FARMS, FUNGI, AND CLIMATE:

DRIVERS OF PATHOGENIC FUNGI ABUNDANCE IN CORN SEEDS

by

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A BIOLOGY HONORS THESIS

Presented to the Departments of Biology and Environmental Science
and the Robert D. Clark Honors College
in partial fulfillment of the requirements for the degree of
Bachelor of Science

June 2017

An Abstract of the Thesis of

Catherine Jaffe for the degree of Bachelor of Science
in the Departments of Biology and Environmental Science to be taken June 2017

Title: Farms, Fungi, and Climate: Drivers of Pathogenic Fungi Abundance in Corn Seeds

Approved: _____
Dr. Brendan Bohannan, Primary Adviser

Seedborne pathogenic fungi in the genus *Fusarium* are prevalent in corn populations and pose human health risks due to their production of carcinogenic mycotoxins. Although much is known about the large scale effects on domestic maize agriculture, less is understood about the impacts of agricultural management and climate on the distribution of microbes such as *Fusarium* that live in and around these plants. Our research aims to better understand how crop management and regional climate affect the abundance and distribution of seedborne fungi, specifically fungi in the genus *Fusarium*. We gathered samples through a citizen science based initiative in which seed savers from a variety of locations in the United States sent us their corn seeds. We used Quantitative Polymerase Chain Reaction (qPCR) to amplify and quantify the abundance of *Fusarium* in theses samples. Using multivariate statistics, we generated a model that explained the relative contribution of factors such as seed type, climate, and agricultural practices to variation in seedborne *Fusarium* abundance. The results of our research may have wide reaching implications due to the ubiquity of *Fusarium*, the potential to impact methods of sustainable agriculture, and the consequences of a rapidly changing climate.

Acknowledgements

This thesis would not have been possible without the support and guidance of so many people. Thank you to Lucas Nebert for sharing his time, expertise, and encouragement throughout this project, his mentorship was invaluable to the completion of this research and my own learning experience. Thank you to Brendan Bohannan for his insightful feedback and guidance. And thank you to the entire Bohannan Lab group for providing some much needed advice on data wrangling. I also want to thank the Undergraduate Research Opportunity Program for funding this work. Finally, a huge thank you to my friends and family for supporting me during my time as an undergraduate.

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Introduction

For thousands of years humans have managed and cultivated many varieties of maize. A foundational source of nutrition and sustenance in cultures across the globe, maize has undergone a dramatic process of domestication and human-selection. Although much is known about the effects of domestication on the genetics and health of corn populations, less is understood about the impacts of agricultural management on the microbial communities living in and around these plants. Although small, these microscopic communities play an essential role in cycling nutrients through the ecosystem, making limiting nutrients such as nitrogen available to maize and defending the plant against pathogenic fungi and bacteria (Johnston-Monje 2011).

My project focuses on one particular genus of fungi, *Fusarium,* which is ubiquitous in both soil and seed environments across the globe. *Fusarium* species can have a variety of ecological functions in the plant, as they can be both endophytic or pathogenic. Although many species are not pathogenic, those that are cause common and widespread diseases (such as Fusarium wilt and Fusarium ear rot) in a variety of grains, including maize. These diseases can significantly reduce yields on a wide range of farms, from small community agriculture to large industrial monocrops. Of particular concern are potentially pathogenic *Fusarium* which produce fumonisins, a group of mycotoxins thought to cause cancer and a variety of other health conditions in humans (Munkvold 2003; US FDA 2001). In addition to its varied ecological roles, *Fusarium* has a variety of infection pathways through which it can enter the plant. These fungi can move from soil to plant via soil-root contact (horizontal transmission) or from parent to

offspring via seeds (vertical transmission) (Johnston-Monje 2011). Thus, potentially pathogenic fungi can reside and proliferate in maize via vertical transmission without causing visible signs of infection, building up through generations of seeds until conditions facilitate an outbreak.

There is a significant body of research on the biotic effects and interactions which can increase the likelihood of *Fusarium* outbreaks in maize. A variety of bacterial endophytes have been shown to suppress species of *Fusarium* and accumulation of associated mycotoxins (Raizada 2015). Additionally, insect related tissue damage and disease in the host plant has been linked to increases in visible signs of *Fusarium* infection (Parsons 2012; Parsons 2010; Cao 2014). There is less research on how abiotic conditions such as climate, host location, and agricultural practices may influence the abundance of seedborne *Fusarium* and thereby the risk for *Fusarium* associated diseases in the host plant. Further, these biotic and abiotic conditions are not entirely independent of each other, and the relative importance of different infection pathways is thought to vary by geographic location (Parsons 2012; Munkvold 2003). As a result, there is a growing need to understand how both variation in agricultural practices and other abiotic factors interact and affect *Fusarium* abundance in corn seeds.

With my research I investigated how climate, maize genetics, and crop management might be influencing the abundance of *Fusarium* in corn seeds. How do variations in precipitation and temperature regimes, agricultural practices, and host genetic background influence the abundance of seedborne *Fusarium*? Which of these individual factors, or their interactions, explain more of the observed variation in

Fusarium abundance? Finally, how can these factors be used to predict which agricultural regions might be at increased risk of *Fusarium* related disease outbreaks?

Seed Type and Plant Phylogeny

Understanding the role of host phylogeny in this seed-fungi-environment system is important for many reasons. Most significantly, maize plants that are more genetically related are more likely to have similar seedborne endophytic microbial communities (Johnston-Monje 2011). Additionally, some research has suggested that the phylogeny of the host plant may change the way abiotic factors influence seedborne abundance of certain fungi (Cao 2014; Parsons 2010). These gene-environment interactions are important to consider as they complicate the way that climate may drive the abundance of seedborne *Fusarium* species.

For my thesis I use two characteristics of maize, corn variety and seed type, as proxies for corn genetics. Using these categorical variables will help us analyze how conditions within the seed and the phylogeny of the host plant might influence the abundance of seedborne *Fusarium*. There were five types of seeds in our samples: Flint, Dent, Flour, Sweet, and Popcorn. Classifications of seed "type" are based on how nutrient storage occurs in the seed. Sweet and flour seeds are the most starchy and sugary types, while popcorn and flint types store nutrients as fatty endosperm. Although these types are not always indicative of evolutionary relationships, there are some general correlations with maize evolutionary development. For instance, the starch composition of the dent seed type is about halfway in between the flint and flour seed, and as a result flint-flour hybrids tend to have dent seed types (Saunders 2009). Maize variety is a convention used by seed producers and farmers to differentiate between

different cultivars. All modern maize varieties are considered a single species, as they can interbreed with each other. Many varieties of corn may have the same seed type, thus variety is a more specific classification than seed type.

