MICROBIAL ECOLOGY OF ARID TERRESTRIAL SYSTEMS

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MICROBIAL ECOLOGY OF ARID TERRESTRIAL SYSTEMS

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Hot desert. Namib Desert, Namibia. Photo by Jean-Baptiste Ramond

Water is usually referred to as the 'Molecule of Life'. It constitutes the most abundant molecule in living (micro)organisms and is also essential for critical biochemical reactions, both for the global functioning and maintenance of Ecosystems (e.g., Photosynthesis) and individual (microbial) cells (e.g., ATP hydrolysis). However, most of Earth's terrestrial environments present deficiencies in bioavailable water. Arid environments cover around a third of the land's surface, are found on the six continents and, with the anthropogenic desertification phenomenon, will increase.

Commonly defined by having a ratio of precipitation to potential evapotranspiration (P/PET) below 1, arid environments, being either hot or cold, are characterized by scant and erratic plant growth and low densities in macro-fauna. Consequently, these ecosystems are microbially mediated with microbial communities particularly driving the essential Na and C biogeochemical

cycles. Due to the relatively simple trophic structure of these biomes, arid terrestrial environments have subsequently been used as ideal ecosystems to capture and model interactions in edaphic microbial communities. To date, we have been able to demonstrate that edaphic microorganisms (i.e., Fungi, Bacteria, Archaea, and Viruses) in arid environments are abundant, highly diverse, different from those of other terrestrial systems (both in terms of diversity and function), and are important for the stability and productivity of these ecosystems. Moreover, arid terrestrial systems are generally considered Mars-like environments. Thus, they have been the favored destination for astro(micro)biologists aiming to better understand life's potential distribution and adaptation strategies in the Universe and develop terraforming approaches.

Altogether, these points demonstrate the importance of significantly improving our knowledge in the microbial community composition (particularly for Fungi, Archaea and Viruses), assembly processes and functional potentials of arid terrestrial systems, as well as their adaptation mechanisms to aridity (and generally to various other environmental stresses).

This Research Topic was proposed to provide further insights on the microbial ecology of hot and cold arid edaphic systems. We provide a detailed review and nine research articles, spanning hot and cold deserts, edaphic, rhizospheric, BSC and endolithic environments as well as culture-dependent and -independant approaches.

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Chapter 2. Cold and arid systems

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ORIGINAL RESEARCH
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Microsite Differentiation Drives the Abundance of Soil Ammonia Oxidizing Bacteria along Aridity Gradients

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Soil ammonia oxidizing bacteria (AOB) and archaea (AOA) are responsible for nitrification in terrestrial ecosystems, and play important roles in ecosystem functioning by modulating the rates of N losses to ground water and the atmosphere. Vascular plants have been shown to modulate the abundance of AOA and AOB in drylands, the largest biome on Earth. Like plants, biotic and abiotic features such as insect nests and biological soil crusts (biocrusts) have unique biogeochemical attributes (e.g., nutrient availability) that may modify the local abundance of AOA and AOB. However, little is known about how these biotic and abiotic features and their interactions modulate the abundance of AOA and AOB in drylands. Here, we evaluate the abundance of *amoA* genes from AOB and AOA within six microsites commonly found in drylands (open areas, biocrusts, ant nests, grasses, nitrogen-fixing shrubs, and trees) at 21 sites from eastern Australia, including arid and mesic ecosystems that are threatened by predicted increases in aridity. Our results from structural equation modeling suggest that soil microsite differentiation alters the abundance of AOB (but not AOA) in both arid and mesic ecosystems. While the abundance of AOA sharply increased with increasing aridity in all microsites, the response of AOB abundance was microsite-dependent, with increases (nitrogen-fixing shrubs, ant nests), decreases (open areas) or no changes (grasses, biocrusts, trees) in abundance with increasing aridity. Microsites supporting the highest abundance of AOB were trees, nitrogen-fixing shrubs, and ant nests. These results are linked to particular soil characteristics (e.g., total carbon and ammonium) under these microsites. Our findings advance our understanding of key drivers of functionally important microbial communities and N availability in highly heterogeneous ecosystems such as drylands, which may be obscured when different soil microsites are not explicitly considered.

Keywords: nitrogen cycle, drylands, biocrusts, nitrifiers, nitrification

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INTRODUCTION

Arid, semi-arid, and dry-sub humid ecosystems (drylands) constitute the planet's largest biome, and support over 38% of the global human population (Reynolds et al., 2007; Schimel, 2010). A major feature of dryland ecosystems is their spatial heterogeneity (Tongway et al., 2001; Maestre and Cortina, 2002). Drylands are characterized by a sparse coverage of plants, which are separated by open areas devoid of perennial vegetation. Plant patches include a wide variety of vegetation types such as grasses, nitrogen (N)-fixing shrubs, and trees, while open areas are often covered by biocrusts (soil communities dominated by mosses, lichens, and cyanobacteria) and support the nests and burrows of soil arthropods (Belnap et al., 2001; Bonachela et al., 2015). Recent studies suggest that each of these soil surface attributes has unique effects on microbial communities and ecosystem processes in drylands (e.g., mineralization; Castillo-Monroy et al., 2010; Hortal et al., 2013; Bonachela et al., 2015; Delgado-Baquerizo et al., 2015). However, until now, their effects have largely been evaluated separately. To date, no previous research has simultaneously evaluated how multiple soil surface features affect both microbial communities and soil variables in drylands, nor have any studies explored the likely mechanisms behind the observed microsite-specific effects on these ecosystem attributes.

Dryland ecosystems are highly vulnerable to climate change and desertification processes (Reynolds et al., 2007; Maestre et al., 2012). Recent studies suggest that the increase in aridity for the late 21st century forecasted for most drylands (Dai, 2013; Feng and Fu, 2013; Huang et al., 2016) will have negative impacts on the cover and richness of vascular vegetation (Maestre et al., 2012; Vicente-Serrano et al., 2012). Conversely, such declines in plant cover could expand the area occupied by open areas and biocrusts by increasing the surface available for colonization and growth of their constituent organisms (Belnap et al., 2001; Thomas et al., 2011). In addition to climate change, human activities such as overgrazing are also expected to have substantial negative effects on plants and biocrusts in drylands (Fuhlendorf and Engle, 2001; Eldridge et al., 2013). These global change drivers will shift the relative abundance of different microsites in these areas (Whitford, 2002; Escolar et al., 2012; Vicente-Serrano et al., 2012), likely altering their microsite-specific effects on microbial communities and ecosystem functioning. Thus, understanding how different soil microsites affect particular microbial communities and associated ecosystem functions is of paramount importance if we are to predict how dryland ecosystems will respond to global change.

Nitrogen is one of the most important factors limiting net primary productivity and organic matter decomposition in drylands (Schlesinger and Bernhardt, 2013). The availability of N for plants and microbes is regulated mainly by particular microbial guilds (see Robertson and Groffman, 2007 for a review). For example, the critical process of autotrophic nitrification, which converts ammonium to nitrite, is driven principally by the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA; Nicol et al., 2008; Verhamme et al., 2011). Understanding the mechanisms that control the abundance of these microorganisms and their effects on soil N availability is thus critical for understanding and managing soil fertility and ecosystem productivity (Robertson and Groffman, 2007). The abundance of AOA and AOB in soils is known to be differentially affected by factors such as pH, organic matter quality and substrate availability (He et al., 2007; Nicol et al., 2008; Wessén et al., 2010; Rasche et al., 2011; Verhamme et al., 2011). Different dryland microsites (vascular plants, open areas, biocrusts, and insect nests), which have unique effects on soil attributes such as nutrient availability, organic matter quality and content (Castillo-Monroy et al., 2010; Hortal et al., 2013; Bonachela et al., 2015; Delgado-Baquerizo et al., 2015), could provide potentially different niches for AOA and AOB. Recent studies have shown that vascular plants can modulate the abundance of soil AOA and AOB along aridity gradients (Delgado-Baquerizo et al., 2013b), and that variations in the abundance of AOA and AOB drive variations in nitrification rates (Adair and Schwartz, 2008; Delgado-Baquerizo et al., 2013b; Hortal et al., 2013; Marusenko et al., 2013) in drylands. However, little is known about the role of plants and other biotic and abiotic features, such as insect nests and biocrusts, as modulators of the abundance of AOA and AOB in response to increasing aridity.

Here, we explore the effects of six markedly different microsites (open areas, biocrusts, ant nests, grasses, shrubs, and trees) on the abundance of *amoA* genes from AOB and AOA and on nitrate availability, and evaluate the mechanisms underlying microsite-specific effects on these variables at 21 sites along an aridity gradient in eastern Australia. As AOB have been previously reported to prefer high substrate concentration under plant canopies (Martens-Habbena et al., 2009; Verhamme et al., 2011; Delgado-Baquerizo et al., 2013b), we hypothesized that their abundance will peak in microsites with the highest ammonium availability. Conversely, because AOA abundance has been previously reported to increase with aridity in both open areas and under vascular plant canopies (Delgado-Baquerizo et al., 2013b), we predicted that AOA abundance would be microsite-independent and be driven by particular changes in soil properties derived from increasing aridity.

MATERIALS AND METHODS

Study Area

This study was carried out in 21 sites from eastern Australia (**Figure 1**; Supplementary Table S1). Locations for this study were chosen to represent a wide range of aridity conditions, including arid (arid and semiarid; $n = 12$; aridity index < 0.50) and mesic (dry-subhumid and humid; $n = 9$; aridity index > 0.50) ecosystems. Total annual precipitation and mean temperature ranged from 280 to 1167 mm and from 12.8 to 19.5°C, respectively. The sites surveyed encompass a wide variety of vegetation types, including grasslands, shrublands, savannas, dry seasonal forests, and open woodlands dominated by trees. Perennial vegetation cover ranged between 18 and 98%, and was dominated by *Eucalyptus* spp., *Acacia* spp., and *Austrodanthonia* spp. All our sites had a well-developed biocrust community

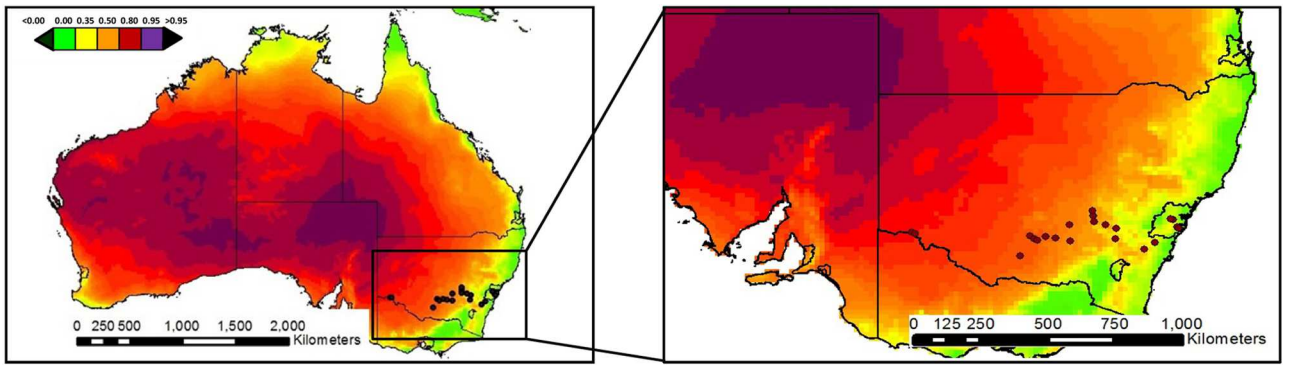

FIGURE 1 | Locations of the study sites. Color patterns indicate aridity (1 – aridity index) gradients. Aridity increases from green to purple in the graphs.

dominated by mosses (*Desmatodon convolutus*, *Barbula calycina*, *Didymodon torquatus,* and *Gemmabryum* spp.).

Sampling Design and Measurements

Soil sampling was carried out in March 2014 according to a standardized sampling protocol. A 30 m × 30 m plot representative of the dominant vegetation was established. The cover of the most abundant microsites (open areas, biocrust, ant nest entrances, grasses, N-fixing shrubs, and trees) at each site was measured using four 30-m transects and the line-intercept method, as described in Maestre et al. (2012). Aridity was determined as 1-aridity index [AI], where AI = precipitation/potential evapotranspiration (UNEP, 2012). Data of the aridity index were obtained from the global aridity map of the FAO (2014).

At each site, three soil cores (0–5 cm depth) were collected under the six most common microsites: open areas, biocrusts, ant nests, grasses (*Austrodanthonia* spp.), N-fixing shrubs (*Acacia* spp.), and trees (*Eucalyptus* spp.). Soil cores were then mixed to get a composite soil sample per microsite (six samples) in each of the sites. A minimum separation distance of 1 m between samples, and between these and plant patches was maintained to remove potential sources of non-independence between samples (Delgado-Baquerizo et al., 2013a). Following field sampling, the soil was sieved (2 mm mesh) and separated into two fractions. A fraction was immediately frozen at −20°C for quantifying the abundance of *amoA* genes from AOB and AOA (hereafter, AOA and AOB abundance). The other fraction was air-dried and stored for 1 month before physicochemical analyses.

We measured organic C, total N, pH, ammonia, and nitrate availability in all the soil samples. We selected these soil properties and nutrient variables because they are largely known to be important drivers of the abundance of AOA and AOB in terrestrial ecosystems (He et al., 2007; Nicol et al., 2008; Verhamme et al., 2011). The concentration of soil organic C was determined as described in Anderson and Ingram (1993). Soil total N was measured with a CN analyzer (Leco CHN628 Series, LECO Corporation, St Joseph, MI, USA). Soil pH was measured for all of the soil samples with a pH meter in soil and water suspension. Ammonium and nitrate were colorimetrically analyzed (Sims et al., 1995) from K_2SO_4 0.5 M soil extracts using a 1:5 soil: extract ratio as described in Jones and Willett (2006). The main soil properties for the different microsites and aridity conditions used in this study are shown in Supplementary Table S1.

Molecular Analyses

We measured the abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA) at each microsite and location using quantitative PCR (qPCR). Soil DNA was extracted from 0.5 g of defrosted soil samples using the Powersoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. qPCR reactions were conducted in triplicate using 96-well plates on an CFX96 Touch™ Real-Time PCR Detection System (Foster city, CA, USA). The *amoA* genes of AOB and AOA were amplified using the primers *amoA1F* (GGGGTTTCTACTGGTGGT)/*amoA2R* (CCCCTCKGSAAAGCCTTCTTC) and *Arch-amoAF* (STAAT GGTCTGGCTTAGACG)/*Arch amoAR* (GCGGCCATCCATC TGTATGT), respectively, as described previously by Rotthauwe et al. (1997) and Francis et al. (2005). Efficiencies for all quantification reactions were higher than 90%, with R^2 values ranging from 0.90 to 0.99. Standards were run in triplicate in each assay, and our standard calibration curve was developed using a serial 10^{-3} and 10^{-9} dilution from 30 ng μl^{-1}. Samples fell within the limits of our standard curve, hence within the detection limit. We generated melting curves for each run to verify product specificity by increasing the temperature from 55 to 95°C. Melting curve analyses resulted in a single peak, confirming the specificity of all amplicons. Actual values of AOA and AOB abundances for the different microsites and aridity conditions used in this study are available in Supplementary Table S1.

qPCR Standard Curve Preparation

The AOA and AOB primers described above were used to amplify *amoA* genes from DNA extracted from composite soil samples. In parallel, both PCR products were cloned into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. One specific clone was selected

for AOA and AOB cultures in order to generate the standard curves. Plasmid DNA was extracted with a Plasmid Mini Kit (Invitrogen), and the insert was sequenced using M13F and M13R primers to check that AOA and AOB were correctly inserted into their respective plasmids (sequences from selected AOA and AOB clones are available in Supplementary Table S1). These sequences were compared to known *amoA* genes in the GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST. This analysis showed that the sequences were >99% similar to known AOA and AOB genes.

Statistical Analyses

We tested for differences across aridity conditions (arid vs. mesic) and microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees) for the abundance of *amoA* genes from AOB and AOA with a two-way ANOVA, with microsite and aridity conditions as fixed factors. Prior to analyses, abundance of *amoA* genes from AOB and AOA and nitrate were log-transformed to improve normality. We conducted *post hoc* analyses (Tukey test) to explore differences in AOB, AOA and nitrate among microsites for those analyses where microsite effect was significant. When interactions between aridity conditions and microsites were significant, we carried our separate *post hoc* analyses for mesic and arid ecosystems. Spearman correlations were used to evaluate the relationship between aridity and both the relative abundance of the different microsites and the abundance of *amoA* genes from AOB and AOA in each microsite. All these analyses were carried out using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

We used structural equation modeling (SEM; Grace, 2006) to evaluate direct and indirect relationships between the different microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees), aridity (1-aridity index), soil properties (pH, C:N ratio, and soil C) and substrate (ammonium) on the abundance of *amoA* genes from AOB and AOA and nitrate availability. Unlike regression or ANOVA, SEM offers the ability to separate multiple pathways of influence and view them as a system, and thus is useful for investigating the complex networks of relationships found in ecosystems (Grace, 2006; Eisenhauer et al., 2015). Thus, this approach was the appropriate tool to evaluate our hypotheses because it allowed us to assess whether microsite effects on AOA and AOB abundance were directly or indirectly driven via nutrient availability and soil properties. The first step in SEM requires establishing an *a priori* model based on the known effects and relationships among the drivers of AOB and AOA abundance and nitrification process. Some data manipulation was required prior to modeling. We examined the distributions of all of our endogenous variables, and tested their normality. Soil C, C:N ratio, pH, ammonium, AOA and AOB abundances were log-transformed to improve normality. In these models, the different microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees) are categorical exogenous variables (Grace, 2006) with two levels: 0 (specific microsite) and 1 (remaining microsites). This approach allowed us to compare the effect of a particular microsite (e.g., ant nests) on the abundance of AOA and AOB compared with the average of the remaining microsites. Categorical exogenous variables can be used in SEM because distributional assumptions do not apply to them (Grace, 2006). The combined effects of nitrifier (AOA and AOB) abundances on nitrate availability were included in our model as a composite variable (Grace, 2006). With a good model fit (see below), we could confidently interpret the path coefficients of the model and their associated *P*-values. A path coefficient is analogous to the partial correlation coefficient, and describes the strength and direction of the relationship between two variables (Grace, 2006). We parameterized our models after data manipulation and used our datasets to test them for their overall goodness of fit. There is no single universally accepted test of overall goodness of fit for SEM that is applicable in all situations regardless of sample size or data distribution. Here, we used the chi-squared test (χ^2; the model has a good fit when $0 \leq \chi^2 \leq 2$ and $0.05 < P \leq 1.00$) and the root mean square error of approximation (RMSEA; the model has a good fit when $RMSEA\ 0 \leq RMSEA \leq 0.05$ and $0.10 < P \leq 1.00$). Additionally, and because some variables were not normal, we confirmed the fit of the model using the Bollen-Stine bootstrap test (the model has a good fit when 0.10 < bootstrap $P \leq 1.00$; Schermelleh-Engel et al., 2003).

We calculated the standardized total effects of microsite, aridity, soil properties and substrate on abundance of AOA and AOB and nitrate availability. The net influence that one variable has upon another is calculated by summing all direct and indirect pathways between the two variables. If the model fits the data well, the total effect should approximately be equivalent to the bivariate correlation coefficient for that pair of variables (Grace, 2006). All the SEM analyses were conducted using AMOS 20.0 (IBM SPSS, Chicago, IL, USA).

RESULTS

The cover of open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees ranged from 0–43%, 0–73%, 0–1%, 0–25%, 0–10%, and 0–70%, respectively. The cover of biocrusts increased ($\rho = 0.67$, $P = 0.001$), while that of ant nests and trees decreased along the aridity gradient studied ($\rho_{\text{ant nests}} = -0.55$, $P = 0.010$; $\rho_{\text{trees}} = -0.74$, $P < 0.001$). The cover of the remaining microsites did not vary significantly with aridity, though clear trends were observed in some cases ($\rho_{\text{bare ground areas}} = 0.35$, $P = 0.120$; $\rho_{\text{grasses}} = -0.41$, $P = 0.064$; $\rho_{\text{N-fixing shrubs}} = -0.40$, $P = 0.069$). Our results revealed important differences in the abundance of *amoA* genes from AOB (but not AOA) among microsites in both arid and mesic ecosystems (**Figure 2**). Ant nests, N-fixing shrubs and trees showed the highest AOB abundance (**Figure 2A**). This was particularly evident for ant nests and N-fixing shrubs under the most arid conditions, as indicated by the significant aridity conditions × microsite interaction ($P < 0.001$; **Figure 2A**). Thus, this interaction provides evidence that the size effect of microsite on AOB differ between xeric and mesic ecosystems. Biocrusts and open areas had the lowest AOB abundance (**Figure 2A**), particularly in the arid sites (aridity conditions × microsite interaction: $P < 0.001$; **Figure 2A**). We did not find significant differences between microsites for AOA abundance, which consistently showed the highest abundance in the most arid

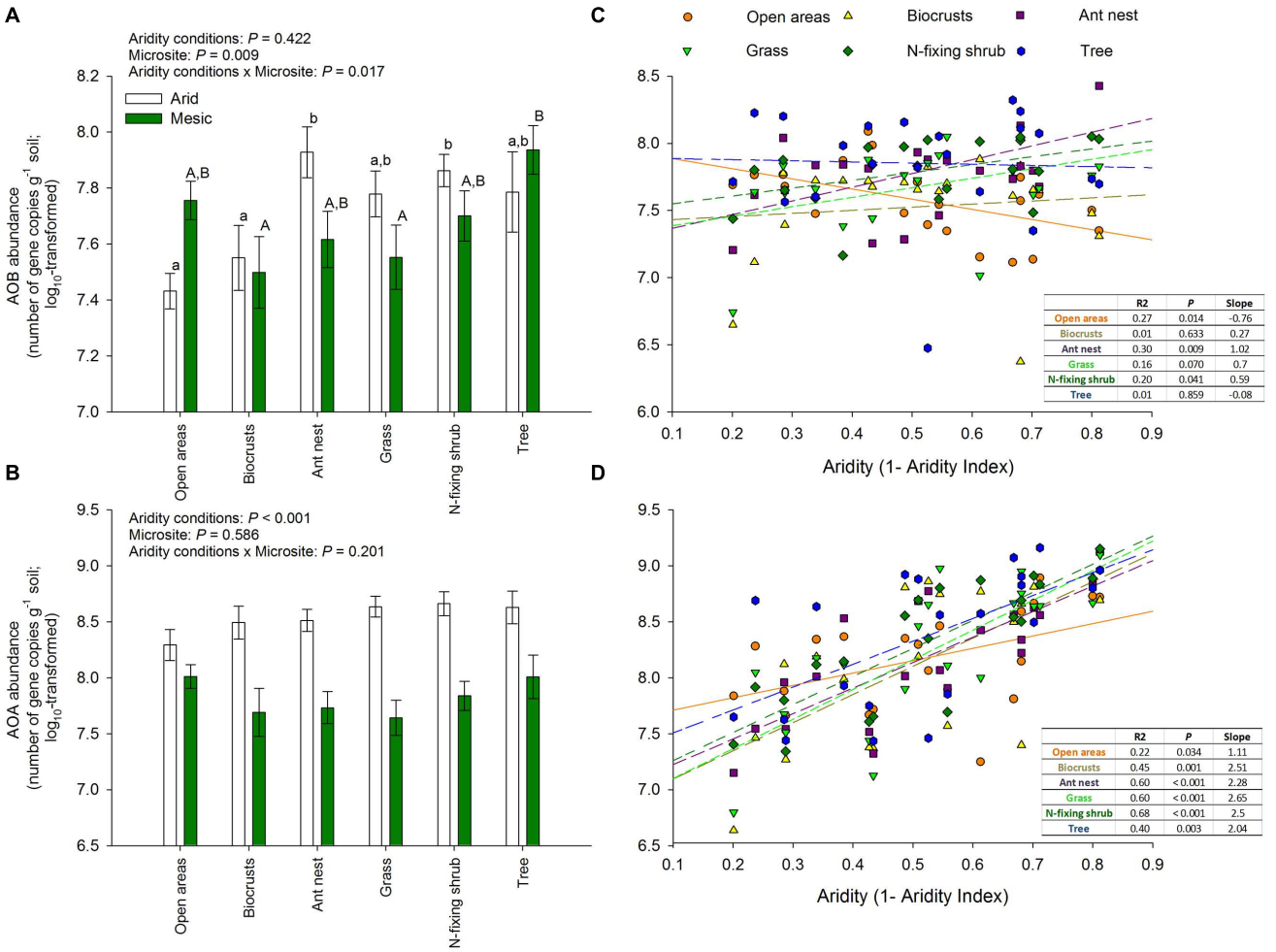

FIGURE 2 | Changes in the abundance of *amoA* genes from AOB and AOA across different levels of aridity. (A,B) Show mean ± SE, $n = 12$ and 9 for arid and mesic ecosystems, respectively. Upper case letters indicate differences between microsites (Tukey *post hoc* test after ANOVA). When interactions between aridity conditions and microsite were found, we conducted *post hoc* analyses independently for arid and mesic ecosystems. In this case, lower and upper case letters are used to show microsite differences separately for arid and mesic ecosystems. **(C,D)** Represent linear regressions between aridity and of the abundance of *amoA* genes from AOB and AOA across different microsites. The solid and dashed lines in **(C,D)** represent the fitted linear regressions.

parts of the gradient ($P < 0.001$; **Figure 2B**). Interestingly, the abundance of *amoA* genes from AOA increased with increasing aridity in all microsites (**Figure 2D**), but the response of AOB to aridity was microsite dependent, with increases (grasses, N-fixing shrubs and ant nests), decreases (open areas) or no changes (biocrusts and trees) in their abundance with increasing aridity (**Figure 2C**). We found the highest nitrate availability under ant nests, followed by trees and N-fixing shrubs ($P < 0.001$; **Figure 3**). Differences between arid and mesic ecosystems were not observed for this variable ($P > 0.05$; **Figure 3**).

Our *a priori* SEM model was satisfactorily fitted to our data, as suggested by non-significant χ^2 values ($\chi^2 = 0.6$–4.2; $P = 0.37$–0.98; $df = 4$), non-parametric Bootstrap *P*-values ranging from 0.46 to 0.97 and by values of RMSEA ranging from 0.00 to 0.03 ($0.53 < P < 0.98$; **Figure 4**). Most microsite effects (i.e., open areas, ant nests, grasses, and trees) on the abundance of *amoA* genes from AOB were indirectly driven by variations in soil C and ammonium availability, suggesting that these predictors adequately explained the abundance of AOB beneath these microsites. Contrary to this, we found a predominant direct positive and negative effect of N-fixing shrubs (**Figure 4E**) and biocrusts (**Figure 4B**) on the abundance of AOB, respectively, indicating that other unmeasured factors may have driven the indirect effects of these microsites on the abundance of *amoA* genes from AOB. Open areas and biocrusts showed a negative direct effect on soil C and ammonium compared with the other microsites, promoting an indirect negative effect on the abundance of AOB and on nitrate availability (via soil C and ammonium; **Figures 4A,B**). Conversely, ant nest and grasses had an indirect positive effect on AOB abundance via their influence on that of ammonium (**Figures 4C,D**), while trees had an indirect positive effect on AOB abundance by affecting soil C, hence the availability of ammonium in soil (**Figure 4F**). For AOA, our SEM results revealed that aridity had the highest

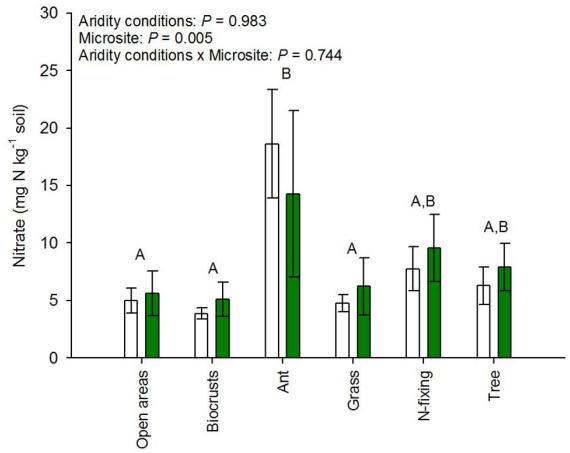

FIGURE 3 | Nitrate availability in the different microsites studied. Data are mean ± SE, $n = 12$ and 9 for arid and mesic ecosystems, respectively. Upper case letters indicate differences between microsites (Tukey *post hoc* test after ANOVA).

direct positive effect on the abundance of *amoA* genes from AOA (**Figure 4**); aridity also had an indirect positive effect on these microorganisms by reducing both the C:N ratio (negatively related to the abundance of AOA), and the amount of soil C (positively related to ammonium; **Figure 4**) and by enhancing soil pH (positively related to AOA abundance; **Figure 4**).

Our SEM models supported the microsite dependence of AOB (but not AOA) abundance in the ecosystems studied, and indicated that both aridity and microsite differentiation were the most important factors predicting the abundance of AOA and AOB, respectively (**Figure 5**). Ant nests, N-fixing shrubs and trees had a total positive effect (sum of direct and indirect effects from SEM) on the abundance of AOB, while open areas and biocrusts showed the highest negative total effect compared to other microsites (**Figure 5A**). Nitrogen-fixing shrubs and trees were the only microsites showing positive total effects on the abundance of AOA, but the magnitude of these effects was about seven-times lower than the total positive effect from aridity on such abundance (**Figure 5B**).

The abundance of both *amoA* genes from AOA and AOB had positive total effects on nitrate availability (**Figure 5C**). Ant nests (+), soil C (+), C:N ratio (−), ammonium (+) and both AOA (+) and AOB (+) abundance were the main predictors of nitrate availability (**Figures 4** and **5**). Aridity had a negative, but weak (i.e., close to 0), effect on nitrate concentration, while the positive effect of AOA abundance on nitrate was higher than that of AOB abundance (**Figure 5**).

DISCUSSION

Microsite-Specific Effects on the Abundance of AOB and AOA

Our study provides empirical evidence that microsite differentiation modulates the abundance of particular groups of microorganisms, such as AOB, in response to increases in aridity. This pattern was not found for AOA, as their abundance was driven mainly by aridity, regardless of the microsite considered. Strikingly, ant nests were one of the microsites with the highest abundance of AOB. Ants are one of the most widespread insect groups globally (Pêtal, 1998; Lenoir et al., 2001), and their activity during nest construction has major effects on multiple soil properties, including clay mineralogy, water infiltration and retention and nutrient cycling (Lenoir et al., 2001; Bonachela et al., 2015; Wu et al., 2015). Ant nests have been reported to support higher concentrations of inorganic N (ammonium and nitrate) and phosphorus due to the accumulation of animal and plant litter (Lenoir et al., 2001; Bonachela et al., 2015; Wu et al., 2015), which can be used by the plants growing near them (Sternberg et al., 2007; Wagner and Nicklen, 2010). They have also been observed to promote mineralization and nitrification processes in soils (Pêtal, 1998; Lenoir et al., 2001). This is particularly important in drylands, where N is one of the most important factors limiting the growth of plants and microorganisms (Austin et al., 2004; Schlesinger and Bernhardt, 2013). Thus, ant nests may act as "resource islands" (*sensu* Reynolds et al., 1999), providing suitable habitat for particular groups of microbes such as AOB, which support the high nitrification rates and nitrate availability reported here and in other studies (Pêtal, 1998; Lenoir et al., 2001; Wagner and Jones, 2006).

We found that the effects of vascular plants on the abundance of AOB varied depending on their functional attributes. The observed differences in the abundance of AOB between microsites may have been due to their different effects on microclimate and soil properties. The surface of soils close to and under trees and N-fixing shrubs receives greater levels of litter than soils adjacent to bare ground, biocrusts or small grasses (Vesterdal et al., 2012; Travers and Eldridge, 2015), and has lower temperature and higher infiltration rates that may lead to improved soil moisture conditions (Breshears et al., 1997; Berdugo et al., 2014; Eldridge et al., 2015). Overall, these environmental changes may provide a refuge for AOB in drylands. Interestingly, although small grasses had a lower effect on AOB abundance than other vascular plants, they still provide a better refuge for AOB than open areas and biocrusts, a response likely linked to the higher ammonium content found under grasses. Our results build up those from Delgado-Baquerizo et al. (2013b), who found that vascular plants can modulate the abundance of AOB in drylands, by emphasizing the role of different plant functional types on the abundance of these microorganisms.

Contrary to the results for AOB abundance, which was highly microsite-dependent, abundance of AOA increased along the aridity gradient studied, and was not affected by any of the microsites considered. Our results mimic those from Delgado-Baquerizo et al. (2013b), who also found increases in the abundance of AOA along an aridity gradient in Mediterranean grasslands dominated by *Stipa tenacissima*. Other studies have also reported high abundance of AOA in drylands (Adair and Schwartz, 2008; Marusenko et al., 2013). Remarkably, our results further suggest that this effect is microsite-independent, and that

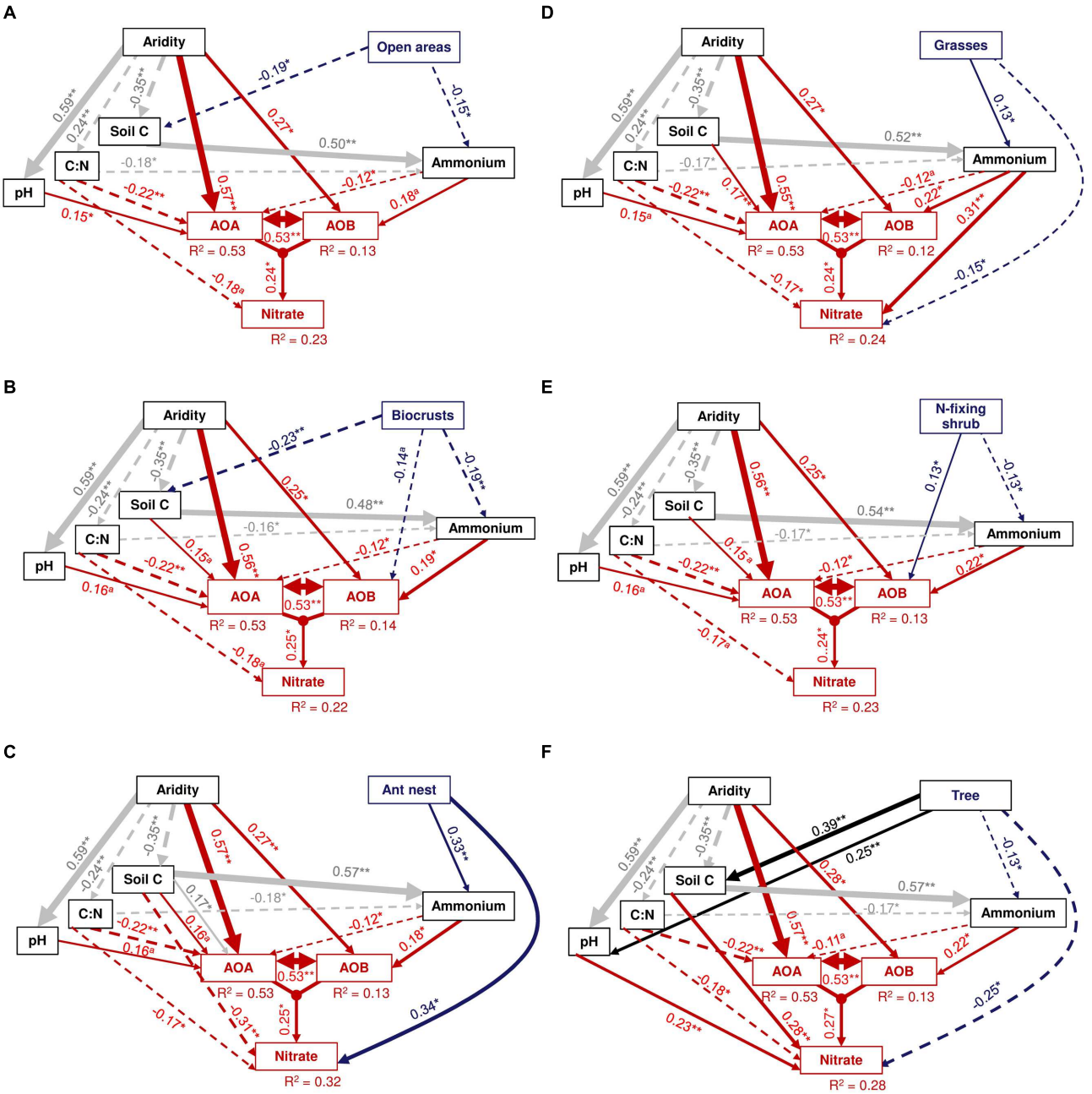

FIGURE 4 | Results from structural equation modeling showing the direct and indirect effects of aridity and different microsites on the abundance of AOB and AOA and on nitrate availability. Each panel represents the model used for each microsite (indicated in the upper right box of each model). Numbers adjacent to arrows are standardized path coefficients, analogous to relative regression weights, and indicative of the effect size of the relationship. Continuous and dashed arrows indicate positive and negative relationships, respectively. The width of arrows is proportional to the strength of path coefficients. Double-headed arrows between AOA and AOB indicate that the abundance of AOA can influence that one from AOB and *viseversa*. The proportion of variance explained (R^2) appears above every response variable in the model. Significance levels are as follows: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

aridity can be the best predictor for AOA abundance in the studied ecosystems. These results further support the notion that AOA often occupy those niches with more extreme conditions (i.e., low water and nutrient availability), where they usually outcompete AOB (Valentine, 2007; Adair and Schwartz, 2008; Moin et al., 2009; You et al., 2009).

