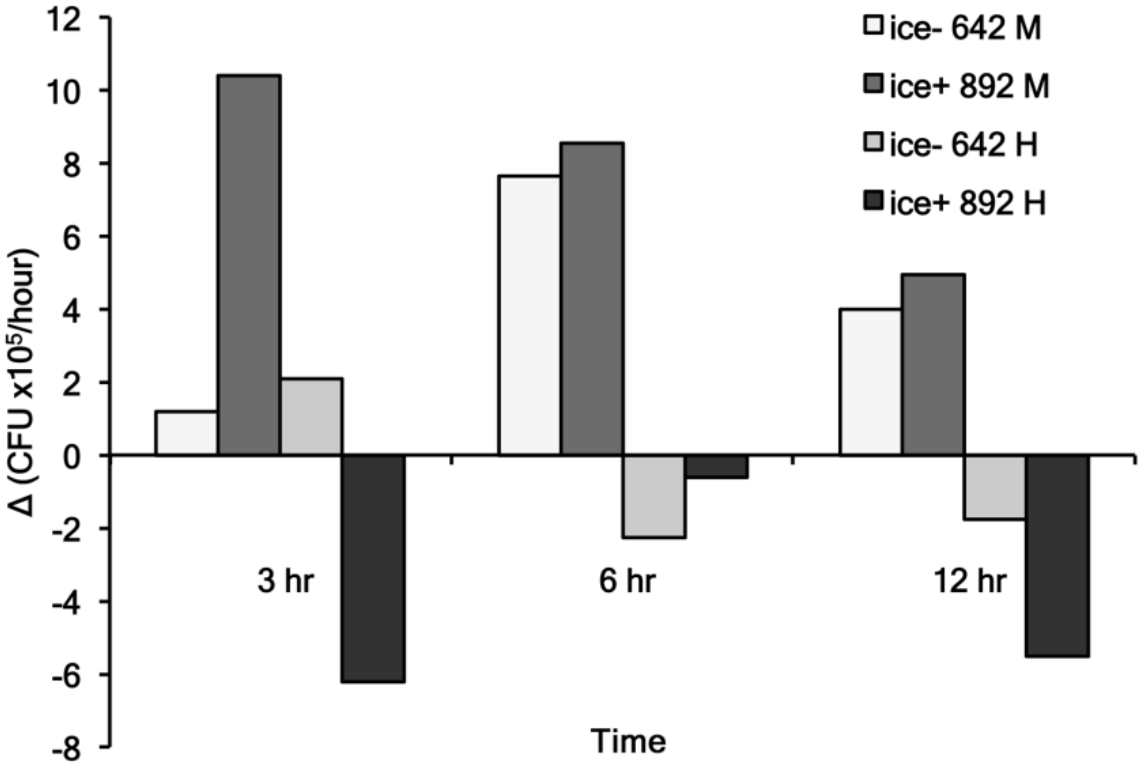

Figure 3.6. Rate of change from initial concentration of samples in the nebulizer measured in CFU for 10 µL aliquots of *Pseudomonas syringae* suspension in 10 mM $MgSO_4$ or H_2O for Ice- and Ice+ strains sampled at 3, 6, and 12 hours (n=6). M = $MgSO_4$ and H = H_2O.

Chapter 4 – Tracking the movement of microbial aerosols from aquatic systems with high-speed video

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Abstract

Aquatic environments contain a great diversity of microorganism, including bacteria. These bacteria are capable of crossing the air-water interface and entering the environments as aerosols. One natural method of aerosol production is wind moving across the surface of a body of water creating droplets that leave the water surface. The droplets are formed by two different mechanisms, either bubble bursting or fragmentation. A laboratory flume was designed to simulate wind moving across the surface of a lake. High speed video was used to capture droplets moving across the air-water interface at four wind speeds (3.5, 4.0, 4.5, and 5.0 m/s). The images were analyzed to determine the diameter as well as initial speed and angle of each droplet as it leaves the water surface. The rate of droplet production increased quadratically with wind speed, while the droplet mass flux decreased slightly before increasing with wind speed. The diameter and speed of the droplets fit gamma distributions. Droplet angle had a narrower distribution at higher wind speeds. The two mechanisms of droplet production (bubble busting and fragmentation) yielded different distributions for diameter, speed, and angle. The capacity for bacterial flux into the atmosphere was calculated based on the droplet production and bacterial concentrations in freshwater systems. The results show significant amounts of bacteria are crossing the air water interface with the potential to be aerosolized. Aerosolized bacteria have the potential to impact the Earth's radiation budget, precipitation processes, and pathogen transport.

Introduction

Microorganisms are present in every type of terrestrial environment and they are aerosolized into the atmosphere from terrestrial surfaces (Burrows et al., 2009). The global mass of emitted bacteria is estimated from data in terrestrial environments to be between 40 and 1900 Gg per year (Burrows et al., 2009). Microorganisms are also found in aquatic environments, both freshwater and seawater and can aerosolize from aquatic surfaces (Blanchard et al., 1981; Gantt and Meskhidze, 2013). Droplets containing microorganisms are produced from the surface of the water, liberating microorganisms (Baylor and Baylor, 1980) into the atmosphere where they may be involved in atmospheric processes, including cloud formation as cloud condensation nuclei (Charlson et al., 1987; Dinger et al., 1970; O'Dowd et al., 1999; Orellana et al., 2011; Park et al., 2014; Pierce and Adams, 2006) or ice nuclei (Baldy and Bourguel, 1987; Baylor et al., 1977a; Bigg and Leck, 2008; Blanchard and Woodcock, 1957; Blanchard, 1989; Blanchard and Syzdek, 1970; Christner et al., 2008; Morris et al., 2014; Prather et al., 2013). Aerosolized microorganisms can also affect the earth's radiation budget (Gabric et al., 2005; Haywood et al., 1999; Park et al., 2014; Slingo, 1990) and pathogen dispersal (Polymenakou, 2012). Once airborne, particles can be carried great distances (Schmale III and Ross, 2015). Baylor and Baylor (1980) report marine algae and diatoms 100 miles downwind from the sea coast in rhyme frost on top of Mt. Washington in New Hampshire (Baylor and Baylor, 1980). Mayol et al. (2014) showed that approximately 10% of microbes in the boundary later at a given time were still airborne 4 days later giving them the potential to travel up to 11,000 km before deposition.

Microorganisms are liberated into the atmosphere from droplets, which are described as fluid volumes bound by immiscible interfaces characterized by an interfacial tension (Bourouiba and Bush 2013). Droplets are produced by several mechanisms including bubble bursting and

spume droplets tearing off of breaking waves by fragmentation (Wu, 1981). Bubble bursting has received the most study and is usually considered the major contributor to droplet production (Baldy and Bourguel, 1987; Wu, 1981). Wind generates breaking waves which inject air below the surface of the water forming a spectrum of bubbles (Bigg and Leck, 2008; Blanchard, 1989). Air bubbles rise and burst at the sea surface producing film and jet droplets which enter the atmosphere (D O'Dowd and De Leeuw, 2007; Sellegri et al., 2006). The size distribution of bubbles in the water and at the water surface has been characterized (Cipriano and Blanchard, 1981a; Spiel, 1998) as well as the relationship between bubble size and the number, size, ejection speed and ejection height of droplets (Blanchard, 1989; Cipriano and Blanchard, 1981b; Spiel, 1995). Size distributions for droplets have been studied as well (Spiel, 1994).

Increasing wind speed is connected to increasing whitecaps and increasing bubble production (Blanchard and Woodcock, 1957). Wind speed varies with height above the water surface making the height at which the wind speed was recorded important when considering the findings. A reference height of 10 m above the water surface is commonly used. According to Blanchard (1989) breaking waves form at wind speeds of 3 to 4 m/s. Hamilton and Lenton (1998) report breaking waves occur at wind speeds above 20 km/hr or 5.6 m/s. The whitecap coverage increases rapidly with more than the third power of wind speed (Blanchard, 1989). Fragmentation droplets form after a threshold wind speed is reached which according to Monahan et al. (1983) is around 10 m/s measured at the reference height of 10 m above the water surface.

Bubble bursting is usually considered the primary means of bacterial aerosolization from aquatic surfaces (Blanchard, 1989). According to Baldy and Bourguel (1987) the bubble population at the air-water interface controls the flux of aerosol into the atmosphere. Wu (1981)

reports that fragmentation droplets are only formed at higher wind speeds, but at high wind speeds bubble bursting increases at a higher rate than fragmentation. Fragmentation droplets also tend to fall back into the water quickly reducing their potential for aerosolization (Wu, 1981). This is likely due to a combination of less vertical angle and larger droplet size. Taking these two factors together bubble bursting may still be the major contributing mechanism for aerosolization even at high wind speeds, but further research is needed.

A variety of experimental methods and modeling approaches have been used to study droplet production. These methods include holography and photography techniques (Koga, 1982; Leifer et al., 2000; Resch et al., 1986), field experiments (Wu et al., 1984), and laboratory experiments with a wind wave tank (Koga, 1981). Monahan et al. (1982) modeled droplet production from a simulation tank and Andreas (1998) developed a numerical model. Several of the experimental studies have focused on one method of generating bubbles, such as entraining air to produce bubbles (Cipriano and Blanchard, 1981a; Wu, 1989), rather than examining natural production of droplets. Less research has been done on fragmentation droplets and examining contributions from the two mechanisms together. Wu (1993) developed a model for fragmentation droplet generation and compared it to a model from a field experiment and another from a wind wave tank. The three models showed considerable variability and wide discrepancies indicating fragmentation droplets are not well understood and further characterization is necessary.

The amount of microorganisms aerosolized from aquatic environments is difficult to estimate. Andreae (1995) and de Leeuw et al. (2011) report global aerosol from the ocean at 1,300–3,300 Tg/yr. Mayol et al. (2014) reports the atmospheric boundary layer contains an estimated 6×10^4 to 1.6×10^7 microbes per m^2 of ocean, but not all of these bacteria necessarily

aerosolized from the ocean. They may have aerosolized from other sources and been transported in the atmosphere. Better estimates of the aerosol production rate - specifically how wind speed influences the aerosolization rate - would be helpful in estimating the impact of aerosolized microorganisms. Bacteria cross the air-water interface in droplets, thus studying droplet production can give a better understanding of bacterial aerosolization from aquatic environments. Bubble bursting droplets and fragmentation droplets have mostly been studied separately. The approach we used sought to simulate the action of the wind and examine the droplets produced by both bubble bursting and fragmentation droplets simultaneously. Towards this the objective of this study was to use high speed video to visualize droplets produced from a wind wave tank at 4 different wind speeds near the critical wave breaking speed. The diameter, initial speed, and initial angle of droplets were determined as well as the production mechanism (either bubble bursting droplet or fragmentation). Information about droplet production was then related to bacteria concentrations in water to further understand aquatic aerosolization processes.

Methods and Materials

Flume Design

A flume was constructed from 0.635 cm Plexiglas with dimensions of 91.4 cm x 3.8 cm x 35.6 cm (lwh) **(Figure 4.1).** The flume was a closed system with one 1.27 cm diameter inlet pipe reaching to the surface of the water and one 1.27 cm diameter outlet pipe exiting near the water surface. The inlet pipe had a nozzle shape designed to direct water away from the side of the flume minimizing droplets landing on the flume side. The flume was filled with water to a height of 9.8 cm. The flume outlet was connected to a vacuum that pulled air across the water surface simulating wind moving on the water surface. The vacuum was controlled with a 5 Amp

FS-5F single pole 120 V rotary dimmer switch (Lutron, Pennsylvania, USA). A 29 series II multimeter (Fluke, Washington, USA) with a resolution of 0.1 V and accuracy of ± 1% + 2 was used to measure the voltage of the dimmer switch. A EA-3010 anemometer (LaCrosse Technology , Wisconsin, USA) with a resolution of 0.1 m/s was used to measure wind speed. The wind speed was measured at 2.5 cm above the water surface and on the downwind edge of the camera's field of view. The outlet valve to the vacuum was adjusted until the anemometer fluctuated by ± 0.1 m/s of the target speed. After an initial calibration was made the wind speed was controlled only with the multimeter. A SpectroLED-14 light (Genray, New York, USA) was placed behind the flume. A Photron FASTCAM Mini UX100 camera (Photron, California, USA) with a micro-Nikkor 105mm f/28 lens (Nikon, New York, USA) was used to record video data at 6250 frames per second in 0.9 second increments. The camera's field of view was 6.6 cm of length by 4.1 cm of height. The entire 3.8 cm width of the flume was in focus. Videos were captured at four wind speeds (3.5 m/s, 4.0 m/s, 4.5 m/s and 5.0 m/s).

Data Analysis

The data were saved as TIFF images and analyzed with a MATLAB image processing technique. The images were made black and white, and a threshold was set to capture the droplets. The centroid of each droplet was determined and the trajectory of each droplet was found across a stack of TIFF images. The diameter of the droplet was determined from the centroid. The speed and angle of the droplet was determined from a line tangent to the droplet trajectory as close to the water surface as possible. The tangent line was determined for each droplet as close to the water as possible to reduce error due to droplet movement being effected by the artificial method of wind generation. The droplets were categorized according to the

mechanism of droplet production: bubble bursting droplet or fragmentation droplet or unknown **(Figure 4.2).** At the 3.5 and 4.0 m/s wind speeds the bubble bursting and fragmentation droplet data sample sizes were both large enough to be further analyzed. The lowest resolution of droplet diameter we were able to calculate was 52 μm based on the resolution of the video.

Results

Rate of droplet production

The rate of droplet production in droplets per second of video are given in relationship to wind speed **(Figure 4.3).** The data fits a second order polynomial with an R^2 value of 0.9715 showing a slight increase in droplet production from 3.5 to 4.0 m/s wind speeds followed by a drastic increase in droplet production at higher wind speeds (**Figure 4.3** and **Table 4.1)**. The mass flux was calculated for each of the four wind speeds dividing the droplets into size groups based on diameter. The mass of droplets with diameters <100 μm and 100-1000 μm increases quadratically with wind speed. The mass of droplets with diameters > 1000 μm decreases slightly at the 4.0 m/s wind speed before beginning a trend of increasing drastically at the 4.5 and 5.0 m/s. Looking at the droplets of all diameters the trend is the same as for the droplets with diameters >1000 μm as their mass is great enough to dominate the whole trend.

Distributions of droplet diameter, speed, and angle

Histograms for droplet diameter at each wind speed (3.5, 4.0, 4.5, and 5.0 m/s) show the best fit with a gamma distribution **(Figure 4.5** and **Table 4.2)**. A log normal distribution (not shown) was also fit to the data, but the gamma distribution was a better representation of the experimental data. The mean droplet diameter increases with wind speed as does the median

droplet diameter, with the exception of the median diameter being higher at 4 m/s compared to 3.5 m/s. Variance also increases with wind speed. Histograms for droplet speed at each wind speed also show a good fit a gamma distribution **(Figure 4.6** and **Table 4.3)**. The mean wind speed is highest at the 4.0 m/s wind speed, followed by the 4.5 and 5.0 m/s wind speeds. The 3.5 m/s wind speed has the lowest mean droplet speed. The shape parameter (k) and scale parameter (θ) are also given to fully characterize the gamma distributions of both droplet diameter and droplet speed. Probability distributions for the droplet angle in polar coordinates for the four wind speeds show a wider angle of distribution in the 3.5 and 4.0 m/s wind speeds compared to the 4.5 and 5.0 m/s wind speeds. Zero degrees is the direction of the wind, which is to the right horizontally and shown in the same orientation on the polar plot **(Figure 4.7)**.