Fusarium and Climate

Abiotic factors such as climate can influence seedborne *Fusarium* abundance by creating favorable or unfavorable conditions for the growth of the fungal population. The degree to which the plant microbiome is influenced by these environmental factors is also debated, and I hope to at least partially address this gap in knowledge with my research. Current understanding of the relationship between climate and seedborne *Fusarium* is complicated by the variability of precipitation and temperature regimes and by regional microclimates. Recent literature has shown that *Fusarium* species increase in abundance in kernel tissue when conditions following pollination are warm and dry (Parsons 2012; Munkvold 2003). The mechanisms behind this observation are unclear, but some scientists have suggested that when maize plants are stressed due to early season drought conditions they are more prone to an outbreak of *F. verticillioides* or other *Fusarium* species (Czembor 2015; Parsons 2010).

From these studies it is clear that broad climatic variables such as annual precipitation and average annual temperature, which do not reflect the temporal aspects of precipitation or temperature, may not be sufficient measurements when examining biological cycles. A solution to this issue is “bioclimatic” variables, which quantify climate variables for specific seasons and time periods (Hijmans 2005). These are the types of variables I use to characterize climate, as I will further explain in my methods.

Fusarium and Agricultural Management

We hypothesized that climate variables interact with agricultural practices to determine the abundance of Fusarium. The term “agricultural practices” encompasses a wide range of methods that may not be shared by all farmers. Such factors include irrigation methods, tilling practices, fertilizer strategies, compost use, cover crops and crop rotations among other practices. Previous studies have shown that planting maize earlier in the season reduces drought stress later on and can result in significant reductions of mycotoxins in maize seeds (Parsons 2012). Additionally, tillage (the practice of disturbing the soil between crops) generally decreases *Fusarium* abundance in soil and in crops by disrupting fungal hyphae systems (Steinkellner 2004; Hofgaard 2016). However, the effect of tillage can be dependent on other management practices including management of surface crop residue. In fact, the reincorporation of infected plant matter back into the soil that occurs during post-harvest tilling has been shown to contribute to increased levels of soil *Fusarium,* which may then be horizontally transmitted into the new round of crops (Wakelin 2008). Thus the relationship between tillage and *Fusarium* warrants further research.

Applications of Citizen Science

My thesis uses a dataset generated from the Microbial Inheritance in Seeds Project, a participatory research project comprised of over twenty seed saving farmers in the U.S. (and a single farm in Ireland) who send in their own maize seeds for microbiome analysis. The project was established by PhD student Lucas Nebert, and so far dozens of the project members have sent in seed samples. All samples used for my thesis come from this project.

Another term for this kind of participatory research process is “citizen science”. There are a variety of definitions for this term, but for the purposes of this research citizen science can be defined as any scientific research in which non-scientists assist with some aspect of the research process from data collection to analysis and presentation. Traditional applications of citizen science have worked to further public engagement with science, increase scientific literacy, and extend the scope of research in conservation biology, ecology, environmental toxicology, and even molecular biology (Dickinson 2012; Hand 2010). In addition to educational and monitoring applications citizen science is becoming an important tool with which farmers and scientists exchange agricultural and technological knowledge (Van Etten 2012). Using citizen science collected data in agricultural and ecological research has benefits and drawbacks. Datasets generated using this method tend to be large, highly variable, and can have a wide geographic range, presenting a unique challenge for developing reliable statistical tests. As a result, these data are best employed for observational studies which serve as a basis for future hypothesis-driven research (Dickinson 2010). To supplement my quantitative investigation I also conducted interviews with some farmers and seed savers involved with the project.

Hypotheses

We hypothesized that climate and agricultural management are important drivers of seedborne *Fusarium* abundance, and may explain more of the observed variation in this abundance than our measures of maize genetics and traits. Of these environmental factors we expected: (1) The amount and timing of precipitation to have a negative relationship with seedborne *Fusarium* abundance. (2) The use of tillage

before planting to have positive interactions with *Fusarium* abundance. (3) We also hypothesized that some maize seed types and/or varieties will be more susceptible than others. We then assessed the effects of each of these factors alone and looked for possible interactions.

Materials and Methods

Sample collection

Seventy-four seed samples were sent in by twenty-three farmers and seed savers from across the United States. Most participants were recruited during conferences hosted by the Organic Seed Alliance, specifically the Organicology Conference and Organic Seed Growers Conference, in Portland, OR (2013) and Corvallis, OR (2014) respectively. Others were recruited through advertising on the website of Adaptive Seeds and the personal project website, Microbial Inheritance in Seeds (www.microbialinheritance.org). Therefore, most corn samples were concentrated in the Pacific Northwest, and were predominantly produced from growers using organic methods.

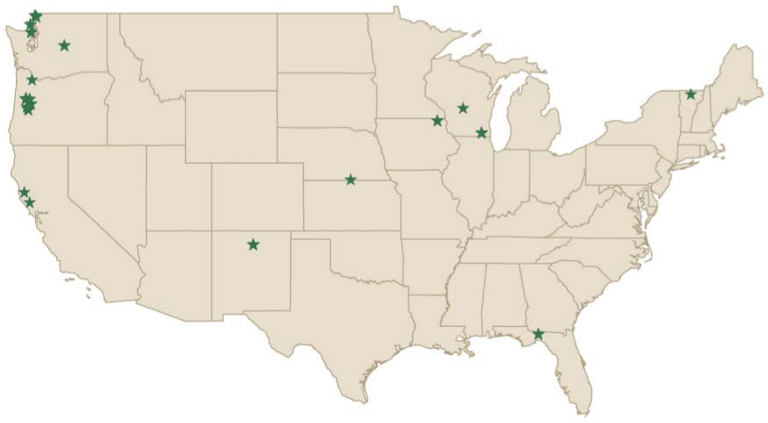

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Figure 1. Locations of Participating Farmers and Seed Savers

Basemap source: ESRI (http://www.esri.com)

Each sample contained 20 seeds. 53 out of the 78 samples were from the Pacific Northwest (Washington, Oregon, and Northern California). Samples were from harvests collected between 2005-2014, although the majority (71 out of 78) were harvested between 2012 and 2014. Although planting and harvesting dates varied by farmer and

location, all seeds are from maize that was planted and harvested between April and October. Seed savers sent in samples on a volunteer basis, meaning that no guidelines were used to select specific farms. As a result, sample collection was not designed to answer specific questions but rather to collect baseline observational data about the composition of the microbial community in the seed.

DNA extraction

The methods for DNA extraction are adapted from Lucas Nebert and the standard MoBio PowerPlant Kit procedures. Twenty seeds were randomly selected from each larger sample to be representative of an entire harvest. To remove surface microbes, seeds were surface sterilized with 3% Bleach for 10 minutes, rinsed and sterilized again with 3% Bleach for 10 minutes. Seeds were then rinsed in 95% Ethanol for 10 minutes, followed by 3 rinses in autoclaved nanopure water. Once dry, samples were aseptically ground in a sterilized Porlex coffee grinder under a sterile flow hood. To sterilize the Porlex grinder, we first disassembled each grinder and washed each part with standard lab detergent. Ceramic and metal grinder components were then flame sterilized and grinders were reassembled under the hood. This sterilization procedure was repeated before grinding each sample.