Microsite as a Modulator of the Abundance of AOB and AOA in Response to Aridity

Our results indicate that microsite differentiation not only modified the abundance of AOB, but also their responses

to aridity. While the abundance of AOA increased with increasing aridity, irrespective of the particular microsite, the response of AOB abundance was microsite dependent, with increases (grasses, N-fixing shrubs, and ant nests), decreases (open areas) or no changes (biocrusts and trees) in abundance with increasing aridity (**Figures 2C,D**). Predicted increases in aridity for the late 21st century in most drylands (Dai, 2013; Feng and Fu, 2013) will negatively impact upon vascular vegetation cover in drylands (Maestre et al., 2012; Vicente-Serrano et al., 2012), and this may increase the proportion of suitable habitat for biocrusts, and thus their cover (Belnap et al., 2001; Thomas et al., 2011). Accordingly, we found a significant decrease in the cover of trees (and clear trends in the same direction in the cover of N-fixing shrubs and grasses), and an increase in the cover of biocrusts, with aridity. The decrease in plant cover and the increase of open areas will likely increase the abundance of AOA at the expenses of AOB due to the high resistance to water and nutrient stresses of the former (Adair and Schwartz, 2008; Verhamme et al., 2011).

Overall, all of our models indicate that the abundance of AOA and AOB is positively related to nitrate availability in our study sites, suggesting that autotrophic ammonia oxidation may be extremely important for N cycling in dryland ecosystems. This is particularly true for AOA abundance, which showed the highest total positive effect on nitrate availability in our models (**Figure 3**). Thus, considering the positive effect of aridity on AOA abundance and the importance of the positive effect of AOA on nitrate availability, our results suggest that AOA abundance could contribute to buffer the direct negative effects of aridity increasing on nitrate availability in drylands (**Figure 5C**; Delgado-Baquerizo et al., 2016). The role of these microorganisms in nitrification is largely supported in laboratory studies (e.g., Verhamme et al., 2011), but our study confirms the importance of the relationship between ammonia-oxidizing microbe abundance and the availability of nitrate under field-based conditions in drylands. Indeed, these results are consistent with reports showing that the relative dominance of nitrate will increase with increasing aridity (Schlesinger et al., 1990; Delgado-Baquerizo and Gallardo, 2011), and suggest that any increase in aridity resulting from climate change (Dai, 2013; Feng and Fu, 2013) may promote losses in nitrate to both the atmosphere and underground water (Robertson and Groffman, 2007; Delgado-Baquerizo et al., 2016).

Mechanisms that Account for Microsite-Specific and Aridity Effects on the Abundance of AOB and AOA

Our SEM approach allowed us to identify the most likely mechanisms controlling microsite and aridity effects on the abundance of AOB and AOA. For example, microsite differentiation indirectly alters the abundance of AOB via organic C and ammonium availability. Thus, the positive effects of ant nests and grasses on the abundance of AOB were indirectly

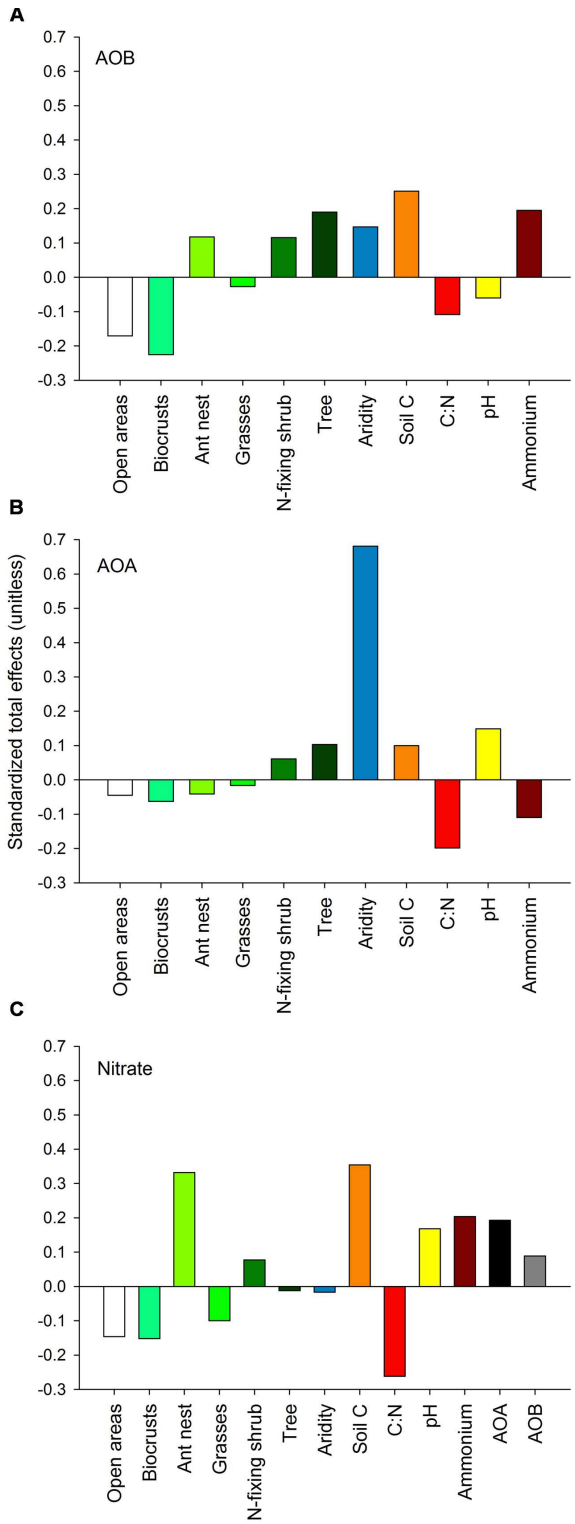

FIGURE 5 | Standardized total effects (direct plus indirect effects) derived from the structural equation modeling, including the effects of aridity and the different microsites evaluated on the abundance of AOB (A) and AOA (B) and on nitrate availability (C). The total effects of aridity, soil C, pH, C:N and ammonium on AOB and AOA abundance and nitrate availability were averaged from all six models in **Figure 4**.

driven by changes in ammonium availability. Ammonium is the main substrate for ammonia-oxidizing organisms (He et al., 2007; Nicol et al., 2008; Verhamme et al., 2011). Thus, compared with the other microsites, the positive effect of ant nests and grasses on ammonium contents observed here and in previous studies (Pêtal, 1998; Lenoir et al., 2001; Wu et al., 2015) may help to explain the positive effect of these microsites on the abundance of AOB. Similarly, the positive effect of trees on AOB may be indirectly mediated by the highest soil organic matter content found in this microsite (vs. other microsites evaluated), which, in turn, increases the availability of ammonium. Conversely, the negative impact of open areas on soil organic matter and ammonium availability may explain the low abundance of these microorganisms found in this microsite.

Interestingly, N-fixing shrubs and biocrusts were the only two microsites showing direct effects on AOB abundance in our SEMs (**Figures 4B,E**). One of these mechanisms could be synergistic effects between microbial communities involved in N cycling that thrive under N-fixing shrubs microsites (Zhang et al., 2015). For example, N-fixing shrubs are well-known to increase the abundance of microorganisms related to N fixing such as *Rhizobium* spp., which may promote the abundance and diversity of AOB by providing higher concentrations of ammonia to these microorganisms (Zhang et al., 2015). The observed indirect positive effect of N-fixing shrubs on nitrate availability via abundance of AOB (**Figure 4**) further suggests that all the N fixed under N-fixing shrubs (i.e., ammonium) may be transformed directly to nitrate, as suggested by the negative direct effect of this microsite on ammonium availability and by the typical high nitrification rates reported for N-fixing shrubs in drylands (e.g., Delgado-Baquerizo and Gallardo, 2011; Delgado-Baquerizo et al., 2011). Conversely, biocrust was the only microsite negatively affecting the abundance of AOB. Thus, while all the effects of open areas on AOB abundance are indirectly driven via the lowest soil C and ammonium availability found in this microsite, other unmeasured factors may indirectly drive the low AOB abundance under biocrusts. For instance, biocrusts often had a higher amount of soil phenols than open areas (Delgado-Baquerizo et al., 2015), which known allelopathic effects on particular soil bacterial communities (Herbert and Bertsch, 1995; Zsolnay, 1996; Saenz et al., 2006), and this could explain, at least in part, the direct negative effect of biocrusts on the abundance of AOB.

Indirect effects of aridity on soil properties such as pH may explain the highest abundance of AOA in the most arid locations. For example, we found a positive indirect effect of aridity on the abundance of AOA mediated by soil pH, which is supported by previous studies suggesting links between soil pH and the dominance of AOA (Nicol et al., 2008; Moin et al., 2009; Gubry-Rangin et al., 2011; Delgado-Baquerizo et al., 2013b). In addition, and contrary to what we found with AOB, the availability of ammonium had a negative direct effect on the abundance of AOA. Negative effect of ammonium availability on the abundance of AOA has been previously reported under laboratory conditions (from ∼155 ug N/g of soil; Verhamme et al., 2011). Both the large positive and direct effects of aridity and the negative direct effect of ammonium availability on the abundance of amoA genes from AOA support the notion that these microorganisms may outcompete AOB under oligotrophic conditions due to their high resistance to water and nutrient stress (Adair and Schwartz, 2008; Verhamme et al., 2011). This would allow AOA to carry out nitrification under the most unfavorable environmental conditions.

Taken together, our results indicate that soil microsite differentiation results in altered abundance of AOB along aridity gradients. The abundance of amoA genes from AOA is mainly driven by aridity in the drylands studied. These results are linked to indirect effects of microsite differentiation on amoA genes from AOB mediated by changes in soil properties such as soil carbon and ammonium availability, and to direct effects of aridity on amoA genes from AOA. These findings advance our understanding about how biotic and abiotic features control small-scale variations in microbial abundance and associated ecosystem processes in highly heterogeneous ecosystems such as drylands. They also indicate that identifying the main microsites promoting the abundance of particular microbial communities is particularly relevant for understanding how ongoing climate change may affect ecosystem functioning in a warmer and drier world.

AUTHOR CONTRIBUTIONS

MD-B designed this study in consultation with DJE, FTM, and BKS. Field data was collected by MD-B and DJE. Laboratory and statistical analyses were done by MD-B. The first draft of this paper was written by MD-B and all co-authors significantly contributed to improve it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00505

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Soil microbial responses to nitrogen addition in arid ecosystems

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The N cycle of arid ecosystems is influenced by low soil organic matter, high soil pH, and extremes in water potential and temperature that lead to open canopies and development of biological soil crusts (biocrusts). We investigated the effects of N amendment on soil microbial dynamics in a *Larrea tridentata-Ambrosia dumosa* shrubland site in southern Nevada USA. Sites were fertilized with a NO_3-NH_4 mix at 0, 7, and 15 kg N ha^{-1} y^{-1} from March 2012 to March 2013. In March 2013, biocrust (0–0.5 cm) and bulk soils (0–10 cm) were collected beneath *Ambrosia* canopies and in the interspaces between plants. Biomass responses were assessed as bacterial and fungal SSU rRNA gene copy number and chlorophyll *a* concentration. Metabolic responses were measured by five ecoenzyme activities and rates of N transformation. By most measures, nutrient availability, microbial biomass, and process rates were greater in soils beneath the shrub canopy compared to the interspace between plants, and greater in the surface biocrust horizon compared to the deeper 10 cm soil profile. Most measures responded positively to experimental N addition. Effect sizes were generally greater for bulk soil than biocrust. Results were incorporated into a meta-analysis of arid ecosystem responses to N amendment that included data from 14 other studies. Effect sizes were calculated for biomass and metabolic responses. Regressions of effect sizes, calculated for biomass, and metabolic responses, showed similar trends in relation to N application rate and N load (rate × duration). The critical points separating positive from negative treatment effects were 88 kg ha^{-1} y^{-1} and 159 kg ha^{-1}, respectively, for biomass, and 70 kg ha^{-1} y^{-1} and 114 kg ha^{-1}, respectively, for metabolism. These critical values are comparable to those for microbial biomass, decomposition rates and respiration reported in broader meta-analyses of N amendment effects in mesic ecosystems. However, large effect sizes at low N addition rates indicate that arid ecosystems are sensitive to modest increments in anthropogenic N deposition.

Keywords: arid ecosystems, nitrogen deposition, microbial biomass, ecoenzyme activity, meta-analysis

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Introduction

Drylands (arid and semiarid lands) comprise about 35% of the terrestrial surface of the western US and 41% of global terrestrial lands (Pointing and Belnap, 2012). Over 35% of the world's human population depends on dryland ecosystems for their livelihood, and this number is increasing (Millennium Ecosystem Assessment, 2003). These resource-limited ecosystems have

low resilience and resistance to abiotic perturbations. Thus environmental changes often have large ecological effects on both regional (Mack and Thompson, 1982; MacMahon, 1987) and global scales (Ahlström et al., 2015). One perturbation of concern is the atmospheric deposition of nitrogen (N), which increases as human utilization of these lands expands. Therefore, it is important for management of dryland ecosystems to understand their vulnerability and response to N deposition.

Globally, the responses of soil microbial communities to experimental N manipulations have been extensively studied, and the subject of several meta-analyses (Knorr et al., 2005; Treseder, 2008; Janssens et al., 2010; Zhou et al., 2014). However, few studies of arid ecosystems had been conducted at the time of writing. There are multiple reasons why arid ecosystem responses to N might differ from those of wetter mesic biomes. Foremost, responses to nutrient amendments are contingent on the annual distribution of soil moisture, which controls plant production and soil processes (Stursova et al., 2006; Ladwig et al., 2012; Collins et al., 2014). In addition, desert soils differ from mesic soils in pH, organic matter concentration, and microbial community composition, both bacterial, and fungal (Porras-Alfaro et al., 2011; Fierer et al., 2012; Steven et al., 2013, 2014; Mueller et al., 2015). Proteolytic and phenol oxidase activities, which are both associated with N mineralization, are greater relative to glycosidase activities (Sinsabaugh et al., 2008; Hofmockel et al., 2010). And fungi are major agents of denitrification (Crenshaw et al., 2008; Chen et al., 2015).

Another contrast between deserts and mesic regions is the presence of biocrusts, a soil surface community of lichens, mosses, cyanobacteria, bacteria, and fungi. Because plant cover is low in deserts, biocrusts are often the dominant living cover (Belnap and Lange, 2003). In very hot deserts, the biomass of these surface crusts is low and dominated by heterotrophic bacteria and fungi. As soil moisture increases, cyanobacteria increase in abundance, followed by mosses and lichens. As rainfall increases, so does the importance of biocrusts in soil stability, the contribution of newly fixed C and N to soils, and the interception of nutrient-rich dust that includes anthropogenically created atmospheric N (Pointing and Belnap, 2012). The fungal networks that integrate biocrusts are able to translocate this N to plants (Green et al., 2008; Zhuang et al., 2014).

Given the low N content of arid soils and generally low rates of atmospheric N deposition at this site and US deserts in general ($\sim$2 kg ha^{-1} y^{-1} in the absence of anthropogenic influence; Fenn et al., 2003), these ecosystems should be responsive to increases in N loading such as those associated with urbanization. The few experimental studies to date show varied responses (Supplementary Table S1). Interpretation is complicated by differences in N dose and duration of experiments, lack of redundancy in the response variables measured, and the spatial heterogeneity of arid landscapes.

We approached these issues two ways. First, we added N to a shrubland ecosystem that covers large areas of the southwestern U.S. Measurements included a broad range of nutrient, biomass and process responses nested within the major structural components of the landscape. Second, we assembled data from other N addition studies conducted in arid ecosystems for a meta-analysis that compared the sensitivity and responsiveness of arid ecosystems to those reported elswhere for mesic ecosystems. Both approaches indicate that arid soils are highly responsive to relatively small increments in N loading and that increased N availability accentuates differences among habitat patches.

Materials and Methods

Study Site

The study site is located in the Lake Mead National Recreation Area (Reno, NV, USA, UTM Zone 11 702869N 3927243E). Three randomly selected 100 m $\times$ 100 m sites, separated by at least 5 km, were chosen within *Larrea tridentata* (creosote bush) – *A. dumosa* (burro bush) dominated shrubland found on lower elevation alluvial fans within a 500 ha area designated as open for study by the U.S. National Park Service. The alluvial soils have an average texture of 80% sand, 13% silt, and 7% clay with a bulk pH of 7.4. Organic carbon content in the upper 10 cm averaged 0.35%. A nearby weather station has documented long-term average annual high and low temperatures of 23.8 and 11.1C, respectively, with an annual average precipitation of 19.8 cm. The nearest National Acid Deposition Program monitoring site (Red Rock Canyon National Conservation Area, 50 km west) reports wet N deposition rates of $<$1.0 kg/ha.

Within each site, fifteen *A. dumosa* plants of similar size, leaf area, and condition were selected. Each plant became the center of a 2 m $\times$ 2 m plot. To simulate N deposition typical of urban impacts, plots were fertilized with a nitrate-ammonium mix at three levels: 0, 7, and 15 kg N ha^{-1} yr^{-1}, added in seven parts every 2 months from March 2012 to May 2013 to mitigate the effects of shock loading, sporadic precipitation and aeolian erosion. Plots were harvested in May 2013. Herein these treatments are referred to as Ambient (Amb), N7 and N15, respectively.

Sampling

Pre-treatment soil and *Ambrosia* leaf samples were collected at each site from within the delineated plots. After the fertilization period, soil and plant leaf samples were again collected from the delineated plots. At both times, soils were collected to depths of 0–0.5 cm (hereafter referred to as "biocrust") and 0–10 cm (hereafter referred to as "bulk soil") from under each plant canopy (hereafter referred to as "canopy" samples) and from the open areas between plants (hereafter referred to as "interspace" samples). Within each plot, soil samples collected on the north, east, south, and west side of the plant at the stem, mid-canopy, and dripline were combined to form a single composite biocrust or bulk soil canopy sample. Interspace biocrust and soil samples were collected between the target *Ambrosia* and its nearest neighbor on the north, east, south, and west. These samples were collected 50–100 cm away from the canopy edge of the target plant and combined to form a single composite biocrust or soil sample for each plot.

Biocrust and bulk soil samples were divided into four parts for analysis of bacterial and fungal abundance, chlorophyll *a*, enzyme activities, and chemistry. All soils were immediately placed on ice until reaching the laboratory. Samples for molecular and enzymatic analyses were frozen at −70°C until analyzed.

Chemical Analyses

Subsamples of the collected soils were air-dried, sieved to 2 mm and mixed; plant leaves were dried and ground, and both soil and plant materials then sent to the Plant and Soil Analysis Laboratory at Brigham Young University in Provo, UT, USA. Organic matter was determined by chromic acid digestion (Walkley and Black, 1934). Sample pH was measured in a saturated soil paste solution (Rhoades, 1982). Total N (TN) was determined by Kjeldahl analysis (Bremner and Keeney, 1966). Available phosphorus (P_{av}) and available K (K_{av}) were extracted with $NaHCO_3$ (Olsen et al., 1954; Schoenau and Karamonos, 1993, respectively).

P_{av} was quantified colorimetrically at 880 nm using the vanadomolybdophorphoric assay (Rice et al., 2012). K_{av} was analyzed using an ICP spectrometer (Johnson and Ulrich, 1959). Soil particle size distributions were determined by the hydrometer method (Day, 1965). Plant leaves were dried, ground, digested with perchloric acid and analyzed for total N (Bremner and Keeney, 1966) and minerals, using ICP spectrometry (Johnson and Ulrich, 1959).

Net nitrification and ammonification rates were calculated by measuring NO_3-N and NH_4-N concentrations in KCl extracts before and after a 10 day incubation at 20C (Finzi et al., 2006). N Mineralization was calculated as the sum of nitrification and ammonification. Net changes were calculated in units of $\mu g\ g^{-1}\ d^{-1}$.

Chlorophyll

Chlorophyll *a* was extracted from biocrust in acetone and quantified by HPLC based on peak areas from a photodiode array detector at 436 nm, using commercial standards (DHI Water and Environment, Denmark; Karsten and Garcia-Pichel, 1996).

Microbial Abundance

DNA was extracted from 0.5 g soil samples collected from Site 1 using the FAST DNA Spin kit for Soil following manufacturer recommendations (MP Biomedicals, Solon, OH, USA). Extracted DNA was quantified using the Quant-it PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA), measured on a BioTech Synergy H1 plate reader.

The DNA concentrations were normalized to 1 ng/μl, and quantitative PCR (qPCR) reactions were conducted in 96-well plates on a CFX Connect Real-Time PCR System (BioRad Laboratories, Hercules, CA, USA), using a modified procedure from Castro et al. (2010). DNA quantitative standards for bacterial and fungal rRNA genes were generated by amplifying the bacterial 16S RNA gene from *Microcoleus vaginatus* (the most common cyanobacterium in regional biocrusts) using EUB 338 and EUB 518 primers (Lane et al., 1985), or amplifying the fungal SSU RNA gene from a *Phoma* sp. culture (one of the most abundant fungi in regional soils) using the nu-SSU-1196F and nu-SSU-1536R primers (Borneman and Hartin, 2000). Amplified fragments were cloned into *Escherichia coli* to generate a single copy bacterial or fungal gene clone for generation of standard curves. For field sample DNAs, duplicate 30 μl qPCR reaction contained 15 ul of iQ SYBR Green Supermix (BioRad Laboratories, Hercules, CA, USA), 1.25 μg/μl BSA (Roche Diagnostics GmbH, Mannheim Germany), 1 μl of normalized soil DNA and 133 nM of each primer (EUB 338 and EUB 518 Bacterial; Fungal Primers: nu-SSU- 1196F and nu-SSU-1536R137). The reactions were amplified using the following conditions: 95C for 3.25 min followed by 40 cycles of 95C for 15 s, annealing temperature for 30 s (55C bacterial or 53C fungal) and 72C for 30 s, followed by a step at 95C for 1 min, and 80 cycles at 55C for 10 s with a 4C hold for dissociation curve analysis.

Soil Enzyme Activities

Biocrust and bulk soil samples were assayed for the potential activities of β-1,4-glucosidase (BG), alkaline phosphatase (AP), alanine aminopeptidase (AAP), and β-1,4-*N*-acetylglucosaminidase (NAG) using flourigenic methylumbelliferyl-linked substrates, following the protocol of Stursova et al. (2006). Aliquots (200 μl) of sample suspensions (1 g sample homogenized in 125 ml of 50 mM bicarbonate buffer, pH 8) were dispensed into 96-well microplates. Each microplate included 16 replicate wells per assay, plus positive and negative controls for quench correction. Microplates were incubated at 21C. Fluorescence was measured at excitation and emission wavelengths of 365 and 450 nm, respectively, using a BioTech Synergy H1 plate reader. Activities were calculated in units of $nmol\ g^{-1}\ h^{-1}$.

Data Analyses

Data were grouped into three categories for multivariate analysis: (i) soil nutrients and N transformation processes, (ii) soil enzyme activities, and (iii) plant chemistry. For soil nutrients and ecoenzyme activities (EEA), we first used permutational MANOVA that included the fixed effects of N treatment, soil collection depth, and collection location including all interaction terms as well as the random effects of site and plot. Plot was nested within site and N treatment. Following normalization of response variables to equalize the measurement scales, perMANOVA was conducted in Primer v. 6.1.10 using 99999 iterations, residuals calculated under a restricted model, and type III partial SS (Clarke and Gorley, 2009). We included *a priori* contrasts to compare the N treatments. For foliar chemistry, the perMANOVA included the fixed effect of N treatment and the random effects of site and plant (nested within site and N treatment).

When multivariate analyses showed significant treatment effects, we followed up with general linear mixed effects models for individual response variables, including the same fixed and random factors described above (restricted maximum likelihood, Proc MIXED, SAS v. 9.3, SAS Institute Inc., Cary, NC, USA). All variables were natural log-transformed to meet

assumptions of normality of residuals and homogeneity of variances, with the exception of ammonification, which did not require transformation. Degrees of freedom of these models varied due to rare incidences of missing data, and the exclusion of one outlier (an ant nest) in the soil chemistry data. For each N treatment, we calculated an effect size as *RII* = (N treatment – Ambient)/(N treatment + Ambient) following Armas et al. (2004) to facilitate cross-response and cross-study comparisons.

The same individual general linear mixed models were used to analyze the responses of soil organic carbon (SOC): Total N and SOC:P_{av} ratios as well as microbial biomass indicators. These variables were log-transformed prior to analysis. Because soil fungi and bacteria data were collected only for Site 1, these models did not include the random effect of site. Chlorophyll data were collected only for the biocrust, so soil depth was not a factor in that analysis.

Mantel tests were conducted to examine relationships among soil nutrient, ecoenzyme, and foliar chemistry response matrices (RELATE function, 99999 permutations, Spearman *Rho*, Primer v. 6.1.10, Clarke and Gorley, 2009). First, across the entire dataset (180 observations) we examined the relationship between soil nutrients/processes and EEA. Second, for the matrices of responses observed at the scale of plants (45 observations), we examined relationships among foliar chemistry, soil nutrients/processes and EEA measured beneath the plant canopy (averaged over the 0.5 and 10 cm soil depths).

Meta-Analysis of Published Studies

Because most studies of arid ecosystem responses to experimental N manipulation have only recently been published, these systems have not been well represented in previous meta-analyses. We created a comparative context for this study by assembling data from 14 studies (this study and 13 others) that included soil microbial responses to N amendment (Supplementary Table S1). N application rates ranged from 5 to 560 kg ha^{-1} y^{-1} with treatment durations of 0.3–10 year.

Eight studies (including our own) included microbial biomass responses, measured variously by phospholipid fatty acids (total, bacterial, fungal), qPCR (bacterial 16S gene copies, fungal 18S gene copies), biocrust chlorophyll, cyanobacteria species richness, muramic acid, and/or microbial biomass carbon or nitrogen (chloroform fumigation/extraction). For each N treatment, defined as a combination of application rate and treatment duration, we calculated the effect size *RII* as described above. For our Nevada study, we used the untransformed data to calculate LS means from the mixed effects model to use in RII. These effect sizes were pooled into a single category called microbial biomass responses.

Ten studies included microbial metabolic responses to N treatment measured using various enzyme assays (β-glucosidase, Ala/Leu-aminopeptidase, phosphatase, β-*N*-acetylglucosaminidase, urea aminohydrolase, invertase, phenol oxidase, peroxidase), BIOLOG substrate induced respiration (total change in absorbance), respiration (CO_2 efflux), net ammonification and/or net nitrification. As above, we calculated an effect size for each N treatment and pooled the results into a single category called microbial metabolic responses.

Regression analysis was used to relate effect size (*RII*) to N application rate (kg ha^{-1} y^{-1}) and cumulative N loading (application rate × duration of treatment). For studies that included multiple measures of biomass or metabolic responses per N treatment, only the mean effect size was used in the regression analyses so that all observations were independent. Analyses compared model fit between linear regression and log-transformation of the N treatment (*x*-axis) using relative r^2 values. Within studies, the number of treatment replicates ranged from 2 to 10 (mean 5.4, Supplementary Table S1). However, we chose not to conduct weighted regressions because of the diversity of response metrics (i.e., weighting by study also biases some response variables over others).

Results

Soil Nutrient Levels and N Transformation Processes

Most soil nutrients and processes were influenced by N addition, with the exception of potassium (K), soil δ15N (**Figure 1B**), and N mineralization (Supplementary Table S2, perMANOVA $P = 0.0003$). Soil N increased by 15 and 30% in the N7 and N15 treatments relative to ambient plots, however, only the N15 response was significantly greater than ambient (**Table 1**; **Figure 1A**).

The effects of N addition varied more strongly with soil depth than with spatial location (Supplementary Table S2: perMANOVA N × Depth $P = 0.027$; N × Location $P = 0.735$). This distinction was most pronounced for net ammonification, which declined from −0.12 (Amb) to −0.33 (N7) to −0.61 (N15) μg N g^{-1} d^{-1} for the biocrust horizon and from −0.16 to −0.27 to −0.30 μg N g^{-1} d^{-1}, respectively, in the bulk soil (**Table 2**; **Figure 2A**, full statistical results in Supplementary Table S2). Net nitrification also responded significantly to N addition but only in the biocrusts (increasing from 1.27 to 2.12 to 2.04 μg N g^{-1} d^{-1}, respectively, **Table 2**; Supplementary Table S2; **Figure 2B**), although the Nitrification × Depth interaction was marginally non-significant ($P = 0.098$). Net N mineralization in the biocrust followed a similar pattern, responding more strongly in the N7 treatment than the N15 treatment (from 1.15 to 2.05 to 1.43 μg N g^{-1} d^{-1}, respectively), but again treatment effects were non-significant over the deeper soil profile (**Table 2**; **Figure 2C**).

Available P (**Table 1**) increased ∼25% with N addition (*RII* = 0.12, **Figure 1C**; Supplementary Table S1), from 11.4 mg/kg to 14.1 and 14.6 mg/kg in the N7 and N15 treatments, possibly as a result of increased acidity generated from nitrification, even though the N treatment did not significantly reduce bulk soil pH, which ranged from 7.1 to 7.4.

N addition also altered nutrient ratios (**Table 1**). For biocrust, the molar SOC:P_{av} ratio was 26% lower under the highest level of N addition relative to N7 and 11% lower relative to

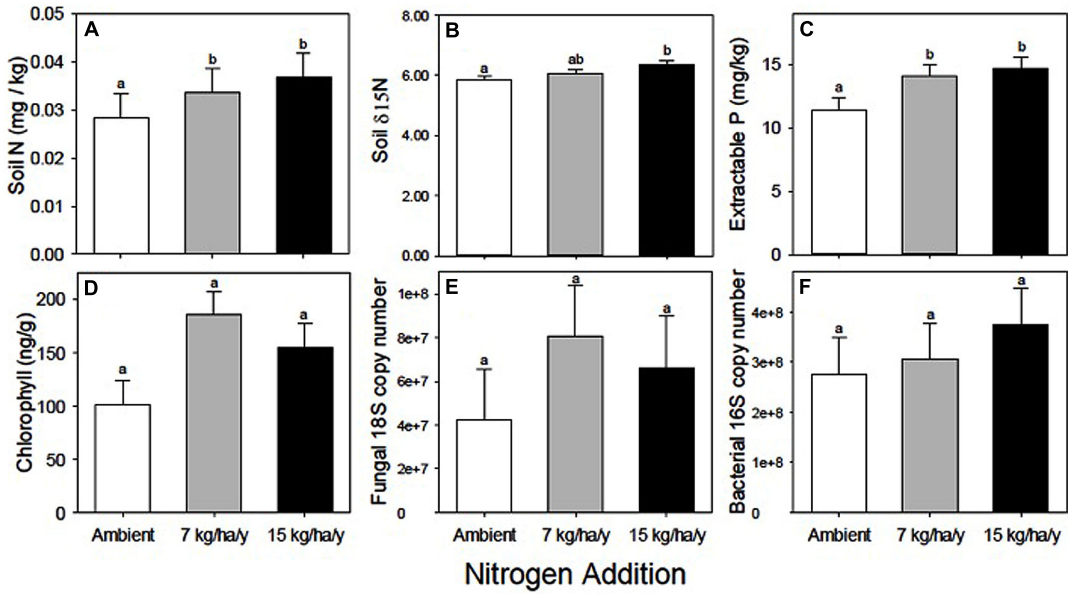

FIGURE 1 | Soil nutrient and microbial biomass responses to experimental N addition. (A) Soil N. **(B)** Soil δ15N. **(C)** Extractable P. **(D)** Chlorophyll *a*. **(E)** Fungal 18S copy number/g. **(F)** Bacterial 16S copy number/g. Different letters indicate significant differences among the N treatments. Data summarized in **Table 1**. Statistical results presented in Supplementary Table S2.

TABLE 1 | Soil nutrient data by horizon, location, and N treatment.

Horizon	Location	N trt	Organic C		Total N		Available P		Available K		SOC:TN		SOC:Pav	
			%	SD	%	SD	ng/g	SD	ng/g	SD	Ratio	SD	Ratio	SD
Biocrust 0.5 cm	Canopy	Amb	0.521	0.271	0.045	0.021	14.07	3.61	257.9	93.0	12.1	3.4	915	360
Biocrust 0.5 cm	Interspace	Amb	0.269	0.121	0.021	0.010	9.87	2.05	115.7	45.8	15.4	8.8	692	281
Soil 10 cm	Canopy	Amb	0.362	0.300	0.029	0.009	10.95	2.95	329.6	98.3	11.4	2.1	887	816
Soil 10 cm	Interspace	Amb	0.128	0.060	0.014	0.004	10.64	3.53	127.1	35.1	11.0	3.7	343	198
Biocrust 0.5 cm	Canopy	N7	0.698	0.309	0.060	0.022	16.03	5.63	275.0	151.0	13.8	4.2	1181	522
Biocrust 0.5 cm	Interspace	N7	0.310	0.135	0.024	0.010	11.16	2.56	112.9	57.0	16.0	8.1	754	352
Soil 10 cm	Canopy	N7	0.297	0.164	0.034	0.016	14.93	6.31	336.9	111.7	9.9	1.8	507	122
Soil 10 cm	Interspace	N7	0.155	0.058	0.017	0.005	14.10	4.23	136.8	65.7	10.8	2.2	306	141
Biocrust 0.5 cm	Canopy	N15	0.594	0.231	0.061	0.025	17.53	6.20	257.5	78.2	12.5	4.9	901	278
Biocrust 0.5 cm	Interspace	N15	0.265	0.161	0.030	0.015	12.78	3.81	127.2	58.7	10.4	3.3	522	220
Soil 10 cm	Canopy	N15	0.329	0.112	0.038	0.011	15.30	4.77	417.7	139.8	10.5	2.7	565	149
Soil 10 cm	Interspace	N15	0.136	0.067	0.018	0.007	12.97	3.79	164.9	29.6	9.2	4.1	292	151

SOC, soil organic carbon; TN, total N; Pav, available P.

the control (N × Depth, $F_{2,124} = 3.12$, $P = 0.0475$, SOC:P_{av}: Amb = 804, N7 = 967, N15 = 711). More generally, the SOC:P_{av} ratio decreased from 709 to 687 to 570 for the Amb, N7 and N15 treatments, but the trend was not statistically significant. The molar SOC:TN ratio showed a marginally non-significant decline with N addition ($F_{2,40} = 2.84$, $P = 0.0705$, C:P: Amb = 12.6 ± 0.8, N7 = 12.5 ± 0.8, N15 = 10.7 ± 0.8).