Distributions of diameter, speed, and angle for bubble bursting and fragmentation droplets

The droplet data at the 3.5 and 4.0 m/s data were divided into categories of bubble bursting, fragmentation, and unknown, based on mechanism of droplet production. The diameter **(Figure 4.9)** and speed **(Figure 4.10)** of droplets from bubble bursting and fragmentation droplets indicate, in general, the fragmentation droplets have a wider distribution while the bubble bursting droplets have a narrower range of distribution focused at the smaller end of the distribution. Polar plots for the angle distributions of bubble bursting and fragmentation droplets indicate the bubble busting droplets in general are between 20° and 100° while the fragmentation droplets are between -40° (320°) and 70° **(Figure 4.8)**.

Discussion

A relationship between droplet production and wind speed has long been suspected (Blanchard and Woodcock, 1957). The data presented here confirms a relationship **(Figure 4.3)**. We also found that droplet production increases quadratically with wind speed over the range of wind speeds studied. This relationship implies that even small increases in wind speed can significantly increase the droplet production and mass emission. For each wind speed the mass of water produced by the droplets per second per cm^2 of water surface was calculated **(Figure 4.4)**. Unlike the rate of droplet production, the mass flux of all size droplets decreases slightly moving from 3.5 to 4.0 m/s before drastically increasing at the 4.5 and 5.0 m/s wind speed. Further insight into this trend was obtained by dividing the droplets into size groups. The mass of the droplets with diameters below 100 μm and between 100 and1000 μm increases with wind speed across all wind speeds and does not show the slight decrease at 4.0 m/s. Even though the rate of droplet production is lower at 3.5 m/s, more large droplets are produced at the 3.5 m/s wind speed leading to the greater mass flux. This finding demonstrates the complex and often competing interaction between parameters that effect droplet production making an estimate of aerosolized bacteria challenging.

The droplet diameters are well represented by a gamma distribution at all four wind speeds. The mean and median droplet diameter increases with wind speed, with the exception of the median diameter at the 4.0 m/s wind speed which is lower than the median diameter at the 3.5 m/s wind speed **(Table 4.2)**. The increase in variance indicates the distribution range of the droplets increases with wind speed. More large drops are produced, but particularly significant is that largest drop size increases drastically. The resolution of the camera lens in this experimental set up made 52 μm the smaller diameter droplet we could measure. The gamma distribution

could be used to estimate the number of droplets with diameters smaller than 52 μm. Smaller droplets are more likely to move through the boundary layer and thus more important for aerosolization. Droplet diameters have previously been studied (Koga, 1981; Resch et al., 1986; Spiel, 1998) as well as the distribution of bubbles before bursting (Leifer et al., 2000). Villermaux (2007) looked at droplet size distributions and fitted Poisson, gamma, lognormal, and exponential fits finding gamma distributions matched the data best. Gamma distributions have previously been seen for droplet diameters in fragmentation droplets breaking off of ligaments of water as well as for natural spray (Bremond and Villermaux, 2006; Ling et al., 2015; Villermaux et al., 2004).

The distribution of droplet speed also showed a gamma distribution **(Figure 4.6)**. The 3.5 m/s data had the lowest mean and median droplet speed with the other three wind speeds showing similar values to each other for both mean and median wind speed indicating, while wind speed makes substantial effects on other parameters, the effect on droplet speed is negligible **(Table 4.3)**.

At higher wind speeds the distribution of droplet angles was narrower **(Figure 4.7)**. For the lower two wind speeds (3.5 and 4.0 m/s) the droplets ranged from below the horizontal (~ -30°) to slightly beyond the vertical (~100°), but at the higher two wind speeds the distribution was more narrow ranging between 0° and 60°. The three parameters (diameter, speed, and angle) were compared in every combination of pairs and no correlation was seen between any of the parameters.

At the two lower wind speeds (3.5 and 4.0 m/s) differences were seen in the angle, diameter, and speed distributions of bubble bursting droplets and fragmentation droplets, which could effect aerosolization. A narrower range of both droplet diameters and speeds was observed

for bubble bursting data. The mechanism of formation for bubble bursting droplets produces droplets within certain parameters while fragmentation droplets have no upper limit in size (Andreas, 1998). The angle for fragmentation droplets is between -40° (320°) and 70° while the bubble bursting droplets range from 20° to 100°. The bubble bursting droplets tend to be ejected closer to the vertical while the fragmentation droplets are torn off of breaking waves with an angle directed near the horizontal, downwind direction. We were not able to determine the mechanism of production for a majority of the droplets at higher wind speed due to the noise of droplet splatter on the flume surface. We observed far fewer bubble bursting droplets at higher wind speed, but this observation may be a reflection of the inability to see them in our experimental set up.

This study examined the contributions to droplet production from both bubble bursting droplets and fragmentation droplets at four wind speeds. Looking at the three parameters together, as the wind speed increases, the diameter range increases, the angle decreases, and the speed doesn't show much change. The rate of droplet production increases quadratically with wind speed. These compensating factors all combine to influence the amount of aerosolized microorganisms. Once droplets leave the water surface they must pass through the boundary layer allowing them to be free to travel through the atmosphere. Further work needs to be done to follow the trajectory of the droplets to determine which droplets are able to pass through the boundary layer into the mixing layer where they may travel great distances as aerosols. The angle, diameter, and speed of the droplet combine to determine the probability of a droplet reaching through the boundary layer. An ideal combination of parameter values could then be determined for aerosolization.

In a natural system droplets may contain bacteria, which can be aerosolized as they cross the air-water interface. An estimated amount of bacteria capable of being aerosolized can be calculated from the droplet flux observed in this experiment. The concentration of bacteria in a lake varies from one lake to another and in different conditions within one lake. The bacterial concentration in lakes is reported to range between 7.2×10^4 and 1.34×10^7 cells/mL (Bird and Kalff, 1984; Coveney, 1982; Field et al., 1980). Taking this range of concentrations, and the droplet flux we observed, an estimate of the amount of bacteria moving across the air-water interface can be calculated. **Table 4.4** shows a low and high estimate based on the rage of bacterial concentrations cited above for three size divisions of droplet size at each of the four wind speeds. While producing the least amount of bacteria, the droplets of diameter <100 μm are most likely to be able to move through the boundary layer and thus most important to examine in the context of capacity to aerosolize bacteria. At wind speed of 3.5 m/s, 0.12 cells leave the water per sec per cm^2 of water surface, this number increases to 0.64, 1.81, and 3.07 for 4.0m/s, 4.5 m/s, and 5.0 m/s wind speed, respectively. Wind speeds are not usually sustained over the whole surface of a body of water, but rather tend to come in an intermittent nature. Given these rates of bacterial flux the capacity exists to aerosolize significant quantities of bacteria, particularly as wind speed increases. Estimates of global aerosolization taken from terrestrial environment measurements in Burrows et al. (2009) as a flux of bacteria per sec per cm^2 of area are 4.35×10^{-3} to 2.06×10^{-2}. It is important to note these are estimates of aerosolized bacteria while the numbers calculated from this experiment are bacteria crossing the air-water interface with the potential for aerosolization. However it does show that aquatic aerosolization has the potential to be a significant contributor to aerosolized bacteria. With the large amount of the Earth's surface covered in water aquatic aerosolization has the potential to be as significant as, or

more significant than, terrestrial aerosolization. Further work can be done on the amount of bacteria present in droplets as it may not be the same as the concentration in the bulk of the water and is likely higher. Baylor et al. (1977b) and Blanchard and Syzdek (1982) found that concentrations of microoroganisms in droplets could exceed the bulk water concentration by 10 to 100 times. These numbers would further increase the amount of potentially aerosolizeable bacteria.

Further understanding of droplet production can give us information about the movement of a variety of particles and molecules across the air-water interface and the fate of the particle once they have crossed the interface. In particular, bacteria move from the water into the air in droplets. With the ubiquitous presence, and great diversity of bacteria in the world, we are just beginning to understand all the negative and positive roles bacteria play throughout a multitude of processes on Earth. Understanding the sources and mechanisms for bacteria to aerosolize will give us greater understanding of long distance atmospheric transport as well as aid in designing management strategies for pathogenic bacteria and can also help shed light on how bacteria may be involved in atmospheric processes.

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References

Andreae, M.O. 1995. Climatic effects of changing atmospheric aerosol levels. *World survey of climatology*. 16:347-398.

Andreas, E.L. 1998. A new sea spray generation function for wind speeds up to 32 m s-1. *Journal of Physical Oceanography*. 28:2175-2184.

Baldy, S., and M. Bourguel. 1987. Bubbles between the wave trough and wave crest levels. *Journal of Geophysical Research: Oceans (1978–2012)*. 92:2919-2929.

Baylor, E., M. Baylor, D.C. Blanchard, L.D. Syzdek, and C. Appel. 1977a. Virus transfer from surf to wind. *Science*. 198:575-580.

Baylor, E.R., and M.B. Baylor. 1980. Surf-to-wind transfer of viruses. *Annals of the New York Academy of Sciences*. 353:201-208.

Baylor, E.R., V. Peters, and M.B. Baylor. 1977b. Water-to-air transfer of virus. *Science*. 197:763-764.

Bigg, E.K., and C. Leck. 2008. The composition of fragments of bubbles bursting at the ocean surface. *Journal of Geophysical Research: Atmospheres*. 113.

Bird, D., and J. Kalff. 1984. Empirical relationships between bacterial abundance and chlorophyll concentration in fresh and marine waters. *Canadian Journal of Fisheries and Aquatic Sciences*. 41:1015-1023.

Blanchard, D., and A. Woodcock. 1957. Bubble formation and modification in the sea and its meteorological significance. *Tellus a*. 9.

Blanchard, D.C. 1989. The ejection of drops from the sea and their enrichment with bacteria and other materials: a review. *Estuaries*. 12:127-137.

Blanchard, D.C., and L. Syzdek. 1970. Mechanism for the water-to-air transfer and concentration of bacteria. *Science*. 170:626-628.

Blanchard, D.C., and L.D. Syzdek. 1982. Water-to-air transfer and enrichment of bacteria in drops from bursting bubbles. *Applied and environmental microbiology*. 43:1001-1005.

Blanchard, D.C., L.D. Syzdek, and M.E. Weber. 1981. Bubble scavenging of bacteria in freshwater quickly produces bacterial enrichment in airborne jet drops. *Limnology and oceanography*. 26:961-964.

Bremond, N., and E. Villermaux. 2006. Atomization by jet impact. *Journal of Fluid Mechanics*. 549:273-306.

Burrows, S., T. Butler, P. Jöckel, H. Tost, A. Kerkweg, U. Pöschl, and M. Lawrence. 2009. Bacteria in the global atmosphere–Part 2: Modeling of emissions and transport between different ecosystems. *Atmospheric Chemistry and Physics*. 9:9281-9297.

Charlson, R.J., J.E. Lovelock, M.O. Andreae, and S.G. Warren. 1987. Oceanic phytoplankton, atmospheric sulphur, cloud albedo and climate. *Nature*. 326:655-661.

Christner, B.C., C.E. Morris, C.M. Foreman, R. Cai, and D.C. Sands. 2008. Ubiquity of biological ice nucleators in snowfall. *Science*. 319:1214-1214.

Cipriano, R.J., and D.C. Blanchard. 1981a. Bubble and aerosol spectra produced by a laboratory 'breaking wave'. *Journal of Geophysical Research: Oceans (1978–2012)*. 86:8085-8092.

Cipriano, R.J., and D.C. Blanchard. 1981b. Bubble and aerosol spectra produced by a laboratory 'breaking wave'. *Journal of Geophysical Research: Oceans*. 86:8085-8092.

Coveney, M.F. 1982. Bacterial uptake of photosynthetic carbon from freshwater phytoplankton. *Oikos*:8-20.

D O'Dowd, C., and G. De Leeuw. 2007. Marine aerosol production: a review of the current knowledge. *Philosophical Transactions of the Royal Society of London A: Mathematical, Physical and Engineering Sciences*. 365:1753-1774.

de Leeuw, G., E.L. Andreas, M.D. Anguelova, C. Fairall, E.R. Lewis, C. O'Dowd, M. Schulz, and S.E. Schwartz. 2011. Production flux of sea spray aerosol. *Reviews of Geophysics*. 49.

Dinger, J., H. Howell, and T. Wojciechowski. 1970. On the source and composition of cloud nuclei in a subsident air mass over the North Atlantic. *Journal of the atmospheric sciences*. 27:791-797.

Field, J., C. Griffiths, E. Linley, R. Carter, and P. Zoutendyk. 1980. Upwelling in a nearshore marine ecosystem and its biological implications. *Estuarine and Coastal Marine Science*. 11:133-150.

Gabric, A.J., B. Qu, P. Matrai, and A.C. Hirst. 2005. The simulated response of dimethylsulfide production in the Arctic Ocean to global warming. *Tellus B*. 57:391-403.

Gantt, B., and N. Meskhidze. 2013. The physical and chemical characteristics of marine primary organic aerosol: a review. *Atmospheric Chemistry and Physics*. 13:3979-3996.

Hamilton, W., and T. Lenton. 1998. Spora and Gaia: how microbes fly with their clouds. *Ethology Ecology & Evolution*. 10:1-16.

Haywood, J., V. Ramaswamy, and B. Soden. 1999. Tropospheric aerosol climate forcing in clear-sky satellite observations over the oceans. *Science*. 283:1299-1303.

Koga, M. 1981. Direct production of droplets from breaking wind-waves—its observation by a multi-colored overlapping exposure photographing technique. *Tellus*. 33:552-563.

Koga, M. 1982. Bubble entrainment in breaking wind waves. *Tellus*. 34:481-489.

Leifer, I., G. de Leeuw, and L. Cohen. 2000. Secondary bubble production from breaking waves: The bubble burst mechanism. *Geophysical Research Letters*. 27:4077-4080.

Ling, Y., D. Fuster, and G. Tryggvason. 2015. Spray formation: an inverse cascade. *arXiv preprint arXiv:1511.04234*.

Mayol, E., M.A. Jiménez Cortés, G.J. Herndl, C.M. Duarte, and J.M. Arrieta. 2014. Resolving the abundance and air-sea fluxes of airborne microorganisms in the North Atlantic Ocean.

Monahan, E., K. Davidson, and D. Spiel. 1982. Whitecap aerosol productivity deduced from simulation tank measurements. *Journal of Geophysical Research: Oceans (1978–2012)*. 87:8898-8904.

Monahan, E.C., C.W. Fairall, K.L. Davidson, and P.J. Boyle. 1983. Observed inter-relations between 10m winds, ocean whitecaps and marine aerosols. *Quarterly Journal of the Royal Meteorological Society*. 109:379-392.