The ground up seeds were thoroughly mixed, and then their DNA was extracted using the MoBio PowerPlant Kit, with the following changes. Seeds were first freeze-thawed by alternating between liquid nitrogen and a hot water bath at 65C to break the cell walls. They were then homogenized in a Fast Prep instrument two times at a speed of 5.5 for 25 seconds each. Included in the vials were .5 ml of 100-micron glass beads, in addition to the provided 1mm metal beads. The homogenates were incubated at 10

minutes in a 65C water bath before continuing with the standard DNA extraction kit procedure.

qPCR

DNA extracts were then used in Quantitative Polymerase-Chain-Reaction (qPCR). qPCR is a standard biomolecular technique used to amplify targeted DNA sequences and quantify the concentration of these target DNA sequences in the original sample. In qPCR, primers (short DNA sequences) are used to target DNA. We used three different sets of DNA primers to target three categories of fungal DNA: Total fungi, the whole *Fusarium* genus, and *F. verticillioides* (a fumonisin producing *Fusarium* species). We used forward and reverse Internal Transcribed Spacer (ITS1F/ITS2) primers to target total fungi, while *Fusarium* specific IGS primers were used to target *Fusarium* (IGS_FumF/IGS_FumR) and *F. verticillioides* (VertF1/VertF2) (Table 1).

Table 1. qPCR Primers

	Primer Name	DNA Sequence	Source
Total Fungi	ITS1-F	CTTGGTCATTTAGAGGAAGTAA	Toju et. al.
	ITS2	GCTGCGTTCTTCATCGATGC	
Total *Fusarium*	IGS_FusF	CGCACGTATAGATGGACAAG	Jurado et al.
	IGS_FusR	GGCGAAGGACGGCTTAC	
Fumonisin Producing *F. verticillioides*	VertF1	GCGGGAATTCAAAAGTGGCC	Patino et al.
	VertF2	GAGGGCGCGAAACGGATCGG	

Before qPCR runs we quantified the concentration of DNA in each extract using Qubit fluorometric quantitation, and diluted each sample according to the measured

concentration. Each 10µl qPCR reaction contained 1µl DNA extract and 9µl of a mixture of forward and reverse primers (200nM), sterile water, and KAPA FAST One-step qt-PCR master mix. Each qPCR reaction contained a 10-minute pre-incubation at 95˚C, followed by 40 cycles of amplification, and finally a melt curve to ensure purity of the amplicon, using a BioRad CFX-96 Real-Time PCR System. The IGS_Fus and VertF genes were amplified using the standard KAPA Fast system protocol: 95˚C for 15 seconds, followed by a 30 second annealing/extension step at 60˚C. Amplification of the ITS1 gene proceeded with 95˚C for 15 seconds, 55˚C for 30 seconds, followed by an extension step of 72˚C. Three negative controls and three positive controls were included with each qPCR run. In negative controls the 1µl DNA extract was replaced with 1µl sterile water. A positive control of *F. verticillioides* of known concentration was used to calibrate the gene copy numbers of ITS1, IGS Fus, and VertF. qPCR runs for *Fusarium* and *F. verticillioides* were triplicated to account for possible error within individual runs. The qPCR for total fungi was duplicated. Outlying data was eliminated from the final data set based on failure to pass quality checks, such as tests for amplicon purity.

Interview Procedures

Before conducting interviews I obtained approval from the Institutional Review Board. Once approved I sent out a recruitment email in early Spring 2016 to members of the Microbial Inheritance in Seeds Project. During Spring and Summer 2016 I conducted a series of phone interviews with seven members of the project to collect

data on the specific details of their agricultural practices and seed saving methods. A copy of the questionnaire used for interviews is attached in the appendix.

Interviews were recorded and then transcribed using the software ExpressScribe. Once transcribed both quantitative and qualitative data were extracted from the interviews. Most quantitative questions were coded as binary responses (yes/no, absent/present). The majority of quantitative responses concerned agricultural practices and inputs such as tilling, fertilizer use, irrigation, organic matter input, pest control, and so on. Some questions were also included for qualitative analysis of seed saving and agricultural practices. These types of questions encompassed general motivations and concerns of the project members such as why certain agricultural practices were used and general challenges in seed saving and maize agriculture.

Climate Data Collection

An emerging method in agricultural citizen science conducted across a wide geographical range is to combine geospatial data with collected data in order to assess possible effects of environmental factors on directly measured variables (Van Etten 2016). I obtained climate data from an open-source, online database generated by researchers at University of California, Berkeley (Hijmans 2005). The authors compiled nationwide measurements of precipitation and temperature and interpolated temperature and precipitation surfaces to be representative of the average climate between 1960-2000. These interpolated data have a spatial resolution of 30 seconds (about $1km^2$). The authors derived 19 bioclimatic variables from these values and made them accessible to researchers via the open-source database Worldclim.org. These variables are termed "bioclimatic" because most are specific to biological and seasonal patterns, and so these

data are specifically tailored for ecological applications. Examples of these variables include “precipitation of the warmest month” and “mean temperature of the coldest quarter”. The United States Geological Survey (USGS) also provided valuable detail on how these variables are both calculated and commonly used in the literature (O’Donnell 2012).

Data Analysis Methodology

Climate Model: Exploratory Factor Analysis

I performed all data analyses in RStudio, a programming platform which uses the coding language R (www.R-project.org). Since many of the 19 bioclimatic variables quantify precipitation and temperature, we anticipated a large amount of collinearity between these variables. To simplify the climate variables and account for underlying seasonal relationships I applied Exploratory Factor Analysis (EFA). The goal of EFA, similar to Principal Component Analysis (PCA), is to reduce the number of independent variables in a given model. To achieve this, EFA takes the independent variables and groups them according to how they co-vary with each other. Unlike in PCA, the resulting groups or “factors” are then representative of latent unmeasured variables which might be driving some of the covariation (Yong 2013).

Both PCA and EFA have been used to simplify climate and atmospheric data, although PCA is the more common of the two methods in these fields (Unkel 2010). In this case, EFA was chosen because we were interested in not only reducing the variables but also identifying any underlying temporal and spatial processes, such as seasonal or regional climate patterns, which might be driving some of the covariation between the climate variables. Similar applications of EFA have been used to reduce

and explain variation in precipitation regimes and other meteorological patterns (Wickramagamage 2010; Tadic 2010). Each factor has a set of factor loadings (Table 1), which are used to calculate factor scores. In this way, the loadings are similar to weights. Once scores are extracted, factors can then be treated as independent, continuous variables to be used in additional analyses such as linear regression and analysis of variance (ANOVA).

