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RESPONSE OF THE SOIL MICROBIOME TO CLIMATE CHANGE

A DISSERTATION APPROVED FOR THE

DEPARTMENT OF BOTANY AND MICROBIOLOGY

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PREFACE

Anthropogenic induced climate change is a problem facing both current and future generations. Central to the issue of climate change, is the exponential increase in atmospheric greenhouse gas concentration that began soon after the rise of the industrial revolution. Driven primarily by the burning of fossil fuels, release of carbon dioxide, nitrous oxides, methane and particulate matter (*i.e.* soot) into the atmosphere has resulted in a concomitant increase in global mean temperatures of ~0.75^{o}C. Climate modeling suggests global mean temperatures will continue to increase 1 to as much as 10^{o}C within the next 100 years. In response to the rise in global temperatures, climate zones are shifting. The shift in climate is evident by tree ring growth patterns, glacial ice melting and the habitat/range extension of plants, small mammals and disease vectors. It is apparent from these examples that slight increases in temperature can have great influence on habitat and species that occupy them. Therefore, it is logical that further increases in temperature will exacerbate the changes already observed.

Much of the work on climate change has focused primarily on increases in CO_2 and temperature, however secondary effects (*i.e.* precipitation patterns) of warming may have greater influence on ecosystem function than increases in temperature. In all ecosystems, water availability drives ecosystem productivity as it is directly coupled to photosynthesis. Productivity of plants (*i.e.* fixation of CO_2 to biomass) is a central regulator of atmospheric CO_2 concentrations. However, alterations to climate, such as precipitation patterns, will have great consequences on primary productivity by interrupting the flow of carbon into the ecosystem. Alterations to rainfall patterns have long been predicted to change under a warming climate and evidence for these changes

are becoming apparent through analysis of long-term rainfall measurements. Of primary concern is the length of time between rainfall events as the amount of rainfall per event has been observed to increase. The increase in time between rainfall events will likely have great influence on the productivity of mesic biomes (for reference, much of Oklahoma would be characterized mesic) as the likelihood of the biome experiencing drought stress increases. The cascading effects of drought on plant communities are well established, however less is understood about how the disruption of carbon flow and low water potentials will affect microbial communities. Much of the "soil microbiome" (*e.g.* soil microorganism community consisting of bacteria, archaea, fungi, and other micro-eukarya) relies either directly (*i.e.* use photosynthate carbon for growth from either root exudation or plant litter degradation) or indirectly (*i.e.* secondary effect such as creation of conditions favorable for growth such as redox or pH) upon plant growth. Plant-microbe interactions play a key role in the geochemical cycling of nutrients such as carbon and nitrogen within the ecosystem, however due to great diversity and abundance of uncultivated microorganisms in soil many of these interactions are left un-elucidated. Great strides in DNA sequencing technology have allowed for a greater glimpse into the soil microbiome, however much is left to understand especially under the effects of a warming climate as heterotrophic bacteria and fungi are key mediators of carbon efflux in soil.

Much of my research has focused on quantifying the diversity and abundance of the soil microbiome and how it responds to increases in temperature and drought. Here I present my primary research that focuses on three elements of the microbiome: *Bacteria*, *Archaea*, and *Fungi.* Chapter one represents the bulk of my work that has been accepted

to International Society for Microbial Ecology (ISME) Journal. This chapter details the sampling transect, methods developed and used to describe the response bacteria to warming and warming under drought. This paper was written with Drs. Lee Krumholz, Mostafa Elshahed, William Howard Beasley, Yiqi Luo, and Xuhui Zhou. In chapters two and three I apply the techniques developed in chapter one to characterize the response of organisms involved in ammonia oxidation (Chapter II) and fungal communities (Chapter III) to warming. These chapters will be completed and submitted as separate manuscripts. Chapter II is written in the short essay format style of the journal Nature Climate Change, while Chapter III is written in the style of the journal Ecology. Finally, within the appendix is a manuscript that is in submission and is written in the style of the ISME Journal. The manuscript encompasses work from a side project that sought to understand how soil microbial communities respond to the chronic exposure of heavy metals chromium and arsenic from leather tanning waste in Pakistan. I was responsible for generating the PCR libraries for pyrosequencing, pyrosequencing data analysis, qPCR, and writing of the paper. This paper was written with Drs. Michael McInerney, Lee Krumholz, Muhammad Faisal and Shahida Hasnain, students Tyler Mitchell and Fariha Zakria Rizvi.

ABSTRACT

Anthropogenically induced climate change is a multi-factor process that will likely exert significant pressure on biogeochemical cycling of important elements such as carbon and nitrogen in all environments. Central to the issue is the increasing temperature associated with the concomitant increase of atmospheric greenhouse gases. To date much work has focused on understanding the response of macro-communities, such as plants, to increasing temperature. However, little attention has been given to microorganisms despite their importance in geochemical cycling of nutrients necessary for plant growth. Thus we sought to understand the dynamics of microbial communities in tallgrass prairie located in central Oklahoma. This thesis focuses on quantifying the response to warming of three main microbial groups found in soil; Bacteria, Fungi and Archaea. Utilizing a sampling time transect that covered two growing seasons beginning in the late summer of 2004 through the spring of 2006, we were able to track microbial population abundance and diversity with quantitative PCR and pyrosequencing methods. Interestingly, beginning in the spring of 2005 the experimental warming site underwent a moderate drought that lasted up until the spring of 2006. We found that in normal precipitation years warming generally increases microbial population size. However when warming was coupled to drought, bacterial and archaeal populations decreased while fungal populations increased. The shift from ample to limited rainfall also had large impacts on bacterial diversity as many groups likely went into dormancy. Ammonia oxidizing (AO) communities were dominated by Archaea, which saw increases in population size under warming but decreased in response to drought. Interestingly, AO Bacteria responded opposite of AO Archaea by increasing in size during the drought. This work highlights the fact that microorganism

populations are dynamic in soil and that under a warming climate, drought events will likely have great influence on the biogeochemical cycling of nitrogen and carbon in grassland ecosystems.

CHAPTER 1

EFFECT OF WARMING AND DROUGHT ON GRASSLAND MICROBIAL COMMUNITIES

SUMMARY

The soil microbiome is responsible for mediating key ecological processes, however little is known about its sensitivity to climate change. Observed increases in global temperatures and alteration to rainfall patterns, due to anthropogenic release of greenhouse gases, will likely have a strong influence on soil microbial communities and ultimately the ecosystem services they provide. Therefore, it is vital to understand how soil microbial communities will respond to future climate change scenarios. To this end we surveyed the abundance, diversity and structure of microbial communities over a two-year period from a long-term *in situ* warming experiment that experienced a moderate natural drought. We found the warming treatment and soil water budgets strongly influence bacterial population size and diversity. In normal precipitation years, the warming treatment significantly increased microbial population size 40-150% but decreased diversity and significantly changed the composition of the community when compared to the unwarmed controls. However during drought conditions, the warming treatment significantly reduced soil moisture thereby creating unfavorable growth conditions that led to a 50-80% reduction in the microbial population size when compared to the control. Warmed plots also saw an increase in species richness, diversity and evenness, however community composition was unaffected suggesting that few phylotypes may be active under these stressful conditions. Our results indicate that under warmed conditions, ecosystem water budget regulates the abundance and diversity of microbial populations and that rainfall timing is critical at the onset of drought for sustaining microbial populations.

INTRODUCTION

The importance of the soil microbiome in cycling of key nutrients such as carbon and nitrogen is well established. However, because of the microbiome's complexity little is understood about how climate change (*e.g.* warming and precipitation patterns) will affect the diversity, abundance, and structure of the community. Climate change is thought to exert pressure on the soil environment and ultimately the soil microbiome through direct and indirect pathways (Shaver, 2000). Direct influence of climate change on bacteria will likely occur through increased seasonal temperature maxima, as temperature has long been known to be a determinative selector of microbial growth (Ratkowsky *et al.*, 1982) and physiological activity of soil respiration (Lundegärdh, 1927). In grassland soils, experimental warming has been shown to stimulate bacterial biomass in winter and spring (Belay-Tedla *et al.*, 2009), however warming negatively affected bacterial biomass (Liu *et al.*, 2009) and 16S rRNA gene abundance (Castro *et al.*, 2010) in summer and early fall respectively, suggesting that warming may have a seasonal effect on the soil community. *In situ* microbial activity, as measured by soil CO_2 efflux, has been shown to be temperature sensitive (Luo *et al.*, 2001), thermally adaptive (Bradford *et al.*, 2008), and constrained by soil moisture (Garten *et al.*, 2009; Liu *et al.*, 2009) indicating that warming is likely also having an effect on the physiology of the microbial community. Soil respiration is a summation of total CO_2 efflux that includes *Eukarya* in addition to *Bacteria* and *Archaea*. Hence, drawing specific conclusions regarding the response of the soil microbial community to climate change using these data may be arguable. Nonetheless, these studies show that climate warming can have a strong influence on the microbiome.

As a result of increased temperatures, a concomitant increase in the frequency and duration of drought events in mesic ecosystems is anticipated (Knapp *et al.*, 2008). The expected exposure to water stress is likely to affect both microbial and plant communities by interrupting key nutrient cycles and plant-microbe feedbacks. Single-factor experiments focusing on precipitation manipulations show that rainfall additions had little effect on the composition of grassland microbial communities except under drought (Cruz-Martinez *et al.*, 2009). In addition, rainfall frequency did not alter microbial community composition in laboratory incubations of grassland soil (Fierer *et al*., (2003). Multifactor warming experiments show that microbial community composition is altered under warming and precipitation additions in a constructed natural grass prairie (Castro *et al.*, 2010). In a recent review, Bardgett *et al.* (2008) suggests that plant-mediated indirect effects of climate change (*i.e.* alterations in plant community physiology or composition) are likely to play a significant role in how the soil microbiome responds to climate change**.** Experimental warming has been shown to influence the phenology (Parmesan, 2006) and extend the growing season of plant communities (Wan *et al.*, 2005). As a result, warming causes increases in plant biomass (shoot and root) (Luo, 2007), however a concomitant increase in water uptake from the soil is needed to account for the increased biomass. Such an anticipated effect from climate warming on the plant community has consequences on the total soil water budget and ultimately will affect the microbiome if frequency and duration of water stress periods increases (Knapp *et al.*, 2008; Porporato *et al.*, 2004). Drought is likely to affect the microbiome due to the combined indirect effect on plants and the direct effects of increases in temperature and decreased soil moisture.

We set out to examine the effects of warming on soil microbial communities at a previously described long-term global warming experimental field site located at Kessler Farm Field Laboratory (KFFL) in central Oklahoma (Luo *et al.*, 2001). However, during our sampling period the site experienced a moderate drought thereby allowing us to evaluate the interactive effects of warming and ecosystem water budgets on microbial communities. We tracked the abundance, diversity and structure of microbial populations before (August 2004), at the onset (April 2005), and during (August 2005, April 2006) a moderate drought, using quantitative PCR (qPCR) and pyrosequencing. Results show that when moisture was abundant, the warming treatment significantly shifted the community structure and furthermore the indirect effect of global warming, specifically the decreases in soil moisture, significantly altered the abundance of the soil microbial community more than the direct effects of warming. Our results therefore suggest that rain fall periodicity and timing, coupled to warming will have a significant impact on microbial communities and will play an important role in regulating microbial carbon and nitrogen cycling.

METHODS

Site Description

The experimental warming site (Wan *et al.*, 2002) is characterized as a tallgrass prairie that receives approximately 90.0 cm of rainfall annually with an average temperature of 16.3^{o}C. The site consists of six paired (control and warmed) 2 x 2 m plots. Warmed plots are heated to ~2.0^{o}C above ambient temperature using infrared heaters suspended above the plot. All warmed plots had been continuously heated for approximately four years prior to the beginning of our sampling. Soil moisture was taken at regular intervals using a portable time domain reflectance (TDR) probe (Wan *et al.*, 2002). Soil samples were taken from unclipped sub-plots (to simulate a native grassland) over a two-year period starting in August 2004 and ending in April 2006.

Soil sampling and DNA extraction

Soil cores were collected from the top 15cm of soil from the unclipped sub-plots of the control and warmed plots using sterile stainless steel coring tools. Two cores were randomly taken from each plot (one core per subplot) combined and stored on ice for transport. The combined core material was then screened for roots, homogenized and stored at -20^{o}C. DNA was extracted in duplicate from soils using the MoBio Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Extraction of DNA from 0.5-gram soil was done according to the manufacturers methods except for the following modification. In lieu of shaking for ten minutes on a flat bed vortex, samples were treated in a Mini-bead beater (Biospec Products Inc., Bartlesville, Ok) for one minute at maximum speed and then placed on ice for one

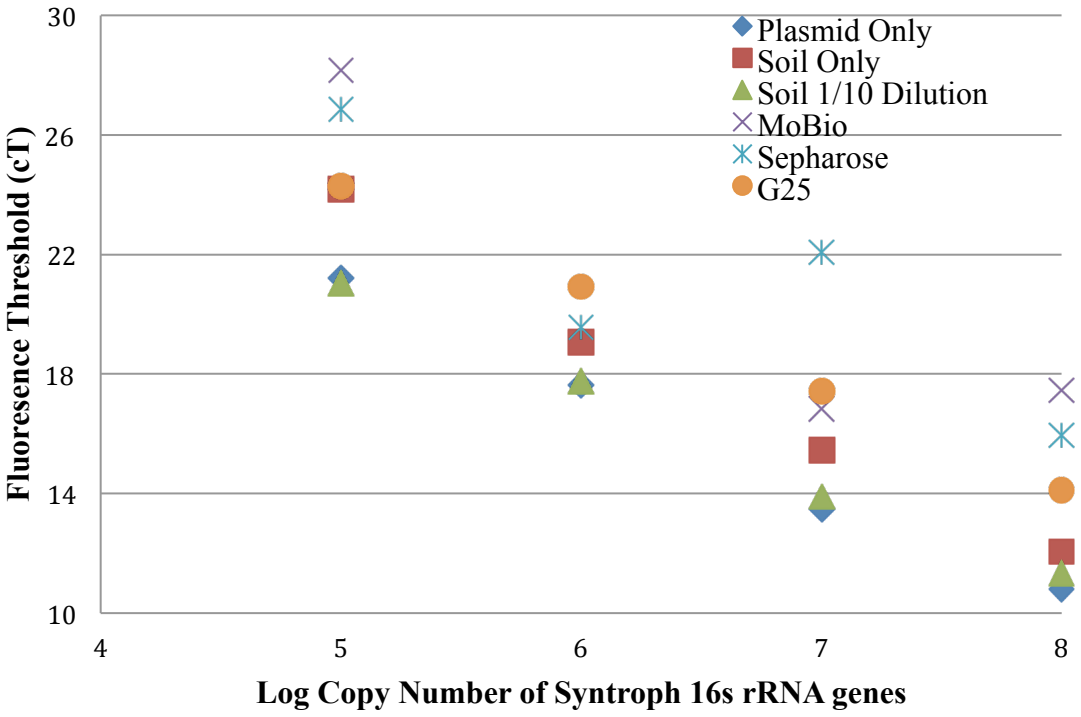
minute. Isolated DNA was then diluted 1/10 in sterile, nuclease-free water and stored at -20^{o}C.

Quantitative PCR (qPCR)

16S rRNA gene copy numbers of targeted phylogenetic groups (Table 1) were quantified over the course of two seasons (August 2004-5 and April 2005-6) in five pairs of plots. qPCR methods were adapted from Fierer *et al.* (2005), with alterations to DNA purification and adaptation to the MyIQ real-time PCR system (Bio-Rad, Hercules, CA). To safeguard against inhibition of qPCR by the co-extraction of humic acids, a series of control experiments testing the efficacy of DNA clean-up methods was performed. To quantify inhibition, a control plasmid was constructed containing the 16S rRNA gene amplified from a pure culture of *Syntrophus aciditrophicus*, an obligate anaerobe not found in soil. The 16S rRNA gene was amplified using the 8F-1492R primer set and subsequently cloned into an Invitrogen PCR-4 Topo-TA vector. qPCR 16S primers specific for *S. aciditrophicus* (qSYN207F 5' CTTGCAACGCCTCACTTA and qSYN467Rev 5' CCGTCAAGTACAAAGGCT) were developed and tested for efficiency (described in the main body of text methods) prior to inhibition tests. Multiple DNA extractions were performed on soil collected from the study site using a MoBio UltraClean Soil DNA Isolation Kit. Extractions were pooled and aliquots were either diluted with PCR grade sterile water or cleaned using a sepharose 4-b column (Jackson *et al.*, 1997) a MicroSpin G-25 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK), or the MoBio PowerClean DNA Clean-Up Kit (MoBio Laboratories, Carlsbad, CA, USA). A known copy number of control syntroph plasmid, a dilution series of 10^{8} – 10^{4} copies•μl^{-1}, was added in a 1:1 ratio to DNA

cleaned using the MoBio PowerClean, Sepharose 4-b column, or Sephadex G-25 column; non-cleaned DNA as well as DNA diluted 1/10 were used. Plasmids suspended in nano-pure water were used as controls. Inhibition was quantified by comparing the cT values of the cleaning methods to that of the control. We found that in our soil, a 1/10 dilution of soil DNA was sufficient to alleviate inhibition, as cT values were equivalent to those of plasmid only and lower than those of soil only (Figure 1). Furthermore, cleaning methods utilizing a column matrix saw a marked increase in cT, indicating a net loss of gene copies. Therefore we choose to dilute DNA 1/10 to alleviate inhibition.

Figure 1. Efficacy of DNA purification methods tested for alleviating qPCR inhibition.



To quantify the 16S rRNA genes, 1/10 diluted DNA from the two extractions from each plot at each of the four time points for a combined total of eighty reactions for control and warmed plots was used (two replicate DNAs x four time points x five plots= 40 reactions per treatment). qPCR was then performed in triplicate on each set of DNAs from both control and warmed plots across the time transect resulting in a total of 240 reactions for each group of bacteria tested. qPCR was done on the same day for each group making sure that threshold values were equivalent for each run. Each reaction mixture (25-µl total volume) consisted of 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 9.5 µl of water, 1.0 µl (10 µM) of each primer, and 2.0 µl of diluted DNA. qPCR generally followed a standard two-step protocol consisting of 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s. Specific qPCR annealing temperatures and times for each primer set are specified in Table 1. In order to quantify the number of gene copies, standard curves were constructed using tenfold serial dilutions of pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) containing a representative amplicon derived from the environment from each of the primer sets. Plasmids were extracted using a Qiagen Plasmid Miniprep kit (Qiagen, Valencia, CA). Plasmids for each primer set were sequenced prior to quantification to ensure specificity of the target. Each plasmid standard was quantified using a Nanodrop spectrophotometer system (Thermo Scientific, Wilmington, DE, USA) then converted to copy number from plasmid molecular weight. PCR conditions were modified for each primer set to reach as closely as possible an amplification efficiency of -3.3. No primer dimers were observed in all primer sets as determined by melting curves of qPCR amplicons. The environmental sample copy number was determined from the

standard curve and subsequently standardized to copy numbers per gram of dry soil. Seasonal averages of qPCR data are given in Table 2. To control for spatial variation in microbial population response at the sampling site, Multilevel Modeling (MLM) was chosen. MLM allowed data to be structured by individual bacterial groups, experimental plot, and time. This permitted us to represent the interactive effect of warming on microbial populations, since plots and microbial populations may not respond similarly to the warming treatment. Log transformed qPCR values were the model's dependent variable, which took into account all six populations plus total bacteria.

Table 1. List of primers and PCR conditions used for the quantitative PCR (QPCR) assay and pyrosequencing.