Morris, C.E., F. Conen, J. Alex Huffman, V. Phillips, U. Pöschl, and D.C. Sands. 2014. Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology*. 20:341-351.

O'Dowd, C.D., J.A. Lowe, M.H. Smith, and A.D. Kaye. 1999. The relative importance of non-sea-salt sulphate and sea-salt aerosol to the marine cloud condensation nuclei population: An improved multi-component aerosol-cloud droplet parametrization. *Quarterly Journal of the Royal Meteorological Society*. 125:1295-1313.

Orellana, M.V., P.A. Matrai, C. Leck, C.D. Rauschenberg, A.M. Lee, and E. Coz. 2011. Marine microgels as a source of cloud condensation nuclei in the high Arctic. *Proceedings of the National Academy of Sciences*. 108:13612-13617.

Park, J.Y., M. Kim, S. Han, S. Lim, G. Kim, and K. Park. 2014. Measurement of insoluble submicrometer particles and biological materials in seawater to investigate marine aerosol production. *Journal of aerosol science*. 75:22-34.

Pierce, J.R., and P.J. Adams. 2006. Global evaluation of CCN formation by direct emission of sea salt and growth of ultrafine sea salt. *Journal of Geophysical Research: Atmospheres*. 111.

Polymenakou, P.N. 2012. Atmosphere: a source of pathogenic or beneficial microbes? *Atmosphere*. 3:87-102.

Prather, K.A., T.H. Bertram, V.H. Grassian, G.B. Deane, M.D. Stokes, P.J. DeMott, L.I. Aluwihare, B.P. Palenik, F. Azam, and J.H. Seinfeld. 2013. Bringing the ocean into the laboratory to probe the chemical complexity of sea spray aerosol. *Proceedings of the National Academy of Sciences*. 110:7550-7555.

Resch, F., J. Darrozes, and G. Afeti. 1986. Marine liquid aerosol production from bursting of air bubbles. *Journal of Geophysical Research: Oceans*. 91:1019-1029.

Schmale III, D.G., and S.D. Ross. 2015. Highways in the sky: Scales of atmospheric transport of plant pathogens. *Annual review of phytopathology*. 53:591-611.

Sellegri, K., C. O'Dowd, Y. Yoon, S. Jennings, and G. de Leeuw. 2006. Surfactants and submicron sea spray generation. *Journal of Geophysical Research: Atmospheres*. 111.

Slingo, A. 1990. Sensitivity of the Earth's radiation budget to changes in low clouds.

Spiel, D.E. 1994. The number and size of jet drops produced by air bubbles bursting on a fresh water surface. *Journal of Geophysical Research: Oceans*. 99:10289-10296.

Spiel, D.E. 1995. On the births of jet drops from bubbles bursting on water surfaces. *Journal of Geophysical Research: Oceans*. 100:4995-5006.

Spiel, D.E. 1998. On the births of film drops from bubbles bursting on seawater surfaces. *Journal of Geophysical Research: Oceans*. 103:24907-24918.

Villermaux, E. 2007. Fragmentation. *Annu. Rev. Fluid Mech.* 39:419-446.

Villermaux, E., P. Marmottant, and J. Duplat. 2004. Ligament-mediated spray formation. *Physical Review Letters*. 92:074501.

Wu, J. 1981. Evidence of sea spray produced by bursting bubbles. *Science*. 212:324-326.

Wu, J. 1989. Contributions of film and jet drops to marine aerosols produced at the sea surface. *Tellus B.* 41:469-473.

Wu, J. 1993. Production of spume drops by the wind tearing of wave crests: The search for quantification. *Journal of Geophysical Research: Oceans (1978–2012)*. 98:18221-18227.

Wu, J., J.J. Murray, and R.J. Lai. 1984. Production and distributions of sea spray. *Journal of Geophysical Research: Oceans*. 89:8163-8169.

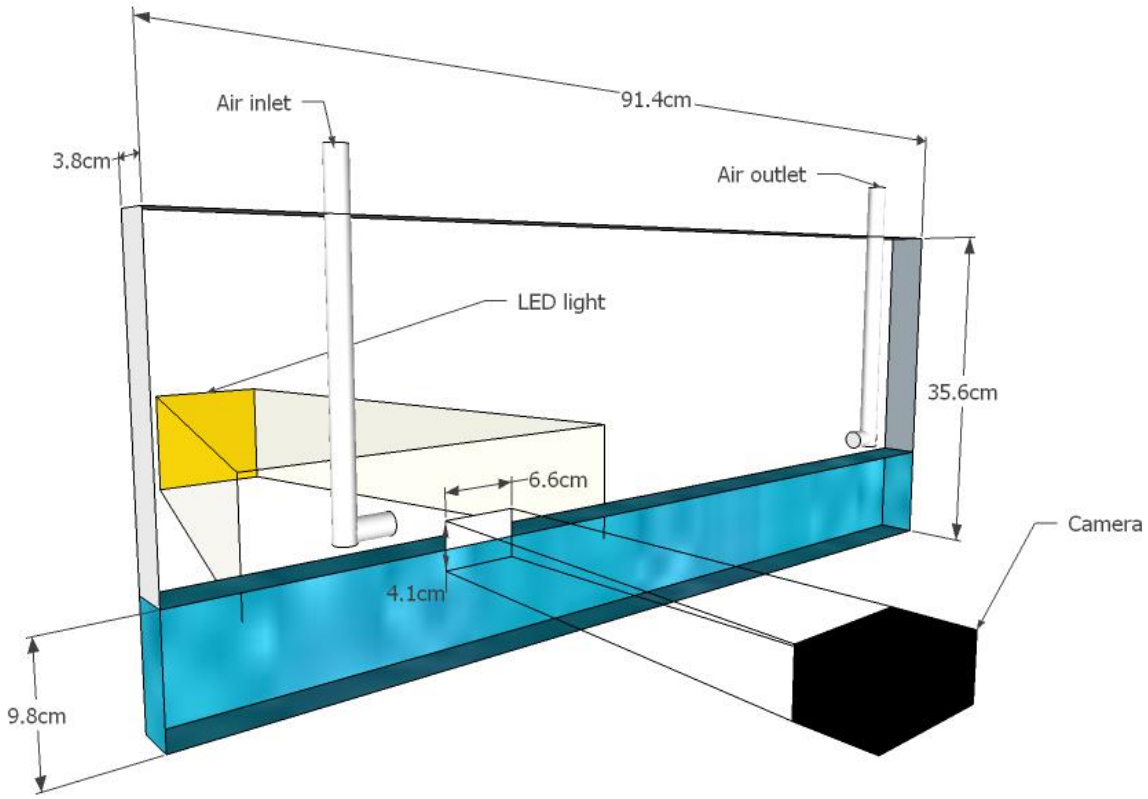

Figure 4.1. Schematic showing the experimental set up with the flume, water in the flume to 9.8 cm depth, LED back light, and Photron high speed camera. The camera viewed an area of 6.6 cm by 4.1 cm at the air-water interface. The air outlet was connected to a vacuum which pulled air across the water surface and the air inlet allowed air to enter the system.

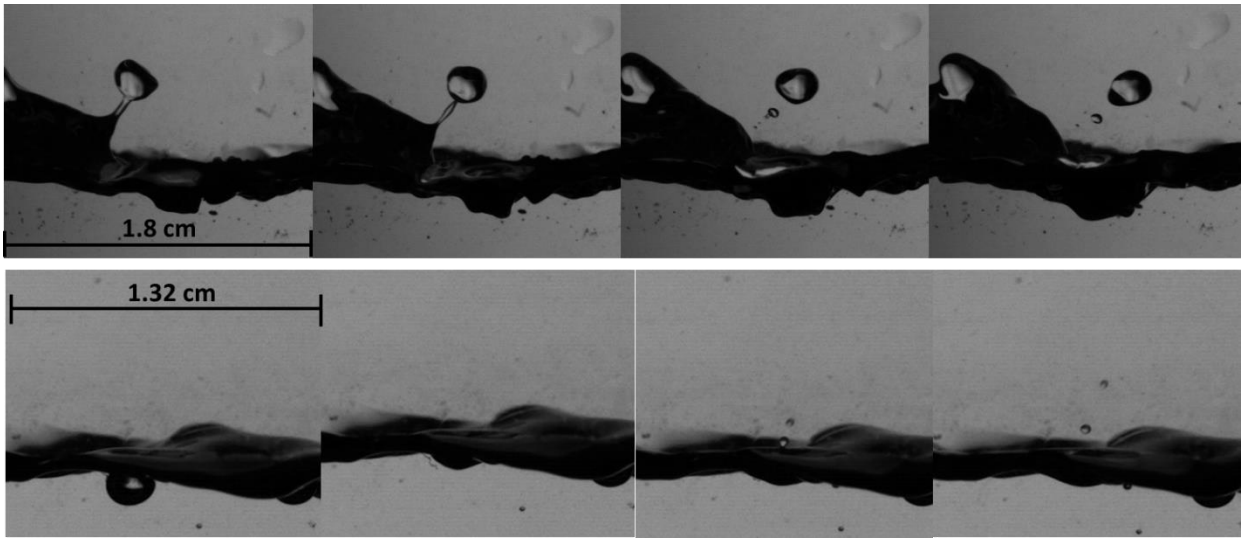

Figure 4.2. Series of high speed images recorded at 6250 fps showing fragmentation droplet formation (top) and bubble bursting droplet formation (bottom). Each series shows four images 10 frames apart.

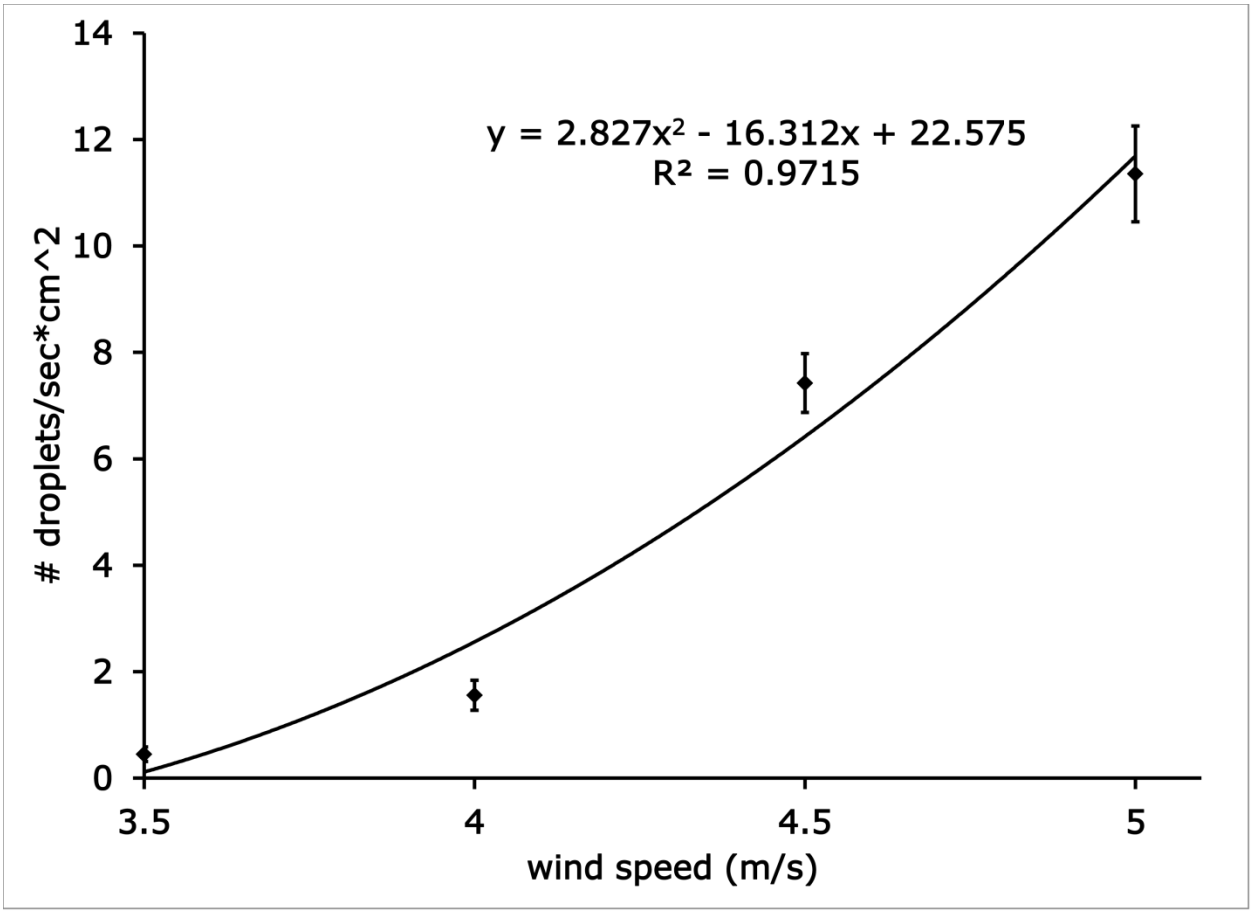

Figure 4.3: Rate of droplet production from high speed video at 6250 fps in a wind wave tank at found wind speeds (3.5, 4.0, 4.5, and 5.0 m/s). The data is fitted with a second order polynomial.