Interview data

From the interviews I obtained an additional dataset for 16 samples. The objective of analyzing this smaller dataset was to gain a window onto the relationship of agricultural practices to fusarium abundance. Though the sample size was small for this subset of data, results from this analysis might provide insight into potentially important agricultural practices that could be studied in future research. Information from transcribed interviews was coded in excel as either qualitative or quantitative variables to be used in further statistical tests. Variables coded as binary were: tilling, cover crop use, livestock presence, and disease presence. Categorical variables obtained from interviews were: tillage intensity, irrigation method, cover crop type, time as agricultural land, and irrigation frequency. The tillage intensity for each sample could be either high, low, or none. Tilling was coded as high intensity if a rototiller or similar instrument was used by farmers, usually resulting in tillage to a depth of greater than 2 inches. Low intensity tillage was 2 inches deep or less, and no-till practices were simply coded as “none”.

Results

Exploratory Factor Analysis

I first ran a series of linear regressions with each of the 19 climate variables as a preliminary screen for relationships between climate and the abundance of seedborne Fusarium. Predictably, including more than a few of these variables in the same model resulted in high collinearity between climate variables (Figure 2). Stepwise selection of the model was not successful in simplifying the number of relevant climate variables.

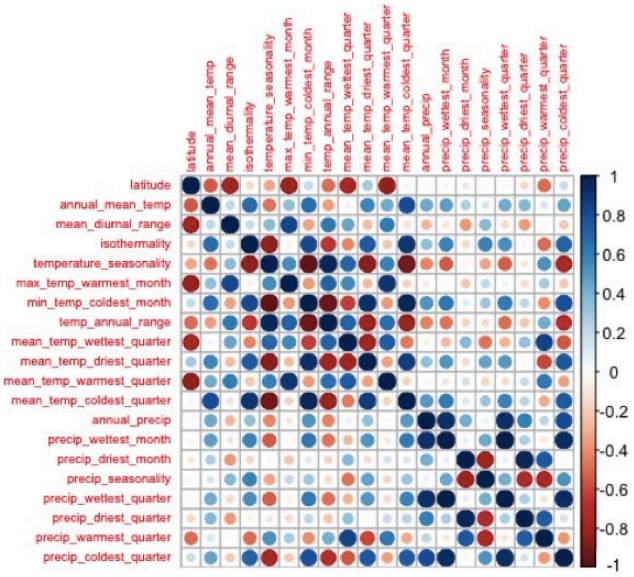

Figure 2. Correlation Plot of Bioclimatic Variables.

The largest and darkest dots indicate strong covariation between variables. As expected, most covariation occurs between precipitation variables and between temperature variables, though there are some notable outliers.

EFA was performed with all bioclimatic variables and latitude. Latitude was included as it also co-varies with temperature and precipitation values. The rotation I used with EFA was “varimax”, which ensures that resulting factors are independent of each other (R Development Core Team, 2013). The score extraction method was

principal axis. The only climate-related independent variable excluded from EFA was longitude, as the two samples from Ireland had longitude values which were dramatic outliers from the rest of the samples. Additionally, when I ran EFA, the loadings for longitude in three of the four factors were almost equal and quite low (< .5) which indicates that longitude does not co-vary significantly with the other climate variables and therefore would not be useful to include in EFA (Yong 2013) A table of loadings is displayed in the Appendix (2).

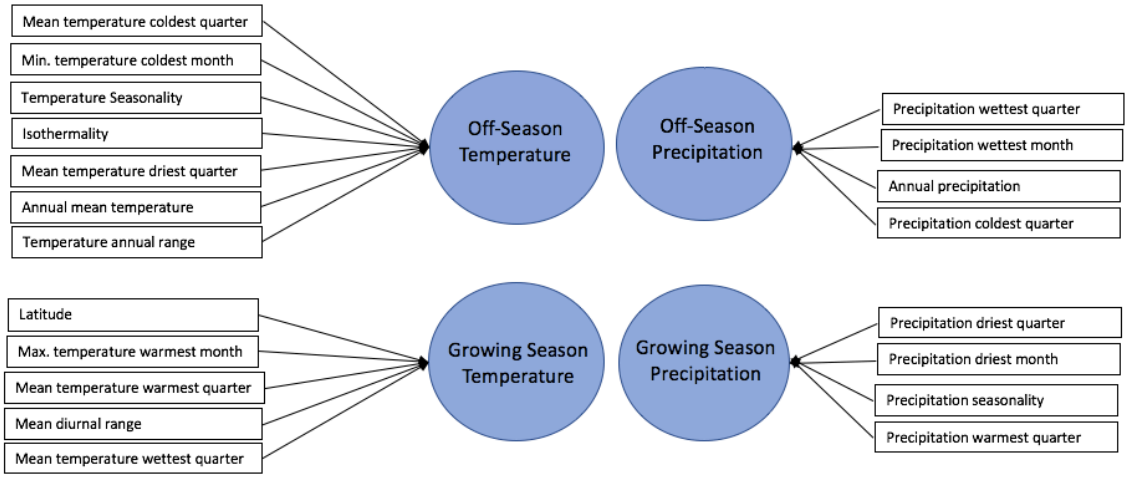

Figure 3. Climate Variables and Associated Factors

Using EFA the climate variables were simplified to four factors: “Off-Season Temperature”, “Growing Season Temperature”, “Growing Season Precipitation”, and “Off-Season Precipitation” (Figure 3). I chose to name these factors according to their relevance to the growing season of maize “Growing Season Precipitation” and “Growing Season Temperature” quantify combinations of rainfall and temperature most favorable for the growth of maize (usually the warmest times of year). A good way to conceptualize these groupings is as distinct temperature and precipitation regimes (Wickramagamage 2010). The factors are not representative of overall precipitation and

rainfall but instead they more closely quantify the timing and intensity of climatic patterns which may be more or less common in certain regions (Figure 4). For example, a sample with a high score for the factor “Growing Season Temperature” was grown in a region with high temperatures during periods of high rainfall, such as Florida. Similarly, samples with high scores for the factor “Growing Season Precipitation” have relatively high precipitation, even during the driest, hottest times of the year.

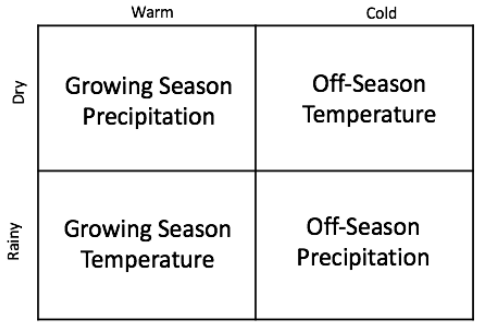

Figure 4. Climate Factor Diagram.

The above figure is a visual representation of how each factor represents a specific temperature-precipitation regime.

Total Seedborne Fungi

The number of ITS copies per ng of total DNA extracted from seeds ranged from 3,430 to 306,000 copies per ng (SD = 35,100). For all statistical analyses involving qPCR, the Log_{10} of copy number was used, to satisfy normality requirements for the statistical tests. There was no statistically significant relationship between the climate factors and total fungi abundance, and no spatial autocorrelation was present. A one-way ANOVA with seed type (Figure 5) indicated that sweet corn had significantly

lower abundances of total fungi, although the variation explained by this relationship is low (8.8%).