Phyla Target	Primer Name	Primer Sequence (5'-3')	Approximate Length (bp)	Annealing Temp (oC)	Annealing Time (sec)*
Total Bacteria	Eub338R2F*[1]	ACHCCTACGGGWGGCWGC	180	54	30
	Eub518R2R*[2]	ACCGMVGKKGCTGGCAC			
Acidobacteria	Acid31F [3]	GATCCTGGCTCAGAATC	487	50	45
	Eub518R	-			
Actinobacteria	Actino243F[4]	CGCGGCCTATCAGCTTGTTG	275	60	30
	Eub518R	-			
Alphaproteobacteria	Eub338F	-	347	53	45
	Alf685[1]	TCTACGRATTTCACCYCTAC			
Crenarchaeota	Cren771F[5]	ACGGTGAGGGATGAAAGCT	186	54	30
	Cren957R[5]	CGGCGTTGACTCCAATTG			
Planctomycetes	Plancto 58F[6]	ACCTAATCCGTACGTTCAGC	460	60	45
	Eub518R	-			
Verrucomicrobia	Ver53F[7]	TGGCGGCGTGGWTAAGA	465	60	45
	Eub518R	-			

* Degeneracy added from original to include more microbial taxa and also used for pyrosequencing
[1](Lane, 1991), [2](Muyzer *et al.*, 1993), [3](Barns *et al.*, 1999), [4](Stach *et al.*, 2003), [5](Ochsenreiter *et al.*, 2003), [6](Liesack and Stackebrandt, 1992), and [7](Stevenson *et al.*, 2004)

Multilevel Modeling Methods and Results

QPCR abundance was evaluated with a variety of competing multilevel models (MLM). Among the models with good fit, three features were consistently exhibited: (A) the warmed treatment had significantly more abundance than the control in the August before the drought began. (B) The warmed treatment had significantly less abundance than the control in the August during the drought. (C) The warmed treatment had significantly more abundance in the first August than in the second August. The models were evaluated with two approaches in parallel. Frequentist MLMs were calculated with the lme4 package in R (Bates and Maechler, 2009). Bayesian MLMs with weak priors were calculated in WinBUGS (Lunn *et al.*, 2000). Both approaches lead to comparable conclusions; the biggest difference was the Bayesian models (which are reported here) had larger standard errors, because the uncertainty in Level 2 parameters was allowed to propagate through the model. We were primary interested in the differences between treatments over time. Each time point had a varying intercept ($\gamma_{0,k}^{\text{Time}}$) and a varying coefficient for Treatment ($\gamma_{2,t}^{\text{Time}}$). In Panel H of Figure 4, the value of $\eta_{\text{Tx},t}^{\text{Time}}$ is plotted for the blue control points, while $\eta_{0,t}^{\text{Time}} + \eta_{\text{Tx},t}^{\text{Time}}$ is plotted for the red warmed points, for t = 1,…,4. The bands represent the 95% Bayesian Confidence Intervals (CI) estimated for each treatment. The criterion variable was the natural log of 16S rRNA gene copy quantity.

Level 2: $$\begin{pmatrix} \alpha_{jkt} \\ \delta_t \\ \beta \end{pmatrix} = \begin{pmatrix} \mu_0 \\ \mu_{\text{Tx}} \\ \mu_{\text{Mois}} \end{pmatrix} + \begin{pmatrix} \gamma_{0,j}^{\text{Bact}} \\ 0 \\ 0 \end{pmatrix} + \begin{pmatrix} \gamma_{0,k}^{\text{Plot}} \\ 0 \\ 0 \end{pmatrix} + \begin{pmatrix} \gamma_{0,t}^{\text{Time}} \\ \gamma_{\text{Tx},t}^{\text{Time}} \\ 0 \end{pmatrix}$$

$$\gamma_{0,m}^{\text{Bact}} \sim N\left(0, \sigma_{0,\text{Bact}}^2\right) = \eta_{0,j}^{\text{Bact}}$$

$$\gamma_{0,j}^{\text{Plot}} \sim N\left(0, \sigma_{0,\text{Plot}}^2\right) = \eta_{0,k}^{\text{Plot}}$$

$$\begin{pmatrix} \gamma_{0,t}^{\text{Time}} \\ \gamma_{\text{Tx},t}^{\text{Time}} \end{pmatrix} \sim N\left(\begin{pmatrix} 0 \\ \mu_{\text{Tx}} \end{pmatrix}, \begin{pmatrix} \sigma_{0,\text{Time}}^2 & - \\ \varrho\sigma_{0,\text{Time}}\sigma_{2,\text{Time}} & \sigma_{2,\text{Time}}^2 \end{pmatrix}\right) = \begin{pmatrix} 0 & +\eta_{0,t}^{\text{Time}} \\ \mu_{\text{Tx}} & +\eta_{\text{Tx},t}^{\text{Time}} \end{pmatrix}$$

Level 1: $y_i \sim N\left(\alpha_{jkt} + (\delta_t)\text{Tx}_i + (\beta)\text{Moist}_i, \sigma_y^2\right)$

$$y_i = \left[\mu_0 + \eta_{0,j}^{\text{Bact}} + \eta_{0,k}^{\text{Plot}} + \eta_{0,t}^{\text{Time}}\right] + \left[\mu_{\text{Tx}} + \eta_{\text{Tx},t}^{\text{Time}}\right]\text{Tx}_i + \left[\mu_{\text{Mois}}\right]\text{Moist}_i + \varepsilon_i$$

Case *i=1,...,1920*; Bacteria phyla: *j*=1,…,8; Plot: *k*=1,…,4; Time: *t*=1,…,4

Controlling for Plot and Bacteria

Several variables were included to control for individual differences and improve the model fit. The 8 different bacterial and archaeal phyla were allowed to have varying intercepts ($\gamma_{0,j}^{\text{Bact}}$); likewise the 4 plots were allowed to have varying intercepts ($\gamma_{0,k}^{\text{Plot}}$).

Soil Moisture

A measure of soil moisture was included as a covariate, but was fixed across the different plots and time points in the reported model (μ_{Mois}). The log of soil moisture was centered for interpretative and computational reasons (Raudenbush and Bryk, 2002; Robert and Casella, 2004). In some of the alternate models, β had a varying slope that was influenced by plot and by time (i.e., $\beta_{jt} = \mu_{\text{Mois}} + \gamma_{\text{Mois},j}^{\text{Bact}} + \gamma_{\text{Mois},t}^{\text{Time}}$). One justification is that the different plots might be expected to respond in nonequivalent ways (e.g., due to due to variations in topology and hydrology between pairs of plots), and the time points may respond differently too (especially when the times are in

different seasons). In these models, β_{jt} was significantly positive for the summer drought time point, which corresponds to the intuition that warming stimulates the microbial community when moisture is abundant. However in the other three time points, β_{jt} was significantly negative indicating that decreased soil moisture coupled to warming is driving the decreases in microbial abundance. Regardless if moisture had a fixed or varying slope in model, Features A, B and C remained significant.

Insensitivity to Prior Distributions and Data Exclusions

Bayesian models require prior distributions to be specified, and Features A, B and C remained significant with all weak priors we evaluated. The models investigated incorporated normal prior distributions for intercepts and slopes, uniform prior distributions for standard deviations, and Wishart distributions for covariance matrices (Sections 13.3 and 17.1) (Gelman and Hill, 2006). One of the 5 plots was excluded from the reported results. We believe its hydrology was substantially different from the other paired plots, and we believe the bacterial abundance and soil moisture patterns reflected this difference. However, measurement of hydrology is difficult and beyond the scope of this thesis but the idea that hydrology is influencing these plot differentially is not far fetched. Despite the observed differences, when the atypical plot was included in the models, Features A, B and C remained significant. The residuals appeared roughly symmetric, normally distributed and homogenous. The confidence intervals (CIs) in the first 8 panels of Figure 4 were calculated with bootstraps (specifically Bias-Corrected and Accelerated CIs) (Efron and Tibshirani, 1993), which can reflect asymmetry and should be more robust to non-normality than parametric CIs. The symmetric parametric CIs were almost indistinguishable from the bootstrap CIs.

MCMC and MLM Details

WinBUGS uses Markov chain Monte Carlo simulation to estimate parameter values (Carlin and Louis, 2009). Six chains were run for 500,000 iterations after 4,000 burn-in interations (models typically converged in less than 1,000 iterations). All parameters had an $\hat{R}$ less than 1.01, indicating agreement among the chains and suggesting convergence to the stationary distribution (Gelman and Rubin, 1992). The critical syntax of the Frequentist model syntax was LogCopyCount ~ 1 + Moisture + Treatment + (1 | Bacteria) + (1 | PlotID) + (1 + Treatment | Time).

Pyrosequencing

PCR libraries were generated using modified 338 forward and 518 reverse primers listed in table 1. Modifications to the primer set included: adding the A-adapter and a unique 8 base barcode to the 5' end of the 338 forward primer while the B-adapter was added to the 5' end of the reverse primer (sequences for A and B adapters, taken from http://www.454.com, 454 Life Sciences, Branford, CT). Duplicate DNA extractions from each plot (5 plots per treatment) were amplified with two PCRs for a total of 20 reactions per treatment (control and warmed) at each of the four time points. The products of the 20 PCR reactions were combined prior to sequencing. DNA from control and warmed plots were amplified using a separate barcode per treatment and time point. DNA was PCR amplified in 50-µl reactions containing (final concentration) 2 µl of 1/10 diluted DNA, 1x Flexi buffer (Promega), 2.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mixture (Promega), 2.5 U GoTaq DNA polymerase (Promega), and 500 nM of the forward and reverse primer. PCR amplification protocol

was as follows: 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min with a final extension of 15 min at 72°C. PCR reactions were screened using gel electrophoresis and positive reactions were pooled (time and treatments kept separate) and concentrated using a speed vac concentrator (Savant DNA120, Thermo Electron Corporation, Waltham, MA, 02454). Concentrated PCR products were then purified using the PureLink™ PCR purification kit (Invitrogen) and purity was confirmed using gel electrophoresis. PCR products were then sent to Engencore (http://engencore.sc.edu/) for pyrosequencing using FLX chemistry.

Data processing of pyrosequencing reads

Raw pyrosequencing data was processed for quality and barcode recovery using the software package Mothur (Schloss *et al.*, 2009) version 1.7.0 (http://www.mothur.org). Clean sequences were then aligned to the Greengenes core sequence set using NAST algorithm (DeSantis *et al.*, 2006a), distances were calculated, and OTUs generated all within Mothur. Representative $OTUs_{0.03}$ were classified (Hugenholtz taxonomy) using the Greengenes database (DeSantis *et al.*, 2006b). Diversity indices (Chao, ACE, Shannon and Simpson) and rarefaction curves were also generated using Mothur. Diversity ordering methods (Renyi generalized entropy and Hulbert family of diversity indices) were chosen because of their insensitivity to sample size and indices were calculated as previously described (Liu *et al.*, 2007; Youssef and Elshahed, 2008). A warming treatment effect was scored as significant if two of the three diversity ordering indices were higher/lower than the control treatment and do not cross. Aligned $OTUs_{0.03}$ for each treatment and time point were lane masked within Mothur and a phylogenetic tree was generated using FastTree (Price *et al.*, 2009)

(FastTree flagged conditions: -fastest, -gtr, -nt). The phylogenetic tree was uploaded to the Fast Unifrac (Hamady *et al.*, 2009) web interface and an analysis of beta diversity (community structure) was performed using the P-test and (un)weighted Unifrac metric. Heatmaps and clustering analyses were generated using the web interface (http://www.hiv.lanl.gov/content/sequence/HEATMAP/heatmap.html) that implements the heatmap tool (heatmap.2) in the gplots package within the statistical program R (http://www.r-project.org/). The relative abundances of the top fifty OTUs from each phyla were used to generate heatmaps using the Euclidean distances. Clustering of the sample times and individual $OTUs_{0.03}$ was done using the complete method (Johnson, 1967).

RESULTS

Rainfall patterns

During the course of our sampling, the experimental site experienced a moderate drought receiving ~47% less rainfall in 2005 (51.5 cm) than 2004 (96.5 cm) (Figure 2), which had received above average precipitation (Luo *et al.*, 2009b). The drought extended through the winter of 2005 and up to the time of sampling in April of 2006. During the months leading up to the start of the drought in April 2005, monthly rainfall totals steadily declined to ~74% of those of the previous spring (February- April cumulative rainfall totals were 19.7 cm (2004) and 10.4 cm (2005) resulting in a net deficit in soil moisture reserves at the beginning of the growing season (Figure 3).

Figure 2. Rainfall amounts by month for 2004-2006. Rainfall amounts were calculated using data collected from the publically available mesonet database (http://www.mesonet.org). Data was taken from the Washington, Ok Mesonet observatory located at Kessler Farm Field laboratories. Lines represent the cumulative rainfall while the bars represent the rainfall totals per month for each year.

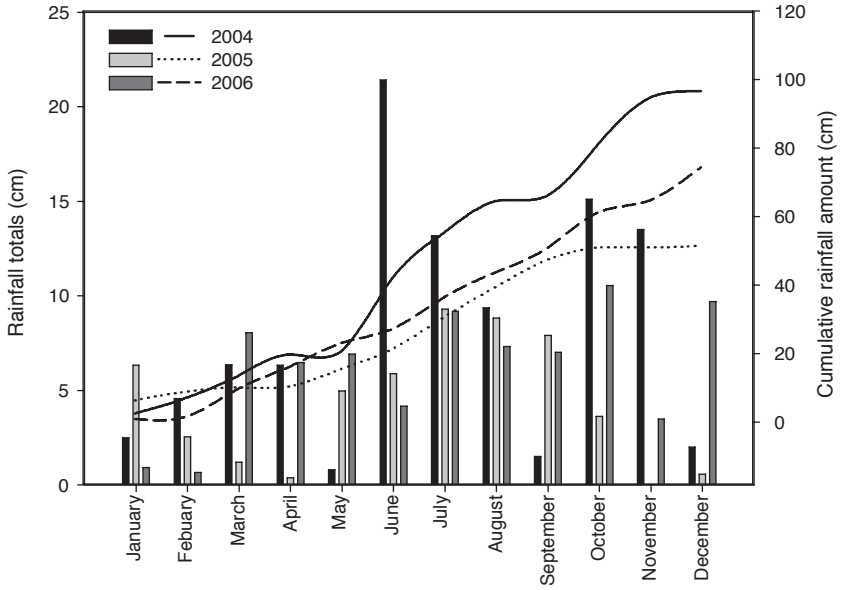
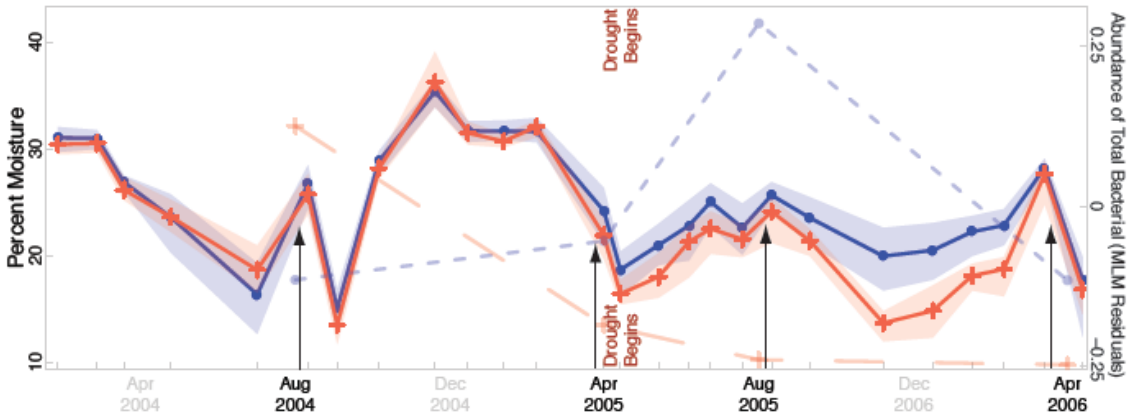


Figure 3. Average soil moisture taken at 10 cm over the two-year sampling period. Red and blue solid lines represent warm and control treatments while red and blue bands represent the standard deviation associated with each time point. Dashed red and blue lines represent the total microbial community modeled abundance from the multilevel model. Solid black lines represent dates at which samples were taken.



Assessment of bacterial population size in control and warmed plots using Quantitative PCR (qPCR)

Quantification of dominant bacterial and archaeal phyla (Total, *Actinobacteria, Acidobacteria,* α-*Proteobacteria, Planctomycetes, Verrucomicrobia* and *Crenarchaeota*) revealed that both warming and warming coupled to drought had a significant influence on soil microbial population size. During the period of above normal precipitation (August 2004), warming increased numbers of all microbial groups tested by 40-150% (Figure 4 A-G). At the onset of the drought (April 2005), the majority of control-plot bacterial and archaeal population sizes were elevated above

those in the warmed plots. Exceptions were members of the *Acidobacteria* and *Verrucomicrobia* (Figure 4 C, G). During the drought period (August 2005), the warming treatment caused a significant decrease (50-80%) in numbers (Figure 4 A-G) of all groups tested. The following spring (April 2006) most of the phyla tested had recovered from the drought and returned back to population sizes comparable to those in April 2005, however most were still below or similar to control plots. Interestingly the warming treatment increased numbers of *Acidobacteria* and *Crenarchaeota* (Figure 4 C, E) populations in April 2006 samples while *Actinobacteria* and *Verrucomicrobia* (Figure 4 B, G) never recovered from the August drought. Bayesian-multilevel modeling results revealed that the warming treatment significantly stimulated microbial populations in August 2004 when soil moisture was abundant. However as the experimental plots transitioned into drought, soil moisture became a limiting factor and when coupled to the warming treatment caused significant decreases in microbial populations (Figure 4 H).

Figure 4. Residual plots of qPCR generated 16S rRNA gene abundance data over time. Residual plots of Total bacteria (A) and individual phyla (B-G) were generated using the baseline average for each plot across all four seasons. Red and blue lines depict the warmed and control plots respectively while the shaded bands depict the standard deviation. (H) Bayesian multilevel modeling takes into account all microbial groups assayed and red/blue bands are the bootstrapped 95% confidence intervals. Raw values are in Table 2.

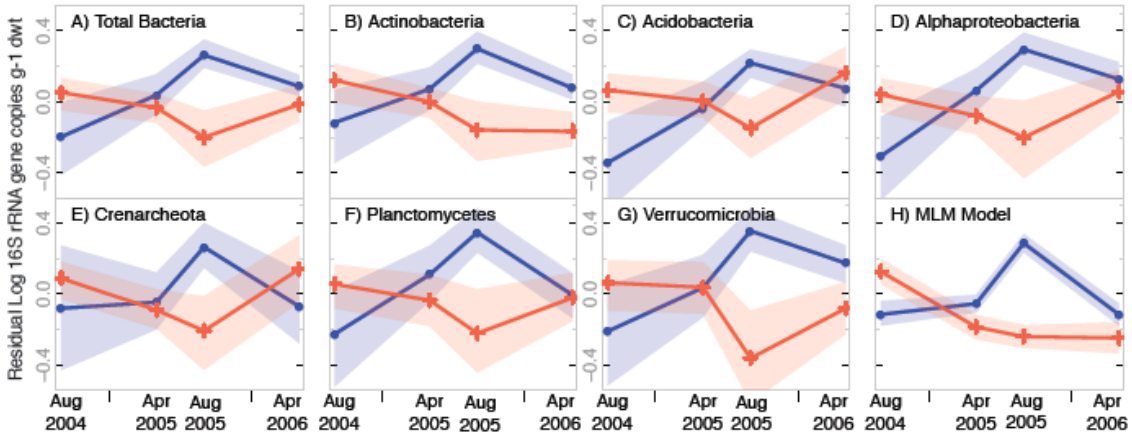


Table 2 Absolute QPCR values. All values including standard deviation are Log_{10} transformed.