Table 4.1. The number of drops total and the seconds of video recorded along with the average number of droplets per second of video per cm^2 of water surface at each of the four wind speeds.

wind speed	3.5	4.0	4.5	5.0
number of drops	122	563	672	771
total video time (seconds)	10.8	14.4	3.6	2.7
#droplets/sec*cm^2	0.45	1.55	7.42	11.36

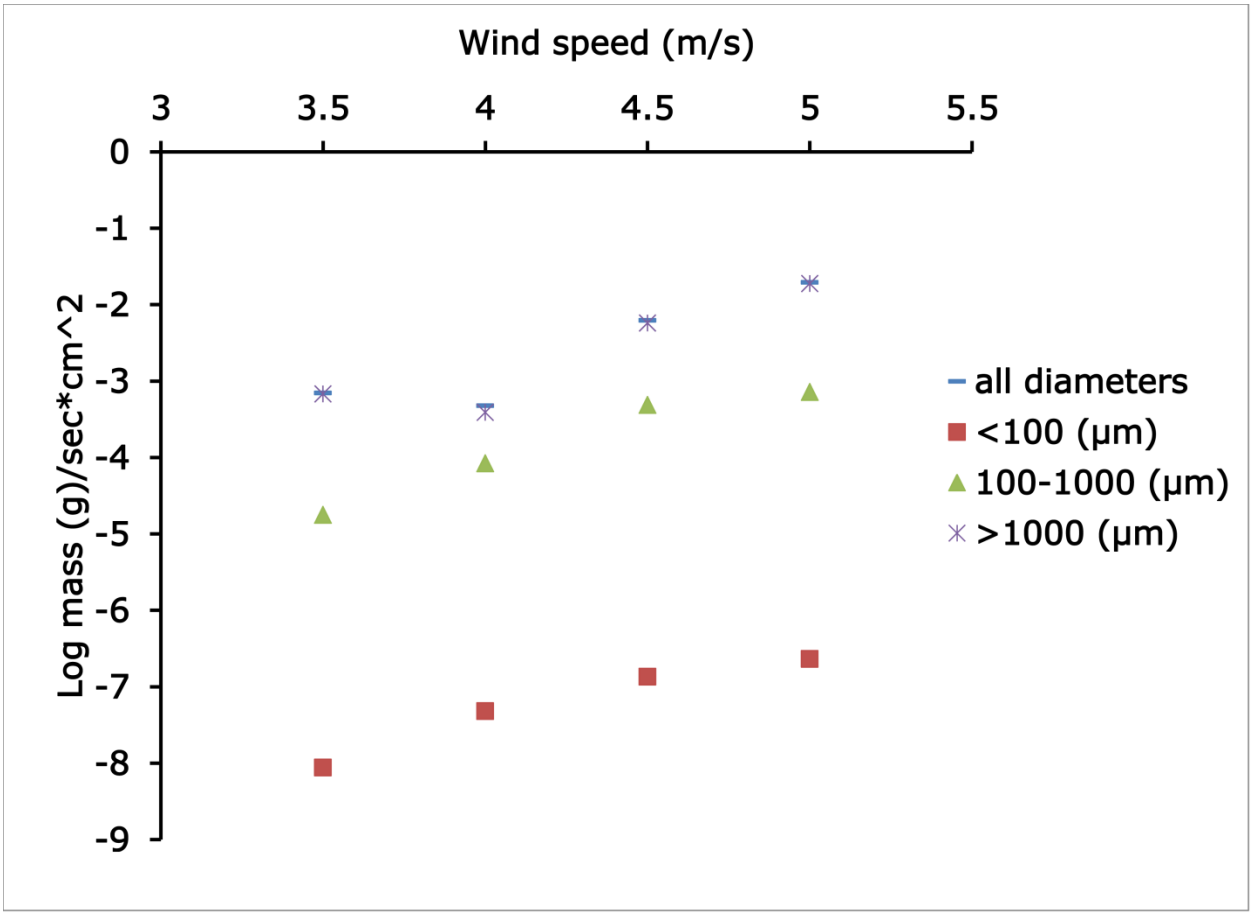

Figure 4.4. Log of mass (g) of water per second per cm^2 of water surface emitted from the water surface in droplets. All droplets are shown as well as three grouping of the droplets based on droplet diameter (< 100 µm, 100-1000 µm, and >1000 µm).

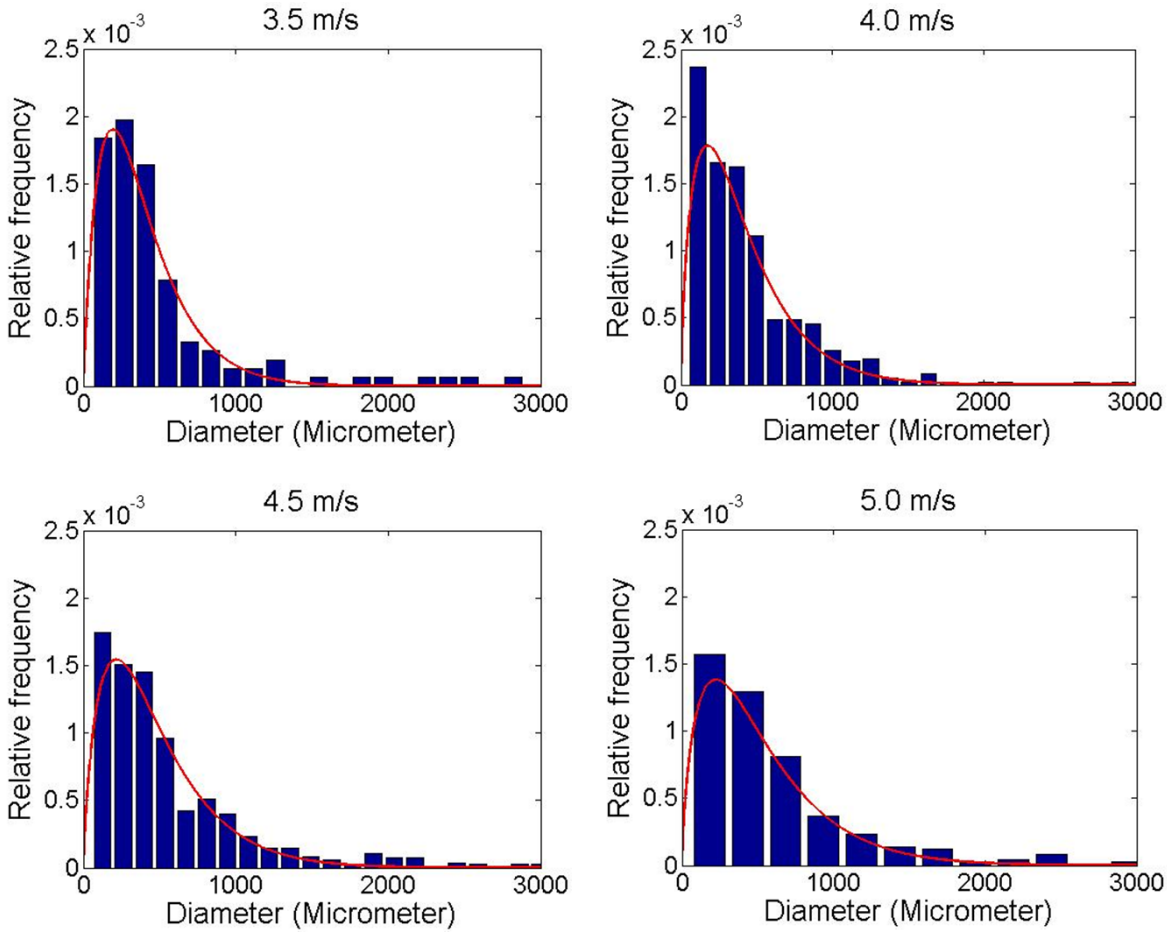

Figure 4.5. Histograms of droplet diameter at each of the four wind speeds (3.5, 4.0, 4.5, and 5.0 m/s) with a gamma distribution fit.

Table 4.2. Parameters for droplet diameter at each of the four wind speeds including shape and scale parameters from the Gamma distribution, variance, mean, median, skewness, and error.

wind speed (m/s)	k	θ	variance	mean	median	error
3.5	2.00	193.60	74856.10	386.66	350.89	0.00004
4.0	1.76	231.47	94078.37	406.44	345.30	0.00005
4.5	1.85	255.60	120585.00	471.77	412.89	0.00003
5.0	1.74	301.63	158229.04	524.58	443.96	0.00002

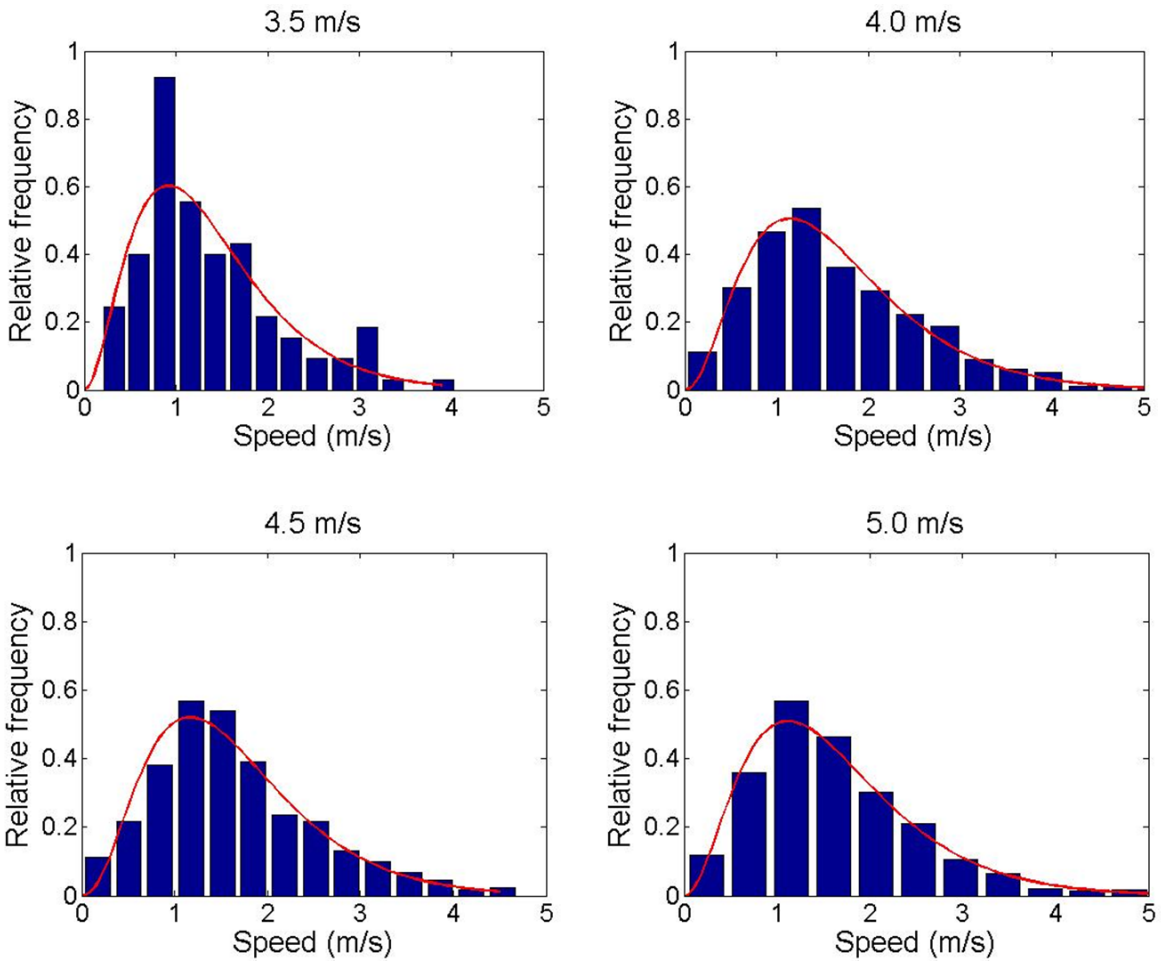

Figure 4.6. Histograms of droplet speed at each of the four wind speeds (3.5, 4.0, 4.5, and 5.0 m/s) with a gamma distribution fit.

Table 4.3. Parameters for droplet speed at each of the four wind speeds including shape and scale parameters from the Gamma distribution, variance, mean, median, skewness, and error.

wind speed (m/s)	k	θ	variance	mean	median	error
3.5	3.07	0.44	0.60	1.36	1.12	0.04117
4.0	3.26	0.51	0.83	1.65	1.46	0.00366
4.5	3.48	0.47	0.77	1.64	1.48	0.00727
5.0	3.19	0.51	0.83	1.63	1.46	0.00404

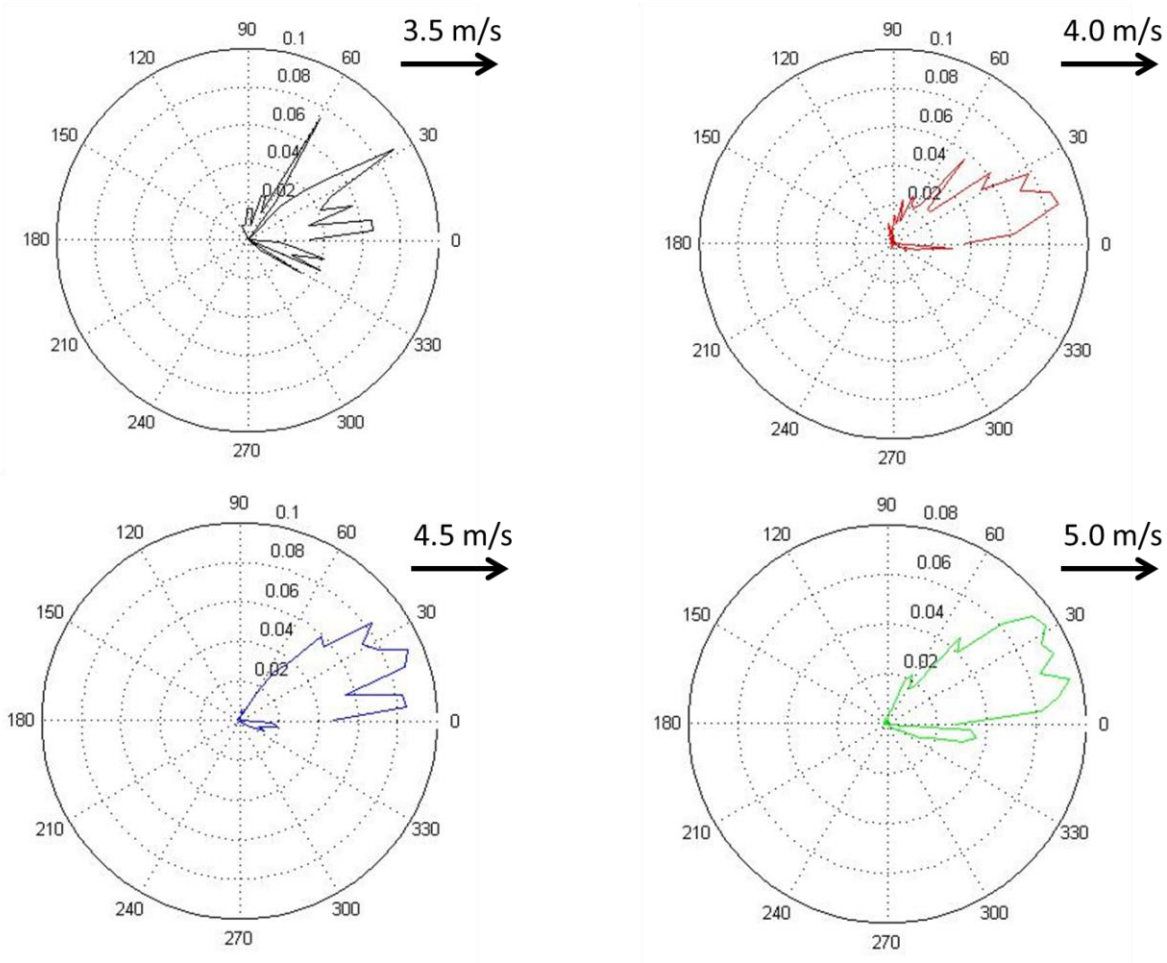

Figure 4.7. Polar coordinates showing distributions of droplet angles at four wind speeds (3.5, 4.0, 4.5, and 5.0 m/s). The arrow shows the direction of the wind.