Other nutrient variables were non-responsive to N addition, but showed significant spatial patterns. SOC was significantly greater beneath the shrub canopy (mean ± SE: 0.47 ± 0.04%) than in the interspace (0.21 ± 0.04%; Supplementary Table S2, $P < 0.0001$) and declined 89% with soil depth from 0.44 ± 0.04% at 0.5 cm to 0.23± 0.04% at 10 cm (**Table 1**; Supplementary Table S2). Soil N was 63% greater in the biocrust (0.041 ± 0.005) than in the bulk soil (0.025 ± 0.005), and 118% greater beneath the shrub canopy (0.045 ± 0.005) than in interspaces (0.021 ± 0.005, **Table 1**; Supplementary Table S2). Available P was 24% greater under the shrub canopy (14.82 ± 0.78) than in interspaces (11.92 ± 0.78, **Table 1**; Supplementary Table S2). Soil K was also greater (139%) beneath the shrub canopy (312 ± 19) compared to the interspaces (131 ± 19, **Table 1**; Supplementary Table S2). Biocrust soils beneath shrub canopies had 400% greater net nitrification rates (3.02 ± 0.16) than interspace biocrust (0.60 ± 0.16);

TABLE 2 | N transformation rates by horizon, location, and N treatment.

Horizon	Location	N trt	Nitrification		Ammonification		N mineralization	
			$\mu g\ g^{-1}\ d^{-1}$	SD	$\mu g\ g^{-1}\ d^{-1}$	SD	$\mu g\ g^{-1}\ d^{-1}$	SD
Biocrust 0.5 cm	Canopy	Amb	2.299	1.700	−0.098	0.289	2.200	1.902
Biocrust 0.5 cm	Interspace	Amb	0.348	0.311	−0.124	0.055	0.224	0.295
Soil 10 cm	Canopy	Amb	0.712	0.575	−0.239	0.236	0.473	0.604
Soil 10 cm	Interspace	Amb	0.113	0.095	−0.095	0.115	0.018	0.141
Biocrust 0.5 cm	Canopy	N7	4.275	2.361	−0.251	0.758	4.025	2.626
Biocrust 0.5 cm	Interspace	N7	0.634	0.320	−0.272	0.132	0.362	0.348
Soil 10 cm	Canopy	N7	0.829	0.801	−0.404	0.436	0.424	1.004
Soil 10 cm	Interspace	N7	0.132	0.144	−0.151	0.122	−0.018	0.195
Biocrust 0.5 cm	Canopy	N15	3.115	1.629	−0.694	0.376	2.421	1.698
Biocrust 0.5 cm	Interspace	N15	0.832	0.569	−0.524	0.175	0.308	0.468
Soil 10 cm	Canopy	N15	1.323	1.694	−0.341	0.377	0.982	1.821
Soil 10 cm	Interspace	N15	0.191	1.252	−0.277	0.273	−0.468	1.373

this difference was stronger (620%) for bulk soils (canopy 0.96 ± 0.16; interspace 0.13 ± 0.16; **Table 2**; Supplementary Table S2, $P = 0.0002$). Net N mineralization showed a similar trend (**Table 2**). Rates for biocrust beneath shrub canopy were 10-fold greater than those for interspace crust (2.88 vs. 0.30), with a stronger pattern across the deeper soil profile (canopy: 0.63, interspace: −0.16).

Fungal, Bacterial, and Cyanobacterial Biomass

None of the estimates of microbial biomass responded significantly to N amendment in either soil horizon, and there was little shift in fungal/bacterial ratios (∼0.15, Supplementary Table S4). However, in nearly all cases, the biomass indicators increased with N addition (**Table 3**; **Figures 1D–F**). Sample sizes for determining treatment effects on fungal and bacteria copy numbers were small ($n = 5$ plots) because measurements were conducted only at Site 1. Fungal copy numbers were 90% greater for the N7 treatment ($RII = 0.31$) and 56% greater for N15 ($RII = 0.22$) relative to the Amb plots (**Figure 1E**). Bacterial copy numbers were 11% greater for the N7 treatment ($RII = 0.05$) and 36% higher for N15 ($RII = 0.15$; **Table 3**; **Figure 1F**). Approximately 3% of the bacterial sequences were cyanobacteria, which is typical for hot, dry deserts (Fierer et al., 2012). Biocrust chlorophyll *a*, which was measured at all three sites, was 82% greater for the N7 treatment ($RII = 0.29$) and 52% greater for N15 ($RII = 0.21$) relative to the Amb plots (**Table 4**; **Figure 1D**).

Independent of responses to N amendment, microbial biomass was greater in the biocrust than in the bulk soil (217% for fungi and 118% for bacteria). Biocrust chlorophyll was 156% higher under the canopy (211.7 ± 17.9) than in interspaces (82.8 ± 17.9).

Soil Enzyme Activity

Collectively, the four soil EEA responded significantly to N addition (**Table 5**; Supplementary Table S3, **Figure 3**: perMANOVA $P = 0.0039$). AAP was the most responsive, increasing with N addition in all four soil fractions. Overall, AAP activity increased by 54% ($RII = 0.21$, Amb $= 21.64 \pm 5.02$, N7 $= 22.83 \pm 5.02$, N15 $= 33.33 \pm 5.02$; Supplementary Table S3). In contrast, β-1,4-*N*-acetylglucosaminidase activity declined 36% across treatments ($RII = -0.22$; Amb $= 5.25 \pm 0.96$, N7 $= 3.87 \pm 0.96$, N15 $= 3.37 \pm 0.96$; Supplementary Table S3).

Like N transformation rates, the effects of N addition on ecoenzyme activity varied with sampling depth (**Table 5**; Supplementary Table S3, **Figure 3**; perMANOVA $P = 0.0045$). This interaction was driven primarily by AP, which declined 54% with N addition in biocrust soil ($RII = -0.37$; Amb $= 29.98 \pm 8.89$, N7 $= 21.01 \pm 8.89$, N15 $= 13.76 \pm 8.89$) but increased 59% for bulk soil ($RII = 0.23$; Amb $= 4.16 \pm 8.89$, N7 $= 13.70 \pm 8.89$, N15 $= 6.60 \pm 8.89$; **Figure 3**). β-1,4-*N*-acetylglucosaminidase showed a similar pattern with a 45% decline with N in the biocrust ($RII = -0.29$; Amb $= 9.00 \pm 1.28$, N7 $= 6.37 \pm 1.28$, N15 $= 4.99 \pm 1.28$) but a 17% increase in bulk soil ($RII = 0.08$; Amb $= 1.49 \pm 1.28$, N7 $= 1.36 \pm 1.28$, N15 $= 1.74 \pm 1.28$). AAP also showed a greater relative response to N in deep soil (116%, $RII = 0.37$) compared to surface biocrust (23%, $RII = 0.10$).

Spatially, all ecoenzymes showed greater potential activity in canopy soils than in interspaces (**Table 5**). β-1,4-*N*-acetylglucosaminidase showed the strongest difference (353% greater in canopy soil) followed by β-glucosidase (107%), AAP (39%), and AP (37%).

Foliar Nutrients

N addition had no effect on nutrient concentrations in leaves of *A. dumosa* (Supplementary Table S5, perMANOVA pseudo-$F = 0.90$, $P = 0.4149$).

Connecting Biomass, Activities, and Nutrients

At the plot scale, the enzyme response matrix was positively associated with the nutrient response matrix, but only beneath the shrub canopy (Mantel $\rho = 0.152$, $P = 0.0313$, $n = 45$ plots), not within the interspaces (Mantel $\rho = 0.088$, $P = 0.1849$). Across all samples, the ecoenzyme response matrix was positively correlated with all estimates of microbial biomass. Fungal copy number showed the strongest positive correlation with

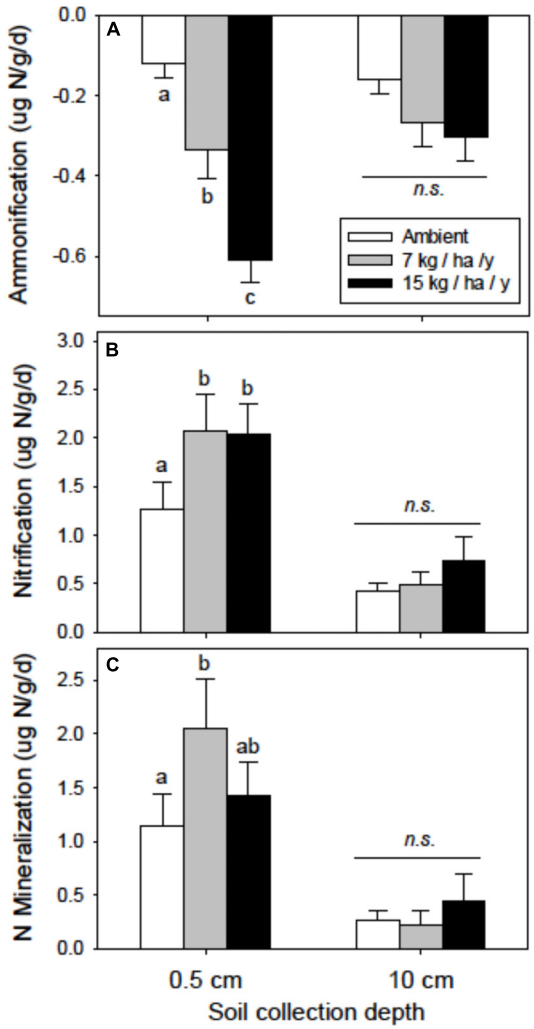

FIGURE 2 | N transformation rates in relation to experimental N addition for biocrust (0.5 cm) and soil (0–10 cm). (A) Ammonification. **(B)** Nitrification. **(C)** N mineralization. Different letters indicate significant differences among the N treatments. Data summarized in **Table 2**. Statistical results presented in Supplementary Table S2.

ecoenzyme NMS axis1 (Spearman $r = 0.39$, $P = 0.0026$, $n = 58$), followed closely by bacterial copy number (Spearman $r = 0.38$, $P = 0.0036$, $n = 58$), then chlorophyll *a* (Spearman $r = 0.37$, $P = 0.0004$, $n = 87$).

Meta-Analysis

Across studies, microbial biomass responses to N treatment were best described by logarithmic regressions (**Figure 4**). For N application rate (kg ha^{-1} y^{-1}): effect = −0.0959 (ln rate) + 0.429 ($r^2 = 0.358$, $n = 26$, $p = 0.00125$). For N load: effect = −0.0779(ln load) + 0.395 ($r^2 = 0.386$, $n = 26$, $p = 0.0007$). The critical points separating positive from negative treatment effects were 88 kg ha^{-1} y^{-1} and 159 kg ha^{-1}, respectively.

Metabolic responses to N treatment were better described by linear regressions, rather than logarithmic (**Figure 4**). For application rate (kg ha^{-1} y^{-1}): effect = −0.00065(rate) + 0.455 ($r^2 = 0.21$, $n = 41$, $p = 0.0024$). For load: effect = −0.00052(load) + 0.0593 ($r^2 = 0.41$, $n = 41$, $p < 0.00001$). The critical points separating positive from negative treatment effects were 70 kg ha^{-1} y^{-1} and 114 kg ha^{-1}, respectively.

Discussion

N Responses and Spatial Heterogeneity

By most measures, nutrient availability, microbial biomass, and process rates were greater in soils beneath the shrub canopy compared to the interspace between plants, and greater in the surface biocrust horizon compared to the bulk soil. Most of these measures also responded positively to experimental N addition, despite the absence of large precipitation events over the study period or evidence of changes in plant production and nutrient content.

One notable exception was AP activity. Generally, phosphatase activity increases in response to experimental N addition because mitigating N limitation increases the relative demand for P (Skujiņš, 1978; Sinsabaugh and Follstad Shah, 2012). In that context, the 50% loss of phosphatase activity in the N-amended biocrusts is anomalous, particularly because activity increased by 50% over the deeper 10 cm profile (**Figure 3**).

Chemical analysis showed that available P increased by 20–50% with N addition in all soil fractions (**Table 1**). These P increases could be the result of acidity generated by increased nitrification, which increased significantly (60–80%) in biocrusts, with a somewhat smaller response for bulk soils. Analysis of these carbonate soils conducted prior to treatment found a total P content of 950 ± 40 μg/g (data not presented), which compared to values of 10–18 μg/g for P_{av} (**Table 1**) suggests a potential for increased P solubilization in response to acidity. Increased P_{av} may also be a product of greater microbial biomass in the N addition treatments. In any case, the divergent phosphatase responses of biocrust and bulk soil indicate that N addition depressed relative P limitation within the biocrust, but increased it for bulk soil, possibly as a result of nutrient competition with plants.

Although phosphatase is the clearest case of divergent responses between biocrust and bulk soil, the general trend extends to other ecoenzymes. Activities tend to show larger and more positive responses in the bulk soil than in the biocrust (**Figures 3** and **4**). This spatial pattern is consistent with the dose-dependent responses observed across studies (**Figure 4**). The surface biocrust likely experienced a greater effective N dose than the underlying mineral soil given the sparse precipitation over the study period. In addition, the mineral soil is the site of plant–microbe interaction, including carbon priming and nutrient competition, which may also affect the responses to N addition.

Only one other aridland study included measurements of the same ecoenzyme responses presented here (Stursova et al., 2006). That study, conducted in semiarid grassland, sampled soil (0–5 cm) beneath bunch grass (grama) canopy and in

TABLE 3 | Fungal and bacterial SSU rRNA gene copy number by horizon, location, and N treatment.

Horizon	Location	N trt	Fungal 18S		Bacterial 16S		Fungi/ Bacteria	
			Copies/g	SD	Copies/g	SD	Ratio	SD
Biocrust 0.5 cm	Canopy	Amb	1.03E+08	6.20E+07	5.82E+08	2.47E+08	0.169	0.056
Biocrust 0.5 cm	Interspace	Amb	2.48E+07	1.27E+07	1.88E+08	2.74E+07	0.140	0.091
Soil 10 cm	Canopy	Amb	3.42E+07	1.12E+07	2.35E+08	4.64E+07	0.146	0.039
Soil 10 cm	Interspace	Amb	7.08E+06	3.65E+06	1.04E+08	2.98E+07	0.066	0.018
Biocrust 0.5 cm	Canopy	N7	2.28E+08	2.17E+08	6.22E+08	2.87E+08	0.345	0.217
Biocrust 0.5 cm	Interspace	N7	1.94E+07	6.66E+06	2.42E+08	1.02E+08	0.089	0.032
Soil 10 cm	Canopy	N7	6.50E+07	4.67E+07	2.34E+08	1.01E+08	0.366	0.440
Soil 10 cm	Interspace	N7	9.14E+06	2.44E+06	1.29E+08	3.51 E+07	0.072	0.012
Biocrust 0.5 cm	Canopy	N15	1.42E+08	1.17E+08	7.18E+08	3.97E+08	0.204	0.105
Biocrust 0.5 cm	Interspace	N15	6.24E+07	9.93E+07	2.87E+08	1.06E+08	0.221	0.335
Soil 10 cm	Canopy	N15	5.82E+07	5.92E+07	3.97E+08	2.44E+08	0.148	0.097
Soil 10 cm	Interspace	N15	7.40E+06	2.10E+06	1.09E+08	8.78E+07	0.094	0.059

TABLE 4 | Biocrust chlorophyll *a* data by horizon, location, and N treatment.

Horizon Location	N trt	Chlor *a*	
		ng g^1	SD
Biocrust 0.5 cm Canopy	Amb	135.0	86.4
Biocrust 0.5 cm Interspace	Amb	68.4	36.8
Biocrust 0.5 cm Canopy	N7	234.2	136.2
Biocrust 0.5 cm Interspace	N7	75.5	55.1
Biocrust 0.5 cm Canopy	N15	265.8	214.9
Biocrust 0.5 cm Interspace	N15	104.4	83.5

biocrust-covered interspaces. β-*N*-acetylglucosaminidase and β-glucosidase activities were similar in magnitude to those reported here, but AAP and AP activities were 3–5 times greater in the prior study. In response to N amendment (10 kg ha^{-1} y^{-1}), AAP activity declined rather than increased, β-*N*-acetylglucosaminidase activity doubled, while AP and β-glucosidase activities showed little response.

Integrating N Responses through Carbon Use Efficiency

The comparison above highlights the need for integrative measures of ecosystem response to N loading, rather than focusing on the causal nexus underlying individual responses. Across the patch mosaic landscape of arid shrubland, nutrient concentrations, microbial activity, microbial biomass and their responses to N addition are often correlated because they are integrated through microbial carbon use efficiency (CUE). CUE is commonly defined as the ratio of microbial growth to assimilation, which is often estimated as the sum of growth and respiration. But CUE can also be estimated from stoichiometric relationships among substrate composition, biomass composition and nutrient acquisition activities (Sinsabaugh et al., 2013). Sinsabaugh and Follstad Shah (2012) proposed that:

$$\mathrm{CUE} = \mathrm{CUE}_{max}[S_{C:N}/(S_{C:N} + K_N)], \text{ where}$$

$$S_{C:N} = (1/\mathrm{EEA}_{C:N})(B_{C:N}/L_{C:N}).$$

TABLE 5 | Ecoenzymatic activity by horizon, location, and N treatment.

Horizon	Location	N trt	AAP		AP		NAG		BG		AG	
			nmol g^{-1} d^{-1}	SD	nmol g^{-1} d^{-1}	SD	nmol g^{-1} d^{-1}	SD	nmol g^{-1} d^{-1}	SD	nmol g^{-1} d^{-1}	SD
Biocrust 0.5 cm	Canopy	Amb	43.0	29.7	38.9	29.2	15.3	14.8	56.7	32.2	2.91	2.89
Biocrust 0.5 cm	Interspace	Amb	31.4	26.5	32.8	26.5	2.94	2.06	26.6	31.2	2.48	7.14
Soil 10 cm	Canopy	Amb	16.0	11.8	6.34	7.08	2.28	1.87	18.5	9.9	1.28	2.64
Soil 10 cm	Interspace	Amb	19.0	14.9	4.43	4.86	0.71	0.55	11.7	12.3	0.31	0.36
Biocrust 0.5 cm	Canopy	N7	34.8	22.7	30.0	32.2	11.4	16.0	55.4	35.5	4.13	5.68
Biocrust 0.5 cm	Interspace	N7	17.6	11.2	16.2	16.8	1.38	1.49	22.4	21.7	0.57	0.58
Soil 10 cm	Canopy	N7	29.7	25.4	15.3	18.2	2.77	2.53	25.1	16.8	1.00	1.53
Soil 10 cm	Interspace	N7	22.4	18.8	17.9	18.3	0.54	0.54	12.4	14.8	0.29	0.42
Biocrust 0.5 cm	Canopy	N15	41.2	23.3	14.8	13.7	7.20	4.69	40.9	23.2	3.31	3.95
Biocrust 0.5 cm	Interspace	N15	33.7	18.1	14.7	15.9	2.79	3.30	23.2	15.0	1.37	1.31
Soil 10 cm	Canopy	N15	32.9	22.2	7.35	11.3	2.58	1.92	23.2	14.7	1.71	1.94
Soil 10 cm	Interspace	N15	34.5	30.5	7.31	4.21	0.90	0.91	11.2	9.3	1.19	1.70

AAP, alanine aminopeptidase; AP, alkaline phosphatase; NAG, β-N-acetylglucosaminidase; BG, β-glucosidase; AG, α-glucosidase.

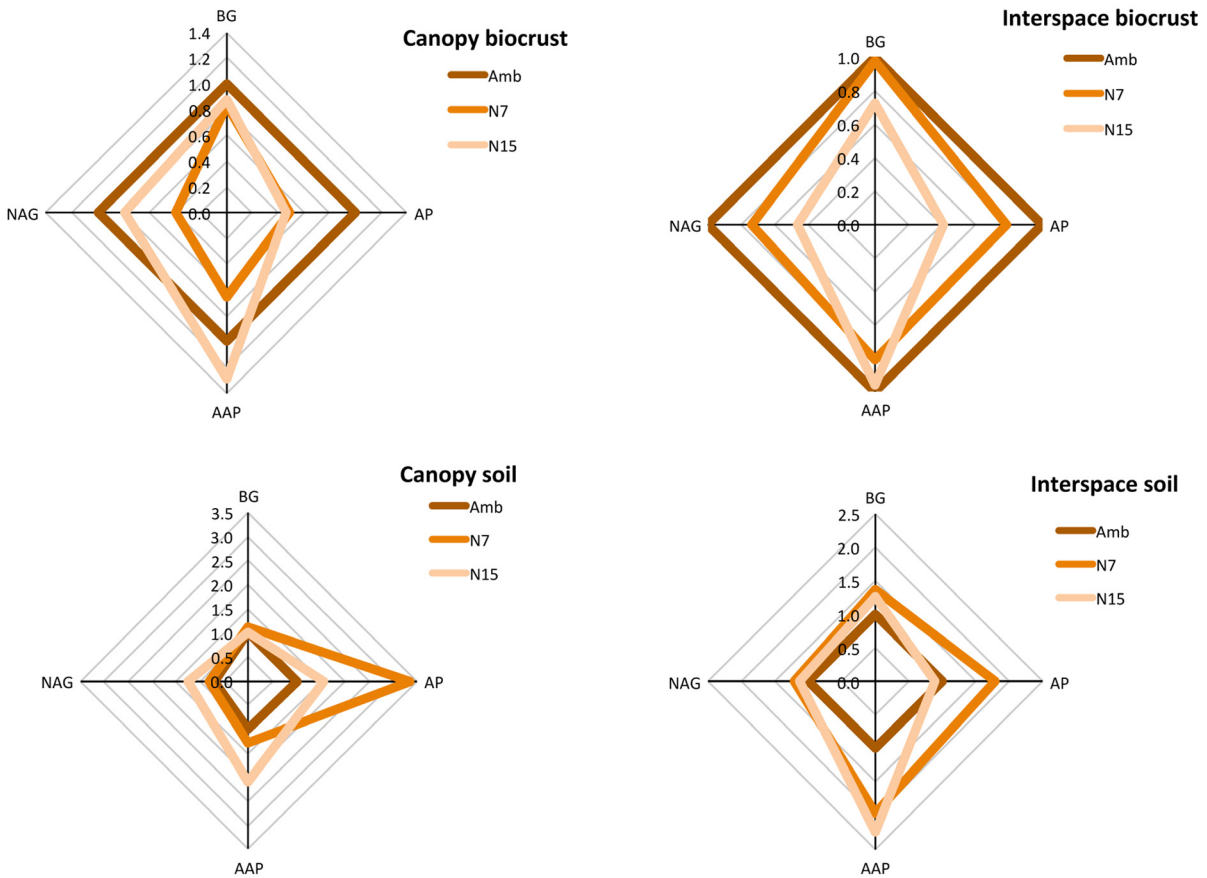

FIGURE 3 | Relative responses of soil ecoenzyme activity to N treatment for biocrusts (0.5 cm) and bulk soil (0–10 cm). BG: β-1,4-glucosidase, AP: alkaline phosphatase, AAP: alanine aminopeptidase, NAG: β-1,4-*N*-acetylglucosaminidase.

Ecoenzyme activities$_{C:N}$ is the ratio of C to N-acquiring ecoenzymatic activities, measured here as the ratio of BG/(NAG + AAP); $B_{C:N}$ is the elemental C:N ratio of microbial biomass; $L_{C:N}$ is the elemental C:N ratio of labile organic matter; K_N is a half saturation constant (0.5); and CUE_{max} is the upper limit for microbial growth efficiency (0.6).

Using data from **Tables 1** and **5,** and assuming a mean value of 8.6 for $B_{C:N}$ (Cleveland and Liptzin, 2007), the estimated CUEs ranged from 0.29 to 0.51 across soil fractions (**Figure 5**). For the N15 treatment, CUE increased in all soil fractions relative to the Amb. The N7 responses were mixed, CUE declined in biocrusts and increased in bulk soils. The CUE estimates for the semiarid grassland study conducted by Stursova et al. (2006) are similar to those for the shrubland (0.29–0.36), but show no net response to N addition after 10 years of fertilization (**Figure 5**). The Lake Mead NRA responses were measured after 1 year of treatment. Based on other cross study comparisons (**Figure 4**), this difference in CUE response between the studies may be the result of the 10-fold difference in cumulative N loading.

Meta-Analysis of Aridland N Addition Studies

To aid decision making regarding ecosystem management and restoration, there has been an effort to define critical loads for N deposition by ecosystem type, where critical load is defined as "the deposition of a pollutant below which no detrimental ecological effect occurs over the long term according to present knowledge" (Pardo et al., 2011). This definition does not differentiate positive and negative responses. Our meta-analysis of aridland studies showed that N amendment had generally positive effects on microbial biomass and metabolic rates at N doses <70 kg ha^{-1} y^{-1} and N loads <120 kg ha^{-1}, and generally negative effects at greater doses and loads (**Figure 4**).

Treseder (2008) conducted a meta-analysis of microbial biomass responses to N amendment, using data from 82 ecosystems (80 mesic and 2 arid). She found no significant differences across biomes, fertilizer types, or methods of biomass measurement. A regression relating ln(response ratio) to N load ($n = 40$, $r^2 = 0.18$, $p = 0.005$) estimated the critical load separating net positive and net negative effects at 200 kg ha^{-1}. Our regression model for aridland ecosystems yielded a similar critical load of 160 kg ha^{-1} (**Figure 3**).

The meta-analysis by Knorr et al. (2005) focused on litter decomposition rates, using data from 24 studies conducted in grassland, forest and tundra ecosystems. Litter decomposition was inhibited by N additions when dose rates were 2–20 times the ambient N deposition level or when litter quality was low

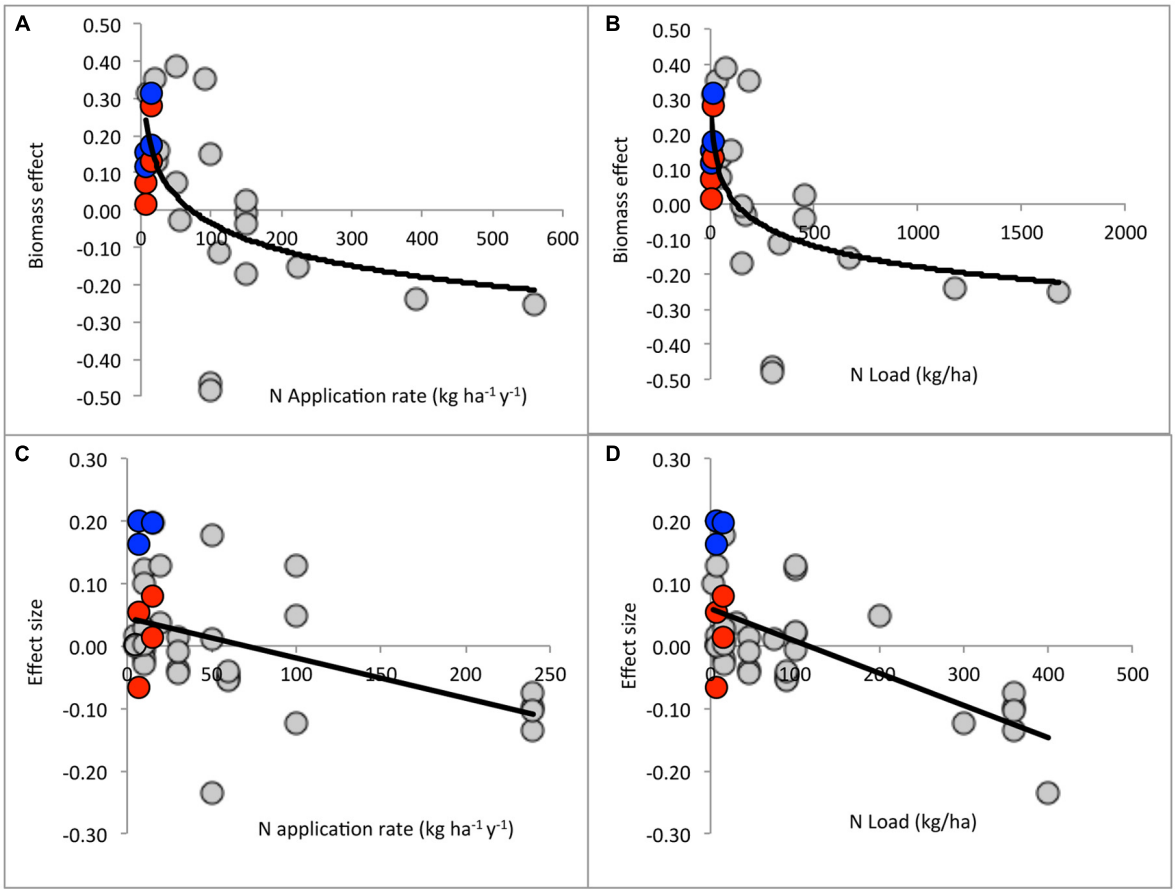

FIGURE 4 | Meta-analysis of aridland microbial responses to N addition. **(A)** Microbial biomass in relation to N application rate: effect = −0.0959 (ln rate) + 0.429 (r^2 = 0.358, n = 26, p = 0.00125). **(B)** Microbial biomass in relation to cumulative N load: effect = −0.0779 (ln load) + 0.395 (r^2 = 0.386, n = 26, p = 0.0007). **(C)** Metabolic rates in relation to N application rate: effect = −0.00065 (rate) + 0.455 (r^2 = 0.21, n = 41, p = 0.0024). **(D)** Metabolic rates in relation to cumulative N load: effect = −0.00052 (load) + 0.0593 (r^2 = 0.41, n = 41, p < 0.00001). Blue circles represent Lake Mead bulk soil responses. Red circles represent Lake Mead biocrust responses. Data from Supplementary Table S1.

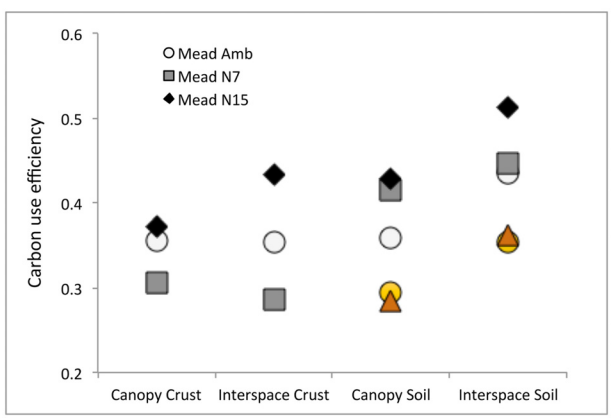

FIGURE 5 | Microbial carbon use efficiency (CUE) by horizon, location, and N treatment. For comparison, the orange circles (Ambient) and triangles (N addition, 10 kg ha^{-1} y^{-1}) are values calculated for a N addition study conducted in semiarid grassland in New Mexico (Stursova et al., 2006).

(i.e., high lignin or humus content). Conversely, low ambient N deposition rates, high litter quality and short experiment duration tended to increase decomposition rates. The conflation of N dose with litter quality effects makes it difficult to estimate a critical value for the positive to negative transition, but dose rates >125 kg ha^{-1} y^{-1} generally inhibited decomposition. For comparison, our regression model for microbial metabolic responses in arid ecosystems estimates the critical dose at 70 kg ha^{-1} y^{-1}.

Janssens et al. (2010) conducted a meta-analysis of N effects on heterotrophic respiration using data from 36 microcosm studies of temperate forest soils. On average, respiration declined by 15% in response to N amendment with a response range of −57 to +63%. Doses >50 kg ha^{-1} y^{-1} generally had negative effects, in comparison with our threshold estimate of 70 kg ha^{-1} y^{-1}.

A meta-analysis by Zhou et al. (2014) analyzed N effects on soil respiration, resolving autotrophic and heterotrophic responses, using data from 295 studies conducted across a broad range of biomes. On average, N addition stimulated autotrophic respiration by 22% but reduced heterotrophic respiration by

13%. A regression relating the response ratio of heterotrophic respiration to N load showed no positive responses, i.e., the response ratio intercept was <1.0. The maximum N dose rate included in the meta-analysis was 74 kg ha^{-1} y^{-1}, a value that approximates our estimated critical point for the transition from net positive to net negative effects.

Across ecosystems, lichens, and bryophytes, which are components of biocrusts, are among the most sensitive responders to increased N deposition with critical loads estimated at 1–9 kg ha^{-1} y^{-1}; critical loads for semiarid grasses and shrubs also fall in this range (Pardo et al., 2011). Within our meta-analysis of arid soil responses, the lowest N application rate was 5 kg ha^{-1} y^{-1} and nearly all microbial responses <15 kg ha^{-1} y^{-1} were positive. These results suggest that arid ecosystems, characterized by low soil N contents and low atmospheric deposition rates, may be among the most sensitive to anthropogenic N deposition.

Conclusion

Despite the difficulties in making direct comparisons among ecosystems, it appears that soil microbial responses to N amendment in arid ecosystems are broadly comparable to those of mesic ecosystems in terms of N saturation. However, it appears they are more sensitive to low dose inputs by some measures and more spatially heterogeneous in their responses. This heightened vulnerability will create many future challenges for land managers, as anthropogenically related N deposition is expected to increase as arid regions become more developed as spaces for living, recreation, and energy development.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00819

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Rhizosphere bacterial communities of dominant steppe plants shift in response to a gradient of simulated nitrogen deposition

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We evaluated effects of 9-year simulated nitrogen (N) deposition on microbial composition and diversity in the rhizosphere of two dominant temperate grassland species: grass *Stipa krylovii* and forb *Artemisia frigida.* Microbiomes in *S. krylovii* and *A. frigida* rhizosphere differed, but changed consistently along the N gradient. These changes were correlated to N-induced shifts to plant community. Hence, as plant biomass changed, so did bacterial rhizosphere communities, a result consistent with the role that N fertilizer has been shown to play in altering plant-microbial mutualisms. A total of 23 bacterial phyla were detected in the two rhizospheric soils by pyrosequencing, with *Proteobacteria, Acidobacteria,* and *Bacteroidetes* dominating the sequences of all samples. *Bacterioidetes* and *Proteobacteria* tended to increase, while *Acidobacteria* declined with increase in N addition rates. *TM7* increased >5-fold in the high N addition rates, especially in *S. krylovii* rhizosphere. Nitrogen addition also decreased diversity of OTUs (operational taxonomic units), Shannon and Chao1 indices of rhizospheric microbes regardless of plant species. These results suggest that there were both similar but also specific changes in microbial communities of temperate steppes due to N deposition. These findings would contribute to our mechanistic understanding of impacts of N deposition on grassland ecosystem by linking changes in plant traits to their rhizospheric microbes-mediated processes.