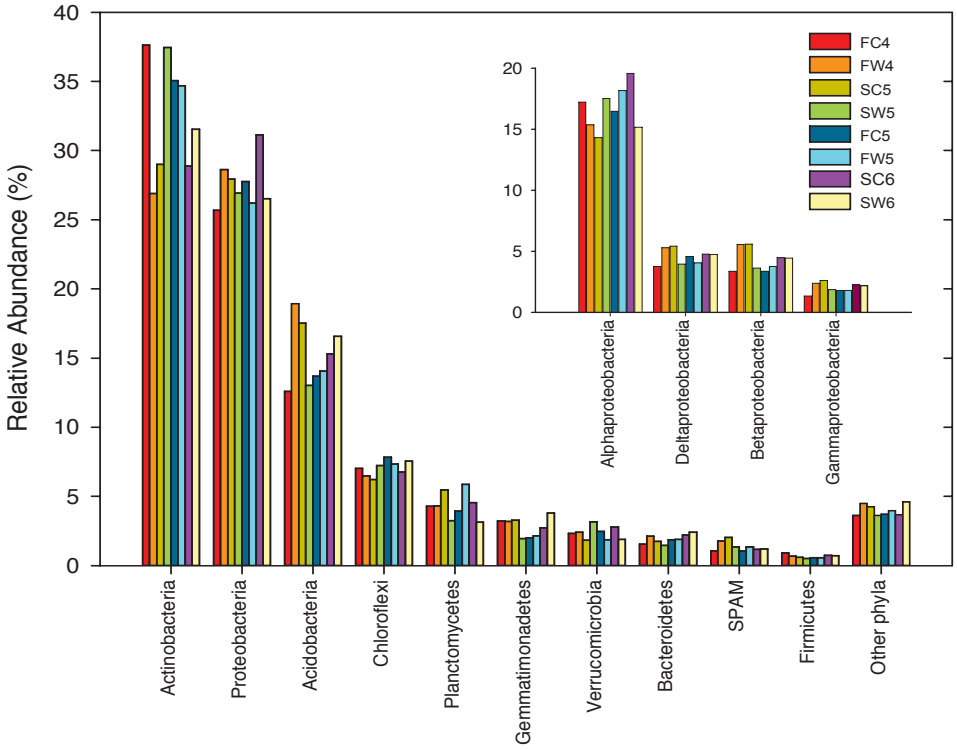
		August 2004	April 2004	August 2005	April 2006
Total	Control	8.986 ± 0.592	9.221 ± 0.551	9.159 ± 0.637	9.063 ± 0.454
	Warm	9.235 ± 0.441	9.150 ± 0.320	9.126 ± 0.564	9.245 ± 0.242
Actinobacteria	Control	6.701 ± 0.612	6.872 ± 0.294	7.003 ± 0.486	6.893 ± 0.202
	Warm	6.987 ± 0.260	6.818 ± 0.212	6.660 ± 0.577	6.650 ± 0.191
Acidobacteria	Control	6.976 ± 0.665	7.284 ± 0.432	7.537 ± 0.277	7.394 ± 0.180
	Warm	7.384 ± 0.453	7.323 ± 0.395	7.170 ± 0.574	7.481 ± 0.289
Alphaproteobacteria	Control	8.180 ± 0.619	8.548 ± 0.686	8.780 ± 0.768	8.612 ± 0.563
	Warm	8.518 ± 0.489	8.406 ± 0.428	8.284 ± 0.685	8.544 ± 0.260
Planctomycetes	Control	6.773 ± 0.783	7.108 ± 0.666	7.345 ± 0.775	6.993 ± 0.607
	Warm	7.054 ± 0.665	6.963 ± 0.497	6.778 ± 0.809	6.978 ± 0.312
Verrucomicrobia	Control	7.356 ±0.865	7.623 ± 0.754	7.920 ± 0.898	7.739 ± 0.730
	Warm	7.629 ± 0.646	7.663 ± 0.492	7.205 ± 0.982	7.486 ± 0.345
Crenarchaeota	Control	4.719 ± 0.842	4.735 ± 0.681	5.045 ± 0.583	4.710 ± 0.327
	Warm	4.869 ± 0.563	4.692 ± 0.657	4.574 ± 0.741	4.992 ± 0.558

Assessment of the diversity and community structure of control and warmed plots using Pyrosequencing

A total of 260,499 sequences were generated resulting in approximately 10,000-45,000 sequences for each treatment and time point (4 control and 4 warmed libraries combined, Table 3). In all control and warmed datasets, phylum level distributions for each of the four time points revealed an overall pattern similar to other soil studies

(Elshahed *et al.*, 2008; Fulthorpe *et al.*, 2008) whereby *Actinobacteria, Proteobacteria,* and *Acidobacteria* were the most abundant phyla represented (Figure 5). When rainfall was abundant (August 2004), the warming treatment increased the relative abundance of sequences of the *Proteobacteria* (11%), *Acidobacteria* (50%), *Bacteroidetes* (35%), *Verrucomicrobia* (3%), and SPAM (68%) (Figure 5). However during drought (*i.e*. August 2005) the warming treatment had little effect on the relative abundance of the dominant phyla with the exception of the *Planctomycetes*, which saw an increase of 48 % over the control (Figure 5).

Figure 5. Relative abundance of dominant bacterial phyla in pyrosequencing generated 16S rRNA genes sequence libraries at the KFFL warming site. Each bar is color-coded and represents a single sampling time. Abundance was normalized by treatment and time. Abundance of *Proteobacteria* are included in the subset. F(C/W)4 = August 2004, S(C/W) = April 2005, F(C/W)5 = August 2005, S(C/W)6= April 2006; C = Control, W =Warmed.



To assess the bacterial diversity of control and warmed plots at various sampling times we chose to employ both traditional species richness estimators and less used diversity ordering methods. In the grassland soil, species richness (Chao1), evenness (ACE), diversity (Shannon Index), and diversity ordering followed a similar pattern. During the non-drought period (August 2004) the warming treatment decreased richness, evenness and diversity; while during the drought (August 2005), warming increased richness, evenness and diversity of the community when compared to the controls (Table 3). Diversity ordering of the most dominant phyla in Kessler Farm soil reveals a similar pattern to that of the total data set with control plots more diverse in August 2004 and warmed plots more diverse in August 2005 (Table 3). Spring time-points (April 2005 & 2006) show that species richness, evenness and diversity patterns showed no clear treatment level effects. Diversity ordering patterns of individual phyla also show spring samples to be variable with most phyla not stimulated by the warming treatment (Table 3).

Table 3. Estimates of species richness and diversity. LCI and UCI are the Lower/Upper confidence interval. F=Fall, C/W=Control/Warmed, and 4/5/6= Year of sampling.

Sample	OTU Cutoff	Total Seqs	No. OTUs	ACE (LCI-UCI)	Chao (LCI-UCI)	Shannon	Simpson
FC4	Unique	28311	12646	71476 (70477-72492)	37460 (35928-39093)	8.550	0.001
	0.03		8902	24833 (24491-25183)	17066 (16464-17715)	8.128	0.001
	0.05		6886	15740 (15507-15979)	11843 (11414-12312)	7.750	0.002
FW4	Unique	11748	6122	35433 (34739-36144)	18504 (17418-19695)	8.155	0.001
	0.03		4680	13840 (13572-14116)	9586 (9098-10128)	7.816	0.001
	0.05		3853	9913 (9712-10120)	7197 (6823-7618)	7.532	0.001
SC5	Unique	34319	13450	58805 (58081-59540)	33842 (32623-35138)	8.633	0.001
	0.03		8713	19486 (19232-19746)	14788 (14315-15301)	8.131	0.001
	0.05		6747	12960 (12776-13150)	10614 (10259-11005)	7.772	0.001
SW5	Unique	19494	9982	87914 (86476-89378)	38156 (36122-40349)	8.429	0.001
	0.03		6997	23572 (23193-23959)	14791 (14155-15482)	7.974	0.001
	0.05		5400	14574 (14321-14834)	10163 (9711-10662)	7.580	0.002
FC5	Unique	32800	14826	95385 (94160-96629)	47355 (45464-49362)	8.749	0.001
	0.03		9836	28652 (28281-29031)	19740 (19034-20500)	8.242	0.001
	0.05		7505	18299 (18039-18566)	13340 (12854-13871)	7.834	0.001
FW5	Unique	45068	22036	152397 (150396-154429)	76477 (73838-79250)	9.170	0.001
	0.03		12939	34936 (34547-35333)	25066 (24302-25880)	8.496	0.001
	0.05		9411	21757 (21482-22037)	16700 (16147-17299)	7.964	0.001
SC6	Unique	40268	17169	109402 (108130-110692)	55536 (53438-57755)	8.800	0.001
	0.03		11142	31404 (31025-31791)	22042 (21306-22831)	8.284	0.001
	0.05		8319	19400 (19141-19665)	14744 (14225-15308)	7.858	0.001
SW6	Unique	48491	20019	102401(10123-103700)	55031 (53308-56843)	8.941	0.001
	0.03		12087	29648 (29312-29990)	22341 (21662-23069)	8.325	0.001
	0.05		8929	18522 (18289-18762)	14987 (14503-15514)	7.894	0.001

To assess how community structure is affected by the warming treatment we chose to use the beta diversity measurements P-test (Martin, 2002) and (un)weighted Unifrac (Lozupone *et al.*, 2006) metrics implemented in Fast Unifrac (Hamady *et al.*, 2009) and traditional community similarity measures Bray-Curtis and Jaccard dissimilarity indices. Pairwise comparisons between warmed and control plots show warming had no significant effect (unweighted Unifrac $P>0.05$) on the overall structure of microbial communities at each time point, however warming did significantly alter lineage specific patterns in two out of the four seasons (P-test: August 2004, $P<0.001$; weighted Unifrac: August 2004, $P=0.032$ and April 2005, $P=0.016$). Transitioning into drought (August 2004 to April 2005) from normal moisture conditions had a significant effect on control and warmed plot community structures (Unweighted Unifrac: Control plots $P= 0.009$; Warmed plots $P=0.007$). Bray-Curtis and Jaccard similarity indices corroborate Unifrac results such that when moisture was abundant (August 2004 and April 2005) the warmed and control plots had low similarity, but as the drought intensified similarity between the two communities increased (Figure 6). Cluster analysis of dominant genus level OTUs from each phyla, revealed that the warming treatment continually altered the community profile such that the control and warmed plots never clustered with one another at any given time point (Figure 7). Heatmaps in conjunction with the cluster analysis allows for visualization of the dominant genera distribution patterns captured at the time of sampling. Interestingly these patterns were either constrained (*i.e.* few $OTUs_{0.03}$ dominant across all time points sampled) as in the *Betaproteobacteria* or highly variable (*i.e.* many $OTUs_{0.03}$ share the dominance but are sporadically distributed across time) like the *Deltaproteobacteria* (Figure 7).

Figure 6. Jaccard and Bray-Curtis community similarity values between warmed and control plots across time for different species (0.03) and genus (0.05) OTU cutoffs.

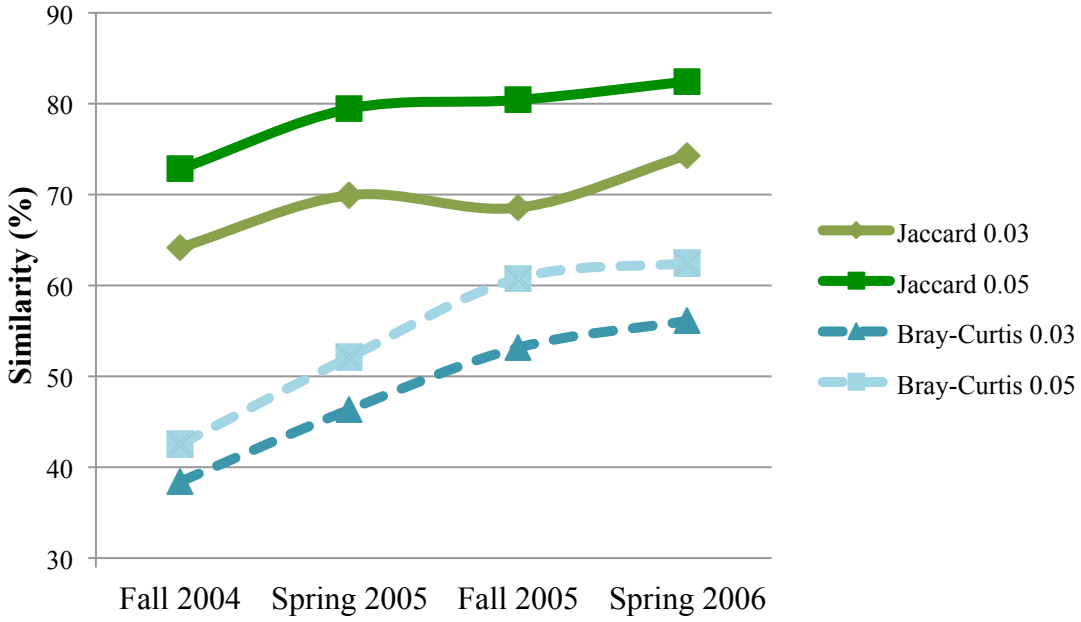
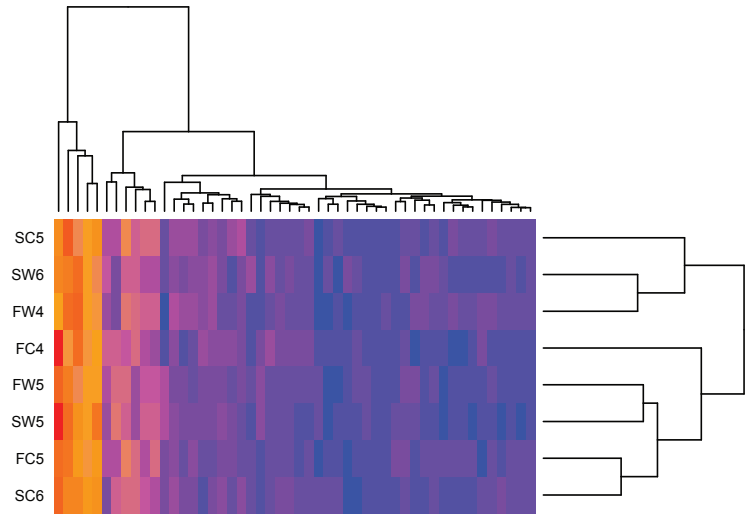
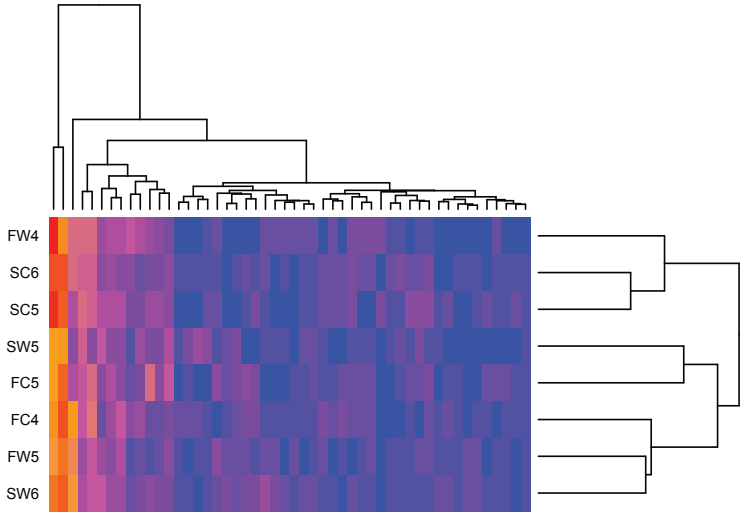


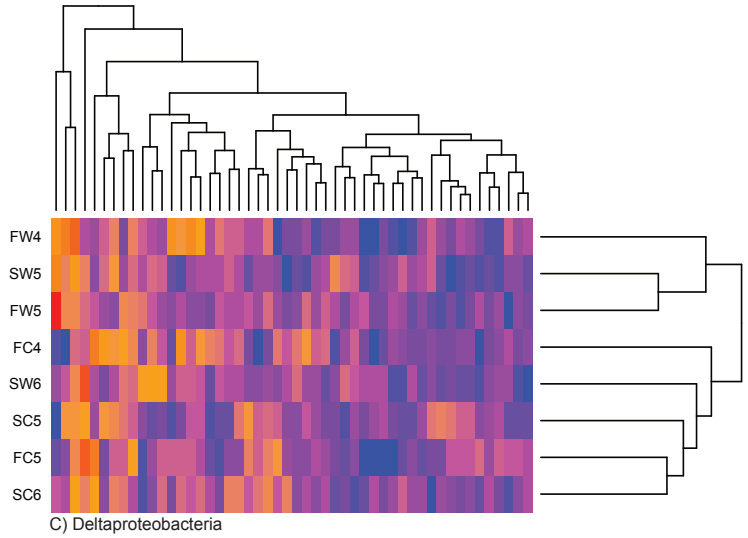
Figure 7. Heatmap and cluster dendrogram of dominant shared genus level $OTUs_{0.05}$ within the *Proteobacteria*. Each column represents a genus level OTU shared across all time points and the color of the box indicates the relative abundance of the OTU at the time of sampling with red depicting high abundance and blue low abundance. Each row represents a sampling date and treatment: F(C/W)4 = August 2004, S(C/W) = April 2005, F(C/W)5 = August 2005, S(C/W)6= April 2006; C = Control, W = Warmed. Dendrograms of the clustering analysis on the top and right of the heatmap depict the clustering of the $OTUs_{0.05}$ (top) and the sampling time/treatment (right side).



A) Alphaproteobacteria



B) Betaproteobacteria



C) Deltaproteobacteria

DISCUSSION

The microbiome is an integral component of soil and is important to maintaining the functioning of all ecosystems. However, our understanding of how these microbial communities will respond to disturbances such as climate change is limited. Therefore we sought to understand how bacterial and archaeal communities would respond to elevated temperatures associated with future, predicted global warming temperatures. Microbial communities were monitored during normal precipitation and drought periods using pyrosequencing and quantitative PCR. Sampling during the transition from adequate precipitation to drought provides a glimpse of how microbial communities respond to the environmental stressors of warming alone and warming under drought.

Effects of warming under normal precipitation

Temperature has long been known to be a determinant for the growth and physiology of microorganisms and may be a determining factor for niche space competition between physiologically similar organisms (Bennett and Lenski, 1993). However, due to the abundance of uncultivated organisms in soil little is known of the temperature optima of the majority of microorganisms. We observed that under normal precipitation conditions (August 2004) the warming treatment had a significant effect on microbial populations and community structure when compared to the control. Under warming conditions microbial population abundance was significantly higher than the controls, however diversity of the warmed plots was markedly lower than the control. Furthermore shifts in lineage specific community structure (unweighted *vs.*

weighted Unifrac) and community similarity (Bray-Curtis and Jaccard indices) affirms that the warming treatment is selecting for a subset of the total warming plot diversity. Assessing a physiological significance to the shift in community structure is difficult because many of the dominant phylotypes within our dataset are uncharacterized. Under warming, the reduction of *Actinobacteria* suggests that increases in respiration associated with higher temperatures (Luo, 2007) may be negatively affecting this group as they are known to be sensitive to increased CO_2 (Goodfellow and Williams, 1983). Furthermore, increases in the relative abundance of *Acidobacteria* may be due to the reduction in *Actinobacteria,* as these groups likely share similar niches. Increased abundance of the *Crenarchaea*, known archaeal ammonia oxidizers (Konneke *et al.*, 2005), suggests this group may be a more important component of the nitrogen cycle in a warmed climate, especially under nitrogen limiting conditions. Recently *Crenarchaea* have been shown to be active at ammonia concentrations far below those that are used by their bacterial counterparts (Martens-Habbena *et al.*, 2009).

Effects of warming at onset of drought

As the plots transitioned from a normal precipitation year into drought (Spring 2005 to August 2005), precipitation patterns at the study site suggest that lack of rainfall prior to the 2005 growing season was more important for long-term survival of microbial communities than summer rainfall. Under decreasing rainfall conditions, the biome must survive on soil moisture reserves (often described as the bucket model (Porporato *et al.*, 2004)) until stocks are replenished through rainfall (Knapp *et al.*, 2008). However under warmed conditions, plant communities respond by developing earlier (Parmesan, 2006), which is evident at our study site by elevated plant biomass in

warmed plots relative to controls (Luo *et al.*, 2009b). The tax on soil moisture reserves due to early onset of plant growth is evident as plant evapotranspiration and increased evaporation rates contributed to the critically low soil moisture levels (Figure 3 March-April 2005) in warmed plots. As a result of the moisture deficit, microbial communities responded to warming differently than when moisture was abundant. Microorganisms in warmed plots were generally less abundant and community structure was significantly different when compared to the control. The significant shift in lineage specific microbial community structure would again suggest the warming treatment is selecting for a subset of the total community. However because plant communities are phenologically advanced ahead of control plots, the shift in microbial community structure may also be due to increased labile carbon pools (Belay-Tedla *et al.*, 2009) and the reduction of moisture. Furthermore significant shifts in community structure from August 2004 to April 2005 in both control and warmed plots would suggest that seasonality is a large contributing factor to the structure of these communities and could correspond to life stage of the plant community.