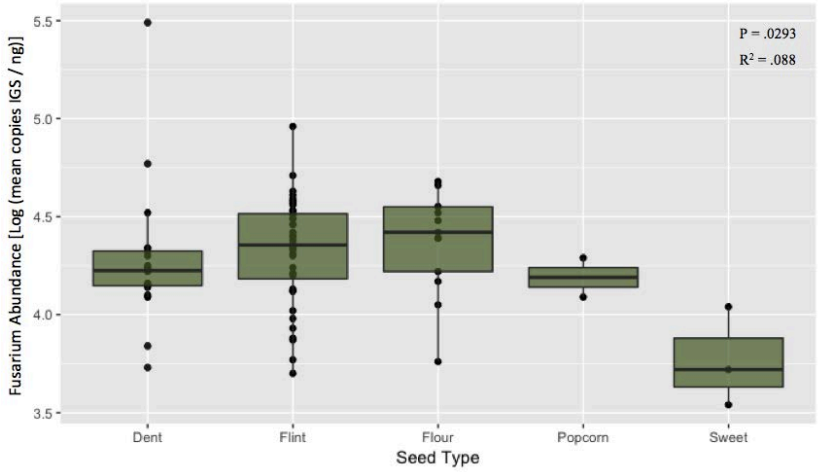

Figure 5. Total Fungi Abundance by Seed Type.

Genus Level Analysis: *Fusarium*

The abundance of IGS_Fus copies per ng of total DNA in each sample ranged from 0.8 copies/ng to 8617.5 copies/ng (S.D. = 1358.0).

Climate

I performed multiple regression with all four climate factors as independent variables. I then used stepwise selection to eliminate non-significant factors until there was no over-fitting of the model. In the resulting model, Growing Season Temperature and Growing Season Precipitation showed significant correlation with seedborne *Fusarium* abundance when both factors were present (Table 2). Growing Season Temperature is positively correlated with seed-borne fusarium abundance, indicating that the locations with highest temperatures during the rainy times of the year had higher *Fusarium* abundances (p = .0001). The Growing Season Precipitation factor was negatively correlated with seed-borne *Fusarium* abundance, indicating that locations

with the least rainfall during their driest, hottest season had higher fusarium abundances (p = .0012). Together, these two climatic factors explained about 17% of the variation in seedborne *Fusarium* abundance.

Table 2. Influence of Climate on *Fusarium* Abundance

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	1.9045	0.0859	22.18	0.0000
Growing Season Temperature	0.1056	0.0252	4.18	***0.0001
Growing Season Precipitation	-0.1577	0.0469	-3.36	***0.0012
Observations	78			
Adjusted R^2	0.168			
P-value	.00037			
Residual Std. Error	0.758 (df = 75)			
F Statistic	8.792 (df = 2; 75)			
Note:	*p<0.1; **p<0.05; ***p<0.01			

To further confirm that the influence of climate on *Fusarium* abundance was specific to the genus and not due to overall fluctuations in total fungi, these regressions were also run with *Fusarium* as a percentage of total fungi (IGS/ITS). Stepwise selection of this model also revealed relationships between *Fusarium* abundance and the same two factors (p = .006, R^2 = .11).

In a one-way ANOVA with seed type, popcorn maize had significantly lower *Fusarium* abundance (Figure 6). Similar to analyses with total fungi, seed type explained only a fraction of the variation in abundance (7.6%). Again, the sample size for popcorn and sweet corn are notably lower than the other seed types.

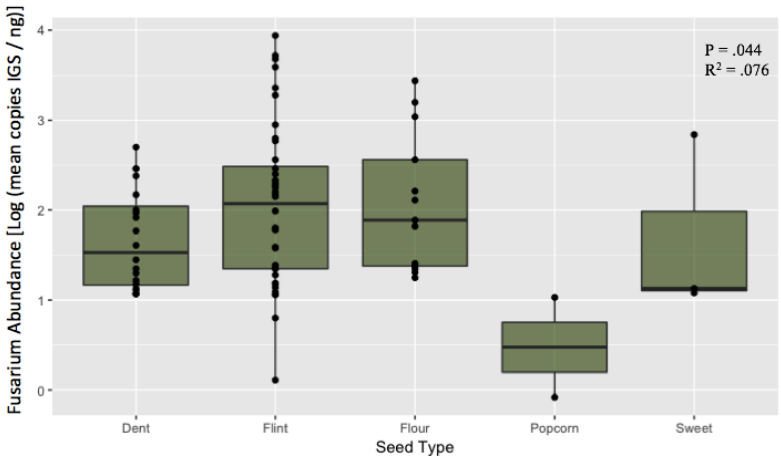

Figure 6. *Fusarium* Abundance by Seed Type

Variety and Climate

To evaluate potential interactions between host genotype and the significant climate factors identified above, I introduced maize variety and seed type as a variables to the climate model and ran linear regressions using interaction terms. Stepwise selection resulted in a model with just climate factors and variety. This model (Table 3) explained 39.6% of the variation in seedborne *Fusarium* abundance.

Table 3. Mixed Model with Climate Factors and Variety

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Growing Season Temperature	1	3.55	3.55	7.93	***0.0078
Variety	23	18.94	0.82	1.84	**0.0490
Growing Season Precipitation	1	2.84	2.84	6.34	**0.0164
GS Temperature:Variety	6	8.18	1.36	3.05	**0.0163
Residuals	36	16.10	0.45		
Observations	68				
P-value	.0058				
Adjusted R^2	0.3958				
Note:	*p<0.1; **p<0.05; ***p<0.01				

Grower

To determine if farm-scale differences may determine seedborne *Fusarium* abundance, I tested whether “grower” could explain variation in IGS_Fus abundance. One-way ANOVA by grower revealed a statistically significant relationship between

the grower of the seeds and the seedborne fusarium abundance. A post-hoc Tukey test showed that high fusarium abundances in the samples from a single grower, P017, drive this difference. Once the three samples from this grower were removed, the "grower" variable lost its explanatory power, indicating that (aside from P017) farm specific variation did not significantly drive seedborne *Fusarium* abundance.

Species-Level Analysis: *F. verticillioides*

45 of the 78 samples contained no detectable *F. verticillioides* (Figure 7), so the raw abundance data for this species were coded as a binary variable (present/absent).

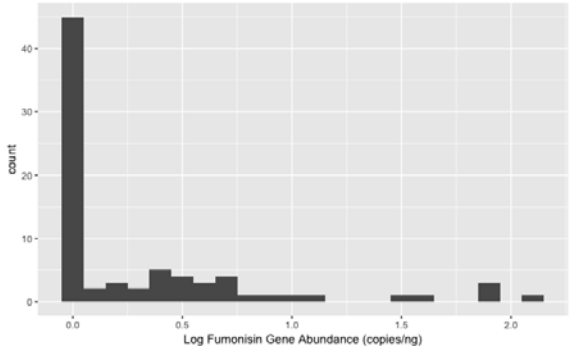

Figure 7. Histogram of *F. Verticillioides* Abundance.