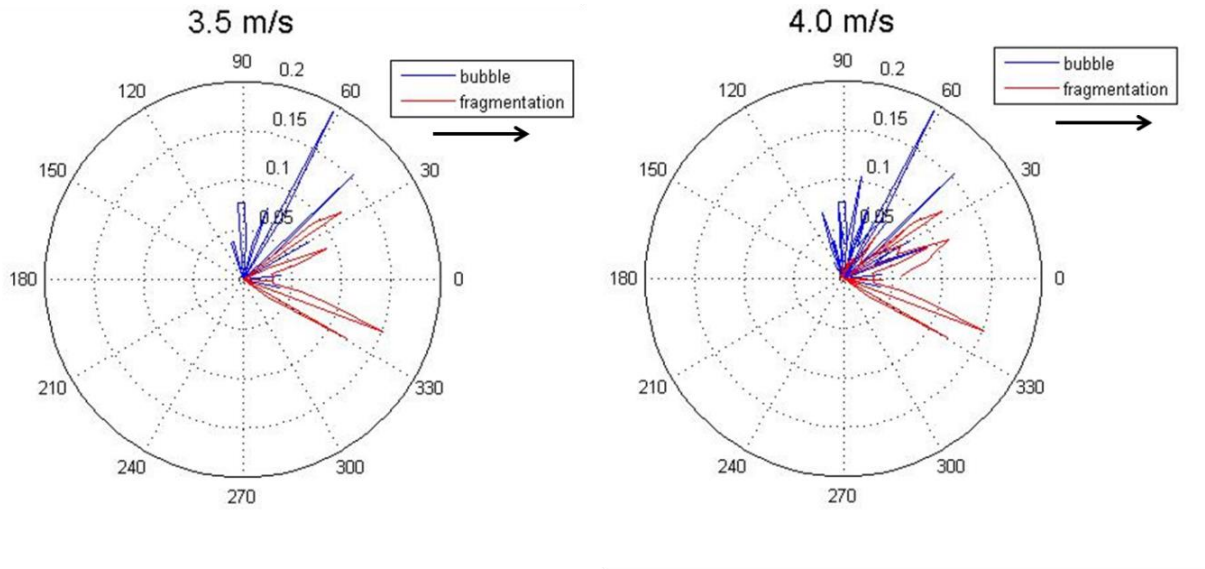

Figure 4.8. Polar coordinates showing distributions of droplet angles at two wind speeds (3.5 and 4 m/s) for bubble bursting droplets (black) and fragmentation droplets (red).The arrow shows the direction of the wind.

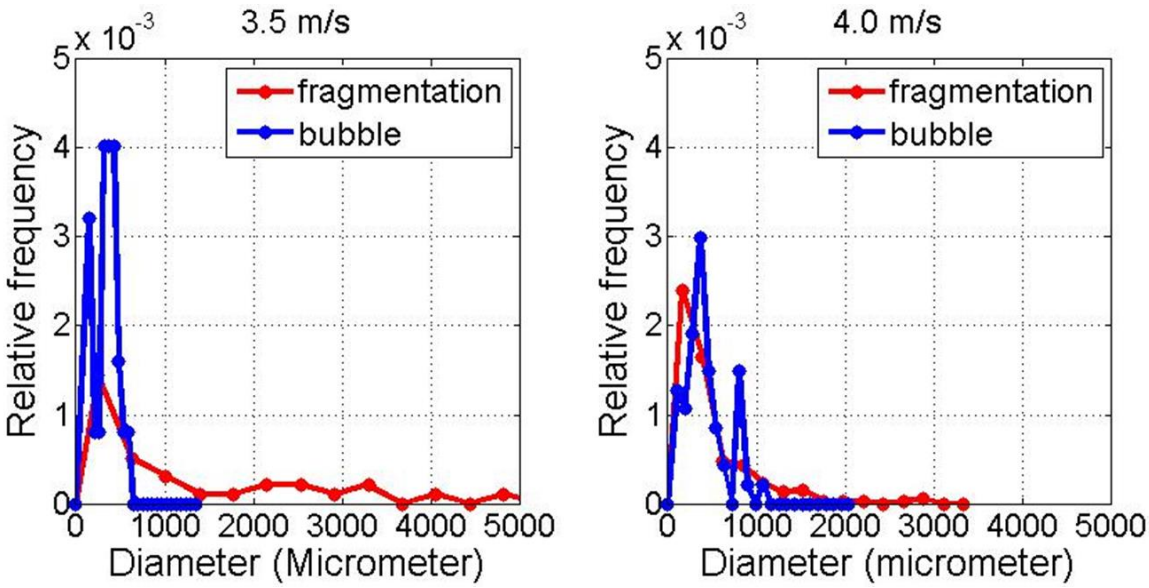

Figure 4.9. Histograms for droplet diameter at 3.5 and 4.0 m/s wind speeds for bubble bursting droplets (blue) and fragmentation droplets (red).

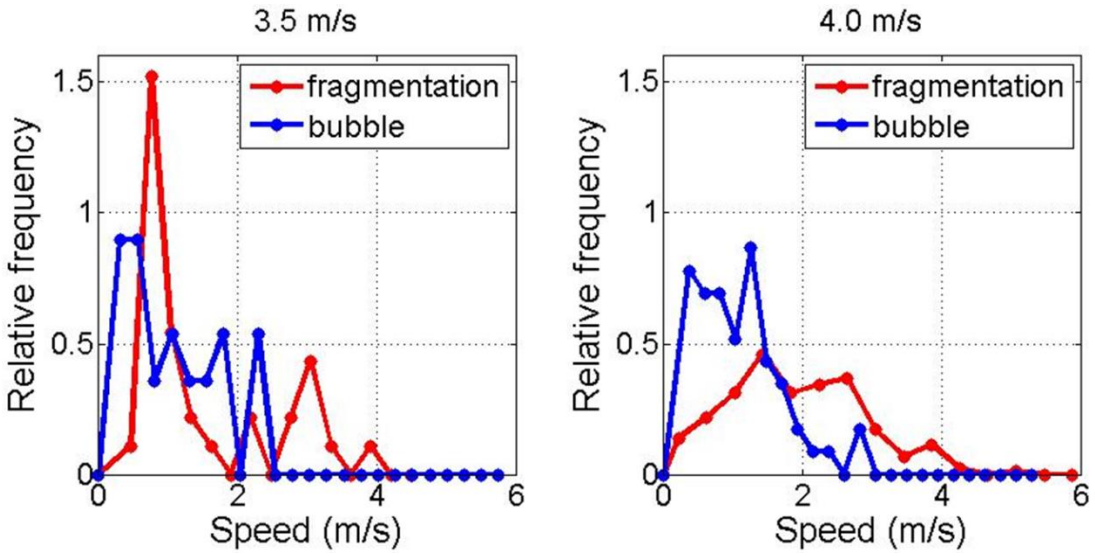

Figure 4.10. Histograms for droplet speed at 3.5 and 4.0 m/s wind speeds for bubble bursting droplets (blue) and fragmentation droplets (red).

Table 4.4. The capacity for bacteria to move across the air-water interface based on the droplet mass flux calculated in this experiment at three different droplet size ranges given the reported bacterial concentrations from literature for freshwater lakes (Field, Griffiths et al. 1980; Coveney 1982; Bird and Kalff 1984).

Wind Speed	<100 µm		100-1000 µm		>1000 µm	
	Lower cells/sec* cm^2	Upper cells/sec* cm^2	Lower cells/sec* cm^2	Upper cells/sec* cm^2	Lower cells/sec* cm^2	Upper cells/sec* cm^2
3.5	0.0006	0.12	1.28	238.24	48.66	9055.70
4.0	0.0034	0.64	6.05	1126.89	28.06	5222.14
4.5	0.0097	1.81	35.01	6516.67	413.13	76888.88
5.0	0.0165	3.07	51.90	9658.75	1352.56	251726.68

Chapter 5 – Ice Ice Baby; Teaching Advanced Secondary School Students about Biological Ice Nucleation and the Global Water Cycle

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Abstract

Innovative immersive experiences are needed in high school to prepare students and enhance interest toward the study of geosciences. There is an increasing need to educate secondary school (high school) students about precipitation processes and their impact on our world. We developed and delivered a unique unit for advanced secondary school students (age 16-18) to explore biological ice nucleation (the process by which biological particles catalyze freezing at warm temperatures) through the lens of a microbial ice nucleator (*Pseudomonas syringae).* The unit used an active learning approach focusing on guided inquiry, open inquiry, and discussion of impacts of ice nucleation on different disciplines. In the guided inquiry portion of the lesson, small groups of students conducted a droplet freezing assay to test ten known samples (abiotic and biotic materials) for ice nucleation activity. In the open inquiry part of the lesson, students collected outdoor environmental samples and tested them for ice nucleation activity. The temperatures at which each of the samples froze were entered and analyzed in a dynamic spreadsheet. Class discussions considered global impacts of ice nucleation on the water cycle, and possible human manipulations (e.g., cloud seeding to create rain) of the system with associated ethical considerations. Evidence of learning was documented through written responses of students. This unique unit was designed to integrate with the Next Generation Science Standards (NGSS) and exposed advanced secondary school students to complex multi-scale systems surrounding water highlighting the importance of critical and ethical thinking at the intersection of biology and engineering.

Introduction

Super cooled water remains liquid at temperatures down to -38° C in the atmosphere. Ice nucleation (the process by which particles catalyze freezing at temperatures above -38° C) is an important part of the global water cycle (Morris et al., 2013), and microorganisms are arguably some of the most important ice nucleators at temperatures above -15° C (Murray et al., 2012). Rain-producing clouds form around an ice nucleus, a particle that catalyzes the freezing of super cooled water at warmer temperatures. Some bacteria, such as *Pseudomonas syringae*, produce an outer membrane protein that serves as an ice nucleus and catalyzes freezing at some of the warmest temperatures known (Wolber et al., 1986). Microbial ice nucleation has been known since the 1970's (Maki et al., 1974), but recent interdisciplinary studies have suggested that these microbes are an intrinsic part of the water cycle (**Figure 5.1**). In particular, *P. syringae* has been found in clouds, rain, and snow. (Sands et al., 1982; Constantinidou et al., 1990; Amato et al., 2007; Morris et al., 2008; Monteil et al., 2014). The bacterium is aerosolized from agricultural environments (Lindemann et al., 1982), and expression of the ice nucleation protein may increase the aerosolization of *P. syringae* from aquatic environments (Pietsch et al., 2015). Once in the atmosphere, the ice-nucleating bacterium could serve as an ice nucleus and initiate precipitation (Morris *et al.*, 2014). The bacterium can then return to earth in rain, thus providing the bacterium with a means of transport and deposition as well as causing rain, which can increase plant growth thus providing greater habitat for the bacterium (Morris *et al.*, 2013). Though ice nucleation is an important part of the water cycle, secondary school curricula often do not consider precipitation processes in detail and their associated impacts on the world. (Libarkin and Anderson, 2005) and (McConnell et al., 2005) studied students entering college and found students are unprepared for introductory geoscience courses due to a lack of training

or misconceptions about fundamental concepts. Innovative immersive experiences are needed in high school to prepare students and enhance interest and positive feelings toward the study of geosciences (Elkins and Elkins, 2007, McConnell and van Der Hoeven Kraft, 2011).

Disciplinary education predominates in secondary education (Adler and Flihan, 1997). The National Science Foundation (NSF) has called for development of Interdisciplinary Research (IDR) approaches in environmental education and research (Vincent and Focht, 2011). The link between ice nucleation and the water cycle is an ideal topic to present interdisciplinary concepts since it spans multiple traditional disciplines. While disciplinary knowledge is important, interdisciplinary pedagogy offers several advantages. First, interdisciplinary curricula encourage students to have the ability to make applications beyond the immediate problem thus developing vital real world skills (Applebee et al., 2007). Second, interdisciplinary research is rapidly expanding in science and engineering and science, technology, engineering, and math (STEM) problems generally are not isolated within a specific discipline (Vincent and Focht, 2011). Third, students must become comfortable with interdisciplinary methods so that they might offer intelligent solutions as next generation humans in our changing world.

Interdisciplinary curricula tend to naturally emphasize problem based learning with an emphasis on scientific inquiry (Capraro and Jones, 2013). Inquiry based learning involves students being presented with questions for which they must design and execute a method to obtain an answer to the question (Bell et al., 2005). Varying levels of guidance can be given to students. In guided inquiry the students are presented with the research question, while in open inquiry the students develop the research question (Banchi and Bell, 2008). Similarly, in problem based learning students work together in small groups to solve a problem (Hmelo-Silver, 2004). These approaches prepare students to tackle real world problems, which are characterized by

questions being asked without a readily available solution or even a known methodology as opposed to memorizing information presented in a traditional lecture format, which does not represent a real world situation. An interactive approach encourages critical thinking over memorization, stimulating greater thought and retention in the students by allowing them to be active participants in the lesson rather than passive observers of a lecture (Ivanitskaya et al., 2002, Adler and Flihan, 1997). In one example, an inquiry-based secondary school curriculum implementing multidisciplinary applications to current issues of science and society showed average gains of 20-25% in science understanding among students (Bybee and Van Scotter, 2007). Other inquiry-based secondary school units have been developed to teach material at the science- engineering interface leveraging inquiry based learning. Bohland et al. (2015) presented a secondary school unit where students used robotic and cyborg cockroaches to explore engineering potential and associated ethical considerations. Pietsch et al. (2014) developed a unit to teach students about the basic principles of flight with emphasis on biological flight including a hands on component of building and flying remote controlled gliders. Bohland et al. (2011) presented an inquiry based lesson involving Lego mazes and a plasmodial slime mold to teach students about barriers that limit movement of organisms. Here, we designed a unit to stimulate inquiry in the students by presenting them with information piece by piece in a way to engage their attention and let them be active participants assembling the pieces like a puzzle. This lesson can be used to prepare students for college level geoscience courses and also fits with the Next Generation Science Standards **(Table 5.3)**. The 2.5 hour unit was designed around the following learning objectives:

1. Develop an understanding of the principles of ice nucleation and recognize the diversity of ice nucleating particles including identifying specific organic and inorganic ice nucleators.
2. Recognize how interdisciplinary research can address complex problems across multiple fields of study using ice nucleation and its relationship to atmospheric processes as examples.
3. Discuss potential societal and ethical implications of human manipulations of the ice nucleation phenomenon.
4. Consider principles of experimental design, including the formulation of testable hypotheses and the inclusion of appropriate positive and negative controls in developing an ice nucleation assay.
5. Design and conduct an ice nucleation experiment with known and unknown samples of undetermined ice nucleation activity.
6. Understand connections between systems of different scale such as microbiology (ice nucleation) and the global water cycle (bioprecipitation).

Methods and Materials

The 2.5 hour unit was designed to use an active learning approach focusing on guided inquiry, open inquiry, and discussion of impacts of ice nucleation on different disciplines. In the guided inquiry portion of the lesson, six groups of four students (24 students total) conducted a droplet freezing assay to test ten known samples (a range of abiotic and biotic materials) for ice nucleation activity. In the open inquiry part of the lesson, students collected different outdoor environmental samples and tested them for ice nucleation activity. This activity was followed by

data entry and analysis, where data from the droplet freezing experiments (the temperatures at which each of the samples froze) were entered and analyzed in a dynamic spreadsheet. Finally, class discussions considered global impacts of ice nucleation on the water cycle, and possible human manipulations (e.g., cloud seeding to create rain) of the system with associated ethical considerations.