Effects of warming under drought

Under drought conditions the warming treatment had the most pronounced effect on microbial population abundance. All warmed plot populations tested in August 2005 decreased in size from the April 2005 samples and when compared to the control plots the decline is a stark contrast (Figure 4). Multilevel modeling results indicate the decline in abundance is attributed to soil moisture at the time of sampling. Furthermore soil conditions in warmed plots created by the early onset of the plant community in conjunction with the lack of rainfall are likely the underpinnings for the decline in

microbial populations. Plant biomass data at the site also suggests that because of the lack of moisture, NPP (net primary production) was decreased relative to controls (Luo *et al.*, 2009b). The potential reduction in labile carbon and nitrogen entering the rhizosphere could also be a partial cause for the decline of microbial phyla such as *Verrucomicrobia* who are known to be heterotrophs (Hedlund *et al.*, 1997) and are sensitive to nitrogen ratios (Keiblinger *et al.*, 2010). An analysis of the effect of warming on community structure showed no significant difference despite the effect on abundance. Bray-Curtis similarity indices indicate that the drought constrained the abundance patterns between warmed and control communities as the plots transition into drought, whereby control and warmed communities became more similar as the drought intensified thus corroborating the community structure results.

Effects of warming transitioning out of drought

The spring 2006 samples present an interesting look at the community because recharging of soil moisture reserves, a process that normally occurs over winter months at the site, was absent. Instead large differences between control and warmed plot soil moisture was kept throughout the fall of 2005 and just prior to the time of sampling in 2006. Abundance data shows that some groups are much more resilient to drought and quickly return to numbers similar to (*Planctomycetes*) or above (*Crenarchaea* and *Acidobacteria*) control plot values, while others are slower to recover (*Actinobacteria* and *Verrucomicrobia*). The differential response of each phylum suggests that restructuring of communities may take long periods of time after extreme stress events such as drought and warming. Furthermore, the ability of microorganisms to lay dormant during periods of stress (Jones and Lennon, 2010) may be vital to the

resiliency of prairie soil microbial communities (Fierer *et al.*, 2003) and may explain why community similarity increased under drought.

Summary

In Oklahoma prairie soil, increases in temperature and the combination of drought and temperature had dramatic negative effects on the abundance, diversity, and structure of soil bacterial communities. Our data suggests that shifts in rainfall patterns, specifically timing and amounts per event, can evoke long-term changes to the microbial community especially under a warming climate. These alterations to the community are likely to have overarching effects on the physiology of the microbiome, as portions of the community lay dormant in response to stress. Furthermore, recovery of microbial communities after perturbations, such as drought, may not occur immediately and thus may disrupt soil carbon and nitrogen cycling. We feel that further rainfall manipulation and warming experiments are needed to help elucidate the complex response of the microbiome as the likelihood of experiencing water-stress events will increase with a warming climate.

CHAPTER II

RESPONSE OF AMMONIA OXIDIZING MICROORGANISMS TO CLIMATE CHANGE

SUMMARY

Oxidation of ammonia to nitrite by *Bacteria* and *Archaea* is an integral step in the biogeochemical cycling of nitrogen that regulates the productivity of the ecosystem. Because ammonia is positively charged it cannot readily migrate within the soil matrix, therefore ammonia-oxidizing organisms are necessary for regulating nitrogen release that would otherwise be inaccessible to plants and heterotrophic rhizosphere-associated bacteria. Anthropogenically induced climate change is predicted to have many interactive and cascading effects upon the soil environment that will likely affect these microorganisms. Therefore it is necessary to understand how ammonia-oxidizing microorganisms (AOM) will respond to climate change. Using a targeted molecular approach, we quantified the abundance of ammonia monooxygenase (*amo*A) genes from *Bacteria* and *Archaea* in soils from a tallgrass prairie subjected to an increase in temperature. During the course of our study, the experimental warming site underwent a moderate drought, allowing for the investigation of the interactive effects of warming and drought. We found ammonia-oxidizing *Archaea* (AOA) were ~2 log-fold more abundant than ammonia-oxidizing *Bacteria*, more responsive to warming, and drought sensitive. During periods of ample moisture, AOA abundance was over two-times greater in warmed plots than control plots, while ammonia-oxidizing bacteria (AOB) saw little change. However, as the experimental site transitioned into drought, AOA populations in warmed plots saw a dramatic decline (~300%) relative to control plots. Conversely, AOB abundance increased (~150%) relative to controls during the drought. Our results highlight the importance of AOA within the soil microbiome and show that under a warming climate in periods of ample moisture, AOA may increase the

bioavailability of nitrogen for plant growth. Furthermore, niche differentiation between AOA and AOB is apparent, as AOB thrived under warming and warming under drought indicating that nitrification continues to be important under all conditions.

INTRODUCTION

Biogeochemical cycling of nitrogen in soil is a microbial mediated process that is tightly coupled to ecosystem productivity (Norby *et al.*, 2010). Anthropogenically induced climate change (IPCC, 2007) will likely exert multiple direct (*i.e.* temperature, carbon dioxide, and rainfall) and indirect (*e.g.* plant mediated) stressors (Bardgett *et al.*, 2008) upon organisms responsible for nitrogen cycling. *Bacteria* have long been recognized for their ability to transform ammonia (Head *et al.*, 1993; Purkhold, 2000; Rotthauwe *et al.*, 1997) and until recently, were thought to be the only organisms capable of ammonia oxidation. However, recent implication of mesophilic *Archaea* capable of AO (Venter, 2004) in oceans and later in soil (Schleper *et al.*, 2005; Treusch *et al.*, 2005) and their ubiquitous distribution and abundance in terrestrial ecosystems (Leininger *et al.*, 2006) suggests that ammonia oxidizing *Archaea* (AOA) are keystone members of terrestrial ecosystems. To date, much climate change work has focused on understanding the constraints of bacterial AO (Horz *et al.*, 2004). Little work has been done to characterize the response of AOA to climate change.

In soil, competition for ammonia is high as plants, heterotrophic bacteria, and AO microorganisms readily take up and utilize ammonia for growth. Thus, the rate of ammonia oxidation to nitrite is constrained by substrate availability. Until recently the conversion of ammonia was predicted to be performed solely by the chemoautotrophic

bacterial groups *Nitrosomonas* and *Nitrosococcus* within the α, β-*Proteobacteria* (Purkhold, 2000). These organisms convert ammonia to hydroxylamine (a precursor to nitrite) using a conserved protein complex ammonia monooxygenase (Arp *et al.*, 2002). Within this protein complex, the catalytic subunit *amo*A has been extensively studied (Kowalchuk and Stephen, 2001) and can replace the 16S rRNA gene as a phylogenetic marker (Purkhold, 2000). Much work has been done with the characterization of AOB in soil (Kowalchuk and Stephen, 2001), however with the discovery of *amo*A-like sequences near soil–related crenarchaeal 16S rRNA genes (Treusch *et al.*, 2005) suggested our understanding of the organisms capable of nitrogen cycling was limited. Since then, AOA have been shown to be ubiquitous within soil (Leininger *et al.*, 2006), suggesting their importance in nitrogen cycling in terrestrial ecosystems. Thus, reevaluation of the environmental roles of AOA and AOB has been undertaken (Di *et al.*, 2010; Erguder *et al.*, 2009; Herrmann *et al.*, 2011; Leininger *et al.*, 2006; Schleper, 2010).

Despite theoretical overlapping niches between ammonia-oxidizing *Bacteria* and *Archaea*, marine archaeal ammonia oxidizers are unique from their bacterial counterparts, because they are specialized at oxidizing ammonia present at lower concentrations (Martens-Habbena *et al.*, 2009). In soil, nitrogen in the form of ammonia is generally thought to be a limiting resource. Thus, high competition for ammonia within the rhizosphere likely creates and maintains ammonia concentrations favorable for AOA growth. Interestingly AOA and AOB are also integral to carbon cycling as both groups couple ammonia oxidation to CO_2 fixation (Brazelton *et al.*, 2010; Di *et al.*, 2010; Jia and Conrad, 2009; Martens-Habbena *et al.*, 2009; Offre *et al.*, 2011; Offre *et*

al., 2009). This work illustrates the fact that AOA and AOB are integral components of the nitrogen and carbon cycles, however because most AOA and AOB in the environment are uncultivated we still have little knowledge of their growth and response to climate change.

We previously reported the sensitivity of bacterial and archaeal populations to warming and the interactive effects of warming and drought in a tallgrass prairie (Sheik *et al.*, 2011). Here we report that the abundance of the archaeal phylum *Crenarchaeota*, whose members in soil are linked to ammonia oxidation (Schleper and Nicol, 2010), was correlated with water availability when subjected to warming. The negative response of *Crenarchaeota* to warming and drought suggests that nitrogen dynamics within the soil may be adversely affected by increasing global temperatures and drought frequency. To this end, we sought to understand the effects of warming and warming under drought on the dynamics of organisms responsible for ammonia oxidation in prairie soils by using a targeted approach focusing on abundance of the functional gene responsible for ammonia oxidation and the diversity of organisms involved with ammonia oxidation.

RESULTS AND DISCUSSION

Quantification of the *amo*A gene in *Bacteria* and *Archaea* using qPCR (Quantitative PCR) revealed several shifts in abundance in response to the warming treatment and warming under drought. Across all sampling time-points, AOA were ~2-log fold more abundant and more responsive to warming than AOB (Figure 1). Increased abundance of AOA suggests that *Archaea* are playing a more important role than bacteria in ammonia oxidation in our soils. Furthermore, the disparity in abundance between AOA and AOB in our soils is not unique as it has been observed in other undisturbed soils (Schauss *et al.*, 2009). An increase in abundance of AOB appears to be linked to disturbance from farming and fertilization practices (Di *et al.*, 2010; Wessen *et al.*, 2011). Because our soils have been relatively undisturbed (>30 years of no farming practice such as tilling) and are not actively fertilized (passive fertilization through atmospheric nitrogen deposition may occur, however it is assumed that deposition would be uniform), the disparity in abundance of AOA:AOB in our soils is not unreasonable. Furthermore, because ammonia mineralization at Kessler Farm is generally low (≈ 0.5-3.5 $g \bullet m^{-2}$ $year^{-1}$) and highly variable (Wan et al., 2005), competition for ammonia is high as plants, micro-eukaryotes, and bacteria all take up ammonia, thus keeping ammonia concentrations low and creating favorable conditions for AOA (Martens-Habbena *et al.*, 2009).

Table 1. Abundance of the *amo*A gene from *Archaea* (AOA) and *Bacteria* (AOB) in control and warmed plots at each sampling time.

	AO Archaea		AO Bacteria	
	Log *amo*A genes • g^{-1} dwt soil ± Std. Error		Log *amo*A genes • g^{-1} dwt soil ± Std. Error	
	Control	**Warming**	**Control**	**Warming**
August 2004	6.09 ± 0.24	6.43 ± 0.19	4.46 ± 0.13	4.47 ± 0.22
April 2005	6.40 ± 019	6.29 ± 0.27	3.92 ± 0.13	4.36 ± 0.25
August 2005	6.70 ± 0.17	6.00 ± 0.16	4.23 ± 0.16	4.22 ± 0.07
April 2006	6.14 ± 0.26	6.17 ± 0.18	4.25 ± 0.22	4.53 ± 0.20

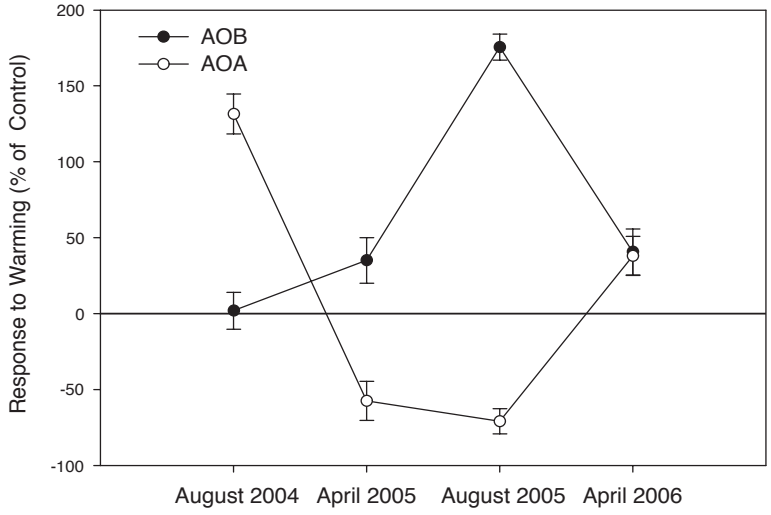
In our soils, AOA and AOB population sizes responded directly to the effects of warming (Table 1) but the observed response to warming was not significant ($P > 0.05$). However, interactions between warming and time were significant for AOA ($F=2.779$, $P= 0.045$) but not AOB. AOB populations showed little response to warming when soil moisture was ample (August 2004), however during periods of drought (Table 1, April 05-August 05) AOB abundance increased in warmed plots. Horz *et al.* (2004) saw similar trends in AOB abundance in response to warming and precipitation manipulations. Conversely, warming only had a positive affect on AOA populations during periods of non-drought (Table 1, August 2004 and April 2006). As the site transitioned into drought (April - August 2005), warming had a significant negative effect on the abundance of AOA (Students T-Test, $P=0.003$) when compared to the control (Table 1). Furthermore, AOA populations in the control plot (Sheik *et al.*, 2011)

reacted opposite of the populations in warmed plots, with increases in abundance seen primarily during the drought (Table 1, April and August 2006). We observed similar trends in crenarchaeal 16S rRNA abundance, thus lending credence that these observed changes in *amo*A are real. Although no significant treatment-level effects were observed, several responses to the warming treatment were seen for AOA and AOB. We feel that because paired plots are spatially separated, subtle variation in the soil habitat (*i.e.* plant and microbe community, soil moisture, soil temperatures, etc…) is likely to exist and possibly drives the large plot-level variation observed in AOA and AOB populations (Table 1, large standard error). Furthermore, large shifts in population size are not anticipated as both AOA and AOB's growth is restricted by substrate availability and exhibit k-selected lifestyles (Nicolaisen *et al.*, 2004). Thus subtle shifts in abundance, despite not being significant, could result in large increases or decreases in ammonia turnover.

Theoretically, competition for ammonia between AOA and AOB organisms is likely, however, Martens-Habbena (2009) show that mesophilic marine AOA are adapted to oligotrophic conditions and are able to utilize ammonia at concentrations far below their bacterial counterparts. This would suggest that niche partitioning (*i.e.* physiological differentiation between organisms that occupy the same niche) between AOA and AOB is rooted in their ability to scavenge ammonia from environments where competition for ammonia exists, such as within the root rhizosphere where both heterotrophic microorganisms and plants are competing for nitrogen (Hungate *et al.*, 2003). In Kessler farm soil, the differential response of AOA and AOB to the warming treatment is strongly driven by water availability. During periods of ample moisture, the

abundance of AOA increased by ~130 % relative to the control plots, while abundance of AOB showed little change (Figure 1). In contrast, as the experimental warming site moved into the drought period (beginning in April & August 2005), the abundance of AOA declined over 300 % in warmed plots from the previous August (Figure 1). In contrast, the warming treatment elevated the abundance of AOB only ~2 % when moisture was profuse (August 2004), but increased the abundance of AOB ~175 % relative to the control plots during the drought (Figure 1, August 2005). Moisture sensitivity of AOA populations has not been previously reported. Our study is the first to track populations *in situ* over time. Nonetheless, the reversal in the abundance of AOA and AOB in our soils under warming and drought suggests that ammonia oxidation may not be hindered under a warming climate and AOB may continue to oxidize ammonia thereby essentially "priming" the soil for when optimal growth conditions returns.

Figure 1. The response of *Archaea* (AOA) and *Bacteria* (AOB) *amo*A genes to the warming treatment. Error bars represent the percent standard error calculated at each time point (±1 SE, n=15)



Increases in AOA during periods of ample moisture and large decreases during drought, suggests that optimal growth of AOA may occur only when heterotrophic bacteria and plants are actively growing. We previously reported similar trends within the bacterial community (Sheik *et al.*, 2011) suggesting that plant mediated effects, such as root exudation, have a large cascading effect on both Bacteria and Archaea. Uptake of amino acids (Ouverney and Fuhrman, 2000), oxidation of ammonia under inhibitory conditions (Jia and Conrad, 2009), differential response to fertilizer source (Schauss *et al.*, 2009) and the genomic analysis of an isolated AOA (Walker *et al.*, 2010) suggest the capability of a mixotrophic lifestyle. These several lines of evidence suggests that some AOA may live a dual lifestyle and because we have only characterized a small portion of the total diversity of AOA within the environment, it stands to reason that species of AOA could have broader physiological capacities than those isolated. Interestingly, the increase in AOA in response to the warming treatment may act as a buffer for CO_2 release from the soil. We previously showed an increase in heterotrophic bacteria to warming (Sheik *et al.*, 2011), however several studies report an acclimation of soil respiration to warming (Luo *et al.*, 2001). Because AOA couple the oxidation of ammonia to the fixation of CO_2 to biomass, any increase in heterotrophic bacterial metabolism, producing both CO_2 and potentially ammonia (Schmidt *et al.*, 2007), could potentially spur the metabolism of AOA thereby suppressing the release of CO_2 from the soil. However, further work is needed to elucidate these types of interactions.

Methods

Site Description

The experimental warming site (Wan *et al.*, 2002) is characterized as a tallgrass prairie that receives approximately 90.0 cm of rainfall annually with an average temperature of 16.3^{o}C. The site consists of six paired (control and warmed) 2 x 2 m plots. Warmed plots are heated to ~2.0^{o}C above ambient temperature using infrared heaters suspended above the plot. All plots had been continuously heated for approximately four years prior to the beginning of our sampling. Soil moisture was taken at regular intervals using a portable time domain reflectance (TDR) probe (Wan *et al.*, 2002). Soil samples were taken from unclipped sub-plots (to simulate a native grassland) over a two-year period starting in August 2004 and ending in April 2006.

Soil sampling and DNA extraction

Soil cores were collected from the top 15 cm of soil from the unclipped sub-plots of the control and warmed plots using sterile stainless steel coring tools. Two cores were randomly taken from each plot (one core per subplot) combined and stored on ice for transport. The combined core material was then screened for roots, homogenized and stored at -20^{o}C. DNA was extracted from soils using the MoBio Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Extraction of DNA from 0.5-gram soil was done according to the manufacturers methods except for the following modification. In lieu of shaking for ten minutes on a flat bed vortex, samples were treated in a Mini-bead beater (Biospec Products Inc.,

Bartlesville, Ok) for one minute at maximum speed and then placed on ice for one minute. Isolated DNA was then diluted 1/10 in sterile, nuclease-free water and stored at -20^{o}C.