Keywords: nitrogen deposition, microbial diversity, rhizosphere, Illumina Miseq, temperate steppe, *Artemisia frigida*, *Stipa kerlovii*

Introduction

Nitrogen (N), an essential mineral nutrient to plant growth, is one of the most limiting factors in many terrestrial ecosystems (Vitousek et al., 2002; Scheible et al., 2004; Asakawa and Kimura, 2008). However, elevated levels of N deposition are changing N inputs and impacting many ecological processes (Sala et al., 2000; Gilliam, 2006). Many ecosystems have developed under tight nutrient cycling and low amounts of available nutrients. In these systems, elaborate plant-microbial mutualisms have developed and are the foundation of ecosystem function (Reynolds et al., 2003).

Nitrogen deposition can disrupt these plant-microbial interactions, which may feedback to alter microbial communities and ecosystem function (Sala et al., 2000; Gilliam, 2006; Martinelli et al., 2006).

Human industrial activities have led to a doubling, on average, of N deposition into terrestrial ecosystems and these inputs are expected to increase in the future (Galloway et al., 2004, 2008; Bodirsky et al., 2014). In China, N deposition has significantly increased over the last three decades, and in some regions are 5X greater than previous decades (Liu et al., 2013). Consequently, the impacts of N input due to deposition of atmospheric N on terrestrial ecosystems warrant further study (Galloway et al., 2004, 2008).

Grassland ecosystems are highly sensitive to N deposition, where long-term N addition has been shown to significantly reduce plant species richness (Stevens et al., 2004; Clark and Tilman, 2008). The semi-arid grasslands in northern China, which are a part of the Eurasian steppe, are exposed to enhanced N deposition rates (Zhang et al., 2008; Liu et al., 2013), and experienced reductions in plant species richness (Bai et al., 2010; Song et al., 2011; Fang et al., 2012; Tian et al., 2015). Several mechanisms have been proposed to explain the decline in species richness by N deposition (Suding et al., 2005; Harpole and Tilman, 2007; Bobbink et al., 2010), and among them are changes in soil microbial activity and biodiversity (Chen et al., 2014; Dean et al., 2014). Nitrogen deposition to a variety of terrestrial ecosystems has also been shown to profoundly affect soil microbial communities (Ramirez et al., 2010, 2012; He et al., 2013; Liu et al., 2014b; Zhang et al., 2014), however, these studies have not emphasized the involvement of root-zone and rhizosphere microbes; rather they have mainly focused on microbes in soils generally. The rhizosphere niche is an important interface for plant-microbes interactions and key to the success of both plants and microbes (Bakker et al., 2013). Microbe communities in the rhizosphere soils differ from those in bulk soils (Berg and Smalla, 2009), thus deserving specific attention for understanding ecosystem responses to N deposition. It is conceivable that microbial communities in the rhizosphere of different plant species may respond more strongly and perhaps, differently, to N deposition. Recently, Dean et al. (2014) found that N deposition affected host-associated plant root-associated fungi, however, there was no broader description of fungal or rhizosphere bacterial communities.

In the present study, we found that plant community shifted from co-dominance by a monocot grass, *Stipa krylovii*, and a dicot forb, *Artemisia frigida*, to exclusive dominance by a monocot grass in an Inner Mongolia steppe after 9-year of N addition. To test whether the microbial communities colonizing *S. krylovii* and *A. frigida* rhizosphere niche respond, and respond in similar or different ways to N addition, we used the high-throughput Illumina Miseq sequencing platform to characterize the rhizosphere microbial communities of the two dominant plant species under varying simulated N deposition rates. The following question was specifically addressed: Whether the rhizosphere microbial communities would be associated with plant host and its response to N deposition rate?

Materials and Methods

Study Site

The experiment was conducted in Duolun county (42°02′N, 116°17′E, 1324 m a.s.l.), Inner Mongolia, China. The area is located in a semiarid temperate steppe where the mean annual temperature is 2.1°C and long-term annual precipitation is 382.2 mm (Yang et al., 2011; Fang et al., 2012). The soil in the area is chestnut (Chinese classification) and Haplic Calcisols (FAO classification). The soil bulk density is 1.31 g cm^{-3} and pH is about 6.84 (Fang et al., 2012). The dominant species in this typical temperature steppe are *A. frigida*, *S. krylovii*, *Cleistogenes squarrosa*, *Allium bidentatum*, *Potentilla acaulis*, *Leymus chinensiss, Salsola collina*, *Carex korshinskyi*, *Melilotoides ruthenica,* and *Agropyron cristatum* (Niu et al., 2008; Fang et al., 2012).

Experimental Design

In the experimental area, 64 plots of 15 × 10 m separated by 4-m-wide buffer strips were established in an 8 × 8 Latin square experimental design. Nitrogen was added as urea (N, 46%) at the midpoint of the growing season (July) every year since 2003. There were eight levels of N fertilization including a control, 0 (N0), 1 (N1), 2 (N2), 4 (N4), 8 (N8), 16 (N16), 32 (N32), and 64 (N64) g N m^{-2} $year^{-1}$ with ambient N deposition of 1.6 g N m^{-2} $year^{-1}$ (Zhang et al., 2008). In the present study, the rhizosphere soil samples were collected from 32 plots with four levels of N fertilization, ambient (N0), 2 (N2), 8 (N8), and 16 (N16) g N m^{-2} $year^{-1}$. The rhizospheric soils from at least two individual plant roots of the two dominant species (*S. krylovii*, *A. frigida*) in each plot were sampled in August 2012 by collecting soils that were adhered to roots after vigorously shaking roots removed from field by a spade as described by Smalla et al. (2001). The rhizospheric soils were sampled from the eight plots under the four N addition levels, and the final three soil samples used for sequencing were obtained by randomly mixing the samples from 3, 3, and 2 plots, respectively. These ensure that the rhizospheric soil samples covered the eight replicates for N addition. Aboveground biomass in one quadrat (1 × 1 m) at each plot was clipped and determined since August 2004 as described by Fang et al. (2012).

Sequencing and Data Analysis

The total genomic DNA was extracted from 0.5 g rhizosphere soils using the SoilGen DNA Kit (CWbiotech Corporation, China) according to the manufacturer's instructions. PCR amplifications were conducted with the 515f/806r (GTG CCAGCMGCCGCGGTAA/GGACTACHVGGGTWTCTAAT) primer set that amplified the V4 region of the 16S rRNA gene (Peiffer et al., 2013). The primer set was selected as it exhibits few biases and should yield accurate taxonomic information. The reverse primer contained a 6-bp error-correcting barcode unique to each sample. The PCR reaction was carried out in 30 μL reactions with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs); 0.2 μM of forward and reverse primers, and about 10 ng template DNA. Thermal cycling

was consisted of initial denaturation at 98°C for 1 min, then 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, elongation at 72°C for 60 s and finally 72°C for 5 min. PCR products were mixed in equal density ratios. Then, mixture PCR products were purified with GeneJET Gel Extraction Kit (Thermo Scientific). Sequencing libraries were generated using NEB Next® Ultra™ DNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit @ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina MiSeq platform and 300 bp paired-end reads were generated.

Pairs of reads from the original DNA fragments were merged by using FLASH-a very fast and accurate software tool which was designed to merge pairs of reads when the original DNA fragments were shorter than twice the length of reads (Magoc and Salzberg, 2011). Sequencing reads were assigned to each sample according to the unique barcode of each sample. Sequences were analyzed with the QIIME software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline (Caporaso et al., 2010; Edgar, 2013). First, the reads were filtered by QIIME quality filters. Default settings for Illumina processing in QIIME were used. Then we used UPARSE pipeline to pick operational taxonomic units (OTUs) by making OTU table. After removal of chimera, sequences were assigned to OTUs at 97% similarity. We picked a representative sequence for each OTU and used the version 2.2 RDP classifier to assign taxonomic data to each representative sequence with default 0.8 as confidence threshold (Wang et al., 2007). Singleton OTUs that appeared in only one sample were removed because they could be potential sequencing errors. In order to compute alpha diversity, we rarified the OTU table and calculated three metrics: Chao1 metric estimated the species richness, the observed species metric was simply the count of unique OTUs found in the sample, and Shannon index. Rarefaction curves were generated based on these three metrics. QIIME calculated unweighted unifrac, which was used to do Principal Coordinate Analysis (PCA).

Sequence Accession Number

The data were deposited in the National Center for Biotechnology Information Sequence Reads Archive with accession number SRS977347.

Statistical Analysis

Considering that the N addition and plant host in the same plot may not be completely independent, we used the linear mixed models to analyze the effects of N addition on the rhizoshpere microbes at four levels with N treatment as the fixed effect and plant host as random effects. For each species, we conducted separate ANOVAs (Dunnett's test) to determine the difference in the aboveground biomass (AGB), microbial diversity and relative abundance of phyla between N0 and different levels of N addition (SPSS 16.0). It was regarded as significant differences when *P*-value was less than 0.05. Square root transformation of the OTUs data and arcsine square root transformation of relative abundance of phyla were done before proceeding with ANOVA and *post-hoc* test. The assumptions of normality and homogeneity of variance were checked prior to conducting the statistical tests.

Results

Nitrogen Addition Reduced and Enhanced Aboveground Biomass (AGB) of Forbs and Grasses

Nitrogen addition for 9 years had contrasting effects on AGB of grasses and forbs, such that AGB of grasses and forbs was significantly ($P < 0.05$) increased and reduced by N addition, respectively (**Figure 1A**). The steppe community was co-dominated by grass *S. krylovii* and forb *A. frigida* in the control plot without N addition. The AGB of *S. krylovii* was enhanced by the N addition, while the same treatment led to a decrease in AGB of *A. frigida* ($P < 0.05$) (**Figure 1B**).

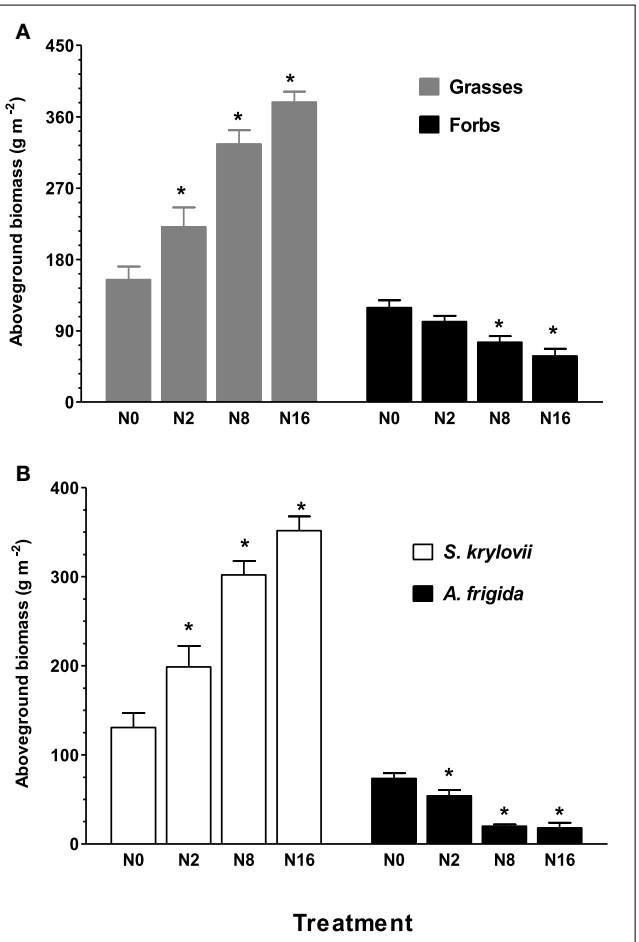

FIGURE 1 | Effect of N addition on aboveground biomass of grasses and forbs (A), and *S. krylovii* and *A. frigida* (B). Aboveground biomass was determined in quadrats (1 × 1 m). N0, N2, N8, and N16 represent N addition rate of 0, 2, 8, 16 g ha^{-1} yr^{-1}. Data are means ± s.e. ($n = 8$). Asterisks on the top of columns indicate significant difference at $P < 0.05$ between N0 and different rates of N addition for each species.

Sequencing and Analysis of Rhizospheric Microbial Diversity

We obtained a total of 1,931,731 clean reads after filtered by QIIME quality filters with default settings (**Table A1**). One sample from N0 plots was later excluded from analysis because of the low quality of the sequence reads. Rarefaction analysis was performed on each soil sample and none of the rarefaction curves reached the plateau phase, suggesting that soils were not sampled to saturation (**Figure 2**).

Linear mixed model analysis indicated that OTU number, Chao1, and Shannon indices of rhizosphere microbes of both *S. krylovii* and *A. frigida* were significantly affected by the N addition (**Figure 3** and **Table 1**). In control plot without N addition, the number of OTUs in the rhizosphere of *S. krylovii* and *A. frigida* was 6036 and 5957, respectively (**Figure 3A**). Nitrogen addition led to similar effects on microbial diversity in the rhizosphere of the two species. As N addition rate increased, the OTU number in both the *S. krylovii* and *A. frigida* rhizospheric niche decreased (**Figure 3A**). For example, the OTU number in the *S. krylovii* rhizosphere was decreased from 6036 to 5698, 5282, and 4583 in response to N addition rate of 2, 8, and 16 g N m^{-2} yr^{-1}. Similarly, the OTU number in the *A. frigida* rhizosphere niche decreased from 5957 to 5700, 5010, and 4567 in response to the same N addition rates (**Figure 3A**). In addition to the number of OTUs, changes in microbial diversity using the Shannon index and the total species richness estimated by the Chao1 index in the two rhizospheric soil samples were also compared among plots treated with different rates of N addition. Shannon and Chao1 indices in the two rhizospheric soils also showed similar trends with increases in N addition rates, such that N addition reduced Shannon and Chao1 indices in the rhizosphere of *S. krylovii* and *A. frigida* (**Figures 3B,C**).

Principal component analysis was used to detect variation in the community composition. As shown in **Figure 4**, the two principal components accounted for 16.62% of the total microbial community variations among the individual samples. The two-dimensional figure showed that the microbial community compositions in both *S. krylovii* and *A. frigida* rhizosphere niche with different N addition rates were distributed separately among each other, exhibiting differences in the microbial community structure. These results suggest that the *S. krylovii* and *A. frigida* rhizosphere microbiome had different composition and that the composition changed along the N-gradient.

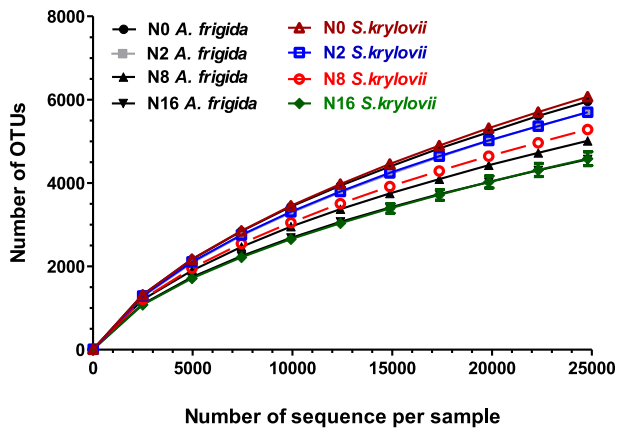

FIGURE 2 | Rarefaction curves of all samples were generated for microbial OTUs which contained unique sequences and were defined at 97% sequence similarities.

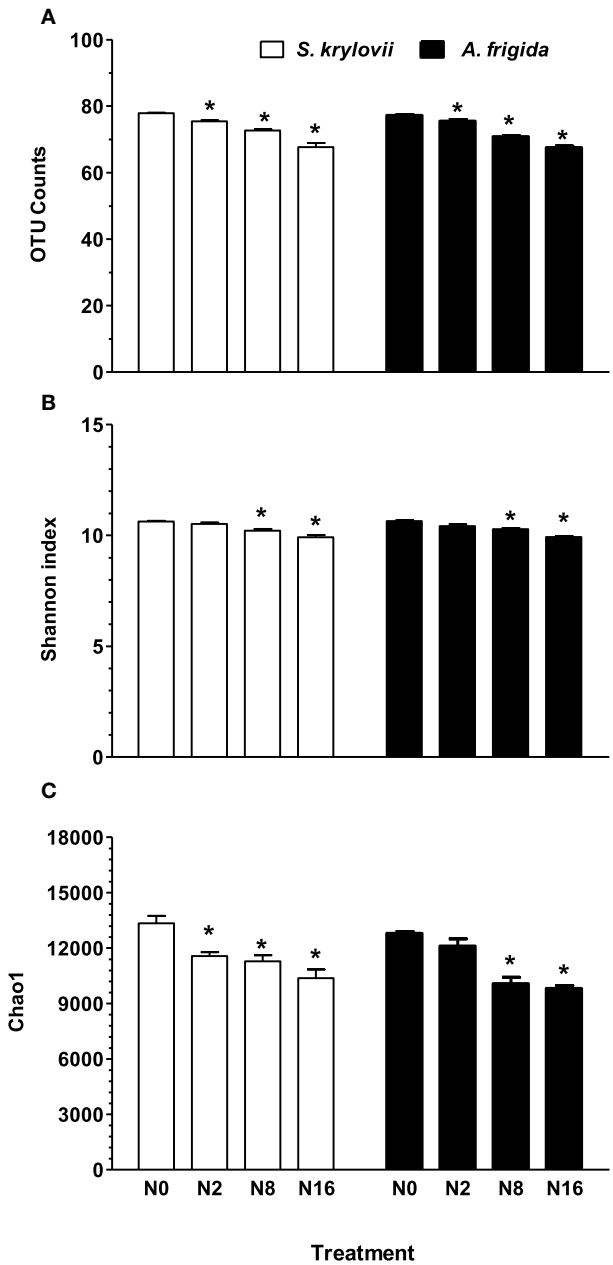

FIGURE 3 | Estimated number of observed (A) OTU counts, (B) Shannon index, (C) Chao1 index of *S. krylovii* and *A. frigida* rhizosphere microbiome across all the N-supplied plots. Data are means ± s.e. ($n = 3$). Asterisks on the top of columns indicate significant difference at $P < 0.05$ between N0 and different rates of N addition for each species.

TABLE 1 | F-value and *P*-value of analysis of variance for the effects of nitrogen addition, species and their interaction (nitrogen × species) on OTU, Shannon, Chao1 indices and the relative abundance of the phyla.

Fixed factors		Nitrogen	Species	Nitrogen × Species
d.f.		3	1	3
OTU	*F*	252.74	7.99	3.18
	P	<0.0001	0.0143	0.06
Shannon	*F*	75.03	0	1.22
	P	<0.0001	0.9624	0.3424
Chao1	*F*	49.09	5.20	4.41
	P	<0.0001	0.0400	0.0239
Acidobacteria	*F*	146.93	13.67	11.94
	P	<0.0001	0.0027	0.0005
Proteobacteria	*F*	19.53	9.94	1.04
	P	<0.0001	0.0076	0.4057
Bacteroidetes	*F*	30.34	0.09	9.87
	P	<0.0001	0.7733	0.0012
Crenarchaeota	*F*	72.80	0.01	11.96
	P	<0.0001	0.9247	0.0005
Verrucomicrobia	*F*	4.03	10.84	0.37
	P	0.0314	0.0058	0.7754
Planctomycetes	*F*	6.24	1.42	3.27
	P	0.0074	0.2555	0.0559
Actinobacteria	*F*	4.71	5.65	4.62
	P	0.0194	0.0335	0.0207
Cyanobacteria	*F*	0.62	0	0.41
	P	0.6168	0.9601	0.7504
Gemmatimonadetes	*F*	12.42	7.81	5.34
	P	0.0004	0.0152	0.0129
Chloroflexi	*F*	16.22	15.54	6.55
	P	0.0001	0.0017	0.0062
Firmicutes	*F*	39.78	21.00	0.28
	P	<0.0001	0.0005	0.8384
WYO	*F*	1.75	24.18	11.48
	P	0.2057	0.0003	0.0006
TM7	*F*	195.38	5.60	7.16
	P	<0.0001	0.0341	0.0044

Effect of N Addition on Relative Abundance of Rhizospheric Microbe

Analysis of the taxonomic groups detected in the soil samples showed that there were a total of 23 phyla in the rhizosphere of both *S. krylovii* and *A. frigida*. The most dominant phyla across all samples were *Proteobacteria, Acidobacteria,* and *Bacteroidetes*, accounting for about 60% of the bacterial sequences (**Figure 5**). In addition, *Verrucomicrobia, Crenarchaeota, Planctomycetes, Actinobacteria, Cyanobateria, Gemmatimonadetes, Chloroflexi, TM7, Firmicutes,* and *WYO* were detected in all the samples with low abundance, while the unclassified and rare phyla accounted for about 6.3% in the samples. In the control plots without N addition, the relative abundance of *Bacteroidetes* and *Crenarchaeota* in the rhizosphere of *S. krylovii* was higher than that in the rhizosphere of *A. frigida* ($P < 0.05$). In contrast, the relative abundance of *Acidobacteria,*

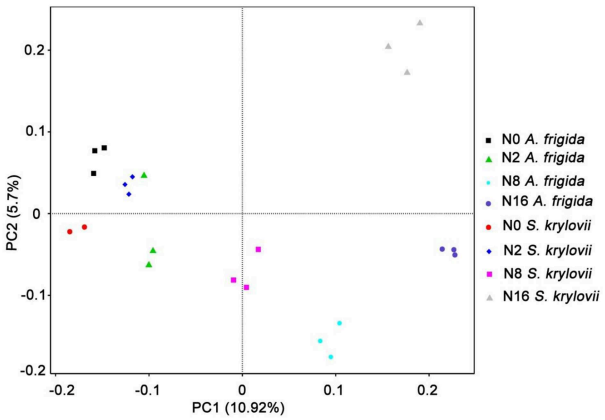

FIGURE 4 | Principle component analysis (PCA) of microbial communities based on OTUs for all samples from *S. krylovii* and *A. frigida* rhizosphere. The first two components were 10.68% and 5.46%, respectively.

Verrucomicrobia, Chloroflexi, and *WYO* in the rhizosphere of *S. krylovii* was lower than that in the rhizosphere of *A. frigida* ($P < 0.05$) (**Figure 5**). These results suggest that difference existed between the microbial communities in the rhizosphere of both *S. krylovii* and *A. frigida* in the control plot.

Nitrogen addition had significant impacts on the relative abundance of the phyla (**Figures 5**, **6**). With the increases in N addition rates, the relative abundance of *Bacteroidetes, Proteobacteria, Gemmatimonadetes, TM7, Firmicutes,* and *WYO* was enhanced in the *S. krylovii* rhizosphere (**Figures 6B,C,G,K–M**). A decrease in the relative abundance of *Acidobacteria, Actinobacteria, Chloroflexi,* and *Crenarchaeota* in the *S. krylovii* rhizosphere niche was detected (**Figures 6A,F,H,J**), while the relative abundance of *Verrucomicrobia, Planctomycetes,* and *Cyanobacteria* in the *S. krylovii* rhizosphere niche remained relatively unchanged (**Figures 6D,E,I**). With the increases in N addition rates, the relative abundance of *Bacteroidetes, Proteobacteria, TM7,* and *Firmicutes* in the *A. frigida* rhizosphere niche was increased (**Figures 6B,C,K,L**). Nitrogen addition had little effects on the relative abundance of *Verrucomicrobia, Actinobacteria, Gemmatimonadetes,* and *Cyanobacteria* in the *A. frigida* rhizosphere niche (**Figures 6D,F,G,I**). By contrast, the relative abundance of *Acidobacteria, Planctomycetes*, *Chloroflexi, Crenarchaeota,* and *WYO* in the *A. frigida* rhizosphere niche was reduced by N addition (**Figures 6A,E,H,J,M**).

Discussion

In this study, we found that 9-year N addition shifted plant community structure from co-dominance by a monocot grass, *S. krylovii*, and a dicot forb, *A. frigida,* to exclusive dominance by a monocot grass in an Inner Mongolia steppe (**Figure 1**). These results concurred with other studies which showed that N enrichment favors more for grass growth than for forb growth (Song et al., 2011; Fang et al., 2012; Zhang et al., 2014). The differential growth responses of the two plants to N addition

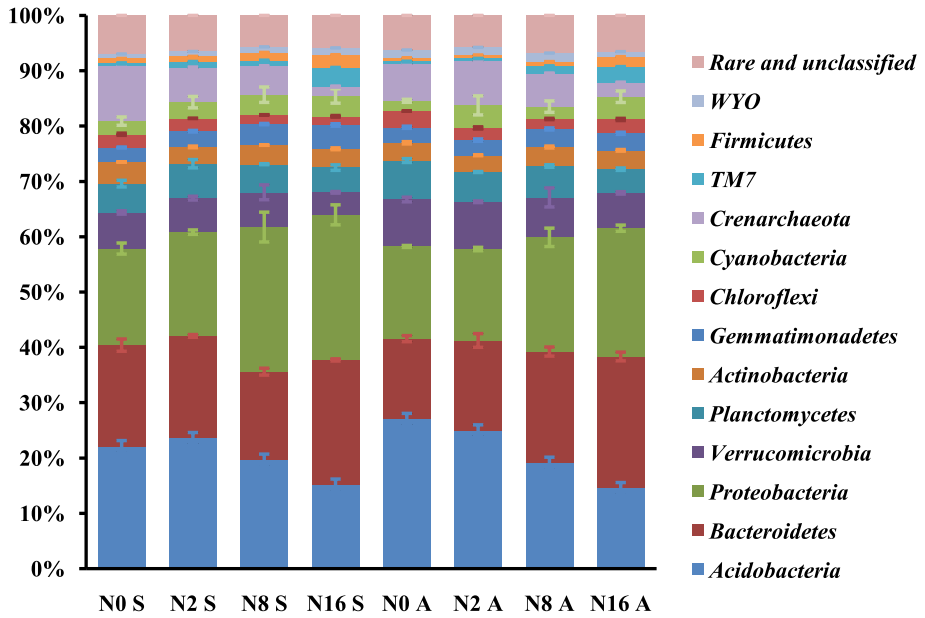

FIGURE 5 | The relative abundance of phyla of *S. krylovii* and *A. frigida* rhizosphere microbiome across all the N-supplied plots (% of total sequence).

prompted us to test whether N addition may also have different impacts on the microbial communities in the rhizosphere of the two species. We thus characterized microbial communities in the rhizosphere of the two dominant species, *S. krylovii* and *A. frigida,* under varying rates of N addition by high-throughput sequencing technique. Our results showed that the relative abundance of most microbial phyla in the rhizosphere of *S. krylovii* differed from that of *A. frigida* under control, ambient N conditions, suggesting that the microbiomes in the rhizosphere of the two species differ intrinsically. Bakker et al. (2013) have suggested that root exudates can explain the differences in the rhizospheric microbiomes of various plant species. In this context, our previous studies showed that *S. krylovii* and *A. frigida* differed in their rhizospheric processes, such that *S. krylovii* roots can exude greater amount of organic anions (malate and citrate) than *A. frigida* roots under ambient N conditions (Liu et al., 2014a). In addition, *Acidobacteria, Bacteroidetes,* and *Proteobacteria* were dominant microbial species in the two rhizosphere microbiomes, accounting for about 60% of the total microbiomes, implying that the three phyla may play important roles in the rhizosphere of the two species.

Several studies have reported that elevated N deposition profoundly impacts the soil microbial communities across different terrestrial ecosystems (Ramirez et al., 2010, 2012; He et al., 2013; Zhang et al., 2014). The microbes in the bulk soils have often been used to evaluate the effect of N addition on composition and biodiversity of bacterial community. However, in contrast to our studies, few studies have specifically investigated the effect of N addition on rhizospheric microbial communities. In a recent study, Zhu et al. (2015) reported that short-term N addition has minimal influence on rhizosphere effect of smooth crabgrass and bermudagrass by T-RFLP and analysis of enzyme activities. In the present study, we examined the rhizosphere microbiomes of *S. krylovii* and *A. frigida* across different N addition rates. We found that the OTU number, Shannon and Chao1 indices in the rhizospheric soils of the two dominant species showed a similar trend in response to N addition. Nitrogen addition reduced Shannon index in the rhizosphere of *S. krylovii* and *A. frigida*, indicating that the rhizospheric microbiomes of the two species became less diverse with increases in N-addition rates. In a similar study, Zhang et al. (2014) reported that long-term N addition had no effects on the overall OTU number of bulk soils in the Inner Mongolia steppe. Given the high heterogeneity of soils and co-existence of multiple plant species in a natural ecosystem, results obtained from monitoring microbes in the bulk soils may not truly reflect the changes in rhizospheric microbial communities. Roots are high in C, so the microbial community closer to the root may be copiotrophic relative to those in bulk soils (Nguyen, 2003; Badri et al., 2009; Gottel et al., 2011). We also found that the relative abundance of most phyla was altered in response to the long-term N addition. The relative abundance of all phyla except *Verrucomicrobia*, *Planctomycetes,* and *Cyanobacteria* in the rhizospheric microbiomes of *S. krylovii* was altered in response to the N addition. In the rhizospheric microbiomes of *A. frigida*, we found that the relative abundance of *Verrucomicrobia*, *Actinobacteria*, *Gemmatimonadetes,* and *Cyanobacteria* remained relatively unchanged by the same N addition. Furthermore, our results showed that the direction of the response of individual phyla to N addition was dependent upon host identity. These findings are in contrast to those previously reported results, where no comparable increases and decreases in response to N addition were observed in the same bacterial phylum of grasslands (Ramirez et al., 2010, 2012; He

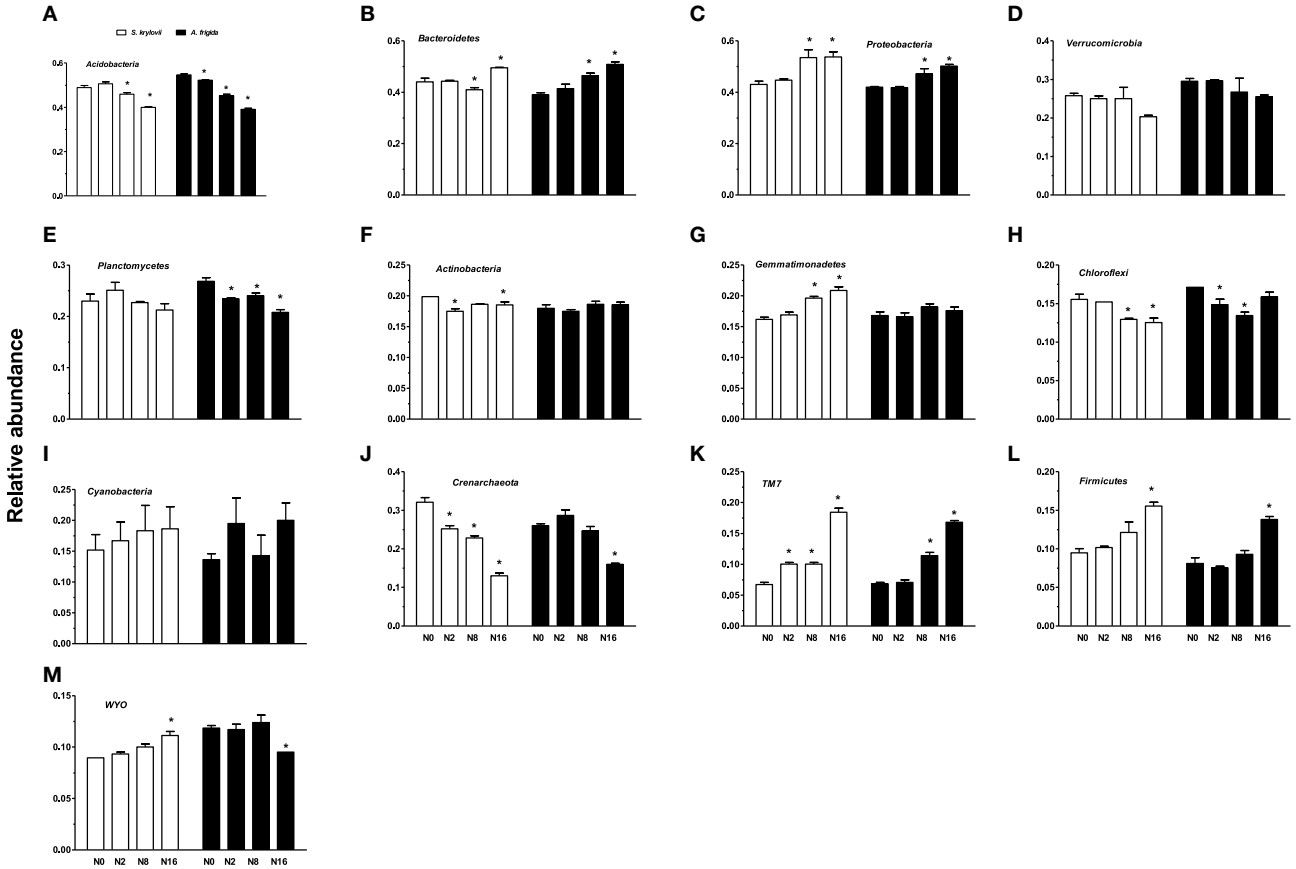

FIGURE 6 | Effect of N addition on the relative abundance of phyla A, of *S. krylovii* and *A. frigida* rhizosphere microbiome across all the N-supplied plots. (A) Acidobacteria, **(B)** Bacteroidetes, **(C)** Proteobacteria, **(D)** Verrucomicrobia, **(E)** Planctomycetes, **(F)** Actinobacteria, **(G)** Gemmatimonadetes, **(H)** Chloroflexi, **(I)** Cyanobacteria, **(J)** Crenarchaeota, **(K)** TM7, **(L)** Firmicutes, **(M)** WYO. Data are means ± s.e. ($n = 3$). Asterisks on the top of columns indicate significant difference at $P < 0.05$ between N0 and different rates of N addition for each species.

et al., 2013; Liu et al., 2014b). The differences in soil sampling methods (rhizospheric soils vs. bulk soils) between our studies and others may partly account for the different results. It is more likely that microbes in the rhizosphere simply behave differently from those in bulk soils.

It has been established that interaction can occur between plant communities and soil microbial communities (Zak et al., 2003; Dean et al., 2014). Many studies have attempted to link the above-ground plant diversity and productivity to below-ground bacterial diversities by characterizing soil bacterial communities (Zak et al., 2003; Dean et al., 2014). Rooney et al. (2006) showed that in agricultural grasslands the response of plants and bacterial communities to sheep urine deposition is dependent on both the concentration of synthetic sheep urine applied and the grass species. The rhizospheric microbial community associated with plant roots is highly diverse, and it is conceivable that the complex plant-associated microbial community is important for plant health (Kyselková et al., 2009; Berendsen et al., 2012). The rhizospheric microbial community may contribute to maintaining plant health directly by releasing pathogen inhibitors, or indirectly by promoting plant growth (Kyselková et al., 2009; Berendsen et al., 2012). More specifically, the phylum *Proteobacteria* is involved in cycling of essential mineral nutrients (Lesaulnier et al., 2008; Chaudhry et al., 2012). For example, Chaudhry et al. (2012) showed that the higher abundance of *Proteobacteria* may contribute to improved soil fertility and plant growth. It has been reported that the phylum *Verrucomicrobia* acts as inhabitants of paddy soil (Asakawa and Kimura, 2008; Do Thi et al., 2012). However, little is known about the function of *Verrucomicrobia* in the semi-arid grasslands. Although its specific physiological functions in the soil remain to be clarified, the wide occurrence of this predominant phylum *Acidobacteria* across all samples may indicate a key role in soil ecosystem functioning. *Firmicutes* is related to plant health and the high abundance of *Firmicutes* can threaten plant fitness (Berendsen et al., 2012; Zhang et al., 2014). These results may indicate that these phyla in rhizosphere soil may be associated with the shift of plant community structure from co-dominance by a grass, *S. krylovii*, and a forb species, *A. frigida*, to exclusive dominance by the grass.