The above histogram of Fumonisin gene abundance on $\log_{10}$ scale shows non-normal distribution of the data. 45 of 78 samples have an abundance of 0 copies/ng.

Climate

Logistic regression was performed with all four climate factors, and stepwise selection was used to eliminate parameters until there was no overfitting of the model (Table 4). Off-Season Temperature and Growing Season Precipitation were the most significant drivers of Fumonisin presence. Together these climate factors explain 11% of the variation in Fumonisin presence.

Table 4. Influence of Climate on the Presence of *F. verticillioides*

	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	-0.3356	0.2401	-1.40	0.1622
Off-Season Temperature	0.2565	0.1064	2.41	**0.0160
Growing Season Precipitation	-0.3754	0.1740	-2.16	**0.0309
Observations	78			
R^2	0.112			
P-value	.0341			
χ^2	6.760** (df = 2)			
Note:	*p<0.1; **p<0.05; ***p<0.01			

Effects of Agricultural Management on *Fusarium* and *F. verticillioides* Abundance

Using the smaller interview dataset containing information on agricultural practices I conducted a series of linear regressions, ANOVA and chi-square tests with these agricultural management variables (Table 5). Linear regressions were used with the only continuous independent variable from this dataset, "farm age". I used ANOVA tests for the remaining categorical independent variables, and chi-square tests to analyze the binary fumonisin presence data.

Table 5. Effects of Agricultural Management on Total Fungi, Total *Fusarium*, and *F. verticillioides*

Explanatory Variable	Total Fungi	Fusarium	Fumonisin Producing
Disease (Y/N)			
Cover Crop (Y/N)		-	
Cover Crop Type		**	
Farm Age (Yrs)			-
Irrigation Frequency (per Month)			
Irrigation Type		*	
Tilling (Y/N)		-	-
Tilling Intensity (Low-Med-High)		-	-
Livestock (Y/N)			
Observations	16	16	16
		Note:	*p<0.1; **p<0.05; ***p<0.01

The direction of relationship between explanatory variable and abundances displayed as +/- for linear regressions (p < .05) and with significance * for ANOVA comparisons.

Total fungi abundance had no significant relationships with any of the interview variables. However, seedborne *Fusarium* abundance showed significant interactions

with tillage use, tillage intensity, and cover crop use (Figure 8). The type of irrigation farmers used was also significant, with seeds grown under overhead irrigation having higher abundances than those grown with drip tape or flood (Figure 8). Tillage and cover crop use were selected for multiple regression using stepwise selection, and together these management variables explained 32% of the variation in this subset of the data (Table 6).

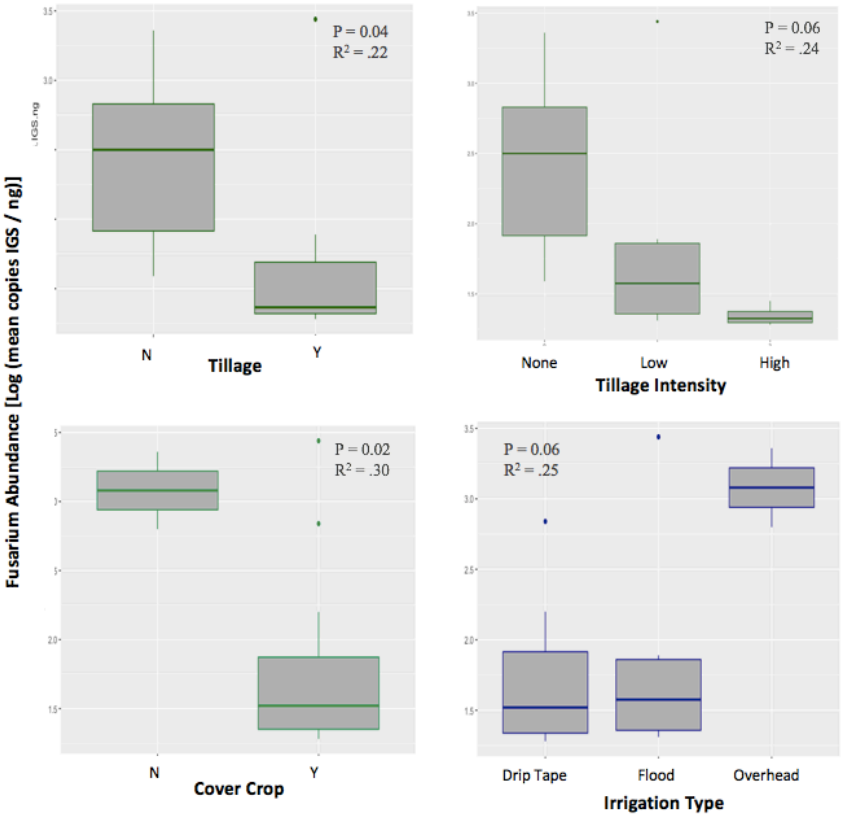

Figure 8. Agricultural Management Effects on *Fusarium* Abundance

Table 6. Mixed Model of Agricultural Practices (*Fusarium* Abundance)

	Df	Sum Sq	Mean Sq	F value	Pr(>F)
tilling	1	2.30	2.30	5.98	**0.0294
cover_crop	1	1.25	1.25	3.25	*0.0948
Residuals	13	5.00	0.38		
Observations	16				
R^2	0.3252				
P-value	.0306				
Note:	*p<0.1; **p<0.05; ***p<0.01				

To assess relationships between fumonisin producer presence and categorical interview variables I used Fisher's Exact test. This test is similar to a Chi-square test, but is more accurate for smaller datasets (R Core Development Team). This test demonstrated that the abundance of seedborne fumonisin producers had a close to significant relationship with tillage intensity (p = .066), with lower intensity tillage resulting in higher amounts of fumonisin producers. Logistic regression using presence/absence data and the variable “farm age” showed that younger farms were more likely to have *F. verticillioides* present.

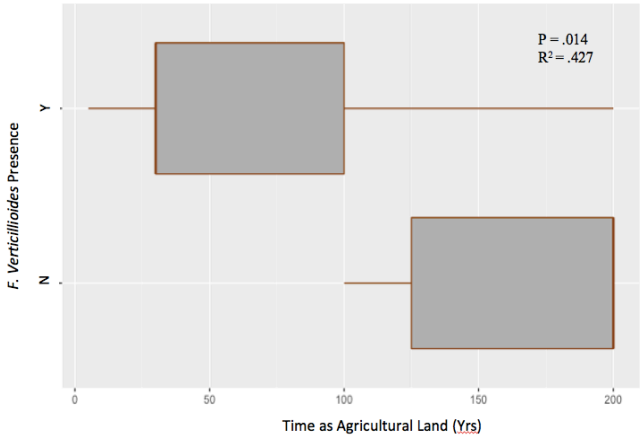

Figure 9. Effect of Farm Age on *F. verticillioides* Presence

Analyses performed with the remaining categorical variables and the binary fumonisin presence data were inconclusive, most likely due to such small sampling size.