Quantitative PCR (qPCR)

*amo*A genes from *Archaea* and *Bacteria* were quantified over the course of two seasons (August 2004-5 and April 2005-6) in five pairs of plots using previously described primer sets. Archaeal *amo*A [amoA-19F (5'-ATGGTCTGGCTWAGACG) (Leininger *et al.*, 2006) and amoA-A616r48Rev (5'-GCCATCCABCKRTANGTCCA) (Schauss *et al.*, 2009)] and bacterial *amo*A [amoA-1F (5'-GGGGTTTCTACTGGTGGT) and amoA-2R (5'-CCCCTCKGSAAAGCCTTCTTC) (Rotthauwe *et al.*, 1997)] was amplified using qPCR methods adapted from Fierer *et al.* (2005), with alterations to DNA purification and adaptation to the MyIQ real-time PCR system (Bio-Rad, Hercules, CA). To quantify *amo*A genes, DNA, extracted from each plot at each of the four time points, was diluted 1/10 to alleviate inhibition and qPCR was then performed in triplicate on both DNAs. (Sheik *et al.*, 2011) saw little variation in gene copy numbers between DNA extractions, thus only one DNA set was chosen for further quantification. Each reaction mixture (25-µl total volume) consisted of 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 9.5 µl of water, 1.0 µl (10 µM) of each primer, and 2.0 µl of diluted DNA. qPCR followed a standard three-step protocol consisting of 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 56(59)°C for 30 s, and 72°C for 1.0 (1:30) min (AOB in parenthesis). In order to quantify the number of gene copies,

standard curves were constructed using tenfold serial dilutions of pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) containing a representative *amo*A amplicon derived from Kessler Farm Soil (AOB) or from the archaeal ammonia oxidizer *Nitrosopumilus maritimus* (Genomic DNA provided by Dr. Liz Carr, University of Oklahoma). Plasmids were extracted using a Qiagen Plasmid Miniprep kit (Qiagen, Valencia, CA). Environmental plasmid ampicons were sequenced prior to quantification to ensure specificity to the *amoA* gene. Each plasmid standard was quantified using a Nanodrop spectrophotometer system (Thermo Scientific, Wilmington, DE, USA) then converted to copy number from plasmid molecular weight. PCR conditions were modified for each primer set to reach as closely as possible an amplification efficiency of -3.3. No primer dimers were observed in all primer sets as determined by melting curves of qPCR amplicons. The environmental sample copy number was determined from the standard curve and subsequently standardized to copy numbers per gram of dry soil. Statistical analysis of the warming response was done using SPSS.

CHAPTER III

FUNGAL COMMUNITY RESPONSE TO CLIMATE CHANGE

SUMMARY

Fungi are integral, diverse members of the soil microbiome, as they initiate/maintain carbon degradation of complex plant-derived macromolecules, mediate carbon flow to the soil and facilitate nutrient uptake to their plant symbionts. Thus, alterations to the structure or abundance of fungal communities, in response to climate change, could have dramatic cascading ecosystem effects as both plants and bacteria will likely be affected. An increase in global mean temperature is anticipated, however little is understood of its potential effects on fungal communities as much of the diversity in soil is yet uncultivated. Thus we sought to understand the response of fungal communities to increases in temperature over the course of two seasons in a tallgrass prairie soil. However during the course of sampling, the experimental warming site underwent a moderate drought allowing us to quantify fungal abundance during a normal moisture year, at the onset of, and abundance only during the drought. We found that during normal moisture conditions, the warming treatment altered fungal community structure, negatively affected alpha diversity, but did not change 18S rRNA gene copy abundance. While at the onset of drought, shifts in community structure but not relative diversity were observed due to the warming treatment. Warming also increased 18S rRNA gene abundance, which was maintained throughout the summer drought. Our data suggest that fungi are drought tolerant and likely are able to fill niches left vacant by bacteria transitioning into dormancy. Furthermore, drought conditions associated with anthropogenic climate change may not have as great of an impact on carbon cycling as previously thought as soil respiration was not observed to decrease during the drought.

INTRODUCTION

The soil microbiome is a highly diverse (Elshahed *et al.*, 2008) and dynamic (Sheik *et al.*, 2011) community of microorganisms that include not only Bacteria but also Archaea, Fungi and other micro-Eukarya. Organisms within the microbiome are responsible for much of the geochemical cycling of nutrients within the soil. Increasing global mean temperatures, as a result of anthropogenic activities (IPCC, 2007), will therefore likely have a direct influence on the microorganisms responsible for nutrient turnover (Davidson, 2006). Other factors associated with warming such as CO_2 concentration, rainfall patterns and plant-mediated carbon flow are anticipated to have large effects on function of the soil microbiome (Bardgett *et al.*, 2008).

Carbon turnover within the terrestrial biosphere is of particular interest (Shaver, 2000) as soil is considered a carbon sink and is responsible for long-term storage of carbon. However much debate centers on whether soil, under a warming climate, will become a source for CO_2 (Luo *et al.*, 2009a) thereby augmenting anthropogenic release, or if secondary environmental factors, such as drought (Knapp *et al.*, 2008) or nitrogen limitation (Luo *et al.*, 2004), will constrain carbon mineralization (*i.e.* degradation of biomass to CO_2), thereby maintaining or enhancing storage capabilities. Temperature has been shown to be a primary contributing factor for carbon release as CO_2 from soil (Lundegärdh, 1927). Still, indirect measures of physiology present a challenge for elucidating the underlying mechanism for the change in physiology.

Niche redundancy in microbial communities is common (Allison and Martiny, 2008) and in soil direct competition is likely partially negated by physiology based

niche partitioning (Jaspers and Overmann, 2004; Lekberg *et al.*, 2007; Schoener, 1974) as growth and ultimately survival of microbial species is bounded by environmental conditions (*i.e.* pH, temperature, carbon source, symbiotic partner…) (Chin *et al.*, 2010; Jaspers and Overmann, 2004). Thus, as the soil environment changes either naturally (*i.e.* diurnal or seasonal) or by perturbation, changes to microbial community's structure are certain as growth is likely optimal for only a fraction of the total community (Jones and Lennon, 2010). In chapter one, we show that bacterial community structure and abundance is sensitive to drought and increase in temperature. *Actinobacteria* are often considered to favor low water potential soils (Goodfellow and Williams, 1983) and are dominant bacterial carbon degraders in soil (Goodfellow and Williams, 1983; Sheik *et al.*, 2011), yet we show that their abundance is sensitive to warming and drought in chapter one. However, because Oklahoma prairie soils have been subjected to past temperature maxima and drought events, niche redundancy theory (Allison and Martiny, 2008) would suggest a portion of the microbiome will be adapted to low-water potentials and/or temperature increases and should respond positively. Thus we chose to investigate the response of fungi in our soils, as they likely compete for niche space occupied by *Actinobacteria* and are thought to be drought tolerant (Griffin, 1963; Toberman *et al.*, 2008).

Fungi are integral players in the carbon cycle as they either degrade carbon directly (saprophytic) (Peay *et al.*, 2008) or can direct carbon flow to the rhizosphere through arbuscular (Drigo *et al.*, 2010) or ecto-mycorrhizal (Selosse *et al.*, 2006) plant root associations. Recently, much research has been directed toward understanding the nature of arbuscular and ecto-mycorrhizal associations (Kranabetter *et al.*, 2009) as the

presence of these fungi have a direct influence on the plant's physiology and growth (Peay *et al.*, 2008). When considering carbon cycling, saprophytic fungi are of particular interest as they are direct mediators of cellulose and lignin degradation (Blackwood *et al.*, 2007; McGuire *et al.*, 2010). Decomposition studies which exclude fungi show an enhanced carbon and nitrogen storage (Beare *et al.*, 1992) suggesting fungi contribute significantly to CO_2 respiration in soil (Vargas *et al.*, 2010). Temperature fluctuations (e.g. due to climate change) has been shown to have a great influence upon rates of carbon degradation (Dang *et al.*, 2009) and to affect the fungal composition of the microbial community. Thus it is important to understand the fungal community dynamics associated with climate change, as they are prominent members of the soil carbon cycle.

Fungal communities, much like bacteria, are tremendously diverse (Buee *et al.*, 2009) and influenced by the physical properties of soil, such as pH (Rousk *et al.*, 2010). Molecular ecology of fungal communities is a relatively new field (Peay *et al.*, 2008). Thus, we know little of how the environment contributes to the structure of fungal communities. Recent work has shown that fungal communities are spatially heterogeneous at relatively small scales (Genney *et al.*, 2006; Robinson *et al.*, 2009; Vandenkoornhuyse *et al.*, 2007) and temporally variable (Schadt *et al.*, 2003). In the context of climate change, much work has focused on the response of arbuscular and ecto-mycorrhizal fungi to climate change (Parrent and Vilgalys, 2007; Vargas *et al.*, 2010), due to their importance in mediating plant nutrient uptake (Lindahl *et al.*, 2007) and drought tolerance (Osonubi *et al.*, 1991). However, total fungal community response to climate change is less understood. Under warming, shifts in substrate

utilization profiles, fungal phospholipid biomarkers, and abundance of fungal ITS genes suggest that community structure and potentially physiology of fungal communities is being affected by climate change (Castro *et al.*, 2010; Zhang *et al.*, 2005). Thus we sought to understand the effects of experimental warming and warming under drought on fungal community structure and abundance in a tallgrass prairie soil. We found fungal community abundance responded positively to the warming treatment at the onset and during the drought suggesting a role replacement with bacteria. Diversity of warmed plot fungal communities was markedly less than the control plot and dominated by *Basidiomycota* when rainfall was abundant. As the site transitioned into drought no differences in diversity were observed, however the control and warmed plot shared few species suggesting a shift in community structure due to warming.

Methods

Site Description

The experimental warming site (Wan *et al.*, 2002) is characterized as a tallgrass prairie that receives approximately 90.0 cm of rainfall annually with an average temperature of 16.3^{o}C. The site consists of six paired (control and warmed) 2 x 2 m plots. Warmed plots are heated to ~2.0^{o}C above ambient temperature using infrared heaters suspended above the plot. All plots had been continuously heated for approximately four years prior to the beginning of our sampling. Soil moisture and respiration was taken at regular intervals using a portable time domain reflectance (TDR) probe and a Li-Cor 6400 (Li-Cor Environmental, Lincoln, NB, USA) (Wan *et al.*, 2002). Soil samples were taken from unclipped sub-plots (to simulate a native grassland) over a two-year period starting in August 2004 and ending in April 2006.

Soil sampling and DNA extraction

Soil cores were collected from the top 15cm of soil from the unclipped sub-plots of the control and warmed plots using sterile stainless steel coring tools. Two cores were randomly taken from each plot (one core per subplot) combined and stored on ice for transport. The combined core material was then screened for roots, homogenized and stored at -20^{o}C. DNA was extracted from soils using the MoBio Ultraclean Soil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA, USA). Extraction of DNA from 0.5-gram soil was done according to the manufacturers methods except for the following modification. In lieu of shaking for ten minutes on a flat bed vortex, samples

were treated in a Mini-bead beater (Biospec Products Inc., Bartlesville, Ok) for one minute at maximum speed and then placed on ice for one minute. Isolated DNA was then diluted 1/10 in sterile, nuclease-free water and stored at -20^{o}C.

Quantitative PCR (qPCR)

18S rRNA genes from Fungi were quantified over the course of two seasons (August 2004-5 and April 2005-6) in five pairs of plots using the previously described 18S rRNA fungal specific primer sets 817F (5'-ATGGTCTGGCTWAGACG) and 1196R (5'-GCCATCCABCKRTANGTCCA) (Borneman and Hartin, 2000). DNA isolated from all experimental warming and control soils was amplified using qPCR methods adapted from Fierer *et al.* (2005), with alterations to DNA purification and adaptation to the MyIQ real-time PCR system (Bio-Rad, Hercules, CA). To quantify fungal 18S rRNA genes, DNA extracted from each plot at each of the four time points was diluted 1/10 to alleviate inhibition and qPCR was then performed in triplicate on both DNAs. Sheik *et al.* (2011) saw little variation in gene copy numbers between DNA extractions, thus only one DNA set was chosen for further quantification. Each reaction mixture (25-µl total volume) consisted of 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 9.5 µl of water, 1.0 µl (10 µM) of each primer, and 2.0 µl of diluted DNA. qPCR followed a standard three-step protocol consisting of 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 56°C for 45 s, and 72°C for 1.0 min. In order to quantify the number of gene copies, standard curves were constructed using tenfold serial dilutions of pCR4-TOPO plasmid (Invitrogen, Carlsbad, CA, USA) containing a representative 18S rRNA gene amplicon derived from an environmental isolate within the *Ascomycota* genus *Galactomyces*. Plasmids were extracted using a Qiagen Plasmid Miniprep kit

(Qiagen, Valencia, CA). Each plasmid standard was quantified using a Nanodrop spectrophotometer system (Thermo Scientific, Wilmington, DE, USA) then converted to copy number from plasmid molecular weight. PCR conditions were modified for each primer set to reach as closely as possible an amplification efficiency of -3.3. Primer dimers were not observed in any of the primer sets as determined by melting curves of qPCR amplicons. The soil copy number was determined from the standard curve and subsequently standardized to copy numbers per gram of dry soil. Data for 16S rRNA gene copies per gram of soil was taken from Sheik *et al.* (Sheik *et al.*, 2011).

Clone Library Construction and Analysis

PCR libraries targeting fungal 18S rRNA genes were generated using previously described universal fungal primers AU2 (5'- TTTCGATGGTAGGATAGDGG) and reverse primer AU4 (5'- RTCTCACTAAGCCATTC) (Vandenkoornhuyse *et al.*, 2007). DNA was extracted from plot five using the FastDNA® SPINKIT for Soil (BIO 101 Corp., Vista, CA 92083) and was diluted 1:10. DNA was then added to 48 µl PCR reactions containing the following (final concentration): 1x PCR buffer (Invitrogen,), 2.5 mM $MgSO_4$, 0.2 mM deoxynucleoside triphosphate mixture (Invitrogen), 3.0 U Platinum *Taq* DNA polymerase (Invitrogen), 400 nM of the forward and reverse primers and 2 µl of 1/10 diluted soil DNA. The PCR amplification protocol was as follows: initial denaturation for 5 min at 95°C followed by 33 cycles of denaturation at 95°C for 30 sec., annealing at 48.5°C for 1 min., and elongation at 72°C for 1 min, and a final elongation step at 72°C for 15 min. (Vandenkoornhuyse *et al.*, 2007). Positive PCR products were cloned using the TOPO-TA cloning kit according to the manufacturer's instructions (Invitrogen Corp., Carlsbad, CA). Plasmids were extracted

from individual clones using a Qiagen Plasmid Miniprep kit (Qiagen, Valencia, CA) and sequenced at Oklahoma Medical Research Foundation DNA Sequencing Core Facility (OMRF, Oklahoma City, OK, USA).

Sequenced clones using forward and reverse primers were quality checked, reverse complemented if necessary, aligned to the Greengenes database (DeSantis *et al.*, 2006b) and catenated to create an 18S gene fragment ~1200 bases in length. Sequences were imported into the phylogenetic software package Mothur (Schloss *et al.*, 2009) version 1.17.0. 64 bit (http://www.mothur.org) for OTU generation, diversity estimates and classification. Sequences were aligned to the Silva core sequence set using NAST algorithm (DeSantis *et al.*, 2006a), chimera checked with Chimera Slayer, distances were calculated with no penalization for end gaps, and OTUs using furthest-neighbor algorithm were calculated at the species-level (97% Similarity) and genus-level (95% Similarity) OTUs and diversity metrics were also calculated. OTUs were classified using NCBI BLAST non-redundant database (Thompson *et al.*, 1997).

Results

In Chapter 1, a full summary of the climatic effects observed at the study site during the course of our sampling can be found. During the course of this study a moderate drought occurred at the study site. Interestingly, the timing of the drought allowed us to view fungal communities during a normal moisture year (August 2004), as the plots went into drought (April 2005), at the peak of the drought (August 2005) and as the site began to transition out of drought (April 2006). This temporal transect is unique in the literature, as we are not aware of any studies that have quantified the long-term response of *in situ* fungal communities to warming and warming combined with drought using a cultivation-independent approaches.

Quantification of fungal response to warming with Quantitative PCR (qPCR)

In our soils, fungal response to the warming treatment was slight, however fungal populations did respond positively to the drought (Fig. 1). Over the course of sampling, control and warmed populations showed a marked increase in population size as the experimental site transitioned into drought. Fungal populations at the onset of drought (April 2005) increased ~ 45 - 190% (control and warmed plots respectively) and remained elevated during the drought ~150 – 250% (August 2005) (control and warmed plots) when compared to the August 2004 samples (Figure 1). Interestingly, no significant treatment-level effects were observed over the course of sampling. The warming treatment did increase the abundance of Fungi during the April 2005 sampling time (Figure 1), however this increase was not significant. Our data shows that control plot fungal abundance did not respond as quickly to the drought as the warmed plots

(Figure 1), as warmed plot fungal abundance was over two-times greater than control plots at the spring 2005 sampling time (Figure 2). Treatment-level effects seen in the spring did not carry over to the following August 2005 sampling times, as control plot fungal abundance was equivalent to warming plots (Figure 1 and Figure 2). In the spring 2006 samples, as the site began to transition out of drought, abundance of fungi in both control and warmed plots returned to levels seen in the August 2004 samples (Figure 1) and no treatment effects were observed (Figure 2). Comparison of the response of fungal and bacterial abundance to warming and warming under drought show a marked contrast between the two kingdoms, as fungi responded positively and bacteria negatively as the site transitioned into drought under warming (Figure 2).

Figure 1. Abundance of fungal-associated 18S rRNA genes in control (blue) and warmed (red) plots. Error bars represent the standard error (±1 SE, n=15) at each time point sampled.

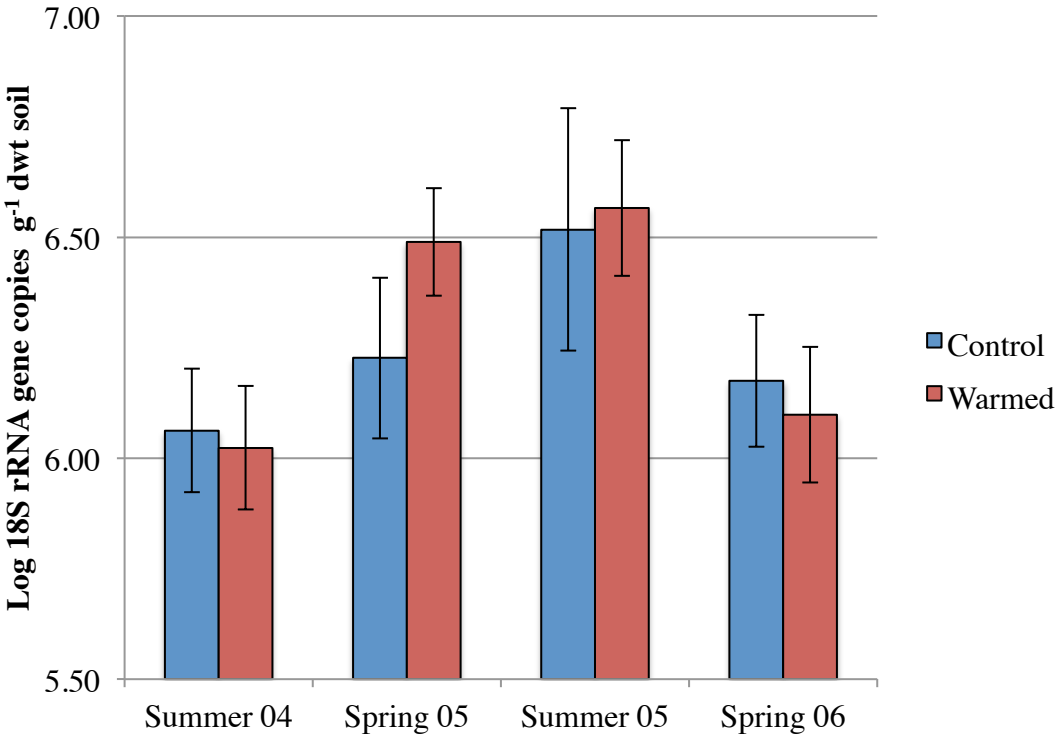
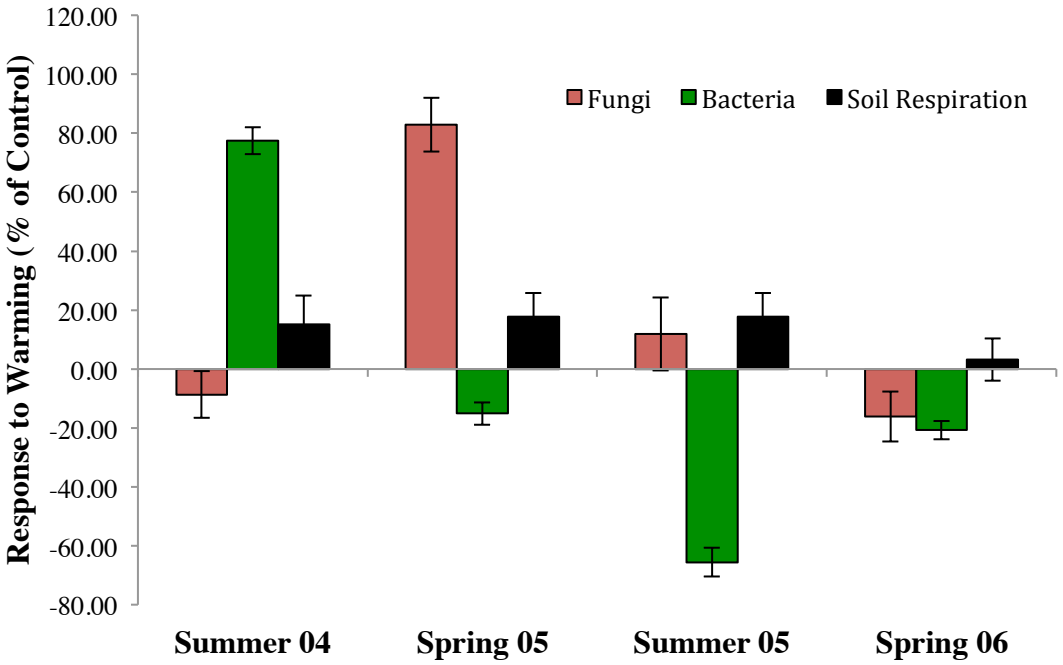