The arbuscular mycorrhiza (AM) is the mutualistic symbiosis between terrestrial plants and fungi. Fungi may play an important

role in driving changes in plant biomass. Goomaral et al. (2013) reported that that *S. krylovii* increased in biomass as soil N availability increased, and this was associated with increased mycorrhizal colonization. Furthermore, altered fungal communities in response to environmental stressors in other systems have been linked to changing plant communities (Deslippe et al., 2012; Semenova et al., 2015). Therefore, further studies to investigate the involvement of fungi diversity and composition in N-induced shifts in species composition in the temperate steppe are warranted.

In the present study, to simulate the effects of N deposition on temperate grassland ecosystems, long-term N addition experiment was conducted in Inner Mongolia steppes by application of urea. The applied urea is first hydrolyzed to ammonia/ammonium by the enzyme urease in soils, and ammonium is further converted into nitrate by ammonia oxidizing bacteria and ammonia oxidizing archaea, leading to increases in inorganic N in soils (Zhang et al., 2012). Previous studies showed that application of urea in the Inner Mongolia steppes led to significant increases in soil nitrate concentrations (Fang et al., 2012). In addition, a similar reduction in plant species richness by addition of inorganic NH_4NO_3 in the Inner Mongolia steppes has been observed (Lan and Bai, 2012; Yang et al., 2012). Therefore, our N addition experiments with urea can simulate the natural N deposition.

In summary, our results demonstrated that long-term N addition had a profound influence on productivity of the steppe and composition of the rhizospheric microbial community of *S. krylovii* and *A. frigida*. These results highlight the importance of rhizospheric microbial communities in control and/or feedback of N addition-invoked steppe communities in response to N deposition. Our findings would contribute to our mechanistic understanding of impacts of N deposition on grassland ecosystem by linking changes in plant traits to their rhizospheric microbes-mediated processes.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Appendix

TABLE A1 | The number of reads before and after quality filtering.

Sample		Before quality filtering	After quality filtering
N0 *S. krylovii*	1	151330	147221
	2	62531	60743
N2 *S. krylovii*	1	37876	36842
	2	88768	86471
	3	71252	69278
N8 *S. krylovii*	1	68660	66952
	2	51083	49787
	3	122252	118084
N16 *S. krylovii*	1	100960	97848
	2	118645	115232
	3	54686	53313
N0 *A. frigida*	1	83187	80841
	2	159845	155362
	3	80904	78636
N2 *A. frigida*	1	102194	99208
	2	77534	75623
	3	80417	78241
N8 *A. frigida*	1	72041	70274
	2	72393	70461
	3	72187	70604
N16 *A. frigida*	1	91416	89042
	2	81153	79019
	3	84889	82649

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Adaptation strategies of endolithic chlorophototrophs to survive the hyperarid and extreme solar radiation environment of the Atacama Desert

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The Atacama Desert, northern Chile, is one of the driest deserts on Earth and, as such, a natural laboratory to explore the limits of life and the strategies evolved by microorganisms to adapt to extreme environments. Here we report the exceptional adaptation strategies of chlorophototrophic and eukaryotic algae, and chlorophototrophic and prokaryotic cyanobacteria to the hyperarid and extremely high solar radiation conditions occurring in this desert. Our approach combined several microscopy techniques, spectroscopic analytical methods, and molecular analyses. We found that the major adaptation strategy was to avoid the extreme environmental conditions by colonizing cryptoendolithic, as well as, hypoendolithic habitats within gypsum deposits. The cryptoendolithic colonization occurred a few millimeters beneath the gypsum surface and showed a succession of organized horizons of algae and cyanobacteria, which has never been reported for endolithic microbial communities. The presence of cyanobacteria beneath the algal layer, in close contact with sepiolite inclusions, and their hypoendolithic colonization suggest that occasional liquid water might persist within these sub-microhabitats. We also identified the presence of abundant carotenoids in the upper cryptoendolithic algal habitat and scytonemin in the cyanobacteria hypoendolithic habitat. This study illustrates that successful lithobiontic microbial colonization at the limit for microbial life is the result of a combination of adaptive strategies to avoid excess solar irradiance and extreme evapotranspiration rates, taking advantage of the complex structural and mineralogical characteristics of gypsum deposits—conceptually called "rock's habitable architecture." Additionally, self-protection by synthesis and accumulation of secondary metabolites likely produces a shielding effect that prevents photoinhibition and lethal photooxidative damage to the chlorophototrophs, representing another level of adaptation.

Keywords: Atacama Desert, carotenoids, endolithic chlorophototrophs, extreme environment, gypsum, scytonemin

Introduction

In the inhospitable environment of extremely arid deserts, microbial life has found refuge in very specific microhabitats (Pointing and Belnap, 2012; Wierzchos et al., 2012a; de los Ríos et al., 2014a). One of these microhabitats is the endolithic habitat, which consists of a network of pores and fissures connected to the surface within translucent rock (Nienow, 2009). Endolithic colonization can be viewed as a stress avoidance strategy, where the overlying mineral substrate provides efficient protection from incident lethal UV radiation and excessive photosynthetically active radiation, thermal buffering, protection from freeze–thaw events, physical stability and enhanced moisture availability (Walker and Pace, 2007; Pointing and Belnap, 2012; Wierzchos et al., 2012a; de los Ríos et al., 2014a). Remarkable examples of lithic habitats are found in the hyperarid zone of the Atacama Desert, in northern Chile. These habitats are as diverse as the insides of halite (Wierzchos et al., 2006; de los Ríos et al., 2010; Stivaletta et al., 2012; Robinson et al., 2015), gypsum (Dong et al., 2007; Wierzchos et al., 2011; DiRuggiero et al., 2013; Vítek et al., 2013; Ziolkowski et al., 2013a), carbonate rocks (DiRuggiero et al., 2013) and volcanic rocks (Wierzchos et al., 2013). The Atacama Desert is known as one of the driest places on our Planet with scarce precipitations as low as 3 mm y^{-1} in its hyperarid core (Houston and Hartley, 2003; McKay et al., 2003; Hartley et al., 2005; Houston, 2006a,b). However, hyperaridity is not solely due to a lack of rainfall (P); it is also related to potential evapotranspiration (PET) and may be defined as a ratio of P/PET of less than 0.05 according to United Nations Environment Program. By this definition, virtually the whole area of the Atacama Desert, between 15 and 30°S and at elevations up to 3500 m a.s.l., may be considered as hyperarid (Houston and Hartley, 2003).

In addition to its extreme dryness, the Atacama Desert holds records for other extreme environmental characteristics. The highest surface ultraviolet (UV) radiations and total solar irradiances ever measured on Earth have been reported from its high altitude "Altiplano" area (Piacentini et al., 2003; Cordero et al., 2014) and pre-Andean Domeyko Cordillera (Rondanelli et al., 2015). The prevalent cloudless conditions and relatively low total ozone column in the Atacama Desert result in extremely high solar radiation, increasing considerably the probability of lethal photoinhibition and photooxidative damage to phototrophs (Solovchenko and Merzlyak, 2008). Adaptation strategies for protection against high solar irradiance were observed in several microorganisms from the Atacama Desert. For example, the thallus of the lichen *Acarospora schleicheri*, which is found in the Altiplano area of Chile, accumulates rhizocarpic acid upon exposure to high levels of UV-B radiation (Rubio et al., 2002). Biomineralization of amorphous silica (sinter) around cyanobacteria filaments has been reported as a significant irradiation shield from the El Tatio geothermal field at 4300 m a.s.l. in the Andes Mountains (Phoenix et al., 2006). A different strategy to avoid stressful light conditions was found within the endolithic habitat of halite (NaCl) rocks. The *Halothece* cyanobacteria (Wierzchos et al., 2006; de los Ríos et al., 2010; Robinson et al., 2015), a unique cyanobacteria colonizing halites synthesizes large amounts of scytonemin, a well-known UV protective molecule (Vítek et al., 2010, 2012, 2014).

When both extreme desiccation and high ambient UV fluxes are combined, such as in areas of the hyperarid core of the Atacama Desert, the surface of rocks can be rendered sterile (Cockell et al., 2008). Under these extreme conditions, a millimeter-thick layer of gypsum has been found sufficient to protect microorganisms from UV-induced killing (Cockell et al., 2008). Microporous and translucent gypsum formations have been reported as lithic substrate harboring endolithic microbial communities in different climates such as moderate (Boison et al., 2004), polar and subarctic (Hughes and Lawley, 2003; Parnell et al., 2004; Edwards et al., 2005; Cockell et al., 2010; Ziolkowski et al., 2013b; Rhind et al., 2014), dry and arid (Stivaletta and Barbieri, 2009; Stivaletta et al., 2010), and dry and hyperarid (Dong et al., 2007; Wierzchos et al., 2011; DiRuggiero et al., 2013; Vítek et al., 2013; Ziolkowski et al., 2013a). In many of the above studies, translucent gypsum was reported to allow photosynthesis while providing protection from excess solar irradiance and retaining sufficient moisture for microorganisms survival. These factors may account for the high biodiversity found in gypsum-hosted communities (see Supplementary Table S1 for a summary of gypsum endolithic communities). However, there is a lack of knowledge regarding the microorganisms vertical organization or the distribution of photosynthetic pigments and secondary metabolites within endolithic community. No studies have addressed the effect of light gradients, or other characteristics of the lithic substrate, on the spatial localization of the different microbial taxa inhabiting this extreme environment.

The aim of the present work was to elucidate exceptional adaptation strategies of chlorophototrophic (i.e., chlorophyll-based phototrophs) microorganisms colonizing the insides of gypsum deposits in the hyperarid zone of the Atacama Desert. These endolithic microbial colonies are taking advantage of the physical and structural properties of translucent gypsum deposits containing sepiolite inclusions. Using a combination of microscopic and spectroscopic analytical methods, molecular and phylogenetic analyses, and micromorphological studies, we provide a detailed characterization of the chlorophototrophic microorganisms colonizing the gypsum endolithic habitat. Together with long-term microclimatic data and the composition and structure of the mineral substrate, we constructed a conceptual model of the vertical succession of microorganisms, together with the distribution of their photosynthetic pigments and secondary metabolites, within gypsum deposits under extremes of aridity and solar radiation.

Materials and Methods

The following is the summary of methods used in this study. More detailed information is provided in Supplementary Materials.

Site Characterization and Sampling

The sampling zone (23°53′S, 068°08′W and 2720 m a.s.l.) was located in the south of the Salar de Atacama basin (**Figure 1A**) in

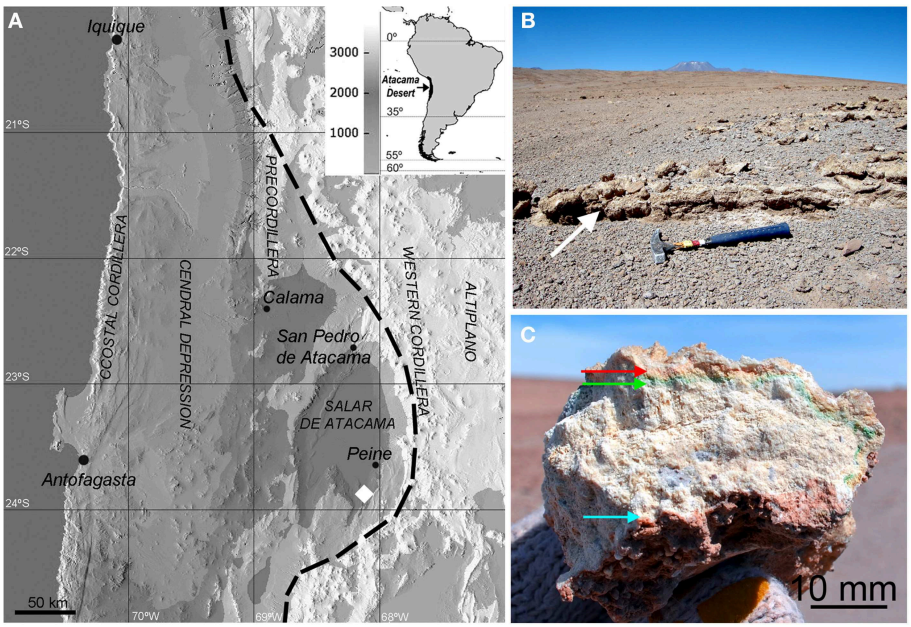

FIGURE 1 | (A) Shaded relief digital elevation map (DEM) showing the sampling area south of the Salar de Atacama basin (white diamond) and the geomorphic units of the central Atacama Desert; dashed line is the limit of the hyperarid area (Houston and Hartley, 2003). **(B)** Sampling area showing gypsum deposits (white arrow); the Socompa volcano is seen on the horizon. **(C)** Cross section of gypsum bearing cryptoendolithic (red and green arrows) and hypoendolithic (blue arrow) microbial colonization.

the north-south trending depression of the Cordon de Lila range, in northern Chile. This depression is mostly covered by volcanic ash material but large gypsum outcrops (1 × 2 m) can be found in several locations (**Figure 1B**). In the field, gypsum deposits were assessed for endolithic colonization by visual inspection of microbial pigments present in fractured samples. These pigments indicate the presence of cryptoendolithic (occupying pore spaces beneath rock surface, Nienow, 2009) and hypoendolithic (colonizing the undermost layer of the rock, sensu Wierzchos et al., 2011) microbial colonization forming horizons beneath the surface and close to the bottom of the gypsum deposits (**Figure 1C**). Colonized gypsum samples (10 × 10 cm) were collected in 2013 in the Cordon de Lila area, sealed in sterile Whirlpacks®, and stored at room temperature in a dark and dry environment until analysis.

Environmental Data Acquisition

Microclimate data were collected 6 km from the sampling site for 39 months (37 for T and RH), from January 20th, 2010 to April 20th, 2013, using an Onset HOBO® Weather Station Data Logger (H21-001) connected to a SolarStream® solar-powered transmitter for data transmission by Iridium Satellite Constellation. Sensors recorded measurements every 30 min intervals. Air relative humidity (RH) and temperature (T) were recorded 25 cm above the rock surface (probe was shaded from the sun) using RH/T sensors (HOBO® S-THB-M002; precision, ±2.5% RH and ±0.2°C T). Solar flux was measured using a photosynthetically active radiation (PAR) sensor for wavelengths of 400–700 nm. PAR data were also indicative of cloud cover at the sampling site. Rainfall was monitored using a Rain-o-Matic 100 (PRONAMIC ApS, Herning, Denmark) tipping bucket gauge (resolution of 1 mm). The presence of liquid water on the gypsum surface was determined by electrical conductivity (EC) using sensor as described by Wierzchos et al. (2013) (see also Supplementary Section S1).

Mineralogy X-ray Diffraction Analyses

The mineralogical composition of the gypsum duricrust was identified by X-ray powder diffraction (XRD) using a Philips X'Pert diffractometer (Philips, Amsterdam, The Netherlands) with graphite-monochromated CuKα radiation (see detailed procedures in Supplementary Section S2).

Petrographic Microscopy

Petrography studies of gypsum duricrust thin sections (30 μm-thick) were conducted using a Nikon Eclipse LV100Pol (Nikon, Tokyo, Japan) polarized light microscope equipped with a Nikon DS-Fi1 digital camera.

Mercury Intrusion Porosimetry

Pore space and pore size distribution of the gypsum samples were characterized by mercury intrusion porosimetry (MIP). Three types of subsamples were analyzed: samples from the surface dense crust (above the colonization zone), samples from the interior of the gypsum deposits (within the colonization zone), and samples from the bottom dense crust (covering the hypoendolithic colonization zone). A PoreMaster 60/Quantachrome (Quantachrome Instruments, Boynton Beach, USA) instrument was used to determine porosity (connected

porosity) of the rock samples in the pore diameter range 0.0036–190.58 μm. A mercury surface tension value of 0.48 N/m and rock-mercury contact angle of 140 dag were used in the Washburn or Laplace equations, respectively.

Measurements of Light Conditions within Gypsum Deposits

The photosynthetic photon fluence rate (PPFR) was measured beneath the gypsum surface at different depths (up to 1.5 mm) with a small (3.5 mm in diameter) spherical sensor. These measurements were performed on wetted gypsum after simulation of a 10 mm rainfall to measure light conditions, inside the rock, when the presence of water would allow photosynthetic activity. Measurements for UVA and UVB radiation beneath the gypsum surface at different depths (up to 1.5 mm) were performed using cosine corrected sensors. Detailed procedures are provided in Supplementary Section S3.

Fluorescence Microscopy (FM)

Small fragments of gypsum showing distinct signs of endolithic colonization with a green/orange layer beneath the rock surface and a green layer just above the bottom of the gypsum sample were scraped and suspended in double-distilled water. Suspensions were vortexed (2 min) and allowed to settle for 1 min before removing the supernatant, which was centrifuged for 10 min at 5000 g. The pellet was then stained with SYBR Green I (SBI) (Molecular Probes), a fluorocromes used for the specific staining of nucleic acids. Bright field images, SBI fluorescence and photosynthetic pigment autofluorescence were visualized in a Zeiss AxioImager D1 fluorescence microscope (Carl Zeiss, Jena, Germany). Detailed procedures are provided in Supplementary Section S4.

Scanning Electron Microscopy in Backscattered Electron Mode (SEM-BSE)

Colonized gypsum samples were processed for SEM-BSE and energy dispersive X-ray spectroscopy (EDS) microanalysis as described in Wierzchos et al. (2011). As the intensity of the BSE signal depends on the mean atomic number of the sample, the SEM-BSE technique identifies heavy metal-stained ultrastructural elements of microbial cells. SEM-BSE was then used in combination with EDS to characterize the minerals associated with specific cell aggregates. Gypsum samples were observed using a scanning electron microscope (DSM960 Zeiss, Oberkochen, Germany) equipped with a solid-state, four diodes BSE detector plus an auxiliary X-ray EDS microanalytical system (Link ISIS Oxford, UK).

Transmission Electron Microscopy (TEM)

Fragments of colonized gypsum material were processed for transmission electron microscopy. Ultrathin sections were visualized using EM910 (Leo, Oberkochen, Germany), CM200 Philips (Philips, Amsterdam, The Nederlands) and analyzed using energy dispersive X-ray spectroscopy (EDS) microanalysis. Detailed procedures are provided in Supplementary Section S5.

Low Temperature Scanning Electron Microscopy

We characterize the features of gypsum deposits and internal micromorphology of microbial cells in their natural hydrated state using low temperature scanning electron microscope (LT-SEM) according to procedures described by Wierzchos et al. (2012b). The LT-SEM (DSM960 Zeiss, Oberkochen, Germany) was equipped with a cryotransfer system (CT1500, Oxford, UK) and with imaging systems using secondary electrons (SE) and backscattered electrons (BSE) detectors, and with an X-ray energy dispersive spectroscopy (EDS) system.

Algal Culturing and Micromorphological Characterization

Fragments of gypsum samples bearing the green/orange colored cryptoendolithic colonization were visualized by stereoscopic microscope with aim to find algal colonies. Small rock fragments inhabited by algae were mechanically scrapped off into Petri dishes poured with agarized BBM (Deason and Bold, 1960) and BG-11 mineral media (Stanier et al., 1971). Algae were inoculated at 20°C under a 14 h/10 h light/dark regime using photon irradiance of approximately 30–50 μmol photons m^{-2} s^{-1} provided by 18 W cool fluorescent tubes (Philips TLD 18W/33). After 5–10 weeks, the agar plates were checked for the presence of algal microcolonies. The algae were investigated using an Olympus BX51 (Olympus, Tokyo, Japan) light microscope with differential interference contrast (DIC). Microphotographs were taken with an Olympus Z5060 digital camera.

Microbial Community Analyses

Environmental DNA was extracted from the top colonization zone of two individual gypsum rocks using the PowerSoil DNA isolation kit (MoBio laboratories Inc., Solana Beach, CA) as described in Robinson et al. (2015). DNA was amplified using the barcoded universal primers 27F and 506R for the V1–V3 hypervariable region of the 16S rRNA gene and amplicons from 3 reactions were pooled together for sequencing using the Illumina MiSeq platform by the Genomics Resource Center (GRC) at the Institute for Genome Sciences (IGS), University of Maryland School of Medicine (Robinson et al., 2015). We obtained a total of 3326 paired-end reads for two rock samples (CL1 and CL2) collected from the same location, with an average size of 436 bp. The QIIME package (v1.6.0) was used to process the Illumina paired-end reads as previously described (Robinson et al., 2015). Sequence reads were normalized to 1662 reads per sample and diversity metrics were calculated based on OTUs at the 0.03% cutoff in QIIME (Caporaso et al., 2010). Nonparametric Mann-Whitney tests were used for statistical analysis. We use the following 18S rRNA primer sets to obtain nuclear sequences from the algae: 82F-1498R (Bachy et al., 2011); ChloroF-ChloroR and BaciF-BaciR (Valiente Moro et al., 2009); Euk328F-Euk329R (Romari and Vaulot, 2004); EUK528f-CHLO02r (Zhu et al., 2005).

Analyses of Photosynthetic Pigments by Raman Spectroscopy

Cryptoendolithic and hypoendolithic colonization zones, harboring phototrophic eukaryotes and prokaryotes, were

examined using point Raman analysis on a Thermo Fisher DXR Raman microspectrometer (Thermo Scientific, Waltham, USA) with a 532 nm diode laser and a Renishaw InVia Reflex Raman microspectrometer using both 785 nm diode and 514.5 nm Ar laser. The later Raman system was used to construct Raman distribution maps of carotenoids within the algal zone using 514.5 nm laser line. The analyses were performed on freshly cut transects of the gypsum substrate. The Raman spectroscopy technique was chosen for its *in situ* approach with small amount of biological material. Detailed procedures are provided in Supplementary Section S6.

Spectrophotometric Analyses

The absorption spectra of acetone extracted photosynthetic and light-protecting pigments (chlorophyll a and b, and carotenoids) were obtained using a HP 8452A Diode Array dual-beam spectrophotometer (Hewlett-Packard, Tokyo, Japan). Absorbance values for characteristic wavelength (maximum peaks for particular pigments) were selected for semiquantitative determination of pigments contents [mg/g of the dry weight (DW) in powdered sample] using the trichromatic equations and extinction coefficients (Lichtenthaler, 1987). Detailed methodology sample processing and pigment extraction are provided in Supplementary Section S7.

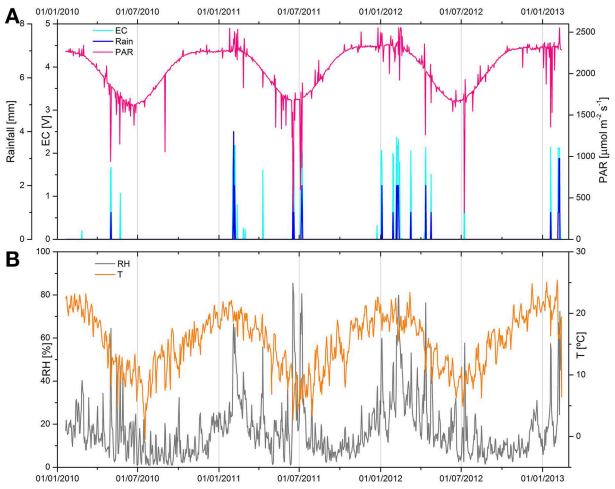

FIGURE 2 | Microclimate data collected from January 20th, 2010 to February 20th, 2013 nearby the Cordon de Lila sampling site. (A) Maximum daily photosynthetically active radiation (PAR, purple line), rainfall precipitations (blue bars indicating mm of rainfall), and cyan bars indicating moisture episodes identified by increase in electrical conductivity (EC) values. **(B)** Medium daily temperature (T, orange line) and medium daily relative humidity (RH, gray line) oscillations.

Results

Microclimate Data

Microclimatic parameters were recoded with a microweather station in close proximity to the sampling site over 39 months (37 for T and RH measurements), starting in January 2010. The maximum daily photosynthetically active radiation, rainfall precipitations, and electrical conductivity measured on the gypsum gypcrete surface, as well as mean the daily air relative humidity and mean daily air temperature oscillation measured at the sampling site are shown in **Figure 2**. Some of this data (first 22 months) was previously reported by Wierzchos et al. (2013). PAR values revealed a very intense solar irradiance over the 3-year period of recording (**Figure 2A**). The mean daily PAR value was 1175 µmol photons m^{-2} s^{-1} and during the austral summer (January, $n = 4$) the mean maximum daily value was as high as 2298 µmol photons m^{-2} s^{-1}. For a number of days, usually in February, spikes of extremely high PAR values, up to 2554 µmol photons m^{-2} s^{-1}, were recoded (**Figure 2A**). These spikes likely originated from light scattering by cumulus cloud formation (Piacentini et al., 2003), although cloudy days were very scarce.

The sum of recorded rainfall precipitation over the three-year period was 88 mm and represented 19 separate episodes (**Figure 2A**). The annual mean precipitation was 27.1 mm y^{-1} with 61% of rain precipitations occurring during the summer months (January-March). The maximum daily rain precipitation was observed on 17th June 2011 with a total of 14 mm of rain between 8:30 a.m. and 11:00 p.m. EC sensors detected liquid water on the gypsum surface mostly in the late evening and until about midnight (**Figure 2A**). Most of these events corresponded to rainfall episodes with the exception of 7 events where moisture was detected by EC readings with no rainfall gauge reading. These 7 events could be the result of intermittent rainfalls below the detection limit of rainfall gauge (<1 mm). Moisture on the rock surface persisted several hours after the rainfall (see Supplementary Figure S1). The total time of moisture at the rock surface, as detected by the EC sensor, was 638 h for the 39 months periods, giving a value of 196 h y^{-1} (about 8 days y^{-1}). These values represent surface water and it is likely that moisture was available inside the gypsum rock for longer periods. Time periods between moisture events were exceptionally long with a maximum of 254 days in 2010. Analyses of our microclimate data indicated that rainfall was the only source of liquid water potentially available for the gypsum microbial communities.

Oscillation of mean daily air temperatures was in accordance with seasonal changes of solar irradiance (**Figure 2B**). The mean T for the 37 month period was 15.3°C. The mean daily maximum T in summer (January-February 2013) was 43.6°C with a maximum of 49.3°C. The minimum recorded T was −7.4°C. Air RH values were remarkably low over the entire recording period. Mean annual RH for 3 years was as low as 16.5% with mean winter and spring seasonal RH values between 7 and 11%. RH above 80–90% was only observed during short and infrequent rainfall episodes.

Mineralogical and Physical Characterization of Gypsum-composed Gypcrete

Gypsum formations at the Cordon de Lila sampling site appear as hard layer deposits on the soil surface, interbedded between layers of ignimbrites and/or filling large cracks on these rocks. Volcanic ashes cemented by gypsum have been previously reported in this region of the Atacama Desert (Bao, 2005). This type of formation is called gypcrete according to the

nomenclature by Horta (1980). The XRD analysis of Ca-sulfate deposits revealed gypsum ($CaSO4{\cdot}2H_2O$) as the major mineral (96–98%) with minor amounts of cristobalite (SiO_2), calcite ($CaCO_3$), and potassium feldspar ($KAlSi_3O_8$). A clay mineral with X-ray reflection at 12.2 Å was identified as sepiolite by *in situ* qualitative and quantitative EDS coupled to high resolution TEM (see Supplementary Figure S2). Study of thin sections with petrographic microscopy (results not shown) revealed dense morphologies with highly compacted large lenticular gypsum crystals in the surface layer (up to 2 mm deep) and in the bottom layer (2–3 mm) of the gypsum deposit. The appearance of these compact layers was indicative of gypsum dissolution and recrystallization processes. The interior of the sample consisted of microcrystalline (50 μm long) lenticular gypsum crystals crossed by dense horizontal bands (0.3–1 mm-wide) of verticalized sub-prismatic gypsum crystals and lenticular gypsum crystals. Sequences of vertically-elongated pores (<2 mm-wide) and gypsum "columns" (0.1–1 mm-wide) were also observed within the interior of the gypsum deposits. These columns were formed by anhedral gypsum crystals and presented evident dissolution features. These petrological observations were consistent with porosity analyses performed with the MIP technique. MIP data showed a total porosity for connected pores (for a 0.0036 μm to 190.58 μm range) of 29.9% ($n = 4$, $SD = 3.9$) for the surface layer, 56% ($SD = 10.1$) for the rock interior, and 25.6% ($SD = 3.8$) for the bottom layer of the gypsum. Pore size distribution curves revealed larger pore diameters, between 0.1 and 190.58 μm, for the gypsum interior than for the surface and bottom layers (**Figures 3A–A″**). The total intrusion volume of mercury was about 0.08 ml g^{-1} for the surface and bottom layers and 0.31 ml g^{-1} for interior of the gypsum deposit. Together, these data support a significantly higher porosity for the interior than for the external layers of the gypcrete.

The transmitted PAR value within the gypsum colonization zone at a depth of 2–5 mm, we measured was 0.1–1% of that of the incident PAR light (**Figure 3B**). Using 2700 μmol photons m^{-2} s^{-1} as the incident PAR value for a sunny day we calculated an absolute PPFR value between 0.2 and 30 μmol photons m^{-2} s^{-1} within the cryptoendolithic colonization zone of the gypsum deposit. No UVA or UVB radiation was detected across a 1.5 mm-thick gypsum layer.

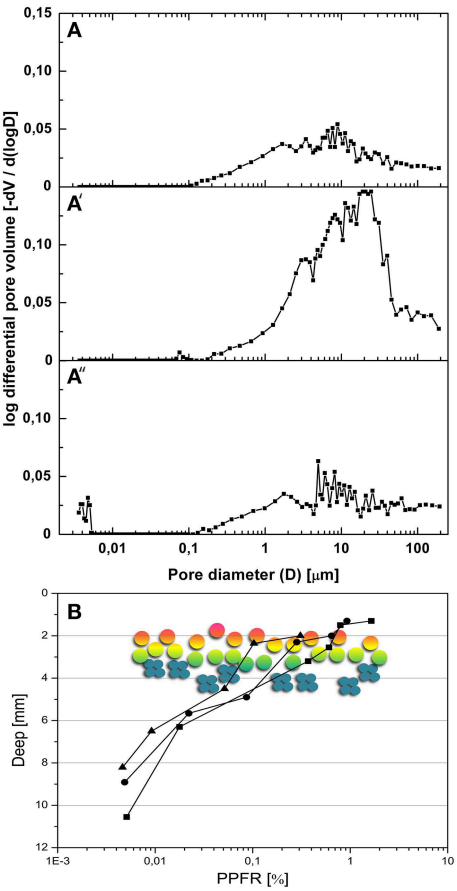

FIGURE 3 | Porosity and light transmission properties of gypsum deposits. (A–A″) pore size distribution curves for surface layer **(A)**, interior **(A′)** and bottom layer **(A″)**; mean curves for four samples. **(B)** Intensity of photosynthetic photon fluence rate (PPFR) within gypsum wet deposits at different depths ($n = 3$); hypothetical position of cryptoendolithic colonization zone is shown by the schematic positioning of green-orange algae (circles) and cyanobacteria (small cyan discs).

Characterization of Endolithic Microbial Communities

Characterization of Endolithic Microbial Communities by Microscopy

In field fracturing of gypsum deposits revealed an abundant microbial colonization, which was clearly visible as a thick 2–6 mm orange-green layer beneath the gypcrete surface (**Figure 4A**). Closer examination of this cryptoendolithic habitat shows the presence of orange-colored algae cells close to the surface and green algae cells just below the orange layer of cells. This transition from green to orange cells was gradual as shown in **Figure 4A′**. DIC microscopy of orange mature algae showed a cytoplasm filled with orange-colored lipids and small loose chloroplasts surrounding the nucleus (**Figures 4B′,C,C′**). Chloroplasts within the green algae cells were denser and found throughout the cytoplasm (**Figures 4D,D′,E**). Green algae aggregate showed some cells with a lower density of chloroplast and starting to take orange color (**Figures 4E,E′**). In some places, it was also possible to observe cyanobacteria aggregates just beneath the algae layer (**Figure 4F**). Cyanobacteria aggregates were also found within the colonization zone at the bottom of the gypsum deposits (**Figure 4G**). This hypoendolithic habitat displayed a dense bottom layer and micro cave-shaped pores within the colonization zone (**Figure 4H**). Fluorescence microscopy revealed potentially viable cyanobacteria cells and cells with degraded photosynthetic pigments (Roldán et al., 2014) within this hypoendolithic habitat (**Figure 4G**).