Discussion

Climate is an Important Driver of *Fusarium* Abundance

These results support the conclusion that climate, principally precipitation and temperature, are primary drivers of seedborne *Fusarium* abundance. The fact that regional climate had little effect on the total abundance of seedborne fungi, but had a significant effect on the abundance of the *Fusarium* genus confirms that the observed effects are genus-specific. The positive relationship between *Fusarium* abundance and Growing Season Temperature indicates that with higher maximum temperatures and warmer temperatures during the wettest times of the year, we might find higher amounts of seedborne *Fusarium* (Table 2). It is important to note that the wettest time of the year is not the same in every location. In the Pacific Northwest, this time period might occur in late Winter or early Spring. While in Florida the wettest months of the year are typically June through September. So, instead of indicating a general time that might facilitate *Fusarium* proliferation, these results point towards a specific combination of precipitation and temperature regimes which could be more or less likely in certain locations.

Additionally, Growing Season Precipitation negatively correlated with *Fusarium* abundance. Thus, high abundances of seedborne *Fusarium* are more likely to be found in when there is especially low rainfall during the driest, hottest times of year. From this data alone it is not possible to determine the precise mechanism behind these correlations. It is well established that maize growing in drier climates is susceptible to higher levels of stress, and certain species of *Fusarium* proliferate more readily in these stressed plants. This hypothesis has support from studies that demonstrate a correlation

between drought stress and certain species of *Fusarium* (Munkvold 2003; US FDA 2001). It is also possible that species of *Fusarium* are more successful than other fungal groups in regions with warmer, drier regional climates (Cao, 2014).

As might be predicted by these genus-level analyses, *F. verticillioides* abundance was also positively correlated with the Growing Season Temperature regime. However, unlike total *Fusarium*, the abundance of *F. verticillioides* was positively correlated with a different precipitation factor, "Off-Season Temperature". This suggests that warmer temperatures during the coldest, wettest times of the year may increase the likelihood of *F. verticillioides* occurring in the seed. The significance of this factor is surprising because it is unlikely that maize plants grown in the Northern Hemisphere would be in the ground during the coldest and wettest times of the year. However, the "overwintering" phase of the crop cycle has been shown to be a key period for the growth of soil *Fusarium* communities. In particular, *F. verticillioides* has been shown to increase in soil abundance when high levels of maize residue are present in the field during this crucial overwintering period (Wakelin 2008). This soil community can then act as inoculum for the next cycle of maize crop, either via networks of fungal hyphae or airborne spores. In support of our findings, *F. verticillioides* spore dispersal from these residues is thought to occur more readily at warmer temperatures, 30ºC being the optimal temperature under laboratory conditions (Munkvold 2003). We did not measure soil abundance of *Fusarium* or *F. verticillioides*, so our ability to draw conclusions about the mechanisms of these climate effects is limited. However, the significance of the Off-Season Precipitation factor for only *F.*

verticillioides indicates that particularly dry off-seasons may uniquely favor the growth of these species of *Fusarium*.

Interactions between Genetic Proxies and Climate

While not as explanatory as climate, proxies for maize genetics (variety and seed type) demonstrated some relationship with the abundance of total fungi and *Fusarium.* Most notably, sweet corn seed types had lower amounts of total fungi and popcorn seed types had less *Fusarium* (Figures 5 and 6, respectively). Seed type is not a variable commonly considered in the literature on *Fusarium* and seedborne fungi ecology. While our study had some over sampling of flint, dent, and flour types, our results still suggest that seed type (or similar genetic proxies) could be included in future studies to fully elucidate effect of host genetics on seedborne *Fusarium* populations.

The high explanatory power of the climate-variety model (39.6%) supports our hypothesis that maize genetics (as measured by maize variety) may mediate the relationship between seedborne *Fusarium* abundance and climate. The possible mechanisms behind this climate-variety interaction are numerous. Many studies in geographically disperse locations have noted that later planting and harvesting generally leads to greater risk of Fusarium related disease and/or concentration of *Fusarium* associated mycotoxins in maize kernels (Parsons 2012; Cao 2014; Blandino 2009). Consequently, varieties which take longer to mature and must be harvested later may be susceptible to higher abundances of seedborne fusarium.

Although maize variety is more specific to evolutionary relationships than seed type, not every variety is well sampled in the dataset for this project. In the samples

used for my research there are over 30 varieties of maize, but only 5 seed types. As a result, some varieties only appear once or twice in the dataset, and this lowers the statistical power of the variety variable. Therefore, while our results are indicative of important gene-environment interactions, they are not conclusive as to the nature of these interactions.

The Effect of Agricultural Practices

With a small sample size (n= 16), the interview data should serve as a purely observational survey for future avenues of research. The non-significance of grower identity (excluding grower P017) confirms that farm-scale differences are not significant drivers of total fungi, total *Fusarium,* and *F. verticillioides* abundance. From our analyses agricultural management proved to be more explanatory than farm-scale differences and genetic proxies (seed type). Tillage emerged as a key agricultural practice which mediates both *Fusarium* and *F. verticillioides* abundance. Those farmers who tilled and with higher intensity had significantly lower abundances of *Fusarium* and the fumonisin producing sub-group (Figure 8). These results are supported by multiple studies, which generally indicates that deeper tillage results in lower abundances of soil *Fusarium* due to the disruption of hyphae (Steinkellner 2004; Hofgaard 2016). The positive effects of tillage are not unanimously supported by the literature. A few studies have provided evidence that breaking up the soil between crop cycles incorporates *Fusarium* colonized plant matter back into the soil, where it can build up to reinfect the next planting of crops and so increase soil and seedborne *Fusarium* (Wakelin 2008). However, our results do not support this alternative hypothesis, most likely because in our study many of the farmers using tillage also

practiced cover cropping and rotation, limiting the amount of maize material that was re-incorporated into the same plot each year.

In support of this theory about cover cropping and tillage, the use of cover crops was negatively correlated with *Fusarium* abundance. This indicates that leaving a field fallow (unplanted) between crop cycles may result in higher levels of seedborne *Fusarium.* As discussed above, this time between crops is a critical time for soil *Fusarium* populations (Wakelin 2008). The significance of cover crop use, regardless of the type of cover crop, also supports the idea that higher soil abundances of *Fusarium* translate into higher seedborne abundances. It should be noted that few of the farmers interviewed left their fields fallow between cycles, as this practice has other benefits including the prevention of soil erosion and maintenance of soil moisture. Like other results from interview data, the small sample size and skew towards one response means that these results are not conclusive. However, it does underscore the importance of cover crop use and suggest this practice should be involved in further studies of *Fusarium* related management strategies.