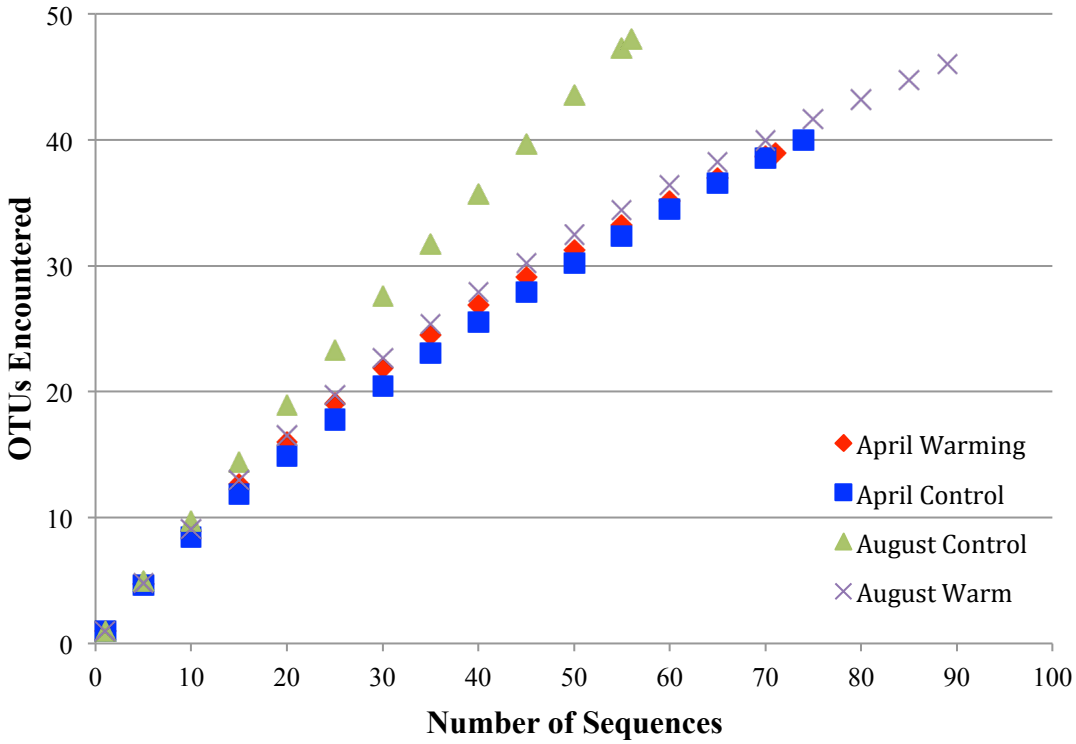
Figure 2. Response of Fungi (red), Bacteria (green) and soil respiration (black) to the warming treatment. Bars represent the response to the warming treatment of fungal 18S rRNA gene copies per gram soil, bacterial 16S rRNA gene copies per gram soil (Data taken from Chapter 1), and soil respiration data collected from the warming site near the time of sampling (± 3 days). Soil respiration data was provided by Dr. Xuhui Zhou. Error bars represent the percent standard error at each time (± 1 SE, n=15 for fungi, n=30 for bacteria, and n=12 for soil respiration)



Clone library analysis of fungal community diversity and identity

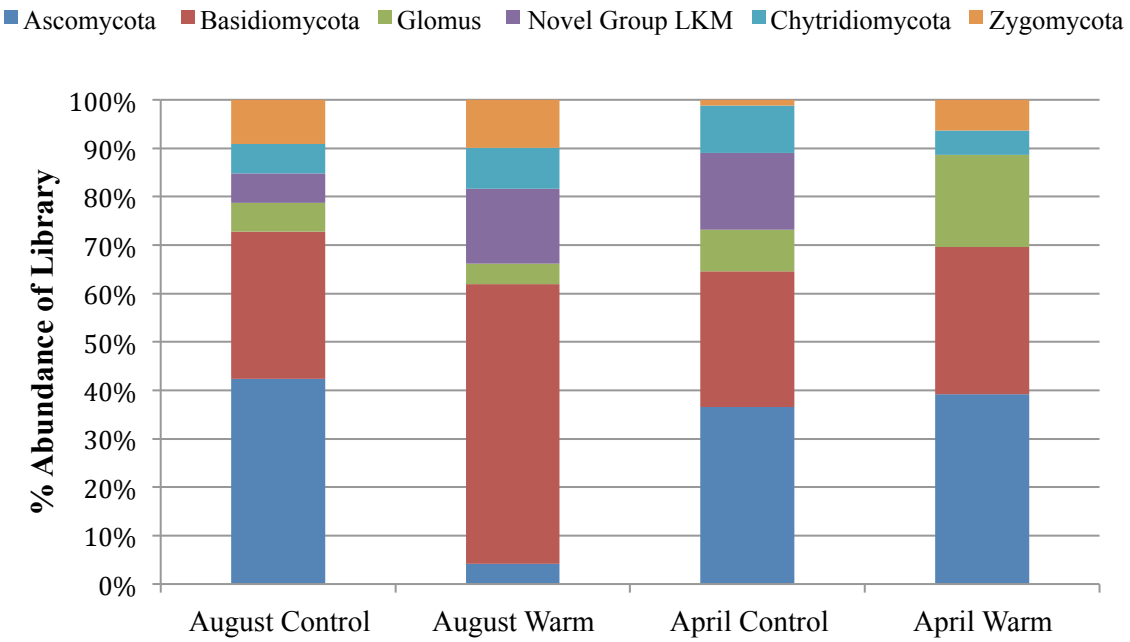
Cloning of fungal associated 18S rRNA genes, revealed a very diverse Dikarya dominated community in both the warming and control plots. A total of 192 clones were sequenced at each of two time points (August 2004 and April 2005) for control and warmed plots. After quality checking and concatenating forward and reverse sequences, fungal 18S libraries ranged in size from 56-89 clones. This resulted in 40-48 species-level operational taxonomic units (OTUs) being generated using a 97 % similarity cutoff (Figure 3). Rarefaction curve analysis shows that the warming treatment significantly reduced the alpha (within sample) diversity during the August 2004 sampling time, however had little effect on the diversity of communities in the April 2005 sampling (Figure 3). Species discovery rates across all samples ranged from approximately 1OTU/clone – 1OTU/2 clones in our libraries.

Figure 3. Rarefaction curve analysis of 18S fungal clones libraries of plot five from August 2004 and April 2005. Species-level operational taxonomic units (OTUs) were generated using a similarity score of 97%.



All samples, both control and warmed, were dominated by dikaryal phyla Ascomycota and Basidiomycota. In response to the warming treatment, Ascomycota saw a decrease of ~35% in relative abundance in August 2004 while Basidiomycota increased to over 50% of the total library (Figure 4). Little change was observed in either Dikarya phyla in the spring 2005 samples. Interestingly, novel phylum LKM was the third most abundant phylum in three out of the four samples and saw a ~10% increase in the August 2004 warming plot library (Figure 4). The arbuscular-mycorrhizal phylum Glomeromycota (*Glomus*) was detected in all four samples and responded positively to the warming treatment in the April 2005 samples (Figure 4). Little seasonal or treatment level change was observed in the relative abundance of the *Chytridiomycota* or *Zygomycota.*

Figure 4. Distribution of fungal phyla and groups identified by clone libraries in control and warmed plot five.



Beta diversity metrics (*i.e.* diversity between plots) show that the structure of fungal communities in the control and warmed plot was very dissimilar from one another. Bray-Curtis and Jaccard metrics show that control and warmed plots in the August 2004 shared few species (Bray-Curtis) and that abundance patterns of fungal species were quite different (Jaccard) (Figure 5). Conversely, the Spring 2005 control and warmed plots saw an increase (~15%) in similarity of both total community (Bray-Curtis) and abundance patterns of species (Jaccard) over the August results (Figure 5). In the August 2004 samples, only five OTUs were shared between warmed and control plots, while in the April 2005 sample, nine OTUs were shared between control and warmed plots (Figure 6). Interestingly, fungal communities from August and the following April bore little resemblance to each other as little to no OTUs were shared between these times (Figure 6). However one caveat to this analysis is that because coverage was low in our clone library, these patterns may be a result of inadequate sampling and not to changes in response to the warming treatment. Further, sequencing is required to validate the beta diversity patterns observed.

Figure 5. Similarity in species level community structure of warmed and control plots using Bray-Curtis (Black) and Jaccard (Grey) indices. Bray-Curtis measures the similarity of the community with presence absence of shared OTUs while Jaccard incorporates abundance weights of shared species. Similarity is based on a 0 to 1 scale, whereby one represents the same community and zero is represents no community overlap.

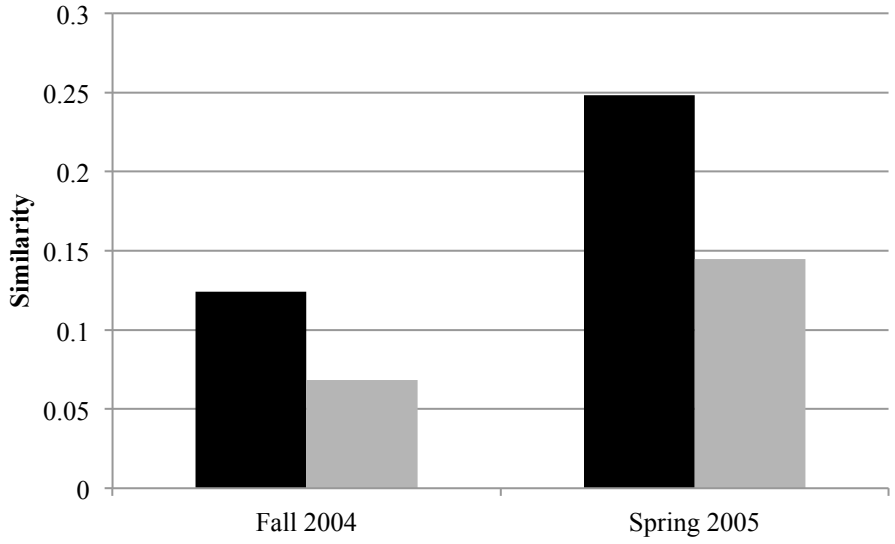
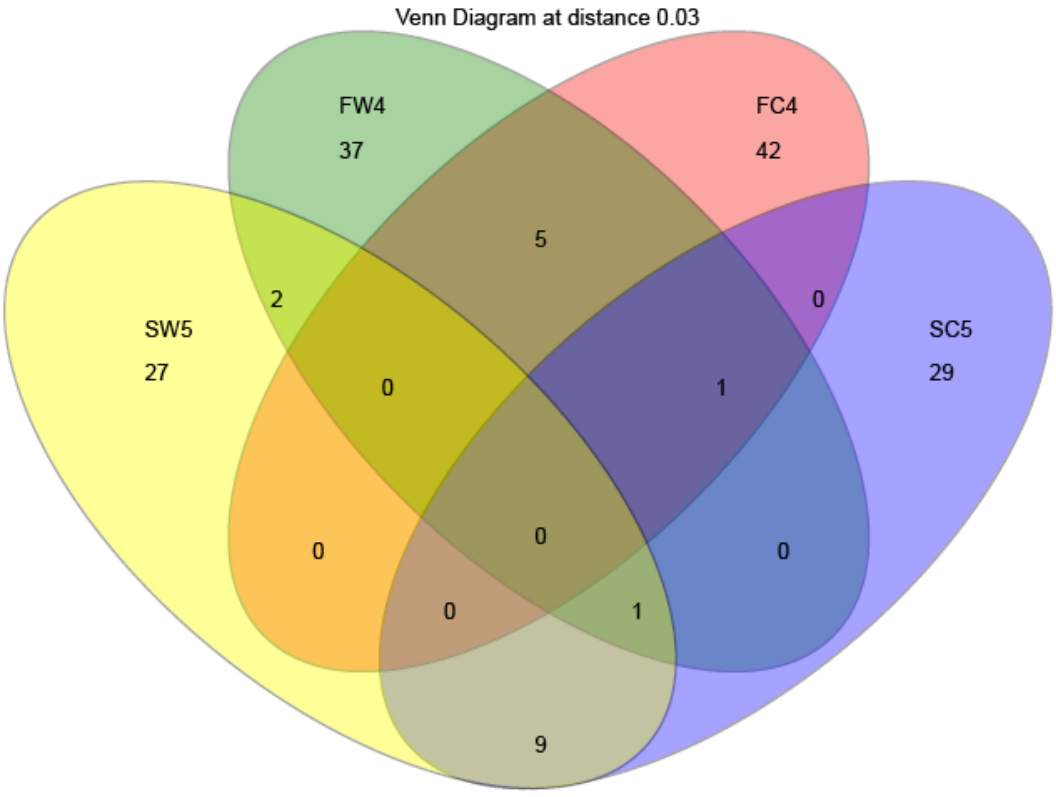


Figure 6. Venn diagram of species level OTUs shared between control and warmed treatments in August and April sampling times. FC4= August 2004 Control, FW4= August 2004 Warmed, SC5= April 2005 Control, and SW5= April 2005 Warmed.



DISCUSSION

Plant mediated carbon sequestration is considered a primary buffer of atmospheric CO_2 concentration (1976). However increasing temperatures from anthropogenically induced climate change, will likely have dramatic impacts on the latency time of carbon within the environment (Luo, 2007) by affecting the microbial catalysts responsible for carbon degradation. Fungi are important carbon mineralizering organisms in soil and are ubiquitous in soil (Peay *et al.*, 2008). They are responsive to warming (Robinson, 2002) and likely drought tolerant (Toberman *et al.*, 2008). Fungi could therefore mediate carbon mineralization when environmental conditions are stressful and increase the oval mineralization rate during warming conditions. Therefore, we sought to open the "microbial black box" and understand how fungal community members respond to warming and warming under drought.

Effects of warming on fungal community diversity and structure

It is well established that diversity of organisms in an ecosystem has direct influence on productivity and stability of the biome (Tilman *et al.*, 2006). Thus subtle changes in diversity, in response to warming, could have long-term consequences on ecosystem function. In Kessler farm soil, the response of fungal alpha diversity to warming was dependent upon the season. Warming only had an effect on fungal community diversity during the August 2004 sampling time, whereby warmed plot diversity saw a marked reduction when compared the control. Interestingly, the disparity in diversity did not carry over to the following April samples. Low community similarity (*i.e.* beta diversity) between warmed and control plots were also observed

during the August sampling time. The marked reduction in alpha and beta diversity during the August time point suggests that slight increases in temperature are having large impacts on the evenness of species (*e.g.* fewer, more abundant species) and the structure of the community. Decreases in fungal diversity in response to perturbation have been reported (Toberman *et al.*, 2008) however few studies have focused on the effects of warming on the total community diversity. Low community similarity between warming plots was anticipated (Genney *et al.*, 2006; Kranabetter *et al.*, 2009; Peay *et al.*, 2008), however large restructuring of the fungal communities at the phylum level was not. The shift in dominance from Ascomycota in the control plots to Basidiomycota in the warmed August sample suggests a physiological shift is occurring in response to the warming treatment. The predominance of Basidiomycetes in the warmed plot may be in response to nitrogen limitation. It is likely that competition for nitrogen during the August 2004 sample was high, as both bacterial populations (See chapter 1) and plant growth (Luo *et al.*, 2009a) were stimulated. Thus, Basidiomycete populations may be outcompeting Ascomycetes (specialized in cellulose degradation) by scavenging nitrogen from nitrogen-rich lignin (Blackwood *et al.*, 2007). Shifts in fungal phospholipid fatty acid profiles in response to warming have been observed at Kessler Farm from early fall samples (Zhang *et al.*, 2005), however fatty acid signature profiles were not reported thus limiting our ability to compare results.

As the site transitioned from August into April, no treatment level effects of alpha diversity of fungal communities were observed. However, community similarity was still very low as few species were shared between control and warmed plots. Dominance patterns observed in Dikarya populations in the fall were absent in April

samples. Strong seasonality effects of fungal populations are evident as few species carry over from fall populations (Figure 5). Seasonality of fungal populations has been shown (Schadt *et al.*, 2003) and suggests that fungal populations are highly dynamic and community turnover may be rapid (Peay *et al.*, 2008). Interestingly, increased relative abundance of arbuscular mycorrhizal group *Glomus* in warmed plots raises the possibility that shifts in plant community phenology (Sherry *et al.*, 2007) may be stimulating the growth of *Glomus.* Arbuscular mycorrhizae are tightly coupled to plant growth as they are dependent upon photosynthate carbon for growth (Selosse *et al.*, 2006) and evidence of their stimulation under warming in the literature has been reported (Rillig *et al.*, 2002). Thus, the increase in Spring *Glomus* supports observations of increased plant biomass in warmed plots and the likelihood that warmed plots are phenogically advanced ahead of controls (Luo *et al.*, 2009a).

Response of fungal abundance to warming and drought

During the course of sampling, the experimental site underwent a moderate drought, however the timing at which the drought occurred was instrumental in controlling bacterial populations in our soils. The April 2005 sampling was pivotal due to an early onset of the plant community (*i.e.* warming allowed plant communities to begin growing sooner than controls) which caused an increase in plant biomass in warmed plots (Luo *et al.*, 2009a). However, because of diminutive rainfall amounts during the early spring, soil moisture reserves were not replenished and the plant communities quickly created drought like conditions in the soil. Bacterial populations at this time began to decline in size and would remain suppressed throughout the summer of 2005 (chapter one). Interestingly, as bacterial populations declined, fungal abundance

responded positively to the warming treatment in April 2005 as 18S rRNA gene abundance was elevated above the control. However, the increase in abundance above the control abundance was short lived, as control plots re-joined warmed plots and remained elevated throughout the summer. The initial increase in fungal abundance in warmed plots suggests that much like in the plant community (Sherry *et al.*, 2007), warming shifts the phenology of fungal populations allowing the community to begin growth cycles earlier. However, because the warming effect on population abundance was short lived, surges in soil respiration associated with the increase in population may only represent a small portion of the total seasonal respiration. Furthermore, little change in soil respiration response was observed between summer 2004 through summer 2005 sampling times as all responded positively to the warming treatment (Figure 2). Little fluctuation of soil respiration suggests that the switch in activity between bacteria and fungi may not result in observable changes in metabolic output (*i.e.* respiration rate). In our soils, evidence for spatial heterogeneity of fungal communities (Kranabetter *et al.*, 2009) was observed as large variation in gene copy numbers were observed between plots. The increase in population size of fungi in warmed plots during the drought is consistent with others findings that fungi are capable of withstanding lower soil water potentials than bacteria (Griffin, 1963; Toberman *et al.*, 2008). Interestingly, as moisture began to return in the April 2006 samples, so did bacterial communities, with fungal communities receding back to levels seen in August 2004. This suggests that fungi and bacteria may be in direct competition for niche space during normal precipitation years, however this remains a speculation as

identification of ecological roles from ribosomal inference are difficult to ascertain for heterotrophs.