The cryptoendolithic habitat appears beneath the 0.5–1 mm-thin hardened surface layer with dense large lenticular gypsum crystals, as shown by the SEM-BSE technique (**Figure 5A**). In many places, large aggregates (0.5–1 mm) of clay mineral identified as sepiolite (SEM-BSE-EDS results not shown) were also observed (dash outline in **Figure 5A**). Algal cells were

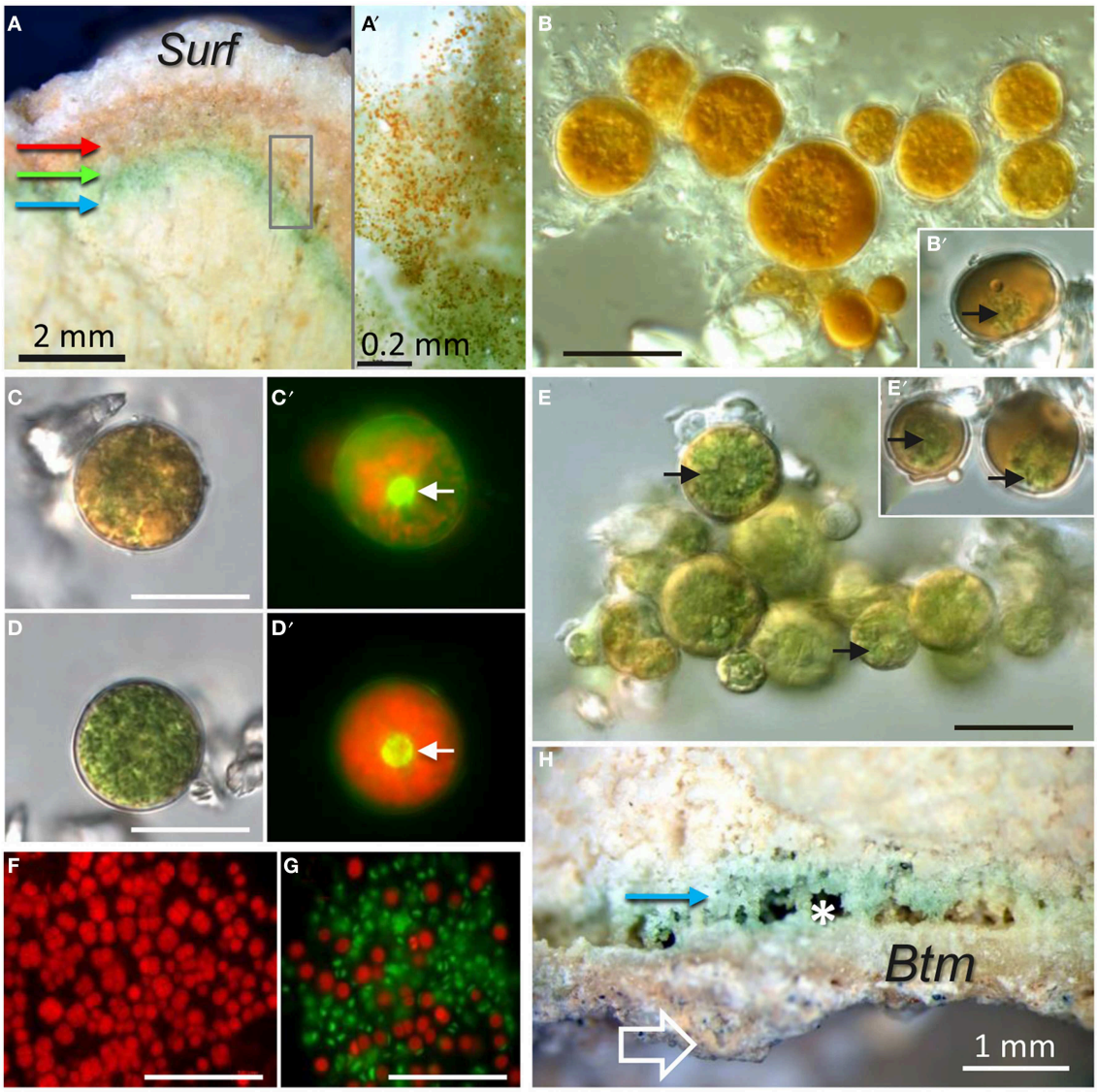

FIGURE 4 | Cross-sections of gypsum layered microbial endolithic colonization. (A) Stereoscopic microscopy view with details in **(A′)** of the orange-to-green cryptoendolithic colonization layer close to the gypsum dense surface (*Surf*); red, green, and blue arrows point to orange algae, green algae, and cyanobacteria colonization zones respectively. **(B)** DIC image of orange mature algae and **(B′)** DIC image insert where some relicts of loose chloroplasts within the mature alga can be distinguish. **(C)** DIC and **(C′)** FM image of orange algae with dispersed chloroplasts [red signal in **(C′)**] surrounding the alga nucleus [arrow in **(C′)**]. **(D,E)** DIC images and **(D′)** FM image of green algal cells with uniformly distributed dense chloroplasts in the whole cytoplasm [red signal in **(D′)**; arrow in **(D′)** point to nucleus and arrow in **(E)** point to chloroplasts; **(E′)** DIC image of green cells in the first step of the carotenogenesis process with less dense chloroplasts (arrows). **(F)** MF image of cryptoendolithic cyanobacteria with red autofluorescence signal. **(G)** FM image of hypoendolithic cyanobacteria; autofluorescence red signal that potentially belong to vital cells and green signal corresponding to cells with degraded photosynthetic pigments. **(H)** Stereoscopic microscopy view of hypoendolithic cyanobacteria colonization zone (blue arrow) within gypsum close to the bottom (arrow) of the rock. Note the dense structure of the bottom (*Btm*) layer and small cave-shaped pores in the hypoendolithic habitat (asterisk). Scale bars = 20 μm, except **(A,A′,H)**.

observed between smaller gypsum crystals, just beneath the hardened surface layer. A detailed view of these algae with a cytoplasm filled by lipoidal globules is shown in **Figure 5B**. Algal cells found deeper in the cryptoendolithic zone contained fewer lipoidal globules and their cytoplasm was mostly filled by chloroplast structures (**Figures 5C–F**). Cyanobacteria cells were mostly found close to sepiolite aggregates (**Figure 5G**) beneath the green algae layer.

Similarly, to DIC microscopy analyses (**Figures 4B**, **6A**), visualization of algae from the orange zone with TEM showed abundant lipoidal globules occupying almost the entire cell (**Figure 6B**). These algae displayed a thick (about 0.5 μm) cell wall. TEM of algae subjacent zone revealed cyanobacteria cells surrounded by electron dense structures, forming concentric sheaths of polysaccharides and abundant extracellular material embedding heterotrophic bacteria (**Figure 6C**). Our TEM

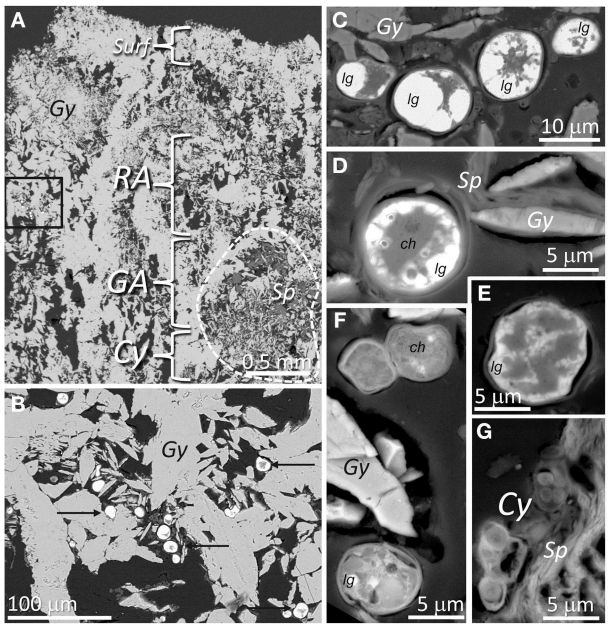

FIGURE 5 | SEM-BSE images of the gypcrete top zone with cryptoendolithic microbial habitat. (A) Image shows surface (*Surf*) dense layer, orange-color algae colonization zone (*RA*), green algae (*GA*) colonization zone, and cyanobacteria (*Cy*) colonization zone close to sepiolite (*Sp*) nodules; square mark area shown in detail on figure **(B)**, where black arrows point to alga cells between lenticular gypsum (*Gy*) crystals. **(C–F)** *In situ* images showing algal cells among gypsum (*Gy*) crystals from higher to lower position within the colonization zone with clearly distinguishable ultrastructural elements such as lipoidal globules (*lg*) and chloroplasts (*ch*). **(G)** Image of cyanobacteria cells (*Cy*) in direct contact with sepiolite (*Sp*).

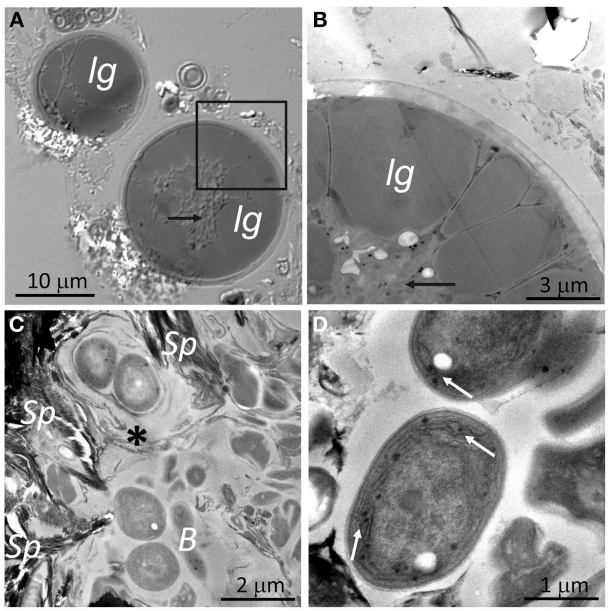

FIGURE 6 | DIC and TEM images of endolithic cells: (A) DIC image and (B) TEM image from zone marked by square in (A) showing algae from the orange zone with lipoidal globules (*lg*) within the cytoplasm (black arrows). (C) Image showing cyanobacteria from the cryptoendolithic zone surrounded by sheaths of polysaccharides (asterisk) with heterotrophic bacteria (*B*) and close to sepiolite floccules (*Sp*). **(D)** Cyanobacteria from the hypoendolithic habitat with thylakoids (arrows).

microscopic observations confirmed that the micromorphology of the gypsum cyanobacteria, in the top layer of the substrate, is similar to that of members of the *Chroococcidiopsis* genus (**Figure 6C**). Note that cyanobacteria from the cryptoendolithic habitat were always observed in close contact with floccules of sepiolite (**Figure 6C**). The mineralogical nature of this clay mineral was identified by *in situ* qualitative and quantitative EDS coupled to high resolution TEM (Supplementary Figure S2). **Figure 6D** shows *Chroococcales* cyanobacteria from the hypoendolithic habitat.

Visualization of cryo-fixed and cryo-fractured samples was essential in observing the gypsum microbial communities *in situ* in their "natural" state after sample rehydration (Ascaso and Wierzchos, 2002). It allowed for the characterization of internal structures in algae cells and that of the extracellular polysaccharidic substances (EPSs) surrounding microbial cells. LT-SEM of fractured algal cells from the green zone showed well defined chloroplasts filling almost the entire cytoplasm (**Figure 7A**). In contrast, algal cells from the orange zone accumulated lipoidal globules (**Figure 7B**). In many cases, we observed rod shaped (1–2 µm long) microorganisms (potentially heterotrophic bacteria) attached to the cell walls of algae (**Figure 7C**). Beneath the algae zone, we found aggregates of cyanobacteria (**Figure 7D**) and an accumulation of heterotrophic bacteria (**Figure 7E**). EPSs with different micromorphologies surrounded all cells. The EPSs associated with algal cells has a honeycomb-like structure (**Figure 7B**) whereas the EPSs in close-contact to cyanobacteria had a slime-like structure (**Figure 7D**). Both types of EPSs were loosely bound to the cells and did not take the shape of these cells. Groups of heterotrophic bacteria were surrounded by capsular-like polysaccharides (**Figure 7E**) that are distinguished according to their thickness and consistency (Rossi and De Philippis, 2015).

The structure of the gypsum hypoendolithic habitat, which did not contain algae, was significantly different than that of the surface cryptoendolithic habitat (**Figure 8**). Sizeable pores (0.2–2 mm in diameter) with compacted walls were located above a compact gypsum bottom layer (**Figure 8A**). The structure of these pores, together with the columns formed by lenticular gypsum crystals and with the dissolution features at the edges of the anhedral gypsum crystals, similar to the "Grating Fabric" defined by Artieda (2013), revealed distinct gypsum dissolution and crystallization processes. The microbial colonization in form of cyanobacteria aggregates covered the walls of the large pores and, unlike in the cryptoendolithic habitat, did not adhere to sepiolite nodules. Many cyanobacteria cell appeared empty and were most likely dead cells (**Figure 8B**).

Micromorphological Characterization of Algae

Growth of algal cells was only observed with solid BG-11 mineral medium. The growth was very slow and the great

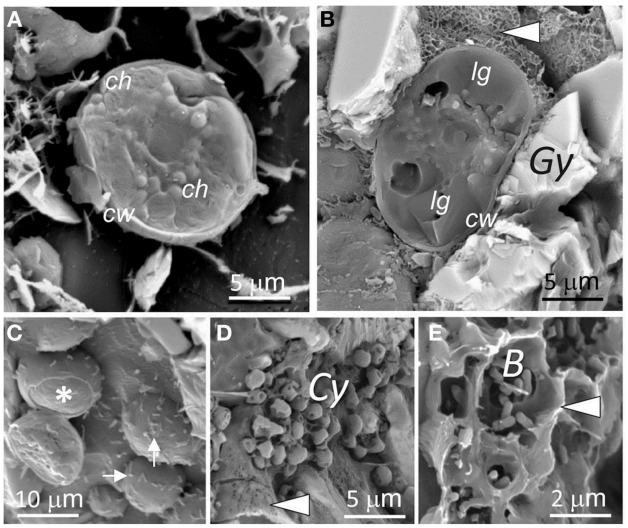

FIGURE 7 | LT-SEM images of endolithic microorganisms and extracellular polysaccharides. (A–C) Fractured algae cells with ultrastructural elements such as: cell walls (*cw*), chloroplasts (*ch*), lipoidal filling (*lg*), and gypsum particles (*Gy*); detached cell wall, asterisk; rod shaped microorganisms (potentially heterotrophic bacteria) attached to algae, white arrows; arrow head in **(B)** point to EPSs. **(D)** Image showing cyanobacteria (*Cy*) aggregates and slime EPSs (arrow head). **(E)** Image showing bacteria (*B*) evolved by capsular EPSs (arrow head).

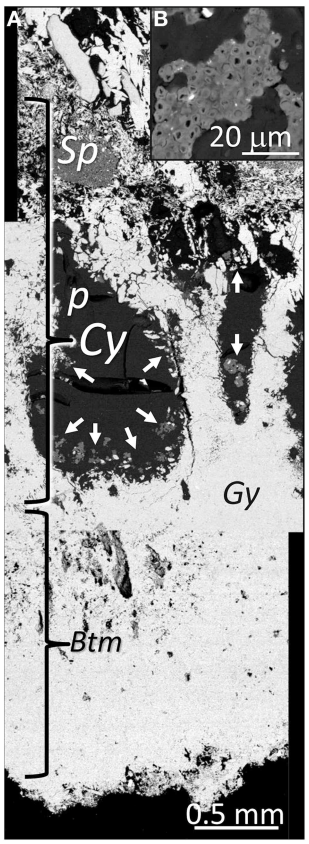

FIGURE 8 | (A) SEM-BSE image of the bottom part of the gypcrete colonized by hypoendolithic cyanobacteria (*Cy*) forming aggregates; pore, *p*; sepiolite nodule, *Sp*; arrows point to cyanobacteria aggregates; hypoendolithic cyanobacteria colonization zone, *Cy*; bottom layer, *Btm*; gypsum, *Gy*. **(B)** Details of cyanobacteria aggregate.

majority of algal cells died within a few weeks of their cultivation. The cultivated cells were globular, uninuclear, 28–50 μm in diameter and with smooth cell walls (**Figure 9A**). The cells possessed a reticulated chloroplast with a complex structure and often with several off-center pyrenoids (**Figure 9B**). Mature cells accumulated large quantities of orange-colored pigments (**Figure 9C**). Asexual reproduction occurred via 12–16 autospores, liberated by gelatinization of the maternal cell wall (**Figure 9D**). The autospores had irregular shape and size of 14–20 μm in diameter. They possessed a chloroplast divided into several polygonal pieces lacking pyrenoids (**Figure 9E**).

Molecular Characterization of Endolithic Microbial Communities

Taxonomic assignment revealed that the dominant phyla in both samples were *Cyanobacteria*, representing between 67 and 83% of all the sequence reads, and *Proteobacteria*, and *Actinobacteria* (**Figure 10A**). The only other photosynthetic phylum, in addition to *Cyanobacteria*, was that of *Chloroflexi* with 1.2–1.6% of sequence reads (**Figure 10A**). All diversity metrics, at a maximum sequencing depth of 1662 sequences per sample, revealed a higher diversity in sample CL1, with 260 OTUs at the 0.03% cutoff, when compared with sample CL2, with 167 OTUs (see Supplementary Table S2). Across the two samples, cyanobacteria included members of the *Chroococcales*, *Synechococcales*, *Pseudanabaenales*, and *Nostocales*; the most numerous were *Chroococcales* (64%) (mostly of the *Chroococcidiopsis* genus), followed by *Synechococcales* (18%), and unclassified *Cyanobacteria* (14%) (**Figure 10B**). No

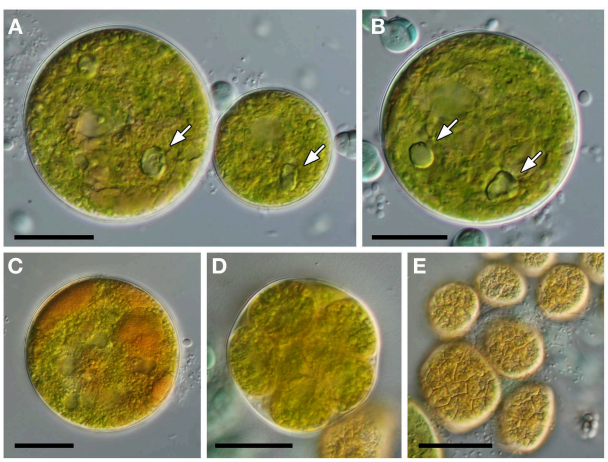

FIGURE 9 | Morphology of cultivated green alga. (A) Size variability of vegetative cells. **(B)** Mature cell with a complex chloroplast and with several pyrenoids marked by arrows (in **A**,**B**). **(C)** Accumulation of orange color pigments. **(D)** Mature autosporangium. **(E)** Irregularly shaped autospores shortly after their liberation. Scale bars = 20 μm.

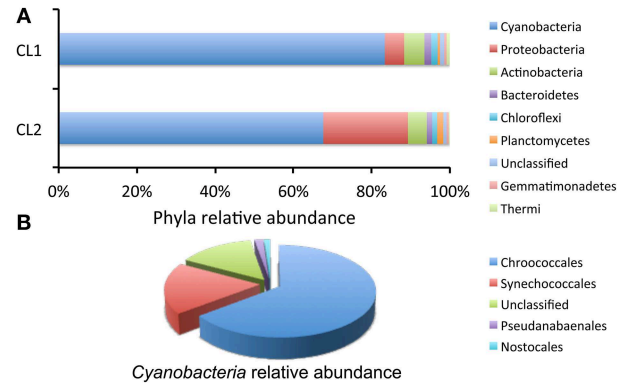

FIGURE 10 | Relative abundance of major taxonomic groups (>0.1% abundance) in the Atacama Desert gypsum community based on 16S rRNA gene sequences. (A) Relative abundance of major phyla from 2 samples (CL1 and CL2). Datasets were of equal size and subsampled at 1662 sequence reads. **(B)** Relative abundance of Cyanobacteria taxa in combined samples.

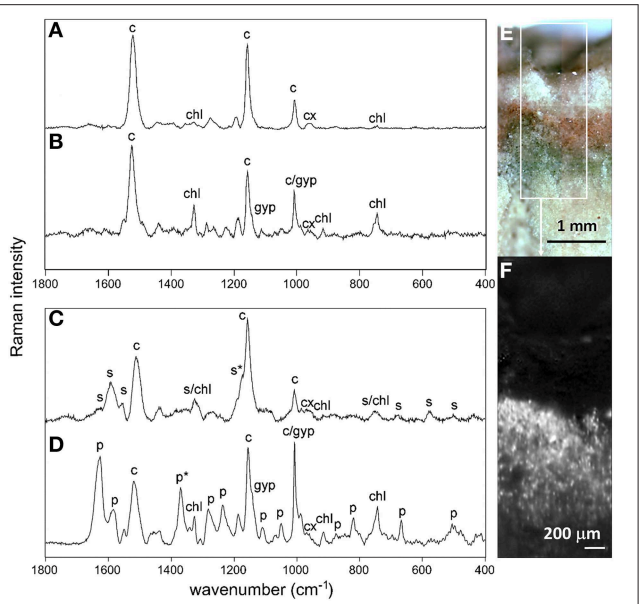

FIGURE 11 | Examples of Raman spectra of orange algal cell (A), green algal cell (B) and cyanobacterial cells from the hypoendolithic habitat containing scytonemin (C) and without scytonemin (D). Spectra were obtained by the 785 nm excitation source; c, carotenoid; cx, carotenoid-xanthophyll; chl, chlorophyll; p, phycobiliproteins; s, scytonemin; gyp, gypsum. Asterisks (*) indicate important corroborative bands for discrimination between scytonemin and phycobiliproteins. **(E)**, microscopy image of cryptoendolithic habitat colonized by orange and green algae; **(F)** Raman distribution map of carotenoid (white spots) of the marked square in **(E)**. For examples of Raman spectra extracted directly from the image see Supplementary Figure S5.

chloroplast sequences from algae were detected indicating a considerable phylogenetic distance away from the *Cyanobacteria* or potential primer mismatches with the plastid's 16S rRNA genes. This was not the case in a previous study were we obtained amplicons from the 16S rRNA gene of the chloroplast from an algae related to the *Dolichomastix* genera (*Mamiellaceae* family) with this same primer set used in this study (Robinson et al., 2015). Despite multiple attempts at PCR amplification, using a range of algal primers, with and without nested primers, we were unable to obtain the 18S rRNA gene sequence of the alga that was visualized by microscopy and analyzed by Raman spectroscopy. Future studies, using metagenome data from the gypsum community, together with our cultivation effort, might provide the genetic characterization of this alga.

The most abundant OTUs from each of the two gypsum datasets were used to build a phylogenetic tree. Most sequences belong to *Cyanobacteria* with closest relatives from arid deserts around the world and from members of the *Chroococcidiopsis* genus (Supplementary Figure S3). Only two OTUs were from *Alphaproteobacteria*, also associated with reference sequences from extreme environments (Supplementary Figure S3).

Raman Spectroscopy of Endolithic Microbial Communities

We obtained Raman spectra for both the cryptoendolithic and hypoendolithic microbial habitats within the gypsum sample (**Figures 11A–D**). Micro-Raman spot analysis of orange (**Figure 11A**) and green (**Figure 11B**) cells from the cryptoendolithic zone revealed spectral records dominated by carotenoid and chlorophyll signals. No phycobiliproteins were detected suggesting that both spectra belong to algal cells. The spectra showed slight differences in wavenumber position of the carotenoid bands between the green and orange cells, with the ν_1(C = C) band position at 1521 and 1519 cm^{-1} (orange cells at 785 and 532 nm) and 1524 and 1518 cm^{-1} (green cells at 785 and 532 nm). These differences were reproducible for other areas of the substrate and were interpreted as a result of selective resonance Raman effect (see Supplementary Section S8.4 section). The Raman distribution map of carotenoids in algae from the cryptoendolithic habitat displayed a higher intensity signal just beneath the gypsum surface and a lower intensity signal close to green algae zone (**Figure 11F**). The method of Raman imaging proved to be extremely powerful for mapping the spatial distribution of strong Raman scatters in geobiological samples (Vítek et al., 2014). Raman signals for cyanobacteria were clearly recognized in the gypsum hypoendolithic habitat. In particular for scytonemin (**Figure 11C**), a UV-screening pigment characterized by the corroborative Raman bands at 1595, 1554 cm^{-1}, and 1173 cm^{-1} (Edwards et al., 2000). The feature around 1322 cm^{-1} is the combination of scytonemin and chlorophyll signals. While brown-green cell aggregates produced a scytonemin signal, no scytonemin was detected within the neighboring blue-green cells (**Figure 11D**). Here, Raman features of carotenoids (at 1518 and 1156 cm^{-1} and Supplementary Figure S6 for Raman identification of carotenoids) and chlorophyll (1325, 915, and 743 cm^{-1}) were detected. Additionally, strong bands for phycobiliproteins also revealed the presence of cyanobacteria. These accessory light harvesting pigments were characterized by the bands at 1627, 1582, 1370, 1283, 1237, 1108, 1050, 874, 820, 667, and 505 cm^{-1} (**Figure 11D**).

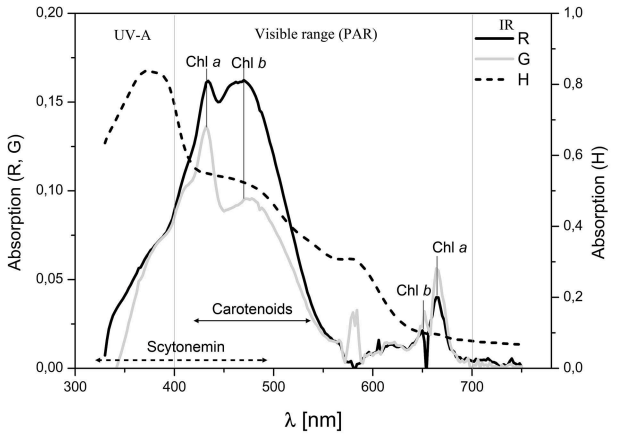

FIGURE 12 | Absorption spectra of acetone pigment extracts in the range of 320–750 nm from different endolithic colonization zones: black line, extract from orange algal zone (R), gray line, extract from green algal zone (G) and dashed line, extract from hypoendolithic zone (H). The characteristic peaks of chlorophyll *a* (663 nm), chlorophyll *b* (647 nm) are observed. Carotenoids and scytonemin absorption ranges are indicated by double arrowed lines.

Characterization of Metabolite Pigments by Spectrophotometry

The absorbance spectrum of pigment extracts obtained from different endolithic colonization zones are shown in **Figure 12**. Extracts from the orange algal cryptoendolithic zone showed high absorption values for the carotenoids absorption range. In contrast, extracts from the green cryptoendolithic colonization zone had lower absorption peaks for carotenoids and a higher chlorophyll absorption peak at 663 nm. This may indicate that the green zone also contains cyanobacteria together with green algae of lower carotenoid content. Extracts from the hypoendolithic zone showed strong absorption in the range of UVA corresponding to scytonemin absorption peaks at 315–400 nm (Proteau et al., 1993). Semiquantitative determination, based on the analysis of absorbance spectrum from extracted pigments, showed that the gypsum orange zone had twice the content of carotenoids and less of both chlorophylls *a* and *b* than the green zone (Supplementary Table S2).

Discussion

How Extreme is the Environment at the Colonized Gypsum Site?

Most of the Atacama Desert is characterized by persistent extremely dry conditions and scarce precipitations in its hyperarid area (Houston and Hartley, 2003; Hartley et al., 2005; Houston, 2006a). Based on an evapotranspiration rate of 2920 mm y^{-1} for the nearby Salar de Atacama basin (Houston, 2006a), and yearly precipitations of 27.1 mm (this study), the aridity index for the Cordon de Lila sampling site is as low as 0.0093, presenting exceptionally hyperarid conditions. The extreme aridity of this location is also expressed by long time-spans between scarce precipitations and also extremely low relative humidity. The mean annual RH value of 16.5% at Cordon de Lila is even lower than the value of 17.3% reported by Azua-Bustos et al. (2015) and recently reported as the driest site of the hyperarid Atacama Desert. Chile's northern Atacama Desert is also known as one of the places on Earth with the highest surface UV radiation (Cordero et al., 2014). The most extreme values for solar total and UV irradiances (1528 Wm^{-2}) were reported for "Puna de Atacama" by Piacentini et al. (2003) and by Cabrol et al. (2014) (43.3 UV index), in a region of high altitude, with mostly cloudless conditions, relatively low total ozone column, and extremely low RH values. Recently, Rondanelli et al. (2015) show that the location of Earth's surface solar radiation maximum is in the Atacama Desert in the region between 24° and 25°S along 69°W, over what is known as the Domeyko Cordillera. While solar radiation is essential for phototrophic life, extremely high levels of UV radiation is harmful to cellular components (Jeffrey et al., 1996; Phoenix et al., 2006), and excessive PAR results in photo-inhibition by damaging the reaction center of photosystem II in photosynthetic microorganisms (Häder, 1986). By combining both hyperaridity and extreme solar irradiance, the Cordon de Lila site with endolithically colonized gypsum studied here holds world records for environmental extremes, leading to the virtual absence of any life form on its rocks and soil surfaces.

How Extreme is the Environment within the Gypcrete Endolithic Microhabitat?

The effectiveness of gypsum in attenuating solar irradiance has been evaluated by several studies. Amaral et al. (2007) reported UV transmission of less than 20% through a 0.5 mm-thick gypsum layer. Effective attenuation of light, leading to transmission of 0.23% to 0.5% (from 300 to 680 nm) of incident light through a 0.9 mm thick Arctic gypsum crust, was reported by Cockell et al. (2010). The transmission of only 0.005% of the UVB and 0.05% of UVA, and 1% of PAR, were reported in a 1.2 mm thick Antarctic endolithically colonized gypsum by Hughes and Lawley (2003). In contrast, a 1 mm thickness of Arctic selenite exhibited only 25% reduction in ambient UV exposure (Parnell et al., 2004), underlying the importance of the micromorphology of gypsum crystals and the compactness of the gypsum layer in solar irradiance attenuation. Our data for the gypsum cryptoendolithic colonization zone, showing no UV transmission and 0.1–1% of PAR transmission, are consistent with previously reported studies. However, when taking into account the extreme solar irradiance at the sampling site and the small zenith angle, between 5° and 25°, experienced at the site when rain water is potentially available (July-March), it is likely that the cumulative dose of PAR within the endolithic habitat is extremely high.

Water is an essential element for the successful colonization of the gypsum deposits. The presence of cracks, fractures and pores in the gypsum substrate allow rainfall water and nutrients to penetrate into the gypcrete. Using EC measurements, we estimated that liquid water was present at the rock surface for less than 10 days per year. However, within the gypsum interior, the moisture regime could be significantly higher. We have observed compact structures and low porosity at the surface and bottom layers of the gypsum deposits that might significantly

decrease water evaporation rates. We also identified gypsum crystal dissolution and crystallization patterns mostly at the bottom layer, indicating the presence of liquid water, within the rock, for some periods of time. These observations lead to what we call the "eggshell" shield effect in which the gypsum interior is protected against rapid water evaporation rates. Moreover, the water can be efficiently trapped and retained inside the gypsum deposits by the sepiolite nodules randomly distributed in the substrate. Sepiolite is a fibrous sheet of magnesium silicate clay with unique physicochemical and rheological properties due to the presence of zeolitic channels in its structure (Leguey et al., 2014). Its nano-porosity induces efficient water absorption and retention up to 0.3 g g^{-1} dry weight (Caturla et al., 1999). We suggest that the sepiolite water retention capacity might increase significantly the fraction of water available to endolithic microorganisms.

Sub-micro Endolithic Habitats and their Colonizers

Our molecular characterization of the gypsum endolithic microbial community revealed a community dominated by cyanobacteria, mostly of the *Chroococcidiopsis* genus. The difference in community structure and composition we observed between samples suggest a high level of heterogeneity in the substrate's architecture, providing sub-micro habitats for colonizing microorganisms. A model of the gypsum deposit "architecture" with its cryptoendolithic and hypoendolithic habitats, gypsum layers, and sepiolite nodules, together with the role played by these structures and by the prevalence of gaseous or liquid water is illustrated in **Figure 13**.

In this model, we hypothesize that the water prevalence states and therefore the moisture regime inside the gypcrete is dependent on the depth, fabric, and others accompany to gypsum minerals. Following rainfall, water fills the fissures of the rock, forming percolation pipes and conducting the water toward the gypsum's bottom layer. There, the water, or rather a calcium sulfate solution, accumulates for longer times, filling the large pores or "micro-caves" we observed. The processes of gypsum dissolution-recrystallization and water evaporation convert the bottom layer of the gypcrete into a compact, thick layer with low permeability and potentially higher UV transmission rates (Parnell et al., 2004). Liquid water is also retained within sepiolite nodules and pore spaces inside the gypcrete, providing sub-micro environments with high moisture until the gypsum is completely dried out.

The first adaptation strategy for microorganisms in hyperarid deserts is the avoidance of extreme external conditions by escaping into the endolithic habitat (Karsten and Holzinger, 2014). The second step is to follow the water, which is essential for the metabolic activity of the primary producers in the community, followed by the level of PAR. This explains the well-defined colonization patterns we observed for the algae and cyanobacteria. We propose that moisture is the key abiotic factor driving the spatial distribution of chlorophototrophs in the gypsum sub-micro habitats, providing there are sufficient PAR levels. The cryptoendolithic habitat is an area with scarce liquid

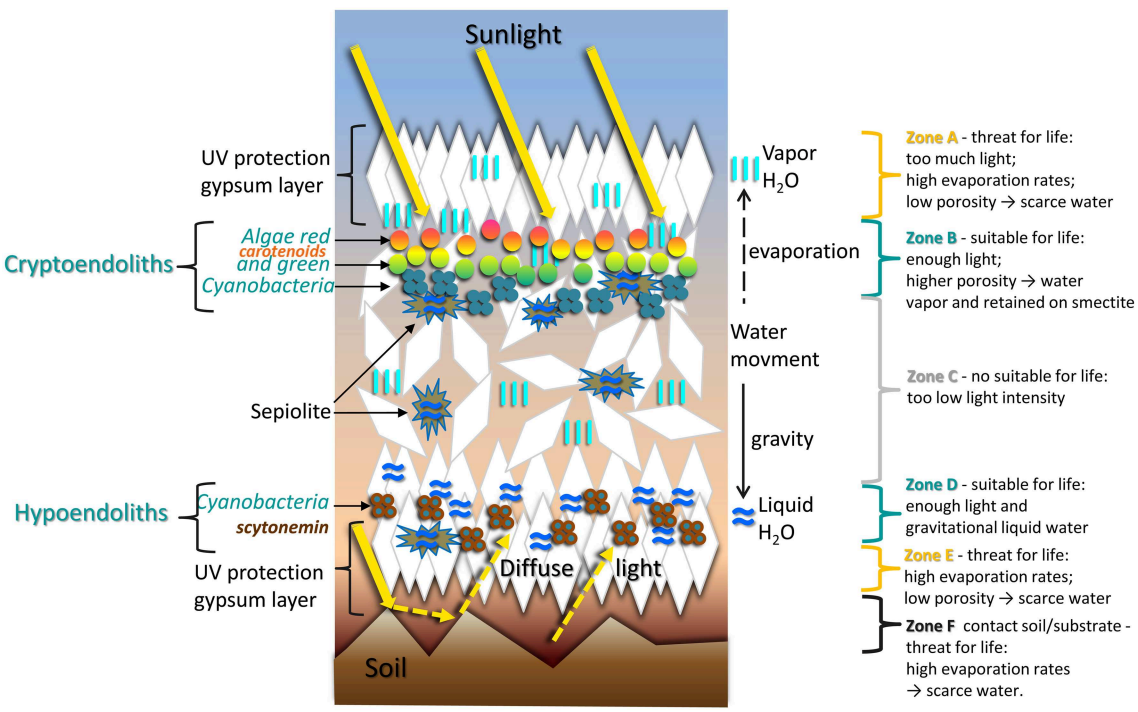

FIGURE 13 | Schematic representation of sub-micro environments with corresponding cryptoendolithic and hypoendolithic habitats within gypsum deposits from the Atacama Desert.

water with the exception of sepiolite moisture islands and pores, with high RH values, among gypsum crystals. Our results show that this habitat is dominated by a layer of green/orange algae and a subjacent layer of cyanobacteria attached to sepiolite nodules. Cultivation of the endolithic green algae showed that the orange- and green-colored layers were formed by the same organism. This alga very probably belongs to the class *Chlorophyceae* (Chlorophyta), known to contain several desert soil algae (Lewis and Lewis, 2005). Morphologically, the gypsum alga resembles the genera *Spongiochloris*, *Neochloris,* and *Deasonia*. However, algae from natural gypsum cryptoendolithic habitat exhibit a distinct suite of morphological features that is unique among all described algal genera. Therefore, we conclude that the alga observed in this study belongs to a new, yet undescribed genus of *Chlorophycean* green algae.

Previous works indicated that, in arid environments, high relative humidity was enough to induce metabolic activity in algae. For example, photobionts from cryptoendolithic lichens inhabiting sandstones of the Dry Valleys of Antarctica begin to photosynthesize at $RH = 70\%$ (Palmer and Friedmann, 1990a) and cultured algae (*Trebouxia* sp.) are capable of photosynthesis at a RH of 80% (Palmer and Friedmann, 1990b). In contrast, endolithic cyanobacteria inhabiting sandstones in the Negev Desert require $RH > 90\%$ for photosynthesis (Palmer and Friedmann, 1990a). These findings may explain the close contact of cyanobacteria with sepiolite nodules within the gypsum. However, the hypoendolithic habitat in the gypsum deposits is an area with periodical occurrence of liquid water after rainfalls hence it is colonized by cyanobacteria colonies covering the walls of micro-caves. The hypoendolithic habitat was first described within the gypsum crusts collected in a less extreme (from the point of view of high night RH values) zone of the Atacama Desert (Wierzchos et al., 2011; Vítek et al., 2013). However, these authors reported the presence of algal *Trebouxia*-like cells aggregates closely associated with fungal hyphae as the main hypoendolithic colonizers of the bottom gypsum crusts, suggesting scarce liquid water within that hypoendolithic habitat.