Part 1: Lecture of microbial ice nucleation discovery story (~5 minutes)

The unit started with a lecture by the lead author on biological ice nucleation (Pietsch, 2015a; https://youtu.be/qPEuWrTbWbE). Ice nucleation by the bacterium *Pseudomonas syringae* was discovered by two groups nearly simultaneously in the 1970s, an atmospheric group and a plant pathology group (Lee Jr et al., 1995). This lesson began with a power point given by the lead author walking students through the history of the discovery of ice nucleation by the plant pathology group in story format. It was observed that corn inoculated with corn leaf powder from plants infected with Northern Corn Leaf Blight showed increased frost damage, but it took a lot of investigation to discover the causal agent of the increased frost damage. The story was stopped before *P. syringae* was revealed as the freezing agent, leaving the students with an unsolved mystery to contemplate as the unit progressed.

Part 2: Ice nucleation demonstration (~15 minutes)

The second part of the unit was a demonstration of the ice nucleation phenomenon (Pietsch, 2015b; https://youtu.be/648QSyHaNII). A 500 mL glass bottle containing 100 mL of sterile nanopure water was placed in an Alpha 12 cooling bath (Lauda, New Jersey, USA) containing ethylene glycol at -8°C for 20 minutes. The bottle was removed from the cooling

bath, and 4 mL of room temperature sterile water was added while the bottle was swished. The bottle did not freeze. Then 4 mL of room temperature water containing a single strain of Ice+ *P. syringae* was added and the bottle was swished. The suspension in the bottle froze instantaneously **(Figure 5.2)**.

Part 3: Lecture continuation (~30 minutes)

In the third part of the unit, students were asked to comment on what they observed in the ice nucleation demonstration and make connections to the story they heard in the opening segment of the lesson. The lecture continued (Pietsch 2015a), progressing through the concepts of super cooled water, homogenous and heterogeneous freezing, ice nucleation, and cloud formation via ice nucleation (Langham and Mason, 1958). This information gave students the background to understand the discovery of microbial ice nucleation by the atmospheric group (Lee Jr et al., 1995), which was then presented in story narrative. Ice nuclei were known to be important in cloud formation, but the group was seeking to identify specific ice nuclei active at temperatures as warm as -3° C (Lee Jr et al., 1995). The two stories of ice nucleation discovery were then merged, and *P. syringae* was revealed to be the causal agent of freezing in both cases. The lecture phase concluded with the life history of *P. syringae* and the concept of bioprecipitation (the idea that Ice+ bacteria could be part of a cycle including making rain) (Sands et al., 1982) and the connection of *P. syringae* to the water cycle was introduced (Morris et al., 2008).

Part 4: Guided and open inquiries (~75 minutes)

In the fourth part of the unit, experiment design concepts were presented to the students including the importance of hypotheses, use of appropriate controls, replication, limiting variation, and recognizing limitations of the experiment design. Students were divided into groups of four, and given the opportunity to conduct a droplet freezing ice nucleation assay. Ten different samples, both Ice + and Ice-, from a variety of organic and inorganic sources were prepared **(Table 5.2)**. The feldspar, montmorillonite, Snomax®, fennel pollen, bee pollen, and button mushroom spores were suspended in sterile water. Vendors and item information are given in **Table 5.2**. The button mushroom spores were obtained by cutting white button mushroom caps (*Agaricus bisporus*) to expose the gills then placing them on a piece of computer paper gill-side down for about 24 hours. The dark colored spores dropped onto the paper and were then suspended in sterile water. The *P. syringae* samples were grown in liquid TSA media for 24 hours and used directly in the assay. These two strains of *P. syringae* are not known to be pathogenic to plants and do not infect humans. The students did not know the ice nucleation activity of the samples. In the open inquiry part of this unit, the students were asked to collect an environmental sample outside the classroom. Based on what they had learned from the unit, they were encouraged to find the 'best' ice nucleator (i.e., the environmental sample that would freeze water at the highest temperature). The students were given 50 mL conical tubes containing 25 mL of sterile water in which to collect environmental samples from the campus area outside the classroom (**Figure 5.3**, top and second panel).

The ten prepared samples were given to each group in 2 mL Eppendorf tubes with 100 µL of sample plus 25 µL of 250 ppm fluorescein disodium salt dye (Thermo Fisher Scientific, New Jersey, USA). The dye causes a change in color of the droplets when freezing occurs, from green (liquid) to yellow (frozen) allowing freezing events to be clearly observed (**Figure 5.3**,

bottom panel). The students were given handouts to guide them through their experiment design (Supplementary Information). They wrote hypotheses, and then selected three of the ten samples plus sterile water as a negative control. All of the ten samples were selected by at least one group. The students pipetted 25 µL of each sample into each of three wells on a 96 well plate making three replicates. Droplets of 12 µL of each sample were loaded onto a Parafilm® M boat floating on the cooling bath (**Figure 5.3**, third panel). The temperature of the bath was set at -2° C during loading of the samples, and then was set to -12° C. The students watched for approximately 30 min while the bath cooled from -2° to -12° C and they recorded the temperature when each drop froze (**Figure 5.3**, third panel). Evidence of learning was documented through written responses of students regarding conclusions, limitations, and future improvements to the unit.

Part 5: Application discussion (~25 minutes)

The freezing temperatures were entered on an Excel spreadsheet projected on a screen visible for the whole class **(Table 5.6)**. A discussion of the student's ice nucleation assay results, including expected and unexpected results, along with ideas for experiment improvements took place. Generation of new hypotheses was also discussed followed by a discussion period talking about various groups and disciplines that would have interests in ice nucleation. Potential conflicts between groups, including ethical implications and human manipulations of the water cycle were considered. The variety of known organic and inorganic ice nucleators was also presented to students.

Part 6: Outdoor environmental sample processing (Post lesson)

The outdoor environmental samples were brought back to the Schmale lab at Virginia Tech and 100µL spread on large plates of TSA. Colonies showing different morphologies were selected and tested for ice nucleation activity. A toothpick was used to inoculate a small amount of each selected colony into 40µL of sterile water. The ice nucleation assay was conducted as described above in the open inquiry experiment, with the exception of the bath temperature which started at -6°C and was lowered to -8°C, and replicates of two instead of three were used.

Next Generation Science Standards

This unit was designed to integrate with the Next Generation Science Standards (NGSS) helping students to meet performance expectations. The NGSS considers three areas of learning: "Practices" describing methods commonly used in scientific study, "crosscutting concepts" that span multiple disciplines, and "disciplinary core idea" central to fundamental understanding (NGSS Lead States, 2013). All three areas are included in this unit as outlined in **Table 5.3**.

Results and Discussion

Ice nucleation and bioprecipitation are ideal systems to be used in an interactive interdisciplinary curriculum for several reasons. First, this system ranges from molecular scale (the ice nucleation protein) to a global scale (the water cycle). A multi-scale system helps students to see the benefits of interdisciplinary research approaches. Second, as an area of new research students were able to think and discuss things that are not yet known giving them a taste of research rather than prescribed lab exercises. Third, the concept of a bacterium causing water to freeze (and the visualization of this phenomenon with the ice nucleation demonstration) is fascinating and naturally increases student interest.

The format of this lesson was successful in presenting the students with pieces of a puzzle and allowing them to assemble the pieces. They responded interactively, indicating they were thinking and putting pieces together as the lecture, particularly the *P. syringae* ice nucleation discovery story, unfolded. Evidence of learning was documented through written responses of students regarding conclusions, limitations, and future improvements to the open inquiry experiment prior to sharing their thoughts in a group discussion. **Table 5.4** is provided to relate each lesson objective to an evaluation method for teachers to evaluate the efficacy of the lesson. Anonymous student paraphrases are provided to show evidence that the objectives were met. A summary of the evaluation for the class as a whole follows. The students were given an Ice+ and an Ice- strain of *P. syringae.* Although the lesson had only talked about *P. syringae* as an ice nucleator, 24% (4/17) of the students correctly concluded that not all strains of the bacterium contain and express the ice nucleation protein. 12 % (2/17) of the students also recognized the great diversity of ice nucleators spanning organic and inorganic substances. Regarding limitations, 53% (9/17) of the students reported that the observation of the droplets freezing could be subjective, 47% (8/17) suggested particles from the air or from breathing could contaminate the samples, and 53% (9/17) of the students reported that frost spreading from one droplet could give a false positive to neighboring droplets. Other limitations identified as evidence of student learning included documented uncertainty about how many different ice nucleating substances are in the outdoor samples (1/17 of the students), the potential for the dye altering the freezing temperature of the droplets (4/17 of the students), and inconsistent temperature across the Parafilm® M boat (3/17 of the students). Appropriate improvements were given for future experiments, with 94% (16/17) of the students suggesting covering the bath to prevent contamination of the samples by particles in the air, 47% (8/17) suggesting an objective

quantitative method of recording when the droplets freeze with one student suggesting use of a high speed camera, 41% (7/17) suggesting spacing the droplets farther apart to prevent premature freezing from frost, and 35% (6/17) suggesting adding more replicates. Students not only mentioned ideas related directly to the material presented, but also ideas tangentially related showing their ability to understand concepts and apply them beyond the immediate application in which these ideas were presented. For example, during the discussion on impacts of manipulating ice nucleation one student commented on increasing local precipitation while other students made the less direct connection that soil salinity could also be altered or species diversity could be impacted.

In the guided inquiry experiment the students were able to use the information they had learned in forming their hypothesis statements. The students were prompted to look for the warmest temperature ice nucleator and all of the groups expected either Snomax® or one of the two strains of *P. syringae* as the warmest temperature ice nucleator (Objective 5, **Table 5.4**). Friendly competition increased student enthusiasm as they sought to find the warmest temperature ice nucleator. The students were excited to fill out the Excel spreadsheet in front of the whole class and compare their results. The ten prepared samples the students used in the open inquiry ice nucleation assay were Ice+ and Ice- as expected based on previous tests by the first two authors as well as literature (Murray et al., 2012; Pietsch et al., 2015). However, the temperature that some of the Ice+ samples froze was a few degrees lower than previous tests. Of the two strains of *P. syringae* given to the students, one was Ice+ and the other was Ice- allowing students to see that both phenotypes of *P. syringae* exist (Objective 1, **Table 5.4**). Six of the eighteen sterile water controls froze at temperatures of -9.7° C and lower, which is likely due to contamination and/or lack of pipetting skills (Objective 4, **Table 5.4**).

In the open inquiry portion of the experiment, the students were able to identify environmental samples that had some of the warmest freezing temperatures observed (Objective 5, **Table 5.4**). Five of the six samples froze **(Table 5.5)**, and surprisingly the freezing temperatures were the warmest temperatures observed for all samples with the exception of Snomax® **(Table 5.6)**. Such warm temperatures suggested a potential link to microbial ice nucleation. These results led us to follow-up experiments back in the Schmale lab at Virginia Tech to isolate and test bacteria from these samples for ice nucleation activity. Multiple colonies of bacteria were isolated from each of the samples, and colonies of different morphologies were tested with an ice nucleation assay. We found ice nucleating bacteria from every outdoor sample, except sample A, with 23% of all the bacteria tested being Ice+ **(Table 5.5)**. At least four different colony morphologies were observed in each of the samples. These results were shared with the biology teacher (Cynthia Bohland). The students were pleased to know that they had found ice nucleators and wanted to know if any of the bacteria they had found were previously unidentified as ice nucleators. There was a sense of excitement and investment in being part of science.

The students were able to identify multiple disciplines that could contribute to ice nucleation and the strength of interdisciplinary research was realized as students were able to perceive how one system is not properly studied from any single discipline (Objectives 2 & 6, **Table 5.4**). They saw how different disciplines would approach one problem differently and focus on different aspects of such a large system. For example, molecular biologists would study the ice nucleation protein, computational modelers would look at predictive patterns, and atmospheric scientists would be interested in applications to weather patterns. Farmers would also be interested in knowing dissemination methods of *P. syringae* as a potential plant pathogen.

Attempts to use this system to manipulate the weather were also discussed, including positive and negative impacts of such intervention. This discussion provided an opportunity to illustrate how nearly every aspect of biology requires ethical considerations (Objective 3, **Table 5.4**), even topics that don't appear to be highly controversial at first glance. Students recognized the difficulty in determining all the consequences of manipulating a global system. Several unexpected negative consequences were raised by the students. One student brought up the idea that increasing rain by cloud seeding could change weather patterns in surrounding areas perhaps decreasing rain in neighboring regions. Another student raised the issue of potential impacts on native flora and fauna. And yet another student suggested that human manipulations could be used with bad intentions, such as using this dissemination method for deliberate spread of a pathogenic bacterium or purposely increasing severe weather events to cause harm in certain areas of the world.

Overall this lesson was effective, and can serve an important role in preparing students for college level geoscience courses as well as allowing them to explore the specific area of biological ice nucleation. The most successful parts of the unit were (1) having the students collect unknown samples outdoors and (2) recording the data from the ice nucleation assay in a dynamic spreadsheet visible to the whole class. There were a few areas that could be modified and/or improved upon in the future. First, students could be given the opportunity design their own controls for the experiments. For example, several students mentioned the dye possibly altering the freezing properties. The students could have tested sample with and without dye to investigate this question. Second, many of the students would have benefited from a short tutorial on pipetting techniques prior to conducting the experiments. Third, the worksheets could include a section for students to write down their individual thoughts about the ethical issues

surrounding biological ice nucleation. Fourth, the freezing bath could have started at a lower temperature (e.g., -5° C) to reduce the amount of time students were watching their droplets freeze.

This unit could be modified in several ways to accommodate different classroom needs. If a cooling bath is not available, a lower cost method could be implemented using beakers with ethanol immersed in an ice bath or dry ice. Temperatures around -6° C can be achieved with the ice bath and colder temperatures with dry ice (http://wiki.bugwood.org/Bacterial_ice_nucleation). Placing an ethanol bath in a freezer and allowing equilibration should allow temperature around -10° C to be achieved. With this method the temperature will gradually rise when the bath is removed for the freezer for testing so the droplets must be placed on the bath immediately upon removal from the freezer. One limitation of this low-cost method is that the temperature can't be controlled as precisely as with the use of a cooling bath. The guided inquiry demonstration could be conducted with smaller amounts of water in glass test tubes submerged in a beaker of ethanol. Small Parafilm® M boats could be floated in the beakers of ethanol to conduct the ice nucleation assay. Discussions could be tailored to emphasize more of either the microbial aspects or environmental aspects of the system. The outdoor environmental samples could also become part of an expanded microbiology lesson. Though we brought the outdoor samples back to the lab for culturing and analysis, this step could easily have been incorporated into a classroom activity, where the students could inoculate media plates in the classroom and then perform follow-up ice nucleation assays to determine if the bacteria grown were Ice+. DNA-based methods could be used to assist in the identification of the cultured microorganisms. The unit can also serve as a part of meeting the performance expectation specified in the NGSS **(Table 5.3)**.