Within Oklahoma soil, we found that population size of fungi responded positively to drought conditions in warmed plots and structure of the communities were greatly influenced by the temperature increase. Our data suggests niche redundancy and differentiation is likely as structure and abundance of fungal communities were affected. Furthermore, little change in respiration implies that metabolic output of the fungal community is able to compensate for the loss of bacteria in the system. However elucidating these interactions are difficult, as much of the microbiome is uncultivated and thus is beyond the scope of this paper. The inability to link function to a species greatly hinders our understanding of the underlying principles of how ecosystems respond to perturbations, such as warming or drought. Nonetheless, we show that fungi are adapted to growth during water stress events and warming has little affect on population sizes of fungal populations.

APPENDIX

CHRONIC EXPOSURE OF SOIL MICROBIAL COMMUNITIES TO CHROMIUM AND ARSENIC FROM TANNERY WASTE ALTERS THEIR DIVERSITY AND STRUCTURE

SUMMARY

Extensive use of chromium (Cr) and arsenic (As) based preservatives from the leather tanning industry in Pakistan has had a deleterious effect on the soils surrounding production facilities. Bacteria have been shown to be an active component in the geochemical cycling of both Cr and As, but in any environment, only a small fraction of the total microbial community is thought to harbor resistance genes. Therefore, we sought to understand the effects that long-term exposure to As and Cr had on the diversity and structure of soil microbial communities. Soils from three spatially isolated tanning facilities in the Punjab province of Pakistan were analyzed by pyrosequencing and quantitative-PCR. The structure and diversity of the microbial community and abundance of microbial 16S rRNA gene were highly influenced by the concentration and presence of hexavalent chromium (Cr(VI)) and arsenic (As). Uncontaminated soils had significantly lower bacterial 16S rRNA gene copy abundance and were dominated by *Actinobacteria*, *Proteobacteria*, and *Acidobacteria.* The three uncontaminated soils were chemically similar and were more similar in species composition to each other than to corresponding contaminated soils. In the presence of either Cr(VI) or As, *Proteobacteria* dominated and *Actinobacteria* and *Acidobacteria* were minor components of the bacterial community. The shifts in community composition were significant and revealed that Cr(VI)-containing soils were more similar to each other than to As contaminated soils lacking Cr(VI). Alpha diversity (within site diversity) was lower in all contaminated soils, and was only significantly, negatively correlated with total Cr, Cr(VI), and pH. None of the alpha diversity metrics was significantly correlated with the presence of As, but the alpha diversity metrics were negatively

impacted as As concentration increased. Our results show that chronic exposure to either Cr or As alters the soil bacterial community allowing a single phylum of bacteria to dominate.

INTRODUCTION

Anthropogenic metal contamination is a problem frequently encountered near long-term industrialized areas (Bååth, 1989). In Pakistan, the leather industry has left soils surrounding production facilities contaminated with chromium (VI & III) and arsenic. Accumulation of Cr (VI) and arsenic in soils is of concern due to their mutagenic and carcinogenic properties in humans (USEPA, 1998a, b) Geochemical cycling of chromium and arsenic can be microbially mediated in both aerobic (Campos *et al.*, 1995; Oremland *et al.*, 2004) and anaerobic (Li and Krumholz, 2007; Marsh and McInerney, 2001) systems. Within the environment, chromium is generally found in two valence states, trivalent [chromite, Cr (III)] and hexavalent [chromate, Cr (VI)] (Kamaludeen *et al.*, 2003). Chromite is generally thought to be non-toxic and poorly mobile because of its low water solubility at neutral pH and its affinity for sorption to iron oxides, clays, and organic matter (Kamaludeen *et al.*, 2003). In contrast, chromate is acutely toxic, mutagenic, teratogenic and carcinogenic (USEPA, 1998b). It is an anion at neutral pH, soluble in water and mobile in soils and sediments (Kamaludeen *et al.*, 2003). Chromate can enter bacterial and animal cells through sulfate transport systems (Ackerley *et al.*, 2004). Cr (VI) resistance is generally thought to occur through the use of a chromium efflux pump (Aguilar-Barajas *et al.*, 2008; Alvarez *et al.*, 1999),

while detoxification (*i.e.* reduction to the insoluble trivalent chromium) is less understood but several genes have been proposed (He *et al.*, 2010).

Inorganic arsenic, much like chromium, is primarily found in two valence states in the environment, pentavalent [arsenate, As (V)] and trivalent [arsenite- As (III)] (Oremland and Stolz, 2005). In contrast to chromium, the reduction of arsenate to arsenite increases the solubility and toxicity, as arsenite is uncharged at pH < 9 (Oremland and Stolz, 2003). Several resistance/reduction mechanisms for arsenate are well described and are reviewed by Oremland and Stolz (2003; 2005). Unlike chromite [Cr (III)], arsenite can be re-oxidized by bacteria through a resistance pathway or utilized by chemolithoautotrophic arsenite oxidizers (Oremland and Stolz, 2005). To date, many of the isolated bacteria capable of resisting or transforming chromium or arsenic are associated with *Proteobacteria*, *Firmicutes*, and *Actinobacteria* (Kamaludeen *et al.*, 2003). Due to the dearth of bacterial isolates with known As resistance mechanisms, it is unclear whether resistance to and transformation of chromium and arsenic is common in underrepresented phyla and in clades within well-characterized phyla.

The diversity of soil microbial communities is exceedingly rich (Elshahed *et al.*, 2008; Fulthorpe *et al.*, 2008; Roesch *et al.*, 2007; Sheik *et al.*, 2011); however, when exposed to metals, alpha diversity (*i.e.* the diversity within a sample) and microbial biomass are commonly observed to decrease (Bååth, 1989). In systems acutely exposed to contamination, diversity may be maintained within the community through either natural resistance (Badar *et al.*, 2000) or the ability of organisms to lay dormant until favorable growth conditions return (Jones and Lennon, 2010). Conversely, chronic

exposure to contamination will likely have deleterious effects on the structure and ultimately the function of the community, as dormancy may not be a useful survival option. The degree of species loss will likely be a function of the mobility of resistance genes (*i.e.* horizontal gene transfer) (Cai *et al.*, 2009) and the behavior of the metal species in the environment (Giller *et al.*, 2009). Soil microbial communities are known to share few species as distance between sites increases (Bell, 2010; Fulthorpe *et al.*, 2008). However, it has been observed that landscape and pH can select for similar communities despite being spatially separated (Lauber *et al.*, 2009). In the face of long-term exposure to stress, restructuring of microbial communities is likely (Odum, 1985), but it is unclear which groups of microorganisms are more tolerant to chromium and arsenic contamination. Therefore we sought to understand the effects of chronic chromium and arsenic contamination on soil bacterial communities from three spatially distant, long-term leather production areas in the Punjab province of Pakistan. We found that in the presence of metals *Proteobacteria* dominated libraries, alpha diversity of bacterial communities were negatively affected, and structure (*i.e.* Beta diversity) of these communities were highly influenced by the presence of Cr (VI).

RESULTS

Geochemistry of Sampling Sites

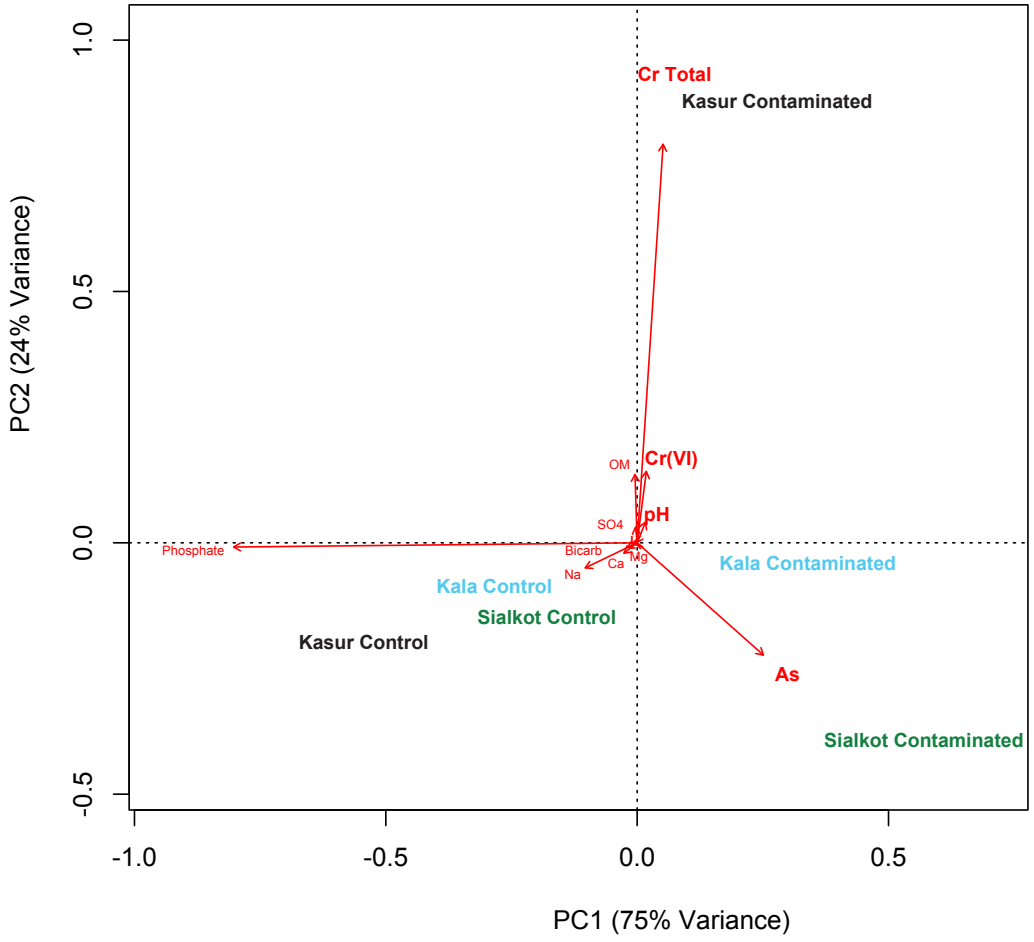
Chromium was detected in all of the contaminated and control sites (Table 1). The majority of chromium detected was the trivalent form. Contaminated soils differed in the presence and amounts of Cr (VI) and As (Table 1). Chromium (VI) and As were not detected in any of the control soils (Table 1). Kasur contaminated soil had the highest levels of Cr (VI) and total chromium, but undetectable levels of arsenic (Table 1). Sialkot contaminated soil had arsenic, but no detectable Cr (VI) and little total chromium. Kala Shah Kaku contaminated soil had high levels of Cr (VI), total chromium and arsenic. Kala Shah Kaku and Kasur contaminated soils also had the highest pH, clay content, and total chromium levels (Supplemental Table 1). Despite being spatially separated by 50-200 km, soil textures were quite similar with the exception of the contaminated Kala Shah Kaku and Kasur soils, which contained a higher silt and clay content than the other soils (Supplementary Table 1). Principal Components Analysis revealed that much of the variance in soil properties and chemistry between the contaminated sites was associated with pH and arsenic, total chromium, Cr (VI) levels, while control soils were correlated with di and monovalent cations, bicarbonate, and phosphates (Figure 1). The pH of all soils was circum-neutral; however contaminated sites were more alkaline (pH 7.0-8.0) than the paired control sites, which were all more acidic (6.4-6.8). Despite the extensive use of arsenic by the tanning industry as a hide preservative, arsenic was detected in only two of the three

contaminated sites (Kala Shah Kaku and Sialkot) with Sialkot having the highest arsenic concentration (Table 1).

Table 1. Location and key geochemical data of contaminated and control study sites.

Site	Contamination Status	Latitude (N)	Longitude (E)	pH	As ($m \bullet kg^{-1}$)	Cr (VI) ($mg \bullet kg^{-1}$)	Total Cr ($g \bullet kg^{-1}$)
Kala Shah Kaku	Control	31 44'44.24"	74 15'54.61"	6.5	0	0	3.82
	Cr (VI) and As-contaminated	31 44'44.32"	74 15'52.92"	7.4	6.6	1.2	6.24
Kasur	Contol	31 06'03.89"	74 27'42.45"	6.4	0	0	1.61
	Cr (VI)-contaminated	31 06'18.53"	74 27'34.17"	8	0	4.21	24.78
Sialkot	Control	32 28'21.10"	74 30'57.53"	6.8	0	0	2.36
	As-contaminated	32 28'18.57"	74 30'59.57"	7	13.9	0	0.23

Figure 1. Principal components analysis (PCA) of soil geochemical data. Paired control and contaminated sites are color-coded. Geochemical data from Supplementary Table 1 were used to generate PCA; however, factors that had little influence on any of the sites (*i.e.* clustered near the center axis) were removed for clarity.



Microbial Community Response to As and Cr Contamination

From the three-paired sampling sites, a total of 232,216 sequences were obtained after quality screening, resulting in and average of 15,000 - 28,000 sequence reads per sampling site (Table 2). One subsample from Kala Shah Kaku (1a) failed to generate enough sequence tags (approximately 1000) after the quality screening and was not further analyzed. Comparative analysis of the control and contaminated sites revealed several large shifts in the relative abundance of many of the dominant phyla. Uncontaminated sites all shared similar phylum level profiles, whereby *Actinobacteria*, *Proteobacteria*, and *Chloroflexi* were the most abundant phyla present (Figure 2). This pattern is similar to phylum level profiles generated by other pyrosequencing studies of soil (Elshahed *et al.*, 2008; Sheik *et al.*, 2011; Youssef and Elshahed, 2008), suggesting that the chosen control sites provided an adequate baseline for comparison to contaminated soils. The contaminated soils all had a similar phylum-level abundance profile that was drastically different from their paired controls. In contrast to the control soils where *Actinobacteria* was the dominant phylum, *Proteobacteria* was the dominant phylum in contaminated soils (Figure 2). Within the *Proteobacteria, Alphaproteobacteria* or *Gammaproteobacteria* were the most abundant classes in all soils. In soils containing either As (Sialkot) or Cr (VI) (Kasur) only, *Alphaproteobacteria* was the most abundant class. However, when both As and Cr (VI)

(Kala Shah Kaku) were present, *Gammaproteobacteria* was the most abundant class (Supplemental Figure 1). The presence of Cr (VI) (Kasur) had a negative affect on the abundance of *Betaproteobacteria* and *Deltaproteobacteria* while the presence of only As (Sialkot) had no effect on either class (Supplementary Figure 2). *Chloroflexi* were more abundant than *Acidobacteria* in control soils (Figure 2). In contaminated soils with Cr (VI) (Kala Shah Kaku and Kasur), *Acidobacteria* were rare members of the community constituting less than 1% of their respective libraries. In Sialkot soils contaminated with Arsenic but not Cr (VI), *Acidobacteria* abundance was more abundant than its paired control.

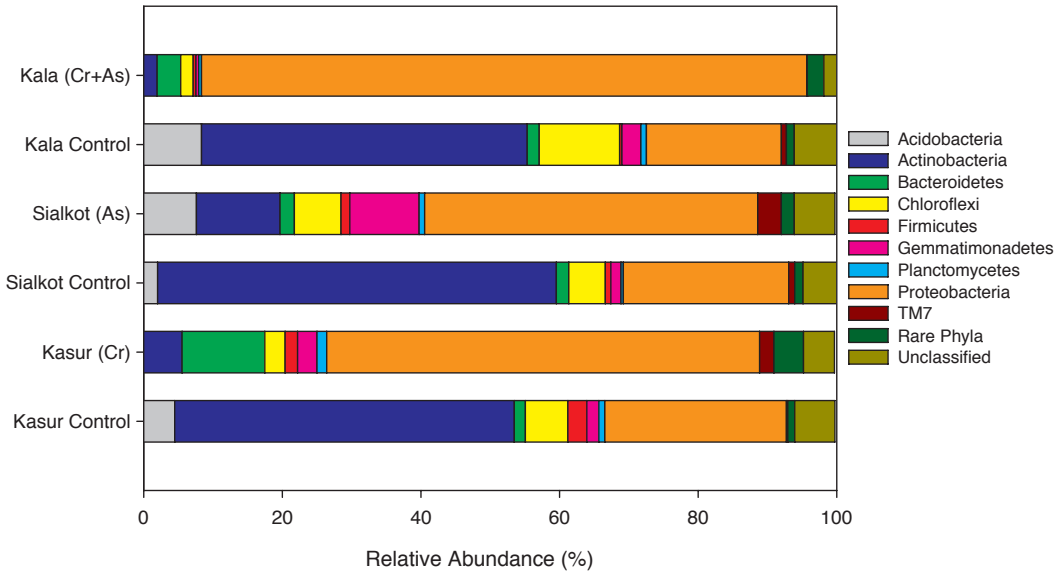
Table 2. Pyrosequencing results, diversity estimates, and 16S rRNA gene copy abundance for each sampling site.

		Pyrosequencing Results		Diversity Estimates[1]			16S rRNA Abundance
Site	Contamination Status	Total Sequences	Total OTUs[2]	ACE	Chao	Shannon	Log copies •g^{-1}dwt soil
Kala Shah Kaku	Control	15610 ± 2730	6095 ± 544	26008 ± 1817	15518 ± 806	7.9 ± 0.01	7.45 ± 0.05
	Cr (VI) +As-contaminated	20907 ± 0	3191 ± 0	9597 ± 0	6794 ± 0	5.9 ± 0	8.89 ± 0.13
Kasur	Control	21745 ± 4737	6555 ± 1076	23970 ± 3825	14908 ± 2425	7.5 ± 0.12	6.33 ± 0.07
	Cr (VI)-contaminated	16221 ± 1576	3754 ± 354	15661 ± 868	9520 ± 908	6.4 ± 0.11	8.81 ± 0.03
Sialkot	Control	28102 ± 3860	8057 ± 547	28055 ± 706	17971 ± 749	7.7 ± 0.01	7.59 ± 0.02
	As-contaminated	23979 ± 315	5758 ± 567	20483 ± 3472	13096 ± 2024	7.3 ± 0.11	7.98 ± 0.03

[1]Diversity Estimators Abbreviations: Abundance-based Coverage Estimator (ACE), Chao's species richness estimator, and Shannon-Weiner Index.

[2] Species level, 97% similarity threshold used to define Operational Taxonomic Units (OTUs).

Figure 2. Phylogenetic distribution of the dominant phyla identified by pyrosequencing. The dominant phyla identified in pyrosequencing libraries are represented by separate colors and are in alphabetical ordered from left to right. Little variation between sub-samples for each site was observed and variation estimates were left out of figure for clarity.



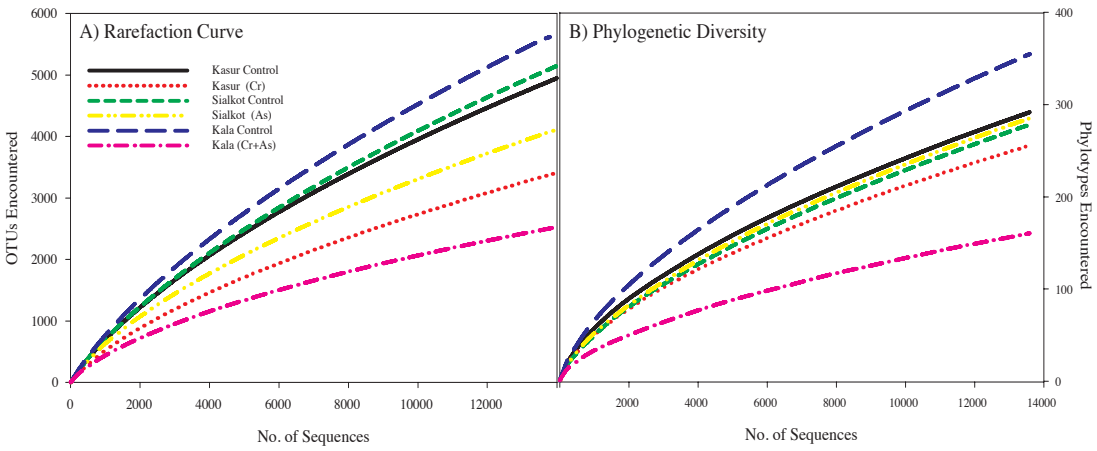
Quantification of total bacterial 16S rRNA gene copies using qPCR showed that the contaminated sites in general had higher 16S rRNA gene copy abundance than did their paired controls (Table 2). Soils with Cr (VI) (Kasur and Kala Shah Kaku) had the highest 16S rRNA gene copy abundance and showed the greatest disparity in 16S rRNA gene copy number compared to their paired control (~1.5- 2 log fold differences) (Table 2).