Light penetration has been considered a primary control for the location and extent of colonization by endolithic phototrophic microorganisms (Walker and Pace, 2007). Endolithic phototrophs from Antarctica (gypsum and sandstone), and other regions around the world, were found to receive as little as 0.01% of incident light (Nienow et al., 1988; Matthes et al., 2001; Hughes and Lawley, 2003). In our study, we did not observed chlorophototrophs below 0.1% of incident PAR in the cryptoendolithic habitat. We suggest that the additional hyperaridity found at the Cordon de Lila sampling site could potential hinder the colonization of deeper substrate layers where PAR levels are below 0.1%. However, despite relatively low values of transmitted PAR, absolutely PAR values were as high as 30 μmol photons m^{-2} s^{-1}, potentially inducing chlorophototrophs to produce abundant secondary metabolites such as carotenoids and scytonemin. High rates of solar irradiance increase considerably the probability of photoinhibition and photooxidative lethal damage to phototrophs (Solovchenko and Merzlyak, 2008). Photoinhibition has been defined as the inhibition of photosynthesis caused by excessive radiance; it may damage the photosynthetic apparatus, causing the (photo-) destruction of the photosynthesizing pigments (Long et al., 1994; Alves et al., 2002). It is therefore likely that the algae and cyanobacteria colonizing the gypsum endolithic habitat have evolved self-protective strategies such as the efficient production of secondary metabolites shielding them against UV and PAR excessive irradiance.

Solar Irradiation Protective Pigments Nature and their Spatial Distribution

Carotenoids are the accessory pigments of the photosynthetic apparatus, which participate in both light-harvesting and photoprotective functions (Nguyen et al., 2013). Some short-chained carotenoids can potentially act directly as UV-screening pigments but are not favored by organisms, whereas ubiquitous carotenoids with nine or more conjugated double-bonds absorb more in the light visible region (Cockell and Knowland, 1999). These long-chain carotenoids are able to cope with toxic reactive oxygen species that can form during photosynthesis, or as the result of UV-irradiation, and which would destroy the biomolecules forming the photosynthetic apparatus (Asada, 1994; Telfer et al., 2008).

From our Raman spectroscopic results, we observe a clear enhancement of carotenoid/chlorophyll intensity ratio of Raman signal in the case of the orange-color algal cells compared to the green-color algal cells. All the band positions may be attributed to a standard C-40 carotenoids, with bands above 1520 cm^{-1} probably due to xanthophyll compounds such as zeaxanthin and lutein, and the band at 1514 cm^{-1} assignable to β-carotene. However, based just on the Raman spectra alone the exact and unequivocal identification of a particular carotenoid in these cells was not possible, as the $\nu 1$ band position can be identical for several carotenoids or different for the same carotenoid in different biological matter (de Oliveira et al., 2010). We interpret the differences in relative carotenoid content (increased within orange algal zone) and carotenoid composition as a response of algae to light-driven stress enhanced with the proximity to the surface. The relative intensity of chlorophyll Raman spectral features differs significantly between green and orange algal cells. The clear characteristic signals of chlorophyll were detected within the green cells using 785 nm laser for excitation, represented by the bands at 1326, 918, and 745 cm^{-1}. On the other hand, spectra from the orange cells revealed none or very weak chlorophyll features. The very weak signal of chlorophyll in the orange cells was the result of decreased chloroplasts content in these cells, which was clearly confirmed by microscopy observations.

The capability to synthesize very high amounts of a complex mixture of secondary carotenoids under environmental stresses is widely spread over the green microalgae, as a defense mechanism against environmental injuries. Secondary carotenoid formation enhances the cell resistance to oxidative stress generated by unfavorable environmental conditions including excess light, UV and PAR irradiation, and nutrition stress and, therefore, confers a higher survival capacity to the cells (Hanagata and Dubinsky, 1999; Orosa et al.,

2000, 2001). High light levels and salinity were reported as main stress drivers enhancing carotenogenesis in the aerial microalga *Scenedemus* sp. (*Chlorophyceae*) leading to the production of xanthophylls as fatty acid esters in the algal cells (Aburai et al., 2015). Moreover carotenoids have been identified as photoprotection agents against photoinhibition and molecular damage of the photosynthetic apparatus (Oren et al., 1995).

Our analytical results obtained by spectrophotometry and Raman spectroscopy demonstrate enhancement in carotenoids and depletion in chlorophyll photosynthetic pigment within orange cells lying close to the gypsum surface. This progressive carotenogenesis process in the case of the cryptoendolithic algae from the Atacama's gypsum deposits is driven by higher PAR light levels toward the gypsum surface, the oligotrophic environment, and the very large periods (up to 9 months) of dehydration followed by desiccation. It was also described that carotenogenesis processes might lead certain algal cells to turn from a green to an orange/red-color (e.g., "blood alga" *Haematococcus pluvialis*, snow alga *Chlamydomonas nivalis* or *Scenedesmus komareki*) due to the accumulation of large amount of carotenoids within the cell (Hanagata and Dubinsky, 1999 see also references therein).

Carotenogenesis generally accompanies larger metabolic changes and morphological modifications, i.e., progressive accumulation of lipoidal globules and/or disappearance of certain cell organelles such as chloroplasts (Hanagata and Dubinsky, 1999). A similar process has been observed in the case of the cryptoendolithic algae, where lipoidal globules that were first formed at the periphery of the cell, progressively propagated toward its inside, eventually filling most of it. The chloroplast was single and parietal in the green cells. As the cells turned orange, the chloroplast divided into several small lobes and was pushed toward the interior of the cell by the accumulating lipoidal globules. However, our knowledge of the cellular localization of secondary carotenoids and the change of cellular ultrastructure associated with their accumulation in other carotenoid-accumulating algae is limited and will be the topic of future studies.

The presence of scytonemin, a well-known UV-screening pigment, in the gypsum hypoendolithic habitat was confirmed by Raman spectroscopy and spectrophotometric analyses. Scytonemin is a typical cyanobacterial metabolite that is extracellularly located in the cyanobacteria exopolysaccharide sheath around cells and cell aggregates, as recently shown for endolithic cyanobacteria colonizing Atacama's halite (Vítek et al., 2014). Scytonemin is known to have photoprotective properties against harmful UV radiation (Garcia-Pichel and Castenholz, 1991; Cockell and Knowland, 1999; Vítek et al., 2014). Periodic desiccation of cyanobacteria in combination with high UV fluxes may further induce scytonemin biosynthesis (Fleming and Castenholz, 2007). This raises the question of how can UV irradiate hypoendolithic cyanobacteria? First, it should be mentioned that hypoendolithic colonization has only been found in these specific gypsum deposits, which were detached from the coarse grain soil underneath, suggesting that diffused solar light could illuminate the gypcrete underneath. In addition, as mentioned above, the bottom gypsum layer shows a very compact structure potentially permitting some transmission of UV (Parnell et al., 2004) from intense solar irradiance.

EPSs and their Role in Reducing Water Stress and UV Radiation

Our results show abundant presence of exopolysaccharides with different micromorphology surrounding algae, cyanobacteria, and heterotrophic bacteria single cells and colonies. EPSs are an important class of biopolymers, which play key protective and structural roles. Under extremely low water availability, large periods of dehydration, and excessive solar irradiance, the protection against water stress and UV and PAR radiation is one of the main roles of EPSs in constrained environments (Rossi and De Philippis, 2015). Although the role of EPSs in reducing water stress has not been fully clarified, they are reportedly involved in maintaining hydration because of their hydrophilic/hydrophobic characteristics (Makhalanyane et al., 2015). Hygroscopic EPSs act as a water reservoir after wetting events (Gorbushina, 2007) and as a possible mechanism for absorption and retention of water vapor, as reported for hypolithic microbial communities from the Dry Valleys of Antarctica (de los Ríos et al., 2014b). In addition, it was described that EPSs may contain UV-absorbing compounds, such as scytonemin (Vítek et al., 2014).

Architecture of a Lithic Substrate

As observed with microscopy techniques, internal structural elements such as porosity, pore-size distribution, presence of large pores and cavities, light transparency, and light scattering properties, dissolution and crystallization features, and sepiolite nodules distribution varied significantly within various locations of the gypsum substrate. All together these structural, physical, chemical and mineral properties give rise to a new understanding of the features and functions relevant to the rock bioreceptive characteristics. We suggest using the term of "rock architecture" instead of "rock structure" to emphasize the functional role of the rock interior. As such, this new concept of the *architecture of a lithic substrate* encompasses all the internal structures of a rock that are essential as a habitat for microbial life. It is about perceiving the rock interior from the existence of porous spaces of different sizes and shapes, interconnected or not; the solid structures that divide and support these spaces, and the minerals and salts that can be transformed. All these components and elements are interrelated and influence one another, thus fulfilling a requisite: they shape a suitable architecture to hold microbial life. The architecture of habitable rocks provides resources (water, light, and nutrients, above all) and guarantees effective protection from excessive evapotranspiration, thus assuring efficient gas exchange. It also provides a long-term stable environment for life. Considering the architecture of a rock provides an integrated view of its potential habitability for endolithic microbial communities. All porous rocks have a structure, yet very few show such a suitable architecture for endolithic microbial colonization, even under extreme environmental conditions, as the Atacama's gypsum do.

Concluding Remarks

Here we report that architectural features of translucent gypsum rocks with sepiolite inclusions provide increased water availability and attenuation of harmful UV and PAR radiation to endolithic microbial communities. Intense PAR radiation induced chlorophototrophic microorganisms to form a unique layering of algae and cyanobacteria within the cryptoendolithic habitat and the synthesis and accumulation of carotenoids in the upper algae zone. Scytonemin pigment is produced by the hypoendolithic cyanobacteria to avoid photoinhibition and photooxidative damages. We found that the spatial distribution of photosynthetic pigments within the gypsum rock such as carotenoids, chlorophylls and phycobiliproteins is linked to different colonization zones and types of microorganisms. The highest concentration of carotenoids was detected in orange-colored algal cells just beneath the gypsum surface, suggesting that the green microalgae use this shielding as a survival strategy against the intensive solar irradiance. This in turn allows green microalgae to colonize a slightly deeper layer in the rock until maturation and transformation into orange-colored algae with high carotenoid content. The same carotenoid-like "umbrella" is also protecting cyanobacteria cells underneath the layer of green microalgae cells. The detection of scytonemin in the hypoendolithic habitat colonized by cyanobacteria can be interpreted as another adaptation strategy against excess of UV and PAR in this unique conformation. The highly specialized microbial assemblages we describe here can be use as model systems to further our understanding of photoprotective mechanisms under extremely unfavorable conditions.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00934

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Namib Desert dune/interdune transects exhibit habitat-specific edaphic bacterial communities

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The sand dunes and inter-dune zones of the hyper-arid central Namib Desert represent heterogeneous soil habitats. As little is known about their indigenous edaphic bacterial communities, we aimed to evaluate their diversity and factors of assembly and hypothesized that soil physicochemistry gradients would strongly shape dune/interdune communities. We sampled a total of 125 samples from 5 parallel dune/interdune transects and characterized 21 physico-chemical edaphic parameters coupled with 16S rRNA gene bacterial community fingerprinting using T-RFLP and 454 pyrosequencing. Multivariate analyses of T-RFLP data showed significantly different bacterial communities, related to physico-chemical gradients, in four distinct dune habitats: the dune top, slope, base and interdune zones. Pyrosequencing of 16S rRNA gene amplicon sets showed that each dune zone presented a unique phylogenetic profile, suggesting a high degree of environmental selection. The combined results strongly infer that habitat filtering is an important factor shaping Namib Desert dune bacterial communities, with habitat stability, soil texture and mineral and nutrient contents being the main environmental drivers of bacterial community structures.

Keywords: deterministic assembly, open soil, habitat filtration, dune transects, environmental gradient, arid ecosystems

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Introduction

Despite extreme surface conditions, notably (hyper-) aridity, wide daily temperature fluctuations, high UV radiation and oligotrophy, edaphic microbial communities have been shown to flourish in desert soils (Makhalanyane et al., 2015). The Namib Desert of south-western Africa is considered to be one of the oldest desert regions on Earth (Eckardt and Spiro, 1999). It is characterized by a wide range of different soil environments including gravel plains, sand dunes, inselbergs, escarpments, river beds, salt pans and playas (Seely, 2012; Eckardt et al., 2013a). In the central Namib Desert, studies have notably determined that local soil physicochemical conditions and climate played a significant role in the assembly of edaphic and hypolithic communities were (e.g., Stomeo et al., 2013; Warren-Rhodes et al., 2013; Ramond et al., 2014; Gombeer et al., 2015).

However, and despite representing over 41% of the total desert land surface (Seely, 2012), the detailed microbial ecology (as determined by modern molecular tools) of the Namib Sand Sea has to date poorly been assessed and essentially focused on fungal communities (Jacobson, 1997; Jacobson and Jacobson, 1998; Jacobson et al., 2015). The dispersal and colonization of mycorrhizal

fungal communities were shown to depend on sand stability and moisture availability, respectively (Jacobson, 1997). And, moisture-activated decomposing fungi were observed on the litter of the perennial Namib dune grass *Stipagrostis sabulicola* and in dune sands (Jacobson and Jacobson, 1998; Jacobson et al., 2015). Recently, a T-RFLP fingerprinting-based study compared the edaphic bacterial community assemblages from multiple soil surface geologies and lithologies of the central Namib Desert. It showed that (i) interdune bacterial communities were significantly different than those of the gravel plains and riverbeds and that (ii) the soil physicochemistry and lithology of the different interdunes sampled were important in structuring their indigenous communities (Gombeer et al., 2015).

Dune morphology and dynamics have in contrast extensively been studied in the Namib Desert. Three geographically distinct dune morphology patterns have been characterized: transverse dunes to the west (i.e., toward the coast), linear and complex linear dunes in the center and star dunes in the east (i.e., inland) (Livingstone, 2013). Similarly, an east/west sand color gradient, from yellowish brown (west) to much redder (east), has been described and attributed to variations in iron oxide and clay mineral concentrations (Walden and White, 1997; Livingstone, 2013). Within dune slopes, a complex moisture gradient has been shown to influence the number of species perennial grasses as well as their growth strategies and distribution (Yeaton, 1988; Seely, 1990). Dunes have also been described to be highly dynamic environments, as they are constantly modified by surface shear forces and mechanical stresses. For example, it has been reported annual transverse dune crest migrations varying from 4 to 56 m (Livingstone, 2013). Wind has also been shown to alter the top of wind-exposed slopes (or stoss slopes) and sand accumulation to dominate along lee slopes (or downwind slopes) (Lancaster, 1985; Eckardt et al., 2013a). The interactions between wind erosion and sediment deposition lead to discernible patterns in grain size and sorting gradients both on individual dune slopes and between dune and interdune areas.

In such a dynamic and heterogeneous habitat, dune microbial communities have thus to cope with numerous stresses, including environmental physical instability, fluctuating soil physico-chemical properties, low water availability, high temperatures, oligotrophy and alkaline pH (typically around 8) (Makhalanyane et al., 2015); all of which have been shown to affect both community diversity and function (Andrew et al., 2012; Kuske et al., 2012; Yu and Steinberger, 2012a; Makhalanyane et al., 2015).

Using the Kahani dune system in the Namib Sand Sea as a model dune environment, we aimed to determine drivers shaping edaphic bacterial community diversity in four dune biotopes (dune top, slope, base, and interdune). We hypothesized that in such a dynamic environment, strong soil physico-chemical gradients would structure bacterial communities; i.e., that deterministic processes would lead to dune habitat-specific communities. Our related null hypothesis stated that high dispersal rates, due to the important dynamicity of dune ecosystems, would lead to homogenized communities (Leibold and Norberg, 2004). To test these hypotheses, we collected 125 soil samples from 5 parallel dune/interdune transects in a single Namib Desert dune system; i.e., from the summit of a western dune to the crest of an eastern dune. For each sample, we recorded 21 edaphic physico-chemical parameters and characterized the bacterial community structure by 16S rRNA gene T-RFLP analyses. We also performed 16S rRNA gene pyrosequencing on representative samples of 7 putative dune habitats found across the dune/interdune transect, namely the east and west dune tops, slopes bases and the interdune, to investigate their taxonomic composition and assess if they presented different assemblages.

Materials and Methods

Study Site and Soil Sample Collection

Soil samples were collected in April 2013 in the Kahani dune system (S 23°35.830′/E 15°01.800′), a typical complex linear dune in the Namib Desert Sand Sea (**Figure 1**). 25 soil samples were collected from each of five parallel (40 m apart) 2 km long dune/interdune linear transects from the west (stoss slope) to the opposite east dune (lee slope) resulting in a total of 125 soil samples (**Figure 1**). Each transect was divided into 4 morphologically distinct zones: dune Top, Slope, Base and Interdune. Sampling points were regularly distributed; every 40 m from the west dune Top to its base, every 200 m in the Interdune area and every 20 m on the eastern dune up to the dune Top, as the west facing dune was typically shorter and steeper than the east facing dune (**Figure 1**). Surface soil samples (0-5 cm depth) were collected (avoiding grasses on the dune Slope to minimize any rhizosphere bias), and each individual sample comprised the pooling of four pseudo-replicate soils in a 1 m^2 area. Samples were stored at 4°C for soil chemistry analysis, at −20°C for molecular analysis and processed immediately for fluorescein di-acetate (FDA) assays.

Soil Physico-chemistry Analyses

All analyses were performed at the Soil Science Laboratory of the University of Pretoria. Prior to analyses of 21 edaphic parameters, the soil samples were sieved (2 mm mesh) and dried overnight at 37°C. pH was measured in 10 g soil slurries (1:2.5 soil/deionized water) with a pH meter (Crison basic 20, Barcelona, Spain) (Eckert and Sims, 1995). Soil nitrate (NO_3^--N) and ammonium (NH_4^+-N) were determined by extraction (2M KCl) and steam distillation with subsequent titration performed as described by Keeney and Nelson (1982). Soil total carbon percentage (C) was measured by oxidizing the organic material with chromic acid and titrating the excess dichromate (Walkley, 1935; Nelson, 1982). Cation exchange capacity (CEC) was determined using an ammonium acetate solution as extractant for the exchangeable and water-soluble cations with steam distillation to separate the ammonia (Rhoades, 1982). Ammonium acetate extraction was also used to measure salt concentrations (Na, K, Mg, and Ca) using inductively coupled plasma atomic emission spectroscopy (ICP-OES) (Spectro Genesis, Spectro Analytical Instruments GmbH, Germany). The Bray-1 method (Bray and Kurtz, 1945) was used to quantify extractable phosphorous (P). Metals (Fe, Al, and Mn) were extracted from 10 g of soil using EDTA buffer and quantified by ICP-OES after filtration through a

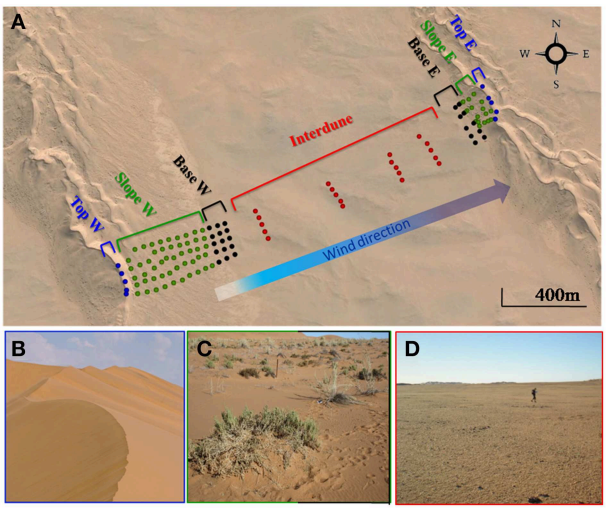

FIGURE 1 | The Kahani dune system sampled in the Namib Desert. (A) Google Maps representation of the sampling site showing the individual soil samples collected. Filled circles represent the 125 soil samples from the 5 dune/interdune transects, from dune Tops (●), Slopes (●), Bases (●) or Interdune (●). E, East; W, West. **(B)** Photograph of the dune Top (west) devoid of vegetation. **(C)** Photograph of the dune Slope (W) with sparse perennial grasses (*Stipagrostis sabulicola* and *Trianthema hereroensis*). **(D)** Photograph of the barren Interdune.

0.45 µm Millipore filter (EMD Millipore Corporation, Billerica, MA, USA).

Particle size distributions were determined using the ASTM protocol D422-63 on 50 g of soil sample (ASTM, 1985). The hydrometer method (Bouyoucos, 1962) was used to measure the size distribution of the silt and clay fractions obtained from the ASTM protocol. Granulometry analysis contributed to the formation of 8 variables: very coarse sand (VCS), coarse sand (CS), medium sand (MS), fine sand (FS), very fine sand (VFS) and the portion of the silt plus clay (SC), silt (ST), and clay (CY).

Fluorescein Di-acetate Degradation Assay

FDA hydrolysis, as an indicator of general bacterial metabolic activity, was measured using the protocol of Green et al. (2006). 0.5 g of soil was mixed with 12.5 mL of sterile *phosphate buffered* saline (PBS, pH 7.4) and 0.25 mL of 4.9 mM FDA dissolved in acetone and subsequently incubated at 28°C for 1 h under constant agitation. FDA hydrolysis was stopped by mixing 40 µL of acetone with 1 mL of soil slurry. After centrifugation (8800 × g, 5 min.) fluorescence was measured with a portable fluorometer (Quantifluor, Promega, Madison, USA), calibrated against a standard curve. FDA activity was expressed as µg fluorescein per g soil per hour (µg fluorescein g^{-1} soil h^{-1}).

Metagenomic DNA Extraction, PCR Amplification and T-RFLP

Metagenomic DNA was extracted from 0.5 g of soil using the PowerSoil™ DNA Isolation Kit (MoBio, West Carlsbad, CA, USA) following the manufacturer's instructions.

PCR reactions were carried out in a Thermo Cycler (BioRad) in 50 µL final volume reactions containing 1X PCR Buffer A, 1X Enhancer, 0.2 mM each dNTP, 0.5 µM of each primer (E9F: 5′-GAGTTTGATCCTGGCTCAG-3′/U1510R: 5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach and Pace, 1995; Hansen et al., 1998), 4% DMSO, 0.5 U of KAPA2G Robust DNA polymerase (KAPA, USA) and 10 ng of template DNA. Thermal cycling conditions were as follows: 3 min denaturation at 95°C followed by 30 cycles with denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and elongation at 72°C for 90 s with a final elongation at 72°C for 10 min. This reaction yielded 1.5 kb amplicons.

For T-RFLP analysis, the forward E9F primer was FAM-labeled and the PCR products were purified using the NucleoSpin Kit™ (Macherey-Nagel, Germany) and digested overnight using *Hae*III (Fermentas, USA). After a second purification, electrophoretic separation of the terminal-restriction fragments (T-RFs) was performed using an ABI3130XL sequencer (Applied Biosystems, USA). The retrieved T-RFLP profiles were analyzed using Peak Scanner 1.0 (Applied Biosystems; http://www.appliedbiosystems.com). True peaks and fragments of similar size were identified and binned using the software R and Perl (Abdo et al., 2006). The term OTU (Operational Taxonomic Unit) is used to refer to individual terminal restriction fragments (T-RF) in T-RFLP patterns, with recognition that each OTU may comprise more than one distinct bacterial ribotype.

Statistical Analyses

Principal Component Analyses (PCA) was performed (*prcomp R* function) on standardized soil chemistry variables and granulometric parameters (Oksanen, 2013). T-RFLP data were Hellinger-transformed (Legendre and Gallagher, 2001) and used to calculate Bray-Curtis dissimilarity matrices, which were further visualized using 3D non-metric multidimensional scaling (3D-nMDS) (Primer 6 software; Primer-E Ltd, UK). Permutational multivariate analysis of variance (PERMANOVA), function *Adonis* (vegan package for R), was used to test for significant differences between sample groups along the dune/interdune transects. Distance based redundancy analysis (dbRDA) was used to determine correlations between bacterial community structures and habitat parameters (e.g., soil chemistry and granulometry) (*vegan* package for R). Negative eigenvalues were transformed by taking the square root of dissimilarities.

An analysis of multivariate homogeneity of group dispersions (variances) (*betadisper* function in vegan package for R) was used to test if one or more of the dune zones was significantly more variable than the others. The test was performed on standardized soil physico-chemical properties and bacterial community structures (relative abundance of OTUs).

Edaphic variables significantly correlated, or "redundant" (based on PCA analysis), were grouped for dbRDA analysis in order to decrease co-linearity and noise (**Table 1**) (Legendre and Legendre, 2012), yielding 15 independent variables: pH, NO_3^--N, NH_4^+-N, C, CEC, Na, K, P, Mg (also representing Ca), Fe (also representing Al and Mn), very coarse sand (VCS), coarse

TABLE 1 | Soil physico-chemistry of the four dune/interdune soil types studied (Top, Slope, Base and Interdune).

Variable	Code	RDA group	Top	Slope	Base	Interdune
SOIL CHEMISTRY						
Phosphorus (mg kg^{-1})	P	**P**	6.54 ± 0.69	9.13 ± 0.30	17.84 ± 0.99	30.04 ± 1.11
Organic carbon (%)	C	C	0.04 ± 0.01	0.04 ± 0	0.05 ± 0.01	0.07 ± 0.001
Ammonium (NH_4^+-N) (μg g^{-1})	NH4	NH4	8.74 ± 0.66	8.23 ± 0.19	8.58 ± 0.17	8.67 ± 0.18
Nitrate (NO_3^--N) (μg g^{-1})	NO3	NO3	6.52 ± 0.43	6.87 ± 0.15	6.79 ± 0.24	6.93 ± 0.24
Potassium (mg kg^{-1})	K	K	2180.30 ± 44.07	1444.51 ± 50.49	2175.13 ± 75.31	2625.23 ± 36.59
Magnesium (mg kg^{-1})	Mg	**Mg**	317.54 ± 9.01	267.20 ± 10.65	581.60 ± 30.52	856.23 ± 18.94
Calcium (mg kg^{-1})	Ca	**Mg**	4914.84 ± 220.18	4725.15 ± 160.22	9375.07 ± 486.57	13702.48 ± 470.59
Sodium (mg kg^{-1})	Na	Na	1634.07 ± 49.26	965.52 ± 29.39	718.06 ± 31.19	508.66 ± 19.96
pH	pH	pH	8.59 ± 0.47	8.30 ± 0.11	7.82 ± 0.20	7.75 ± 0.26
Cation exchange capacity ($cmol^+$ kg^{-1})	CEC	CEC	5.31 ± 0.58	5.11 ± 0.11	5.69 ± 0.16	6.40 ± 0.22
Aluminium (mg kg^{-1})	Al	**Fe**	22.34 ± 1.08	21.07 ± 0.59	35.59 ± 2.23	56.93 ± 2.47
Iron (mg kg^{-1})	Fe	**Fe**	8.19 ± 0.24	7.12 ± 0.14	10.30 ± 0.6	14.46 ± 0.91
Manganese (mg kg^{-1})	Mn	**Fe**	3.10 ± 0.03	3.36 ± 0.05	5.24 ± 0.25	8.22 ± 0.28
GRANULOMETRY						
Very course sand (%)	VCS	VCS	0.05 ± 0.04	0.34 ± 0.08	0.63 ± 0.09	0.58 ± 0.15
Coarse sand (%)	CS	CS	0.61 ± 0.84	6.43 ± 0.7	4.64 ± 0.83	1.30 ± 0.12
Medium sand (%)	MS	MS	26.56 ± 7.42	57.56 ± 1.43	36.14 ± 2.78	16.62 ± 1.20
Fine sand (%)	FS	FS	70.60 ± 7.32	31.12 ± 1.46	44.02 ± 2.22	44.31 ± 1.26
Very fine sand (%)	VFS	**VFS**	1.01 ± 0.28	3.83 ± 0.31	11.53 ± 1.44	29.24 ± 0.98
Silt & Clay (%)	SC	**VFS**	0.01 ± 0.01	0.09 ± 0.01	1.39 ± 0.26	4.57 ± 0.29
Silt (%)	ST	**VFS**	0.00	0.05 ± 0.03	0.13 ± 0.06	0.77 ± 0.15
Clay (%)	CY	**VFS**	0.00	0.01 ± 0.01	0.76 ± 0.1	1 ± 0

*All values are given as the mean of ± standard error. In bold are indicated the variables grouped for the RDA analysis presented in **Figure 7**.*

sand (CS), medium sand (MS), fine sand (FS) and very fine sand (VFS) (representing silt plus clay [SC], silt [ST] and clay [CY]). Tukey's HSD (honest significant difference) test was used to test for differences in averaged bacterial activity between dune areas.

454 Pyrosequencing

Extracted metagenomic DNA samples from 7 dune zones (Tops E+W, Slopes E+W, Bases E+W and Interdune) were pooled. Bacterial 16S rRNA gene amplicons were generated with the primer set 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′)/519Rmodbio (5′-GTNTTACNGCGGCKGCTG-3′) and sequenced using a Roche 454 FLX titanium next-generation sequencer by Mr DNA Laboratories (Shallowater, Texas, USA). The sequences are available at the NCBI Sequence Read Archive under the accession number SRP059482.

Pyrosequencing Data Analyses

Analyses were carried out using the mothur software package (Schloss et al., 2009). Sequences with ambiguity, and/or with homopolymeric stretches longer than 8 bp, or shorter than 200 bp were removed with no barcode and primer mismatches accepted. This resulted in reads with sizes ranging from 232 to 314 bp. Chimeras were removed using Perseus (Quince et al., 2011). Sequences were aligned to the reference SILVA database and clustered into operational taxonomic units (OTUs) at the species (97% similarity) level using the average neighbor settings in mothur. Each OTU was assigned a taxonomic classification with reference to the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/index.jsp). Collector's curves were produced using the Chao1 diversity index and the coverage using Good's coverage estimator. The community composition of the sand dune zones was compared with a UPGMA tree based on the Jaccard index. Indicator species analysis was conducted using the *multipat* function of the indicspecies package in R.

Results

Dune Soil Physico-chemistries

Principal Component Analysis of 21 physico-chemical parameters from all 125 independent soil samples (**Figure 2A**) along the five dune-interdune transects (**Figure 1**) shows Top and Slope samples clearly separated along the PC2 axis (**Figure 2A**), explaining 10.7% of the sample variation. Dune Base samples cluster between Slope and Interdune samples (**Figure 2A**). Top and Slope samples clearly separate from those of the Interdune along PC1, which explains the majority of physico-chemical soil variation (48.4%). The most significant result to emerge from our analysis is the observation that each of the four sites examined, namely [dune] Top, Slope, Base and Interdune, was distinct and significantly different (*adonis* $R^2 = 0.477$, $p < 0.001$) with respect to soil physico-chemistry. The test for multivariate homogeneity of group dispersions (*betadisper*) showed that variations in soil physico-chemical

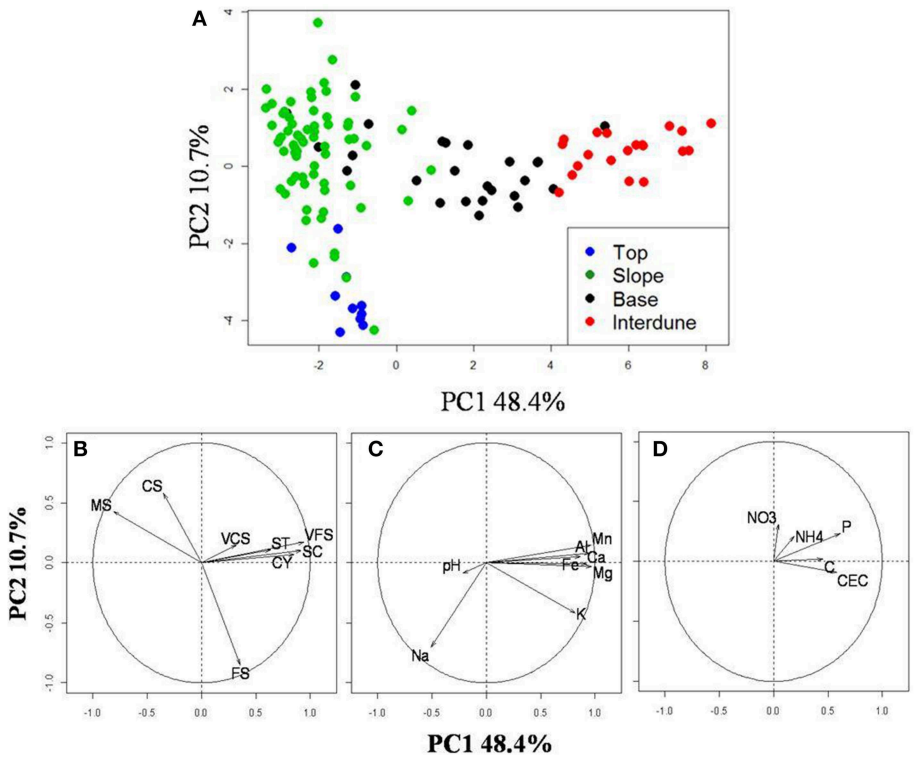

FIGURE 2 | Analysis of the dune edaphic physico-chemical parameters. (A) Principal component analysis (PCA) on 21 standardized edaphic variables from the 125 soil samples collected from the dune Tops (●), Slopes (●), Bases (●), or the Interdune (●).Vectors indicate the strength (length) and direction (arrow orientation) of single variable gradient. **(B)** Soil granulometry gradients; **(C)** Metals, minerals and pH and **(D)** the other soil properties measured. The code corresponding to each variable is described in **Table 1**.

properties within dune zones were not significantly different ($p = 0.14$). No statistically significant differences in physico-chemical soil properties were found between dunes slopes exposed to different wind regimes (i.e., the lee or stoss slopes) (data not shown).

Soil texture analysis (**Figure 2B**) also shows separation of the four dune zones with an important fraction of fine sand in the Top zone and proportionally more coarse and medium sands in the Slope and Base areas (**Table 1**). The Interdune was characterized by a higher proportion of very fine sand, silt and clay than the other zones of the dune (**Table 1**). Dune Top samples had an elevated Na content and a higher pH (**Table 1**). Concentrations of minerals (K, Ca and Mg) and metals (Fe, Al) were lowest on dune Slopes (**Figure 2C**) and highest in the Interdune samples (**Table 1**) and inversely correlated with pH (**Figure 2C**). All other edaphic properties measured, including C, P, pH, CEC, NO_3^--N, NH_4^+-N, and VCS (**Table 1**), were only very weakly correlated with the PC1 axis (**Figure 2D**) and do not appear to contribute to the dune/interdune physico-chemical gradients. Data for the dune Base (**Table 1**) was generally intermediate between Slope and Interdune. To conclude, this comprehensive dune soil physico-chemistry analyses clearly defined four dune biotopes; i.e., Top, Slope, Base and Interdune.

Dune Zone-specific Bacterial Community Structure and Diversity

16S rRNA gene T-RFLP analysis yielded between 12 and 105 OTUs per individual sample (α-diversity) for each of the 125 samples. The *betadisper* function for multivariate homogeneity indicated that variances in the relative abundance of OTUs within dune zones were not significant ($p = 0.59$). Dune Tops showed the lowest bacterial diversity (41 OTUs), while the Slope was the most diverse (80 OTUs) followed by the Base and Interdune with 62 and 53 OTUs, respectively. Only 16 OTUs (15%) were shared by the bacterial communities of the four dune biotopes (Figure S1A), with samples from the Slope showing the highest number of unique OTUs (16). The 3D-NMDS plot showed that dune bacterial communities cluster according to their soil biotope of origin; i.e., Top, Slope, Base and Interdune (**Figure 3**). This was confirmed by PERMANOVA analysis (*adonis* $R^2 = 0.31$, $p = 0.001$) and consistent with the analysis of soil physico-chemistry (**Figure 2A**).