The third significant agricultural practice in our results is irrigation type, as those farmers using overhead irrigation had higher abundances of seedborne *Fusarium.* One hypothesis behind this observation could be that the other two methods of irrigation, drip tape and flood, might limit the exposure of the corn kernels and husk to moisture late in the season and after harvesting. In addition to these management focused variables, the older farms in this dataset were less likely to have fumonisin producers present in their seeds. The role of soil history in *Fusarium* ecology remains

an important avenue for future research, as the literature on this subject is relatively sparse.

Implications for Agriculture and Beyond

Our results indicate that in addition to facilitating high *Fusarium* abundances in the soil, no-till systems may also result in high levels of seedborne *Fusarium*, including fumonisin producers. Thus while beneficial for soil conservation, future applications of no-till agriculture should be implemented with caution. Further, the combination of tillage and cover cropping is a promising strategy for mitigating build up of seedborne *Fusarium*. There is documented potential for agricultural practices to successfully mitigate *Fusarium* outbreaks, even in conditions favorable for growth of *Fusarium* (Blandino 2009). Our research would support further investigation of cover crop and tillage as core practices to be incorporated together into small and large scale *Fusarium* management in maize agriculture.

Agricultural practices which supplement the resiliency of food systems against *Fusarium* related disease will become increasingly needed in the face of a rapidly changing climate. Climate change predictions for the Pacific Northwest include warmer, wetter winters and drier, hotter summers, with precipitation during summer projected to decrease by up to 30% by 2050 (Mote 2014). According to our results, these conditions are particularly favorable to *Fusarium*, and may increase the risk of *Fusarium* outbreaks in Pacific Northwest maize agriculture.

These scientific data concerning the importance of water to the ecology of *Fusarium* and to maize agriculture were reflected in many of my conversations with farmers. Almost all interviewees independently identified water as their main

environmental concern despite living in geographically varied locations. Whether it was water contamination from nearby roadways or impending drought, these farmers had water at the forefront of their farming concerns. Across the United States, drought is projected to be one of the most critical and immanent impacts of climate change (U.S. Global Change Research Program). The results of this research suggest that seedborne *Fusarium*, specifically *F. verticillioides,* may do exceptionally well (particularly in the Pacific Northwest) as growing seasons become drier and warmer with climate change. That the farmers handling these seeds everyday are already thinking about water and drought should highlight the urgency of this issue to researchers and policymakers. Although there are a multitude of ecological factors to consider, the potential for this genus of fungi and its associated mycotoxin producing species to have unique responses to a changing climate certainly warrants further research.

Future Directions: Ongoing Citizen Science

Our project demonstrates that citizen science serves an impressive array of purposes in scientific research. First, it provides a useful mechanism through which we can, as scientific researchers, collect data from a range of locations and agricultural sources. The farmers investment of time, both in growing these seeds and in sharing their management knowledge, was invaluable to the data collection for this project. However, this kind of citizen science is also a powerful tool through which the citizens (in this case, farmers) can collect information from us. Interviewees were consistently curious about the function and health of the microbiota of their seeds and eager to share their own stories, experiences, and hypotheses. In the words of one interviewee: "Farmers really want to learn more from other people and want to teach more too. We

all want to say: “look what I figured out!” and “what did you learn?”. That these exchanges of experimental knowledge are already part of farmers dialogue demonstrates how agricultural citizen science is so well suited to use as a tool of science communication. The interviews proved to be a critical tool for maintaining this communicative link with farmers and facilitating effective exchanges of knowledge. The farmers near-unanimous concern about water also lends a compelling narrative to the implications of this research. This is a perspective that citizen science is uniquely able to provide.

From this collaborative and interdisciplinary investigation we found that climate may be a significant driver of seedborne *Fusarium* abundance, and thus can be a useful predictor of agricultural regions most at risk of increased *Fusarium* related disease with a changing climate. We also suggest that specific agricultural practices, specifically tillage and cover crop use, have the potential to mitigate some of these adverse effects, though significantly more research and experimentation has to be done to confirm the effectiveness of these strategies at a regional or local level. Finally, our research demonstrates that agricultural citizen science is a promising technique with which to investigate *Fusarium* ecology and microbial ecology in general, as it has the capacity to engage the public in scientific issues, guide more directed research questions, and produce more readily applied scientific knowledge.

Appendix

Interview Questionnaire

General Background and Knowledge

1. How long have you been a farmer? A gardener?
2. Where do you grow your food? Describe your farm.
    a. How big is your farm?
3. How long have you been growing corn? How long have you grown corn on this land?
4. Which corn varieties do you grow? How did you come to grow these varieties? Your favorite?
5. When and how did you get into seed saving?
6. When did you begin saving corn seeds?
7. What traits do you select for (or would like to select for) when you save seeds?
8. Have you experienced any challenges to saving corn seeds?
9. Where do you get your seeds? Who do you give/sell your corn seeds to?

We're investigating microorganism abundances in the seeds you save, and how management practices on your farm might affect these abundances. So we have a few questions on your farming practices and the management history of your land.

1. How was the soil on your farm managed in the past? For example: was the land previously a forest, grassland, another type of farm? Were other crops grown there in the past?
    a. How long has your property been a farm? If possible, include the time before you owned the land.
2. What is your irrigation strategy?
3. What is your attitude towards tilling?

. How often do you till your soil while growing corn?

4. What is your general fertilizer strategy for growing corn?

. What type of Fertilizer do you use? If possible, provide brand name.

5. Would you say your soil is high in organic matter?
6. How do you incorporate organic matter into the soil (compost, manure, cover crops)?
7. Do you practice crop rotation? If so, how often and with which other plants (besides corn)?
8. What practices and/or inputs do you use to deal with pests and disease?

. Are you aware of Fusarium fungi? If so, what management methods do you use?

9. What are your thoughts on the microbial communities on your farm?
10. What do you see as the biggest environmental challenges to your farm?
11. Where would you like to see this project go? What other information would you like to receive from this research?

EFA Loadings Table

Table 4: Factor Loadings

Bioclimatic Variable	PA1	PA2	PA3	PA4
mean temp. coldest quarter	0.96			
min temp. coldest month	0.91			
temp. seasonality	-0.86			
isothermality	0.85			
mean temp. driest quarter	0.83			
annual mean temp.	0.77			
temp. annual range	-0.76			
latitude		-0.96		
max temp. warmest month		0.93		
mean temp. warmest quarter		0.88		
mean diurnal range		0.83		
mean temp. wettest quarter		0.66		
precip. driest month			0.96	
precip. driest quarter			0.94	
precip. seasonality			-0.88	
precip. warmest quarter			0.83	
precip. wettest quarter				0.95
precip. wettest month				0.93
annual precip.				0.91
precip. coldest quarter				0.79

Note: Only loadings greater than .6 are displayed. Factors are displayed in the following order: “Off-Season Temperature” (PA1), “Growing Season Temperature” (PA2), “Growing Season Precipitation” (PA3), and “Off-Season Precipitation” (PA4)

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