This unique unit exposed advanced secondary school students to complex multi-scale systems surrounding water and highlighted the importance of critical and ethical thinking at the intersection of biology and engineering. Students were able to see firsthand the types of research questions scientists and engineers are currently dealing with, the methods that are used to answer those questions, and the value of considering the range of impacts of potential discoveries. It is important to stimulate student interest in complex interdisciplinary problems that are important areas of current research. This exposure is important to increase student interest in STEM fields at the secondary school level as well as begin training young scientists and engineers in the methods they would use in STEM careers. Even for students not destined for STEM careers, being aware of current research topics with global impacts will make them better citizens (Jacobson and Wilensky, 2006). There is a critical need to educate secondary school (high school) students about precipitation processes and their impact on our world.

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References

Adler, M., and Flihan, S. 1997. The Interdisciplinary Continuum: Reconciling theory, research and practice. *National Research Center on English Learning & Achievement,* University at Albany, State University of New York.

Amato, P., Demeer, F., Melaouhi, A., Fontanella, S., Martin-Biesse, A.S., Sancelme, M., Laj, P. and Delort, A.-M. 2007. A fate for organic acids, formaldehyde and methanol in cloud water: their biotransformation by micro-organisms. *Atmospheric Chemistry and Physics,* 7:4159-4169.

Applebee, A.N., Adler, M., and Flihan, S. 2007. interdisciplinary curricula in middle and high school classrooms: case studies of approaches to curriculum and instruction. *American Educational Research Journal,* 44:1002-1039.

Banchi, H., and Bell, R. 2008. The many levels of inquiry. *Science and children,* 46:26.

Bell, R.L., Smetana, L., and Binns, I. 2005. Simplifying inquiry instruction. *The Science Teacher,* 72:30-33.

Bohland, C.E., Collver, M., Lally, D., and Schmale III, D.G. 2015. Exploring ethics in autonomous systems through cyborg roaches and robotic insects. *The Science Teacher.*

Bohland, C.E., Schmale III, D.G., and Ross, S.D. 2011. Caging the blob: using a slime mold to teach concepts about barriers that constrain the movement of organisms. *The American Biology Teacher,* 73:537-541.

Bybee, R.W., and Van Scotter, P. 2007. Reinventing the science curriculum. *Educational Leadership,* 64:43-47.

Capraro, M.M., and Jones, M. 2013. Interdisciplinary STEM project-based learning. *STEM Project-Based Learning,* 51-58: Springer.

Constantinidou, H., Hirano, S., Baker, L., and Upper, C. 1990. Atmospheric dispersal of ice nucleation-active bacteria: the role of rain. *Phytopathology,* 80:934-937.

Elkins, J.T., and Elkins, N.M. 2007. Teaching geology in the field: significant geoscience concept gains in entirely field-based intriductory geology courses. *Journal of Geoscience Education,* 55:126.

Hmelo-Silver, C.E. 2004. Problem based learning: what and how do students learn? *Educational psychology review,* 16:235-266.

Ivanitskaya, L., Clark, D., Montgomery, G., and Primeau, R. 2002. Interdisciplinary learning: process and outcomes. *Innovative Higher Education,* 27:95-111.

Jacobson, M.J., and Wilensky, U. 2006. Complex systems in education: scientific and educational importance and implications for the learning sciences. *The Journal of the learning sciences,* 15:11-34.

Langham, E., and Mason, B. 1958. The heterogeneous and homogeneous nucleation of supercooled water. *Proceedings of the Royal Society of London. Series A. Mathematical and Physical Sciences,* 247:493-504.

Lee Jr, R., Warren, G.J., and Gusta, L.V. 1995. Biological ice nucleation and its applications. *American Phytopathological Society.*

Libarkin, J. C., and Anderson, S.W. 2005. Assessment of learning in entry-level geoscience courses: results from the geoceicne concept inventory. *Journal of Geoscience Education,* 53:394.

Lindemann, J., Constantinidou, H.A., Barchet, W.R., and Upper, C.D. 1982. Plants as sources of airborne bacteria, including ice nucleation-active bacteria. *Applied and environmental microbiology,* 44:1059-1063.

Maki, L.R., Galyan, E.L., Chang-Chien, M.M., and Caldwell, D.R. 1974. Ice nucleation induced by *Pseudonomas syringae. Applied microbiology,* 28:456-459.

McConnell, D.A., Steer, D.N., Owens, K.D., and Knight, C.C. 2005. How students think: implications for learning in introductory geoscience courses. *Journal of Geoscience Education,* 53:462.

McConnell, D.A., and van Der Hoeven Kraft, K.J. 2011. Affective domain and student learning in the geosciences. *Journal of Geoscience Education,* 59:106-110.

Monteil, C.L., Bardin, M., and Morris, C.E. 2014. Features of air masses associated with the deposition of *Pseudomonas syringae* and *Botrytis cinerea* by rain and snowfall. *The ISME journal,* 8:2290-2304.

Morris, C.E., Conen, F., Alex Huffman, J., Phillips, V., Pöschl, U., and Sands, D.C. 2014. Bioprecipitation: a feedback cycle linking Earth history, ecosystem dynamics and land use through biological ice nucleators in the atmosphere. *Global change biology,* 20:341-351.

Morris, C.E., Monteil, C.L., and Berge, O. 2013. The life history of *Pseudomonas syringae*: linking agriculture to Earth system processes. *Annual review of phytopathology,* 51:85-104.

Morris, C.E., Sands, D.C., Vinatzer, B.A., Glaux, C., Guilbaud, C., Buffière, A., Yan, S., Dominguez, H., and Thompson, B.M. 2008. The life history of the plant pathogen *Pseudomonas syringae* is linked to the water cycle. *The ISME journal,* 2:321-334.

Murray, B., O'Sullivan, D., Atkinson, J., and Webb, M. 2012. Ice nucleation by particles immersed in supercooled cloud doplets. *Chemical Society Reviews,* 41:6519-6554.

NGSS Lead States. 2013. Next generation science standards: for states, by states. Washington, DC: National Academies Press.

Pietsch, R.B., Bohland, C.L., and Schmale III, D.G. 2014. To fly or not to fly: teaching advanced secondary school students about principles of flight in biological systems. *Journal of Science Education,* 49:53-63.

Pietsch, R.B., David, R.F., Marr, L.C., Vinatzer, B., and Schmale III, D.G. 2015. Aerosolication of two strains (ice+ and ice-) of *Pseudomonas syringae* in a Collison nebulizer at different temperatures. *Aerosol Science and Technology*, 49:159-166.

Sands, D., Langhans, V., Scharen, A., and De Smet, G. 1982. The association between bacteria and rain and possible resultant meteorological implications. *Idojaras*.

Vincent, S., and Focht, W. 2011. Interdisciplinary environmental education: elements of field identity and curriculum design. *Journal of Environmental Studies and Sciences*, 1:14-35.

Wolber, P. K., Deininger, C. A., Southworth, M. W., Vandekerckhove, J., Van Montagu, M., and Warren, G. J. 1986. Identirication and purification of a bacterial ice-nucleation protein. *Proceedings of the National Academy of Sciences,* 83:7256-7260.

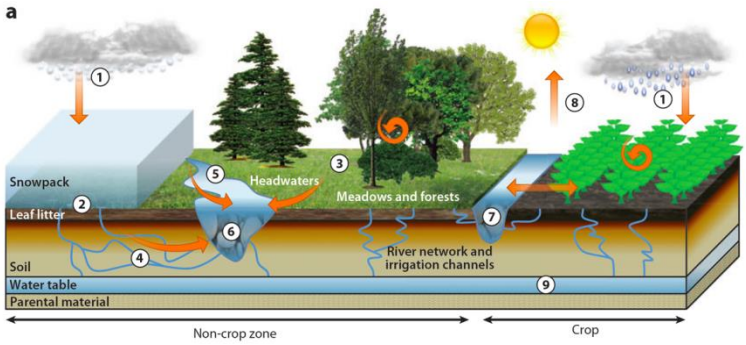

Figure 5.1. The bacterium *Pseudomonas syringae* has been found in clouds and snow (1), snowpack (2), leaf litter and grass (3), subsurface water (4), rivers and lakes (5), epilithic biofilms (6), irrigation water from rivers (7), aerosolized in the atmosphere (8), and ground water (9). These findings provide a link between ice nucleation and the water cycle. (Morris et al. 2013. The Life History of Pseudomonas syringae: Linking Agriculture to Earth System Processes. *Annual Review of Phytopathology*. 51:85-104. Reproduced with permission from Annual Reviews).

Table 5.1. Supplies and estimated cost for this unit for a group of students.

Item	Vendor	Item #	Quantity needed	Cost for one group of students
Freezing bath	Lauda	Alpha 12	1	$2585
96 well plates (100)	Sigma	CLS9018	1	$360
500 mL bottles	Sigma	CLS1399500	1	$55
50 mL conical tubes (500)	Fisher	05-539-5	1	$250
2 mL Eppendorf tubes (pack of 500)	Fisher	05-408-138	11	$70
Flourescein, disodium salt (100 grams)	Fisher Acros	173241000		$23
Parafilm® M (4 X 125')	Sigma	P7793	1	$60
10-100 µL pipette	Cole Parmer	SC-07859-07	1	$181
Pipet tips 20-200 µL (1000)	Cole Parmer	UX-07909-19	100	$28
100-1000 µL pipette (optional)	Cole Parmer	SC-07859-11	1	$181
Pipet tips 100-1000 µL (optional) (1000)	Cole Parmer	UX-07909-21	100	$36.50

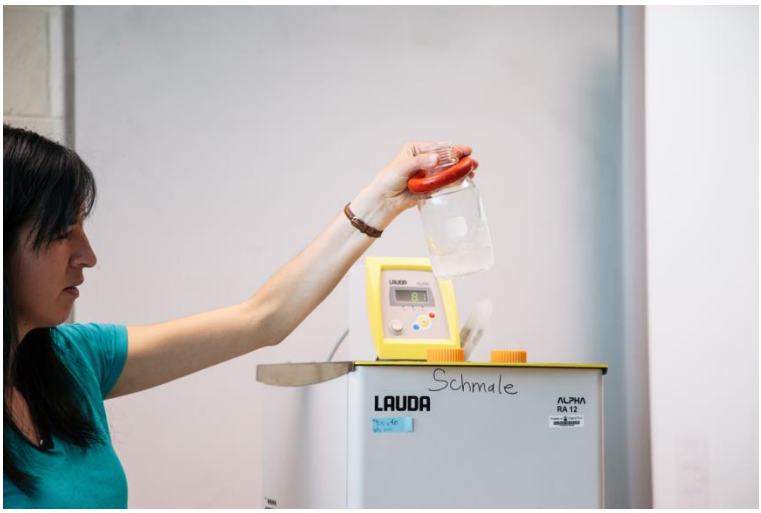

Figure 5.2. Ice nucleation demonstration showing freezing of -8° C super-cooled water by addition of *Pseudomonas syringae*.

Table 5.2. Ice+ and Ice- samples prepared for the students to use in the open inquiry experiment.

Sample Number	Sample Name	Description	Concentration	Item #	Source
Control	Sterile deionized water				
	Sample collected from outside				
1	Tap water				Lab
2	Mineral water				Kroger
3	Feldspar	Inorganic, igneous rock, mineral	0.0005 g/mL		READE advanced materials
4	Montmorillonite	Inorganic, clay	0.5 mg/mL	69866	Sigma
5	*P. syringae* strain 1 (642)	Bacterium isolated in Blacksburg, VA	Suspension grown in TSA for 24 hours		Vinatzer lab, Virginia Tech
6	*P. syringae* strain 2 (892)	Bacterium isolated in Blacksburg, VA	Suspension grown in TSA for 24 hours		Vinatzer lab, Virginia Tech
7	Snomax®	Inert bacteria	0.05 mg/mL		Snomax® International
8	Fennell pollen	Pollen	10 mg/mL	1245	My Spice Sage
9	Bee pollen	Pollen	0.3 mg/mL	618	Brushy Mountain Bee Farm
10	Button mushroom spores	Fungus	Unknown; spores from 1 mushroom cap/2mL		Kroger

Table 5.3. This unit integrates with the Next Generation Science Standards and can be used as a part of achieving the performance expectations listed below (NGSS Lead States. 2013).

Standard	**Performance expectation**	
HS-ESS2-2	Analyze geoscience data to make the claim that one change to Earth's surface can create feedbacks that cause changes to other Earth systems.	
HS-ETS1-3	Evaluate a solution to a complex real-world problem based on prioritized criteria and trade-offs that account for a range of constraints, including cost, safety, reliability, and aesthetics, as well as possible social, cultural and environmental impacts.	
Dimension	**Name and NGSS code/citation**	**Specific connection to classroom activity**
Science and engineering practices	**Analyzing and Interpreting Data:** Analyze data using tools, technologies, and/or models in order to make valid and reliable scientific claims or determine an optimal design solution. (HS-ESS2-2) **Constructing Explanations and Designing Solutions:** Evaluate a solution to a complex real-world problem, based on scientific knowledge, student-generated sources of evidence, prioritized criteria, and tradeoff considerations. (HS-ETS1-3)	Students perform an ice nucleation assay to determine the best ice nucleator using known and unknown samples. The experiment design is discussed to evaluate strengths and weaknesses. The data the students gathered from the ice nucleation assay is used in a discussion of the potential impacts both natural and manipulated of ice nucleators of various sources.
Disciplinary core ideas	**Earth Material and Systems:** Earth's systems, being dynamic and interacting, cause feedback effects that can increase or decrease the original changes. (HS-ESS2-2) **Developing Possible Solutions:** When evaluating solutions, it is important to take into account a range of constraints, including cost, safety, reliability, and aesthetics, and to consider social, cultural, and environmental impacts. (HS-ETS1-3)	The potential for ice nucleating bacteria to be part of a positive feedback cycle generating rain is discussed. Manipulations of a bioprecipitation cycle are discussed including negative and positive impacts to various effected groups.
Crosscutting concept	**Stability and Change:** Feedback (negative or positive) can stabilize or destabilize a system. (HS-ESS2-2)	Bioprecipitation hypothesis is presented illustrating how organisms as small as bacteria can have a significant part in a global scale feedback cycle.

	Influence of Science, Engineering, and Technology on Society and the Natural World: New technologies can have deep impacts on society and the environment, including some that were not anticipated. Analysis of costs and benefits is a critical aspect of decisions about technology. (HS-ETS1-3)	Human interferences in the ice nucleation process is discussed including possible consequences that were not considered as well as how much testing would be sufficient to determine if negative consequences would occur.

Table 5.4. Method of mastery, evaluation method, and example student response after the unit was taught for each of the 6 objectives as well as summative evaluation questions for the whole unit.