Shifts in Diversity due to Contamination

The diversity within each site (alpha diversity) was highly dependent upon the type and extent of contamination. The contaminated sites all showed a 14 to 38% reduction in the number of total $OTUs_{0.03}$ relative to the paired controls when corrected for the total number of sequences in each library (Table 2). The presence of both As and Cr (VI) together had the greatest effect on diversity, whereby a 38% reduction in alpha diversity was observed at the Kala Shah Kaku contaminated site. In contrast, the diversity in the Sialkot contaminated soil, which had arsenic only and in the Kasur contaminated soil, which had Cr (VI) only, declined by 14% and 24%, respectively (Table 2). Calculation of species richness (Chao), evenness (ACE), and diversity (Shannon) indices all confirmed the decrease in diversity within the contaminated soils when compared to control sites (Table 2). Rarefaction curves corroborated the results seen with species richness estimators confirming that exposure to arsenic, chromium (VI), and the combination of the two caused a significant reduction in microbial diversity (Figure 3a). Species richness estimators are sensitive to sample size (Liu *et al.*, 2007; Youssef and Elshahed, 2008). However, the decline in microbial diversity was not due to unequal sampling because Faith's phylogenetic diversity, which is less sensitive to sampling size

(Lozupone and Knight, 2008), along with rarefaction curve analysis showed the greatest decrease (55%) when both As and Cr (VI) were present (Figure 3b). The second largest decrease in Faith's phylogenetic diversity occurred in Kasur soils, which are contaminated with Cr (VI) and only a 12% reduction in Faith's phylogenetic diversity was observed in the arsenic contaminated Sialkot soil. Interestingly, the presence of As but not Cr (VI) affected total diversity but not phylogenetic diversity (Figure 3 b).

Figure 3. Alpha-diversity analysis using rarefaction (A) and phylogenetic diversity (B) of contaminated and control soils. Each soil is represented by color and pattern. Rarefaction analysis focuses on discovery of novel species level (97%) Operational Taxonomic Units (OTUs) while Phylogenetic Diversity focuses on the discovery of novel phylogenetic branches.



To test which environmental factors were contributing to the variation in microbial diversity, Pearson correlation coefficients were calculated. Total Cr, Cr (VI), and pH, content were all significantly-negatively correlated with Chao, ACE, and

Shannon metrics ($P<0.05$, $N=9$) (Table 3). Total chromium was more significantly correlated with diversity than Cr (VI); however, the strongest correlation to diversity of all environmental factors tested was pH. The presence of arsenic was negatively correlated to all diversity metrics, but these correlations were not significant (Table 3). Interestingly, "phylogenetic" diversity, despite being negatively affected by metal presence, was not significantly correlated to the type of metal contamination. The only positively correlated environmental factor was phosphates, but the correlation was not significant.

Table 3. Effect of metal contamination, pH and soil organic matter on diversity and evenness metrics of the soil communities[1].

α-Diversity Metric[2]	pH	Organic Matter	As	Cr (total)	Cr (IV)
Chao	**	ns	ns	*	ns
ACE	**	ns	ns	*	*
Shannon	***	ns	ns	*	*
Phylo Diversity	ns	ns	ns	ns	ns

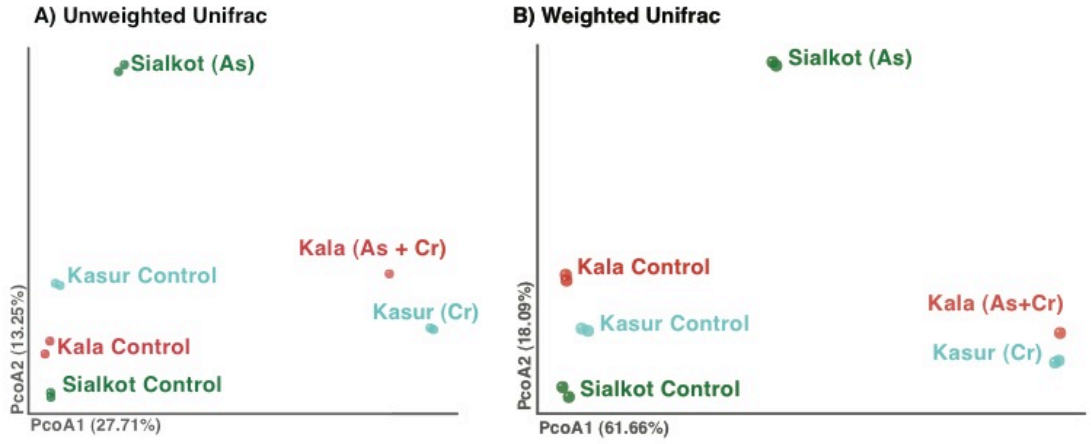
[1] Significance was assessed using a two-tailed t-test (n=9) and p-values are represented by: * ≤ 0.05, ** ≤ 0.01, and *** ≤ 0.001.

[2]Abbreviations: Abundance-based Coverage Estimator (ACE), Chao's species richness estimator, Shannon-Weiner Index, Faith's Phylogenetic Diversity, and ns, not significantly different.

In order to compare the structure of microbial communities between sites and observe effect of Cr (VI) and As had on community structure, beta diversity metrics were calculated. We chose to use the phylogenetic tree-based method, Unifrac, as it has

been shown to be a robust measure of community similarity (Lozupone *et al.*, 2010). Unweighted (total diversity) and weighted (lineage specific diversity) Unifrac analyses confirmed that chronic exposure to Cr (VI) and As significantly altered the microbial community structure (P=0.002 for both weighted and unweighted algorithms using an individual sample analysis with 500 permutations) (Figure 4 a & b). Both the unweighted and weighted forms of the algorithm yielded similar results. Both Unifrac analyses showed that control soils had similar community structures despite being spatially separated (Figure 4). Furthermore, the community structure of the Sialkot contaminated site, which had As and not Cr(VI), was very different from the other two sites, both of which had Cr(VI) but only one which had As (Figure 4). The presence of Cr (VI) appeared to be a greater selective force on structuring the microbial community than was As as evidenced by the clustering of Kala (Cr+As) with Kasur (Cr) contaminated sites by Unifrac analyses. Cr (VI) contamination accounted for 28 and 62% of total variance by unweighted and weighted Unifrac analysis, respectively, compared to As contamination, which accounted for 13 and 18% of total variance by unweighted and weighted Unifrac analysis, respectively.

Figure 4. Effect of As and Cr(VI) contamination on β-diversity of the microbial communities from control and contaminated sites. The first two coordinate axes from a principal coordinate analysis (PCoA) were plotted using the unweighted (A) and weighted (B) Unifrac algorithms. Colors represent paired control and contaminated sites.



DISCUSSION

In Pakistan, the extensive use of chromium and arsenic by the leather tanning industry and historical lack of environmental regulation has left soils near metropolitan areas inundated with these hazardous metal contaminants. Bioremediation strategies targeting metal transforming bacteria are often chosen due to their relatively low cost and environmental impact when compared to chemical treatments (Lloyd and Lovley, 2001). However, stimulating microbially mediated metal reduction will have competing outcomes. Although the reduction of Cr(VI) to Cr(III) leads to detoxification and precipitation, As(III) is more mobile and toxic than As(V). Thus, it is important to understand the effects of long term arsenic and chromium contamination on the resident microbial communities to develop effective remediation strategies.

Alpha diversity (*i.e.* the species richness within a sample) of microbial communities has been correlated with ecosystem stability and functionality (Giller *et al.*, 1997; Girvan *et al.*, 2005). In our study, we observed a marked reduction in bacterial alpha diversity (Shannon Index) in each of our contaminated soils relative to their paired control (Table 2). Reduction in alpha diversity is a commonly observed phenomena in metal contaminated sediments (Gough and Stahl, 2011) and soils (Bååth, 1989). Odum (1985) suggested that the reduction in diversity is rooted in the organisms' inability to physiologically cope with the stressor and, in our soils, the presence of both Cr (VI) and arsenic had the most pronounced effect on the diversity (Figure 2). Furthermore, soils with Cr (VI) or both Cr (VI) and arsenic saw a marked reduction in "phylogenetic diversity" that was not observed in the Sialkot contaminated soil that had

As but not Cr (VI) (Figure 3). The reduction in alpha diversity is likely not pH dependent despite being significantly correlated with pH (Table 3), as soils with circum-neutral pH are relatively similar in total diversity (Lauber *et al.*, 2009). Thus, the reduction in total and phylogenetic diversity in the presence of chromium (VI) suggests that many microbial species could not cope with Cr (VI) stress. This may be because the resistance genes for chromium (VI) may be less mobile throughout the community than arsenic resistance genes (Cai *et al.*, 2009).

Phylogenetic analysis of species level OTUs (97% similarity cutoff for Operational Taxonomic Units) revealed a significant shift in dominance from *Actinobacteria* in control soils to *Proteobacteria* in contaminated soils (Figure 3). The shift in phylum level dominance was independent of the site and suggests that *Proteobacteria,* as a group, may be the most metal tolerant organisms found at metal contaminated sites. Previous chromium and arsenic studies showed that *Proteobacteria* capable of metal transformation are routinely cultivated (Kamaludeen *et al.*, 2003) and identified using molecular tools (Bouskill *et al.*, 2010; Kourtev *et al.*, 2006; Margesin *et al.*, 2011). Additionally, Odum (1985) suggests that r-selected organisms (rapidly reproducing), such as *Proteobacteria* (Fierer *et al.*, 2007; Hugenholtz, 2002), are favored after a stressor is applied to an ecosystem, which is perhaps a reason for their dominance. The distribution patterns of *Proteobacteria* differed between sites with either *Alphaproteobacteria* or *Gammaproteobacteria* being dominant (Supplemental Table 3). Several novel groups within these *Proteobacteria* classes were predominant in Cr soils (Supplemental Table 3), suggesting that they may play an important role in our

soils and that their presence may be a stabilizing factor as these novel but abundant groups can be highly adapted to dealing with extreme environments (Spain *et al.*, 2009).

It is unclear whether the decrease of *Actinobacteria* is attributed to chromium or arsenic toxicity. Some *Actinobacteria* have been implicated in metal cycling (Kothe *et al.*, 2010), but the *Actinobacteria* could have been negatively impacted by higher than normal soil moisture (Goodfellow and Williams, 1983), as the contaminated soils were routinely exposed to waste effluents. The loss of *Acidobacteria* in contaminated soils may stem from an increased pH rather than the presence of Cr (VI) as pH is known to be a strong regulator of *Acidobacteria* abundance and diversity (Jones *et al.*, 2009). The loss of diversity among k-selected groups (highly adapted ecological groups) such as *Actinobacteria* and *Acidobacteria* (Fierer *et al.*, 2007) may decrease the stability of the system, particularly because these groups are thought to produce extracellular enzymes necessary for complex carbon degradation that likely supports growth of other microorganisms (McCarthy and Williams, 1992; Ward *et al.*, 2009). Nevertheless, the prominent shift in phylogeny towards *Proteobacteria* suggests that resistance to chromium and arsenic are widespread within this phylum, and that novel families within the *Proteobacteria* may play important ecological roles.

It is clear in our soils that chronic exposure to chromium and arsenic reduced diversity and shifted phylogeny (Figures 2 & 3 respectively). However, we were also interested in whether the structure of bacterial communities is conserved over a wide geographic area, especially in response to exposure to contaminants. Past work showed that few species are shared across spatially separated soils (Fulthorpe *et al.*, 2008). Yet environmental factors, such as pH (Lauber *et al.*, 2009) and salinity (Lozupone and

Knight, 2007), have been shown to exert selective pressures on the microbial community, which increase the similarity between spatially isolated communities. In our study, the beta-diversity (*i.e.* community similarity between sites) of control soil communities were remarkable similar to each other despite being spatially separated. Furthermore, control soil communities were clearly different from their paired contaminated soil, thus supporting differences seen in phyla distributions. The bacterial community in soils with Cr (VI) were more similar to each other than to the Sialkot bacterial community, whose soil contained As but not Cr(VI) (Figure 4). The similarity of the bacterial communities in Cr (VI) contaminated soils, despite their spatial isolation, suggests that the presence of Cr (VI) selected for groups of microorganisms that had resistance to Cr (VI) rather than those that acquired Cr (VI) resistance. Because few species were shared among the different bacterial communities, the similarity of community profiles, in both control and contaminated soil, is likely driven by family and genus level similarities. The conservation of familial lines amongst contaminated soils suggests that resistance may be a phenotypic trait within these groups; however, physiological conservation is less likely and, despite family and genus level similarities, ecosystem function may be different.

Our study sought to understand the spatial relationships of bacterial communities exposed to chromium and arsenic and the effect that long-term metal exposure has on the community structure and diversity and cell abundance. Despite being spatially isolated, soils exposed to chromium (VI) were similar in structure and each saw a large reduction in diversity. Interestingly, Cr (VI) presence appears to be a much stronger selector of community structure than As because the Kala Shah Kaku

soil that had both Cr (VI) and As was more similar to Kasur contaminated soil which had Cr (VI) but not As. Nonetheless, the marked reduction in microbial diversity across all contaminated soils and changes in phylogeny observed in the soils will likely have important effects on the ecosystem function and ultimately the restoration of the biome, as the physiological diversity of these soil microbial communities was also likely affected. However, inferring the functionality of the community is difficult as many of the bacteria identified in our study are uncultivated. The physiological response of these microbial communities as remediation and ultimately restoration of the biome proceeds will depend on the intrinsic genetic potential that still remains within the microbiome.

METHODS

Sampling site, collection and DNA extraction

Three sampling sites near the University of Punjab in Lahore, Pakistan were selected based on their longstanding history (>40 years) of industrial waste disposal primarily from the chrome tanning industry. The three sites are spatially separated by 50-200 km and are located within or near the cities of Lahore, Sialkot, and Kasur (see Table 1 for habitat type and coordinates). We employed a paired sampling design, such that control soil samples were collected near contaminated sites (< 0.5 km) and had similar soil properties. Contamination and soil type were taken into consideration and minimized as much as possible when choosing control and contaminated site pairs. Soil was collected from each site in 0.1 m^2 blocks using a sterile shovel, placed into gamma-sterilized polyethylene bags, transported on ice back to the laboratory, and stored at -20^oC. Soil blocks were broken apart, homogenized and partitioned for DNA extraction

and soil characterization. Soil texture, chemistry, and metal analysis was performed by the Pakistan Council Scientific and Industrial Research (PCSIR) (Lahore, Pakistan) soil-testing lab. DNA was extracted from 7.0 g of homogenized soil taken from the upper 20 cm of the block from each site using a MoBio PowerMax soil DNA isolation kit (MoBio, Carlsbad, CA, 92010, USA). DNA was precipitated and dried according to the manufacturers directions and then shipped to the University of Oklahoma for further analysis. All DNA was resuspended in 2.0 ml of sterile nuclease free water.

Pyrosequencing and Quantitative PCR

PCR libraries were generated using the modified 338 (5'ACHCCTACGGGWGGCWGC) forward and 518 (5'ACCGMSGKKGCTGGCAC) reverse primers (Sheik *et al.*, 2011). Modifications to the primer set were done according to Hamady *et al.* (2008), which included: adding the A-adapter, 'CT' linker and a unique 8 base barcode to the 5' end of the 518 reverse primer while the B-adapter and 'TC' linker was added to the 5' end of the forward primer (sequences for A and B FLX adapters were taken from http://www.454.com, 454 Life Sciences, Branford, CT). Pseudo replicates from each site were created for DNA from control and contaminated sites by using two separate barcodes per site. DNA from each site was PCR amplified in four 100-µl reactions that contained (final concentration) 4 µl of 1/10 diluted DNA, 1x Hot Start buffer (Fermentas, Glen Burnie, MA, USA), 2.0 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphate mixture (Fermentas), 3.0 U Maxima Hot Start *Taq* DNA polymerase (Fermentas), and 500 nM of the forward and reverse primer. PCR amplification protocol was as follows: 5 min at 95°C, followed by 30 cycles of 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min with a final extension of 15 min at 72°C.

PCR reactions were screened using gel electrophoresis and positive reactions were pooled (site and treatment kept separate) and concentrated using standard sodium acetate/ethanol method. Two volumes of 100% ethanol and 1/10 volume of 3M sodium acetate (pH=4.5) were added to pooled PCR products, incubated at -80^{o}C for 30 min, and centrifuged for 30 min at 4^{o}C. PCR precipitate was dried and resuspended in 50 µL of sterile nuclease free water. Concentrated PCR products were screened using a 2% agarose gel and bands of appropriate size were extracted and recovered using a Freeze 'N Squeeze DNA gel extraction spin column (Bio-Rad, Hercules, CA, 94547, USA). Purified products were quantified using a NanoDrop 1000 (NanoDrop products, Wilmington, DE, 19810, USA) and pooled at equal concentrations. PCR products were then sent to Engencore (http://engencore.sc.edu/) for pyrosequencing using FLX chemistry.

A full description of Quantitative PCR (QPCR) methods for the universal bacterial primer set 338F-518R can be found in Sheik *et al.* (2011). Briefly, a plasmid standard containing the 338-518 16S rRNA gene fragment from *Escherichia coli* was quantified using a NanoDrop 1000 (NanoDrop Products), diluted to 10^{9} copies per µl and then ten-fold serially diluted. The serial dilutions were used to generate a standard curve to calculate the gene copy number in the environmental samples. QPCR was performed in triplicate on each soil using the Bio-Rad MyIQ real-time PCR system (Bio-Rad, Hercules, CA). Each reaction mixture (25-µl total volume) consisted of 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 9.5 µl of water, 1.0 µl (10 µM) of each primer, and 2.0 µl of diluted DNA. QPCR generally followed a standard two-step protocol

consisting of 5 min at 95°C, followed by 40 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s.

Data processing of pyrosequencing reads

Raw pyrosequencing reads were binned by barcode, quality screened by using an average minimum quality score of twenty, and trimmed of primer sequence using the RDP pyrosequencing pipeline (http://pyro.cme.msu.edu/index.jsp). Sequence reads were then imported into the phylogenetic software package Mothur version 1.14.0. 64 bit (Schloss *et al.*, 2009) (http://www.mothur.org) for OTU (Operational Taxonomic Unit) generation, diversity estimates and classification. Before alignment, sequences with more than eight homopolymer nucleotides and outside of our length requirement (140-180 bases) were removed. Within Mothur, sequences were aligned to the Silva core sequence set using NAST algorithm (DeSantis *et al.*, 2006a), chimera checked with Chimera Slayer, sequence distances calculated with no penalization for end gaps, OTUs clustered using furthest neighbor algorithm, and classified within Mothur using Greengenes taxonomy and the Silva database. $OTUs_{0.03}$ (Species level Operational Taxonomic Units) taxonomy was obtained by consensus using a cutoff of 60%. Alpha diversity metrics (Abundance-based coverage estimator (ACE), Chao, Shannon-Weiner Index, rarefaction curves, and rarified phylogenetic diversity) were also calculated with Mothur. In order to calculate the beta diversity with Unifrac (Lozupone and Knight, 2005) and the alpha diversity metric, Faith's phylogenetic diversity (Faith, 1992; Faith *et al.*, 2009), a phylogenetic tree was generated with the program FastTree version 2.1 (Price *et al.*, 2009) using representative Silva aligned and lane masked $OTUs_{0.03}$ from each environment. Unifrac analysis was done with the Fast Unifrac web interface

(Lozupone *et al.*, 2010). Pearson correlation coefficients for environmental factors and diversity estimates were calculated in excel with the CORREL correlation function and tested against a null Student's *t*-test model (Sokal and Rohlf, 2003) using a two-tailed distribution to assess significance ($P \leq 0.05$).

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