Lesson Objectives Evaluation Matrix				
Objective	**Method of mastery**	**Evaluation**	**Timeline**	**Anonymous student response**
1. Develop an understanding of the principles of ice nucleation and recognize the diversity of ice nucleating particles including identifying specific organic and inorganic ice nucleators	Story of the discovery of ice nucleation and following lecture. Guided and open inquiry ice nucleation assay	Student questionnaire	Prior and post lesson	• Strain 2 has IN protein • Not sure what IN is in the mixture of outdoor sample
2. Recognize how interdisciplinary research can address complex problems across multiple fields of study using ice nucleation and its relationship to atmospheric processes as examples	Discussion of multiple approaches to studying ice nucleation and possible applications	Student questionnaire	Prior and post lesson	• Species diversity impacts should be considered if ice nucleation was manipulated
3. Discuss potential societal and ethical implications of human manipulations of the ice nucleation phenomenon	Class discussion of factors to consider in regards to possible human manipulations of ice nucleation	Student questionnaire	Prior and post lesson	• Increasing rain in one area could change weather patterns and negatively impact a nearby area. • Manipulations could be used purposely for negative impact
4. Consider principles of experimental design, including the formulation of testable hypotheses and the inclusion of appropriate positive and negative controls in developing an ice	Discussion of experiment design followed by students designing an ice nucleation assay experiment	Student worksheets	Prior, during, and post lesson	• The ability to draw conclusions is limited by the control freezing” • Spacing droplets farther apart would reduce frost

nucleation assay				contamination • Dye could alter freezing temperature
5. Design and conduct an ice nucleation experiment with known and unknown samples of undetermined ice nucleation activity	Students perform ice nucleation assay with known and unknown samples	Student worksheets	During lesson	• Outdoor samples froze at highest temperature
6. Understand connections between systems of different scale such as microbiology (ice nucleation) and the global water cycle (bioprecipitation)	Presentation of bioprecipitation hypothesis and discussion of its implications	Student questionnaire	Post lesson	• Weather patterns could possibly be manipulated by controlling ice nucleators. • If ice nucleators were manipulated changes in precipitation could change soil salinity

Summative Evaluation

Evaluation Questions	Data Collection	Timeline
Has the unit changed teacher and student perceptions about the importance of ice nucleation?	Teacher and student follow-up surveys	Post lesson
To what extent does student motivation to participate in STEM careers increase as a result of participating in this project?	Student follow-up surveys	Post lesson
Do students demonstrate an increase in practicing noncognitive skills (i.e., communication, critical thinking, collaboration, and creativity)?	Student pre- and follow-up surveys	Prior and post lesson
Do students and their teacher(s) demonstrate an increase in understanding integration of disciplines in an interdisciplinary system?	Student and teacher pre- and follow-up surveys	Prior and post lesson

Figure 5.3. Students collecting outdoor environmental samples to test for ice nucleation activity (top panel), the students recording the freezing temperatures of freezing for their samples on the ice bath (middle panel), ice nucleation assay showing yellow (frozen) droplets and green (liquid) droplets (bottom panel).

Table 5.5. The ice nucleation assay results from the student's outdoor environmental samples and results of ice nucleation assay for bacteria isolated from the samples.

Description of sample	Mean freezing temperature	% of bacteria that froze at -9° C	Morphology of samples that froze
Mulch	-7.8°C	0.0% (0/9)	
Dandelion seed/fruit	-5.2°C	12.5% (1/8)	white
Dandelion flower	-8.4°C	37.5% (3/8)	yellow, orange
Mud	-7.7°C	0.0% (0/8)	
Tree leaf	Did not freeze	83.3% (5/6)	white, yellow
Dandelion leaves	-8.8°C	23.5% (4/17)	yellow, white, pink

Table 5.6. The freezing temperatures from the ice nucleation assay with samples the students prepared.

Sample number	Sample name	Freezing temperature in degree C																				Average freezing temp in degree C
control	Pure water	-11.9	-10	-10.7	-11.9	-9.7	-11.9	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	NF	-11.02
1	Tap water	NF	NF	NF																		
2	Mineral water	NF	NF	NF	NF	NF	NF															
3	Feldspar	-11.7	-11	NF																		-11.35
4	Montmorillonite	-11.5	-10.1	NF																		-10.80
5	*P. syringae* strain 1	NF	NF	NF																		
6	*P. syringae strain 2*	-11.4	-11.4	-11.9	-11	-9.7	NF															-11.08
7	Snomax	-5.8	-5.7	-5.5	-5.8	-5.8	-5.8															-5.73
8	Fennell pollen	-9.8	-10.5	-9.2	-9.9	-10.1	-11.4															-10.15
9	Bee pollen	-11.1	-10.4	-11.4	-11	-11.2	-8.6	-9.2	NF	NF												-10.41
10	Mushroom spore	-10	-10.1	-11.4	-9.5	NF	NF	NF	NF	NF												-10.25
A outdoor	Mulch	-7.8	-7.8	-7.8																		-7.80
B outdoor	Dandelion seed/fruit	-4.9	-5.3	-5.4																		-5.20
C outdoor	Dandelion flower	-8.9	-8.5	-7.8																		-8.40
D outdoor	Mud	-7.1	-8.1	-7.8																		-7.67
E outdoor	Tree leaf	NF	NF	NF																		
F outdoor	Dandelion leaves	-8.7	-9.2	-8.4																		-8.77
NF = not frozen																						

Video 1. Ice Ice Baby: Lecture. This is the lecture portion of a unit on ice nucleation and the water cycle for advanced secondary school students (ages 15-18). This unit was designed to teach about precipitation processes and their impact on our world while exploring ice nucleation through the lens of a microbial ice nucleator. The lecture includes a freezing demonstration showing the ice nucleation ability of *Pseudomonas syringae* (referred to as corn leaf powder to fit in the context of the lecture). The unit includes guided and open inquiry sections where students test known and unknown samples for ice nucleation activity.
https://youtu.be/qPEuWrTbWbE

Video 2. Ice Ice Baby: Freezing Demonstration. This is the freezing demonstration associated with a unit on ice nucleation and the water cycle for advanced secondary school students (ages 15-18). This unit was designed to teach about precipitation processes and their impact on our world while exploring ice nucleation through the lens of a microbial ice nucleator. The demonstration shows the ice nucleation ability of and Ice+ strain of *Pseudomonas syringae*. The unit includes guided and open inquiry sections where students test known and unknown samples for ice nucleation activity. https://youtu.be/648QSyHaNII

Ice nucleation assay student handout Group number: _____

Objective:_____

Hypothesis:_____

Experiment design

Droplet size:_____

Replicates:_____

Control:_____

Samples

Sample Number	Sample Name	Description
Control	Sterile deionized water	
	Sample collected from outside	
1	Tap water	
2	Mineral water	
3	Feldspar	Inorganic, igneous rock, mineral
4	Montmorillonite	Inorganic, clay
5	*P. syringae* strain 1	Bacteria
6	*P. syringae* strain 2	Bacteria
7	Snomax®	Inert bacteria
8	Fennell pollen	Pollen
9	Bee pollen	Pollen
10	Button mushroom spores	Fungus

Choose 3 samples from numbers 1-10 above, plus control, plus outdoor sample for a total of 5 samples.

Go outside and pick a sample you think might be a good ice nucleator (examples: water, soil, leaves, etc.). Place the sample in the 50 mL tube with an orange cap provided to your group.

What was your outdoor sample description including location?_____

Place the droplets on the Parafilm boat with the bath at -2° C. Set the bath to -12° C and watch for freezing as the temperature changes. As the drops freeze they color will change becoming less yellow.

Results:

Record if each sample froze or not, and if it froze, at what temperature.

Sample	Replicate 1	Replicate 2	Replicate 3
Example: 4	Froze @ -8° C	Froze @ -9° C	Not frozen @ -12°C
Control			
Outdoor sample			

What can you conclude?_____

What are the limitation of this design?_____

What could be improved in the future?_____

Chapter 6 – Conclusions and Future Directions

This research has shown that ice nucleating strains of *Pseudomonas syringae* were abundant and diverse in Claytor Lake, VA at all sampling locations and all sampling days. These bacteria have the potential to aerosolize into the atmosphere. Temperature does not seem to affect aerosoilzation rate, but viability of aerosolized bacteria does increase with temperature. This finding indicates the potential effect of seasonal temperature variation on aerosolization. The Ice+ phenotype (bacteria that are expressing the ice nucleation protein) seems to increase aerosolization rate and viability, demonstrating that similar bacteria don't aerosolize at the same rate and particularly important to the bioprecipitation hypothesis, the Ice+ phenotype may increase aerosolization. The flume study showed that wind speed affects droplet parameters in a complex manner. The mechanism of droplet production as well as the droplet diameter, speed, and angle all influence the probability of a droplet being aerosolized. With higher wind speeds, the mass of droplets increases which could also increases the amount of bacteria in an aerosol. However, the droplet size also increases and larger droplets are less likely to reach through the boundary layer as aerosols. Given average concentrations of bacteria in a freshwater lake significant amounts of bacteria can cross the air-water interface in droplets due to the action of wind.

In the future, this work could be extended to other lakes and rivers. Water samples could be collected from other bodies of water representing a range of aquatic habitats. Factors that can contribute to spatio-temporal distribution patterns could be examined, such as dissolved oxygen, pH, bacterial residence time, and depth of thermocline. It would be beneficial to sample bodies of water across all seasons in a year to determine if there are seasonal changes to the concentration of *Pseudomonas syringae* or the percentage of Ice+ bacteria. Weather conditions

could also be examined to see if there is a correlation with the concentration of *P. syringae* and percentage of Ice+ colonies at different depths. Rain has been sampled for Ice+ strains of *P. syringae*, and the sequences of the strains were compared with the sequences of strains that were found in Claytor Lake. A rain collection could be conducted simultaneously in the same location as the lake collection and the sequences compared. If the lake was sampled before, during, and after a rain event along with collection of the rain, the information could indicate how significant the contribution of rain (and thus bacteria transported over long distances via precipitation) is to a local population of *P. syringae*. In the current experiment, the samples that grew on the *Pseudomonas* selective media could be further characterized to determine if they are *P. syringae*. This information would give a more complete understanding of how the Ice+ phenotype is distributed among a population of *P. syringae.*

The droplet experiment in the flume focused on the production of droplets at the surface of the water. This information helped determine how many bacteria have the capacity for aerosolization. Bacteria must cross the boundary layer to enter the atmosphere and travel freely as aerosols. Further work can be done using the data collected in this flume experiment to model the projected trajectory for each droplet determining which droplets will cross the boundary layer and be subject to transport as an aerosol and which will return to the water immediately. Several factors related to the droplet and ambient conditions combine to determine the potential for each droplet to move into the atmosphere (Burk, 1984; Cipriano and Blanchard, 1981; Fairall et al., 1983).

In the nebulizer experiment, we used an Ice- and Ice+ strain from the same phylogenetic subgroup, and though we found the Ice+ strain produced more aerosol, further work is needed to determine if the difference in aerosolization is actually due to the Ice+ phenotype. Several more

strains of Ice- (not expressing the ice nucleation protein) and Ice+ *Pseudomonas syringae* could be tested to confirm if the difference in aerosolization rate is caused by the Ice+ phenotype. The ice nucleation activity gene could be knocked out in the Ice+ strain. Also an Ice+ and Ice- *Escherichia coli* could be developed and aerosolized with the same method used in the nebulizer experiment (Corotto et al., 1986; Kozloff et al., 1991). These are all methods that would help determine if the observed differences in aerosol production are caused by the Ice+ phenotype or other differences in the strains. If the Ice+ phenotype does increase the aerosol production, further research could be done to determine what the cause of the difference is. The ice nucleation protein may create differences in electrostatic interaction and this may influence its interaction with water thus possibly influencing aerosolization (Tinsley et al., 2000).

There are still many areas of microbial aquatic aerosolization that have not received much study. One such area involves aerosolization from ice. Bacteria, including *Pseudomonas syringae*, are found in ice cores from the Arctic (Wilhelm et al., 2012). It is not known what happens to the bacteria when the ice melts. The bacteria may aerosolize from ice directly as melting occurs, or bacteria may aerosolize from the water of melted ice. An experiment could be conducted where bacterial suspensions are frozen and then allowed to melt. A particle counter could be used to count aerosolized bacteria. This experiment could be conducted with the melt water being drained off, and without removal of the melt water. The difference is aerosolization between these two methods could be used to determine if aerosolization directly from ice or from the water of melted ice contributes more significantly to aerosolization. Ice+ and Ice- bacteria could be tested separately to see if viability and aerosolization are effected by the ice nucleation phenotype. If Ice+ bacteria are better able to withstand freezing, this could be evidence for the Ice+ phenotype enhancing viability in atmospheric conditions.

The interdisciplinary inquiry based high school unit sets an example that could be used as a model for curriculum development. High school curricula with this format could prepare students for future research and jobs in STEM fields by presenting current research topics with an interdisciplinary inquiry method.

Given the large amount of water surface on Earth and the amount of bacteria present in aquatic environments, understanding aerosolization processes has important applications in understanding, and possibly manipulating atmospheric processes, as well as understanding pathogen dispersal. Ice+ bacteria are more efficient ice nucleators than inorganic particles, but found in lower concentrations. Understanding the role of Ice+ bacteria in atmospheric processes involves determining if the higher efficiency compensates for the lower concentration. One step towards this understanding is investigating the origins of atmospheric bacteria. Towards this end this research has examined the concentration and diversity of Ice+ *P. syringae* in a freshwater lake, examined factors that can influence aerosolization process, as well as looked at droplet production and found the potential for significant amounts of Ice+ *P syringae* to be aerosolized from a freshwater lake. As our understanding of the involvement of Ice+ bacteria in precipitation processes increases, there may be a future possibility of distributing Ice+ bacteria to promote rain production in a controlled process. Although the focus of this research was specifically *P. syringae,* the information about *P. syringae* in aquatic environments and droplet production could easily be applied to understanding aerosolization and dispersal of other microorganisms. *Pseudomonas syringae* can be a plan pathogen and other pathogens are found in aquatic environments as well. Greater understanding of pathogen transport and dispersal patterns could allow for better predictions of pathogen infection as well as leading to more efficient management strategies.

References

Burk, S.D. 1984. The generation, turbulent transfer and deposition of the sea-salt aerosol. *Journal of the atmospheric sciences*. 41:3040-3051.

Cipriano, R.J., and D.C. Blanchard. 1981. Bubble and aerosol spectra produced by a laboratory 'breaking wave'. *Journal of Geophysical Research: Oceans (1978–2012)*. 86:8085-8092.

Corotto, L., P. Wolber, and G. Warren. 1986. Ice nucleation activity of Pseudomonas fluorescens: mutagenesis, complementation analysis and identification of a gene product. *The EMBO journal*. 5:231.

Fairall, C., K. Davidson, and G. Schacher. 1983. An analysis of the surface production of sea-salt aerosols. *Tellus B*. 35:31-39.

Kozloff, L., M. Turner, and F. Arellano. 1991. Formation of bacterial membrane ice-nucleating lipoglycoprotein complexes. *Journal of bacteriology*. 173:6528-6536.

Tinsley, B., R. Rohrbaugh, M. Hei, and K. Beard. 2000. Effects of image charges on the scavenging of aerosol particles by cloud droplets and on droplet charging and possible ice nucleation processes. *Journal of the atmospheric sciences*. 57:2118-2134.

Wilhelm, R.C., K.J. Radtke, N.C. Mykytczuk, C.W. Greer, and L.G. Whyte. 2012. Life at the wedge: the activity and diversity of Arctic ice wedge microbial communities. *Astrobiology*. 12:347-360.