Pyrosequencing analysis of 16S rRNA gene PCR amplicons yielded a total of 5204 OTUs (defined at 97% sequence similarity, **Table 2**; Figure S2B), of which 2741 (47.3%) were singletons. Dune Tops (E+W) showed the highest number of unique OTUs (1154) followed by the dune Bases (1170), Slopes (971), and the Interdune (775) samples. Only 88 OTUs (2%) were shared

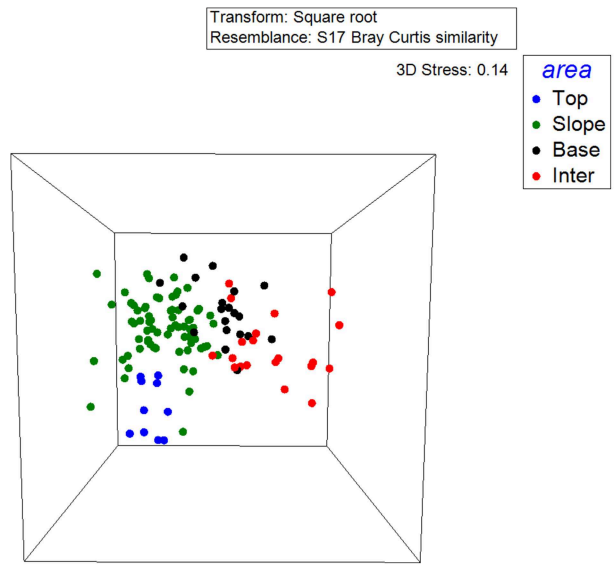

FIGURE 3 | Three-dimensional non-metric multidimensional scaling (3D-NMDS) plot of Bray-Curtis similarity matrixes of Namib Desert dune bacterial community structures determined by 16S rRNA genes T-RFLP. Points represent the composition of a community in 3D multidimensional space, and the distance between any two points represents the difference between those two communities. The good quality of the ordination is indicated by a low stress value (3D stress = 0.14).

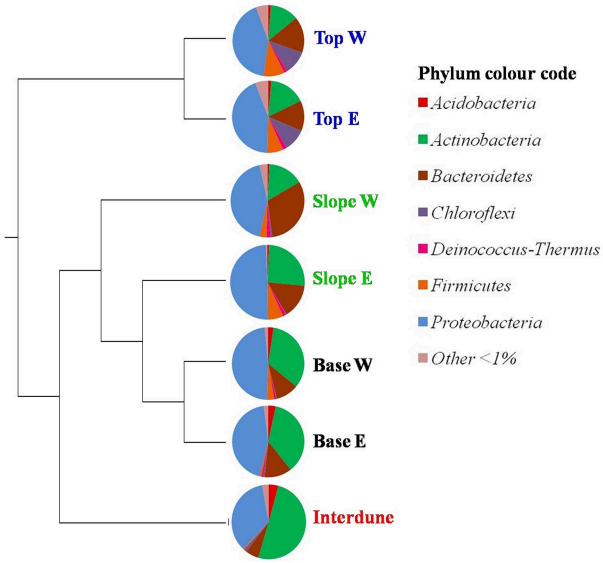

FIGURE 4 | UPGMA cluster analysis comparing the bacterial community composition in the seven *a priori* defined zones of the Kahani dune system. E, East; W, West. The Pie charts depict dune zone-specific bacterial community composition at the phylum level (90% similarity cut-off).

TABLE 2 | Distribution and diversity of OTUs (97% cut-off) in the seven dune zones studied.

Dune zone	Reads	Coverage	Observed OTUs	Chao1
Top E	6899	0.965	782	983.71
Top W	11,709	0.964	1193	1663.27
Slope E	7089	0.939	884	1461.33
Slope W	9669	0.933	1307	2234.56
Base E	7671	0.907	1329	2507.43
Base W	6836	0.905	1209	2289.56
Interdune	4653	0.836	1251	2769.02

E, East; W, West.

between the four dune biotopes, which is broadly consistent with the T-RFLP analysis (Figures S1A,B).

After resampling this dataset for sequence consistency (n = 4653 reads), collector's curves using the Chao1 index indicated that dune Tops (E+W) and Slopes (E+W) samples reached near saturation (Figure S2). UPGMA analysis shows that bacterial communities cluster strongly in relation to their dune biotope of origin (**Figure 4**), supporting the T-RFLP analysis. However, while bacterial T-RFLP fingerprinting did not differentiate the east/west dune slopes (**Figure 3**), pyrosequencing analysis detected minor differences (**Figure 4**) which could be artifactual and related to the low number of samples analyzed.

A total of thirty bacterial phyla were observed in the dune samples (Table S1), with only seven (*Acidobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Deinococcus/Thermus*, *Firmicutes*, and *Proteobacteria*) showing relative abundances greater than 1% (**Figure 4**). *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes* were the dominant phyla in the 7 dune environments studied (Table S1). Bacterial phyla habitat-filtering could be observed with *Proteobacteria* dominating sand dune samples (i.e., Top, Slope and Base), ranging from 42 to 49%, and *Actinobacteria* dominating the Interdune biotope (51%; **Figure 4**). Moreover, the *Chloroflexi*, *Firmicutes*, and *Acidobacteria* (**Figure 4**) as well as the *Alpha-*, *Beta-*, *Gamma-*, and *Deltaproteobacteria* (**Figure 5**) displayed dune habitat-specific relative abundances: *Chloroflexi*, *Firmicutes*, *Alpha-*, *Gamma-*, and *Delta-proteobacteria* presented decreasing abundances from dune Top to the Interdune, while *Acidobacteria* and *Betaproteobacteria* presented the opposite trend (**Figure 4**). Similarly, the three most abundant genera of the dominant *Actinobacteria* (*Geodermatophilus* sp., *Blastococcus* sp., and *Arthrobacter* sp.) and *Proteobacteria* (*Microvirga* sp., *Massilia* sp., and *Novosphingobium* sp.) phyla displayed dune habitat specific abundances (Table S2). Altogether, these results strongly suggest habitat-filtration to be a determining process defining dune bacterial community assemblies.

This is confirmed by the fact that, apart from the dune Tops, each dune zone presented different and significantly associated ($p < 0.05$) bacterial indicator species (Dufrêne and Legendre, 1997). Two actinobacterial OTUs were associated with the Interdune: OTU24 from the genus *Geodermatophilus* sp. and OTU88, a *Modestobacter* sp. Three actinobacterial genera were also significantly linked to the eastern dune Slope (*Kineococcus* sp. [OTU73 and OTU77], *Arthrobacter* sp. [0TU16] and *Rathayibacter* sp. [0TU46]). Contrastingly, the two west dune slope indicator species were from the *Flavisolibacter* sp. (OTU6) and *Microvirga* sp. (OTU23) genera. Finally the dune

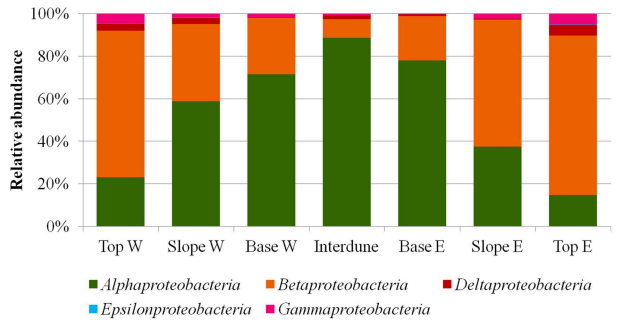

FIGURE 5 | Relative abundances (%) of the different proteobacterial classes in the seven *a priori* defined zones of the Kahani dune system.

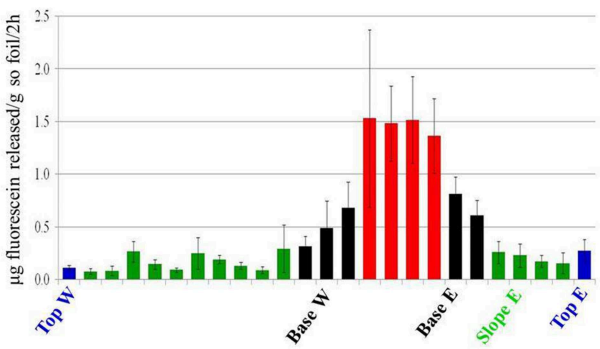

FIGURE 6 | Fluorescein di-acetate (FDA) hydrolysis values showing the average potential bacterial activity (µg fluorescein g^{-1} soil h^{-1}) along the dune/interdune transect. Each column of the histogram shows the mean and standard deviation of the values from the 5 parallel samples of each transect. The transect profile is represented from west (W) to east (E). Blue, Top; Green, Slope; Black, Base; Red, Interdune.

Bases (east and west) were characterized by indicator species OTU74 from the Acidobacteria Gp16 class and OTU85 from the genus *Kineococcus* sp.

Dune Zone-specific Metabolizing Bacterial Community

FDA hydrolysis was used as a proxy for the active bacterial metabolism (Chrzanowski et al., 1984). Active bacterial communities were detected in all dune areas studied (**Figure 6**), with the highest activity detected in the Interdune samples (average values 0.7–0.75 µg fluorescein g^{-1} soil h^{-1}). Lowest activity was observed in Top and Slope samples (0.05–0.15 µg fluorescein g^{-1} soil h^{-1}) with Base samples showing intermediate values (0.15–0.4 µg fluorescein g^{-1} soil h^{-1}). The results of the Tukey's HSD test on the FDA hydrolysis data indicated that there were only three statistically distinct functional bacterial communities in the dune system: Interdune, Base and Slope/Top (**Figure 6**).

Identification of Environmental Factors Shaping Dune Bacterial Community Structures

To further investigate relationships between abiotic factors and bacterial community structure (i.e., T-RFLP results), we performed redundancy analysis (RDA; **Figure 7**). RDA indicated that of the 21 soil attributes measured, 15 parameters were significant in explaining bacterial community structure variability ($p < 0.01$). However, taken together they only explain 21% of this variability. These included mineral [Na, K, P, NH_4], metallic [Fe, Mn, Al] and granulometry factors [VCS, CS, MS, FS and (VFS/SC/ST/CY)], and nutrients [P, NH_4]. Particle size distribution of the sand dune soils was an important environmental determinant to emerge from the RDA analysis. Our data (**Table 1**) show very clear grain size segregation, with the finest sands, silts and clays predominantly in the Interdune samples and the medium and coarser grained sands on the dunes themselves. The data indicated that fine grains, silts and clays [VFS, SC, ST, CY] are positively correlated with metals [Fe/Mn/Al], minerals [Ca/Mg] and some nutrients (C, P, NO_3^--N, NH_4^+-N) (**Figure 7** and Figure S1C).

Two main clusters of bacterial community samples were separated along the RDA axis1 as shown on **Figure 7**: the dune Base and Interdune bacterial community samples (cluster 1) and the dune Slope and Top samples (cluster 2). The bacterial community structures from cluster 1 were mainly driven by VFS, VCS and NH_4^+-N, K, Fe, and P contents while those of cluster 2 by MS and Na contents. Communities from both clusters were further separated along the RDA axis2 according to their dune biotope of origin, with granulometry parameters [FS and CS] differentiating the structures of the Interdune and dune Base communities as well as the ones from the dune Top and Slope (**Figure 7**).

Overall, these results demonstrate that environmental gradients in the four defined biotopes (i.e., dune Top, Slope, Base, and Interdune) significantly impact the bacterial community structures of dune/interdune transects.

Discussion

The geomorphology and the macro-fauna/flora of the 34,000 km^2 area of the Namib Sand Sea have been extensively studied (Seely and Pallett, 2008; Livingstone, 2013). However, arguably the most productive component of this depauperate environment, the edaphic microbial community (Makhalanyane et al., 2015), has received little attention at either local (a unique dune system) or regional (multiple dune systems) scales (Jacobson, 1997). Moreover, the drivers of desert dune microbial community assembly generally remain unclear.

This study was designed to evaluate the importance of deterministic processes (e.g., habitat filtering) in influencing bacterial communities in the Namib Sand Sea dune system. We provide a comprehensive analysis of a localized site, with 125 soil samples collected from five parallel 2 km dune/interdune transects. All samples were characterized by 16S rRNA gene T-RFLP fingerprinting and the determination of multiple physico-chemical parameters. The use of multiple parallel transects was intended to minimize the effects of local heterogeneity and spatial unevenness, and to generate statistically robust and representative datasets (Prosser, 2010). 16S rRNA gene

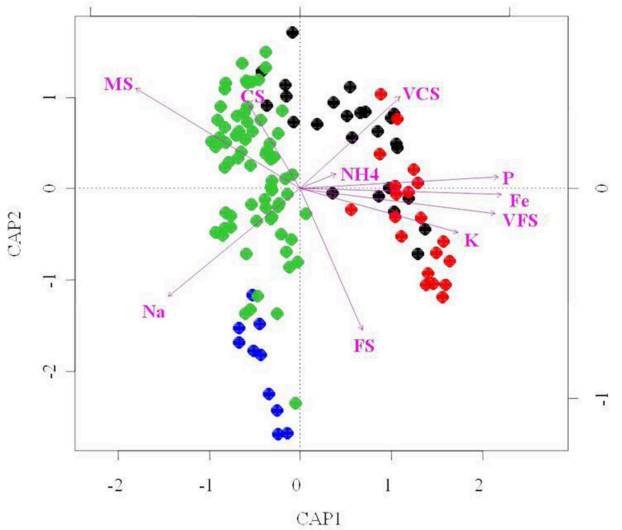

FIGURE 7 | Redundancy analysis (RDA) bi-plot of bacterial diversity and physico-chemical parameters. Only the environmental variables that significantly explained variability in microbial community structures are fitted to the ordination (arrows). The direction of the arrows indicates the direction of maximum change of that variable, whereas the length of the arrow is proportional to the rate of change. Blue, dune Top; Green, dune Slope; Black, dune Base; Red, Interdune.

amplicons from representative samples were also pyrosequenced to provide more insight into the phylogenetic composition of dune/interdune transects and evaluate if *a priori* defined niches would present different community assemblages.

Soil Properties of the Namib Desert Dune/Interdune Transect Define Four Biotopes

The physico-chemical characteristics of soils along the Namib Desert dune-interdune transects clustered into four distinct datasets (**Figure 2**; **Table 1**): dune Top, Slope, Base, and Interdune. Granulometry data were typical for Namib Desert dune systems, with the highest proportion of fine sand on dune summit; a phenomenon commonly attributed to segregation of aeolian deposits from the gravel Interdune zone, and general concentration of coarse sands toward the dune Base (Lancaster, 1981). High Na content in the dune top samples is thought to be the result of salt capture from marine fog, the commonest source of moisture in this particular Namib Desert environment (Abrams et al., 1997; Hachfeld, 2000; Eckardt et al., 2013b). The high metal content recorded in the Interdune zone is likely to reflect the underlying geology, which includes red Tsondab sandstone (Besler, 1996), but may also be a consequence of the higher proportion of the fine soil particle fractions (VFS, SC, ST, and CY), the presence of which reduce percolation and enhance mineral accumulation and persistence (Chamizo et al., 2012).

Bacterial Community Structures, Diversities and Potential Function Suggest Habitat Filtration in Dune/Interdune Transects

A total of 30 bacterial phyla were detected in Namib Desert dune soils and all samples were dominated by *Proteobacteria*, *Actinobacteria*, and *Bacteroidetes*, which has also been observed in other desert systems (Makhalanyane et al., 2015). Namib Desert dune samples showed similar phylogenetic profiles to dune systems from the Gobi (Mongolia) and Taklamaken (China) deserts (An et al., 2013), but with lower OTU diversity than dune slope and interdune of the Gurbantunggut (China) desert (Li et al., 2015). The latter, however, was characterized by cyanobacteria-rich biological soil crusts (BSC), which were not observed in the Namib Sand Sea at the time of sampling.

Both 16S rRNA gene T-RFLP (**Figures 3**, **7**; Figure S1A) and pyrosequencing (**Figures 4**, **5**; **Table 1** and Table S1; Figure S1B) data strongly support the concept of niche-partitioning/habitat-filtering in the Namib Desert dune/interdune system (Dumbrell et al., 2010), as both the structures and phylogenetic compositions of the bacterial communities demonstrated similar clustering than those of the physico-chemical characteristics (**Figure 2**). This was further supported by the low number of OTUs (15% from T-RFLP and 2% from pyrosequencing) shared by all dune biotopes (Figure S1). Taken together, these results strongly imply that habitat filtering, possibly *via* environmental physico-chemical gradients, is a major driver of bacterial community composition in dune ecosystems (Brankatschk et al., 2013; Liu et al., 2014).

While bacterial community T-RFLP fingerprinting and pyrosequencing showed highly comparable overall microbial community patterns (**Figures 3**, **4**), pyrosequencing data showed minor differences in bacterial community structures between the east and west dune slopes (**Figure 4**). As the edaphic characteristics of these dune aspects were not significantly different, it is suggested that other environmental parameters, such as aeolian disturbance (Thomas and Dougill, 2007) or solar radiation (Jacobs and Sundin, 2001) differentials, could account for these differences.

Dune/Interdune Transect Habitat-filters

Distance based RDA analysis showed that 15 edaphic parameters, including mineral [Na, K], metallic [Fe/Mn/Al] and granulometry factors [VCS, CS, MS, FS and (VFS/SC/ST/CY)], and nutrients [P, NH_4] were strongly correlated with bacterial community structure variability in dune soil environments (**Figure 7**, **Table 1**). Using Na as a proxy for soil salinity, we conclude that this factor plays an important role in directing dune microbial community compositions. Variations in salinity have previously been shown to be a strong bacterial habitat filter in Namib Desert gravel plain soils and hypoliths (Stomeo et al., 2013). The importance of soluble salts in dictating bacterial species abundance is supported by the observation that halotolerant microorganisms are readily isolated from interdune soils in the Negev Desert (Yu and Steinberger, 2012b) and Thar Desert sands (Sharma et al., 2013).

The role of K as a habitat filter may be related to intra-cellular osmoregulation (Fierer et al., 2012) while that of metals [Fe/Mn/Al] could be linked to their bioavailability, typically controlled by solubility and/or adsorption onto mineral surfaces (Cornell and Schwertmann, 2003). In this resource-limited desert environment, it was unsurprising to identify the nutrients P

and NH_4 as environmental drivers of dune bacterial community structures. The bioavailability of phosphorus and nitrogen has indeed previously been found to impact desert soil microbial functional and structural properties (Austin et al., 2004; Bell et al., 2014). We note that the use of P-Bray I to quantify total P underestimated the P contents in these mildly alkaline soils (Bowman and Vigil, 2002). In future studies, a more appropriate method such as the sodium bicarbonate procedure (Olsen et al., 1954) should be used to properly evaluate the bioavailable P contents in these desert soils and its potential as a factor shaping dune bacterial communities. The precise role of these dbRDA-determined environmental parameters in shaping dune bacterial community structures and functions could be further tested in controlled experiments (e.g., micro- or mesocosms; Lan et al., 2014).

The single most important environmental determinant to emerge from these data is the desert dune soil particle size distribution. Specific microbe-particle associations have already been demonstrated (e.g., Zhang et al., 2007; Carson et al., 2010). This could be related to the fact that differences in soil structure can manipulate soil nutrient status (Zhang et al., 2007; Carson et al., 2010). Our data do indicate that fine grains, silts and clays [VFS, SC, ST, CY] are positively correlated with metals [Fe/Mn/Al], minerals [Ca/Mg] and some nutrients (C, P, NO_3^--N, NH_4^+-N) (**Figure 2**). This suggests that the relationship between bacterial diversity, soil structure and nutrient contents needs further investigation in dune soils as subtle differences in sand mineralogy and micronutrient contents has been shown to significantly impact edaphic community structuring (Welz et al., 2014).

We noted that 79% of the variation in soil bacterial community structures remains unexplained (**Figure 5**), although this is not uncommon in microbial ecology studies (e.g., Cottenie, 2005; Dumbrell et al., 2010). The high proportion of unaccounted variation might be attributed to other deterministic environmental factors not recorded in this study (e.g., wind transport, effect of sand burial stress or changes in humidity, and/or other soil physicochemistries) or the effects of microbial interactions (Vellend, 2010; Caruso et al., 2011). We also cannot rule out the role of stochastic processes in the assembly of dune communities, as, at a global scale, it has been shown significantly drive bacterial community assembly in deserts (Caruso et al., 2011).

The Namib Desert sand dunes are physically dynamic environments with high levels of wind erosion and dispersion (Eckardt et al., 2013a; Livingstone, 2013). It could be argued that in apparently well-connected habitats such as those of the Namib Sand Sea, dispersal rates should be sufficiently high so as to lead to homogenization of microbial communities due to mass-mixing effects (Leibold and Norberg, 2004). However, our bacterial fingerprint results and phylogenetic analyses show that only a limited number of OTUs were shared across all four dune habitats and that the number of OTUs unique to each area and OTUs shared by contiguous habitats was also low (Figure S1). This suggests that aeolian transport and cellular dispersal is not a substantial driver of sand dune bacterial community structure. However, it cannot be completely excluded. Indeed, in the dune Top zone, which experiences the highest wind disturbance (Eckardt et al., 2013a), the lowest levels of bacterial activity were observed (as determined by fluorescein diacetate hydrolysis: **Figure 6**) and no indicator species was identified. It could be argued that this zone does not represent a stable community and that the detected activity could be derived from microorganisms bound to wind-transported mineral grains (Yamaguchi et al., 2012).

Phylogenetic Composition of Namib Desert Dune Biotope Soils in Relation to Pedogenesis and Ecosystem Development

Many studies have shown that microbial communities evolve with the development of ecosystems, over short (weeks) or long (years/hundreds of years) temporal scales (e.g., Ramond et al., 2012; Ferrenberg et al., 2013; Jangid et al., 2013). For example, in a 6500 year dune soil chronosequence, *Betaproteobacteria*, *Bacteroidetes*, *Actinobacteria*, *Cyanobacteria*, and *Firmicutes* were shown to dominate "young soil ecosystems"; i.e., recent or often-disturbed soil environments (Jangid et al., 2013). Contrastingly, the relative abundances of *Acidobacteria*, *Alphaproteobacteria*, and *Planctomycetes* were higher in "aged soils"; i.e., more structured and stabilized environments (Jangid et al., 2013). Similarly, continuous physical disturbance has been shown to influence community composition in cold desert biocrust soils (Kuske et al., 2012), with continuously trampled soils showing decreasing cyanobacterial abundances with corresponding increases in *Actinobacteria*, *Chloroflexi*, and *Bacteroidetes*, compared to undisturbed soils.

As previously observed in a 6500 year dune soil chronosequence (Jangid et al., 2013), *Betaproteobacteria*, *Bacteroidetes*, and *Firmicutes* were more abundant in the more dynamic dune Tops and Slopes, while the relative abundances of *Acidobacteria* and *Alphaproteobacteria* were higher in the more stable Interdune soils. *Cyanobacteria* were almost exclusively detected in the stable Interdune soils (albeit in proportionally very low percentages: 0.46%), which were dominated by *Actinobacteria*, comprising over 50% of the interdune community (Table S1). Our results are remarkably consistent with the comparative data obtained from disturbed and stable biocrust systems (Kuske et al., 2012) and suggest that the degree of stability or perturbation of the Namib Desert soils may be one of the key deterministic factors contributing to the high proportion of unaccounted variation in community structure of the Namib Desert dune systems.

Concluding Remarks

Our results clearly showed that, in dune/interdune transects of the Namib Desert, soil physico-chemistry define four dune zones, namely the dune Tops, Slopes, Bases and Interdune. Furthermore, we demonstrated that each of these zones presented unique bacterial communities. Altogether, this strongly suggested that these communities were selected through habitat filters, i.e., environmental deterministic

factors (Dumbrell et al., 2010). The measurement of 21 environmental factors enabled us to define that habitat stability, soil texture and mineral and nutrient contents were influencing dune/interdune transect bacterial communities. However, almost 80% of the dune/interdune bacterial community's variability remained unexplained indicating that other unmeasured deterministic factors or processes, such as species' interaction, dispersal and/or stochasticity (Vellend, 2010), may be involved. Consequently, to further investigate bacterial community assembly and its driver(s) in dune systems, such study should be extended to multiple dune/interdune environments.

Author Contributions

SR, JR, and DC designed the experiment. SR performed the lab work and analyzed the data with some help from JR. JR, BJ, SR, MS, and DC participated in writing the manuscript. DC contributed to the reagents, materials and analysis tools required. MS' field knowledge was necessary in the design experiment.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.00845

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Soil bacterial and fungal community responses to nitrogen addition across soil depth and microhabitat in an arid shrubland

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Arid shrublands are stressful environments, typified by alkaline soils low in organic matter, with biologically-limiting extremes in water availability, temperature, and UV radiation. The widely-spaced plants and interspace biological soil crusts in these regions provide soil nutrients in a localized fashion, creating a mosaic pattern of plant- or crust-associated microhabitats with distinct nutrient composition. With sporadic and limited rainfall, nutrients are primarily retained in the shallow surface soil, patterning biological activity. We examined soil bacterial and fungal community responses to simulated nitrogen (N) deposition in an arid *Larrea tridentata-Ambrosia dumosa* field experiment in southern Nevada, USA, using high-throughput sequencing of ribosomal RNA genes. To examine potential interactions among the N application, microhabitat and soil depth, we sampled soils associated with shrub canopies and interspace biological crusts at two soil depths (0–0.5 or 0–10 cm) across the N-amendment gradient (0, 7, and 15 kg ha^{-1} yr^{-1}). We hypothesized that localized compositional differences in soil microbiota would constrain the impacts of N addition to a microhabitat distribution that would reflect highly localized geochemical conditions and microbial community composition. The richness and community composition of both bacterial and fungal communities differed significantly by microhabitat and with soil depth in each microhabitat. Only bacterial communities exhibited significant responses to the N addition. Community composition correlated with microhabitat and depth differences in soil geochemical features. Given the distinct roles of soil bacteria and fungi in major nutrient cycles, the resilience of fungi and sensitivity of bacteria to N amendments suggests that increased N input predicted for many arid ecosystems could shift nutrient cycling toward pathways driven primarily by fungal communities.

Keywords: dryland, shrubland, soil fungal community, soil bacterial community, global change, biological soil crusts, microhabitat, ribosomal RNA

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Introduction

Nitrogen (N) deposition due to human activities has increased dramatically and is predicted to rise with human population growth (Galloway et al., 2008). The effects of N additions on soil microbial communities have been well-studied in mesic forests in the eastern U.S., but the effects of adding N

to dryland ecosystems are poorly understood. Arid lands have distinct characteristics compared to forests, with multiple limiting nutrients, including water, N, and P, and commonly have low inputs of carbon (Collins et al., 2008; Sterflinger et al., 2012), and it is unlikely that microbial responses to N can be accurately inferred from patterns documented in more mesic systems, particularly given documented interactions between soil properties and N responses. Historically, arid regions have received lower natural N inputs (∼1–2 kg ha^{-1} yr^{-1}; Phoenix et al., 2006), but it is not known if these lower levels have similar impacts comparable to higher inputs to more mesic regions. In addition, the native microbial communities in drylands are highly dissimilar from those found in mesic environments (Fierer et al., 2003; Dunbar et al., 2012). The effects of experimental climate change on soil microbial communities can depend upon the composition of the native community (Hawkes and Keitt, 2015), suggesting that variation among ecosystems could alter patterns of community structure and response to N deposition. Urban areas are sources of atmospheric N and are increasing in arid ecosystems (Galloway et al., 2004); thus, a future increase in N deposition is likely in these regions. Understanding soil microbial community responses to additional N input will provide insight on how we may expect the functions of dryland soils to change in response to future conditions, and how we may effectively manage nitrogen inputs into drylands to maintain productivity and resilience of microbial communities.

Drylands are typified by high heterogeneity in seasonal climate, the distribution of nutrients across the landscape, and physical microhabitats. Many hot dryland ecosystems experience strong seasonal shifts, with pronounced dry and rainy seasons and large fluctuations in temperature (Collins et al., 2008). These systems are also characterized by widely spaced vegetation, with biological soil crusts (biocrust) occupying the soil surface between the vascular plants (Belnap, 2003). Habitat heterogeneity has been shown to promote beta diversity across small geographic ranges for plants, insects, and microorganisms (Amarasekare, 2003), where differences in climatic factors are minimal. Previous studies of microbial community biomass and composition in dryland systems suggests these features are strongly correlated with differences in microhabitat (Garcia-Pichel et al., 2003; Schade and Hobbie, 2005; Bates and Garcia-Pichel, 2009; Steven et al., 2013, 2014), which can, in turn, shift function and subsequently alter microbial responses to changes in climate such as altered precipitation (Johnson et al., 2012; Delgado-Baquerizo et al., 2014). In addition, at a broad scale, microbial metabolic pathways have also been shown to differ between rhizosphere and biocrust soils (Steven et al., 2014).

Although most dryland soils do not have highly weathered soils with characteristic organic horizons, soil nutrients are highly stratified by depth (Pointing and Belnap, 2012). In addition, drylands are characterized by highly patchy distribution of vegetation (Collins et al., 2008), leading to high levels of heterogeneity at both vertical and horizontal scales. A full understanding of the microbial response and ecosystem effects of N addition in arid systems therefore requires examination of multiple microhabitats and soil depths. Strong vertical stratification of communities across shallow soil horizons has also been found for bacterial communities in dryland ecosystems (Kuske et al., 2002; Steven et al., 2013), with significant differences in communities between plant and crust-associated soils (Steven et al., 2013), but next generation sequencing technologies now provide the means for in-depth analysis of microbial community shifts. Using high throughput sequencing, we examined fungal and bacterial community response to N addition within two microhabitats: the plant root zone and interspaces soils covered by biocrusts. Soils were sampled at two depths in each microhabitat (0–0.5 cm surface veneer, and 0–10 cm soil).

Previous studies have documented patterns of bacterial or fungal composition in arid lands (e.g., Kuske et al., 2002; Bates et al., 2012; Steven et al., 2013, 2014), but have not examined the effects of N. In addition, few studies have utilized high-throughput sequencing methods to characterize microbial communities within drylands (Makhalanyane et al., 2015). Correlated shifts in bacterial communities, catabolic profiles, and metagenomic surveys have been shown in experimental N sites (Amarasekare, 2003; Fierer et al., 2012), but with no concurrent analysis of the fungal community. Although bacterial biomass generally exceeds that of fungal biomass in arid ecosystems (Bates and Garcia-Pichel, 2009), the majority of biogeochemical cycles in terrestrial ecosystems are driven by both bacteria and fungi (van der Heijden et al., 2008), so an understanding of the responses of both bacterial and fungal communities is needed to predict long-term ecosystem effects of N inputs. Consequently, we conducted compositional analysis of these two communities, combined with examination of nutrient transformations (Sinsabaugh et al., 2015), to provide insights into both the community- and ecosystem-level effects of N deposition on drylands.

Materials and Methods

The study site is located within the Lake Mead National Recreation Area in southern Nevada (36.009 N, 114.797 W). Two species of shrubs, *Larrea tridentate* and *Ambrosia dumosa* comprise the dominant vegetation, interspersed with patches of poorly developed biological soil crusts (biocrusts). Soils are alluvial, with high sand content (80%), low organic carbon (0.35%) and slightly basic pH (7.4 in bulk soils). Mean high and low annual temperatures are 23.8°C and 11.1°C, respectively, with mean annual precipitation of 19.8 cm based on data collected from a nearby weather station.

Within a 100 × 100 m plot, fifteen 2 × 2 m subplots were established, centered around similarly-sized *Ambrosia dumosa* shrubs. To examine the effects of N on plant and soil microbial communities, N was experimentally manipulated at two levels: 7 and 15 kg^{-1} ha^{-1} yr was added, along with an ambient control, hereafter referred to as N7, N15 and ambient. Nitrogen was applied in the form of ammonium nitrate in water every 2 months from March 2012 to May 2013, when soils were harvested.

To examine potential impacts of N amendment on bacterial and fungal community composition, we sampled soils from the two microhabitats (shrub and interspace) across two soil depths (surface and sub-surface) (**Figure 1**). Surface soils were

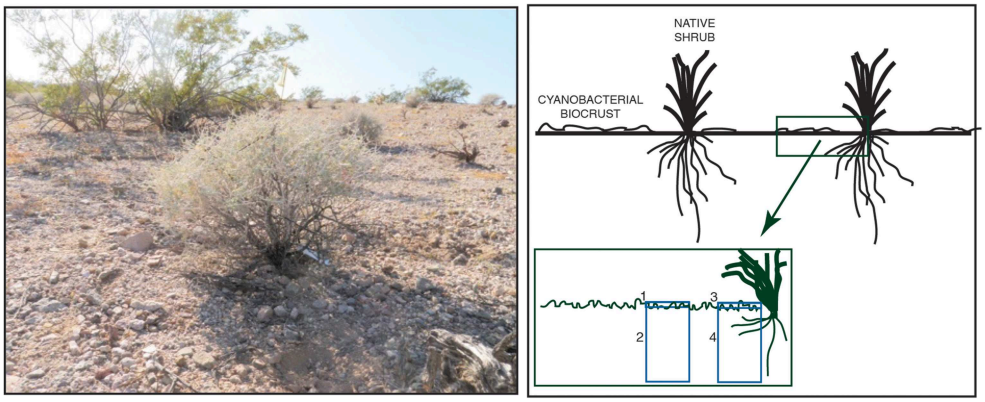

FIGURE 1 | Left. Photo of *Ambrosia dumosa* shrub and surrounding interspace at the field site. **Right.** The sampling design used to examine bacterial and fungal communities in different locations in the soil.

sampled to a depth of 0.5 cm, and sub-surface soils were sampled to a depth of 10 cm. Five replicates per N treatment were collected from four sampling locations: shrub canopy surface at 0–0.5 cm depth (canopy biocrust), shrub canopy bulk soils at 0–10 cm depth (canopy bulk), interspace surface at 0–0.5 cm depth (interspace biocrust), and interspace bulk soil at 0–10 cm depth (interspace bulk). A total of 60 samples were collected. Soils were homogenized by hand and total nucleic acids were extracted from 0.5 g of soil using the MP Biomedical FastDNA for Soil kit (MP Biomedical, Santa Ana CA, USA) according to the manufacturer's instructions.

Bacterial and fungal communities were targeted for Illumina high-throughput sequencing using a two-stage PCR approach described previously (Mueller et al., 2014). The V3-V4 region of the bacterial 16S rRNA gene was amplified using the F515-R806 primer pair (Bates et al., 2010), and the D2 hyper-variable region of the fungal LSU rRNA gene was amplified using the newly designed LR22R primer (TAGCGMACAAGTASMGTGA) with the LR3 primer as the reverse (http://sites.biology.duke.edu/fungi/mycolab/primers.htm). Samples were barcoded to facilitate multiplexing using a combinatorial approach (Gloor et al., 2010), with a unique 6 bp barcode inserted into both the forward and reverse primer. Illumina-specific sequences were added in a second PCR. Amplicons were cleaned using the MoBio –htp UltraClean 96-well plate clean up kit according to the manufacturer's directions, but with a modification to reduce the amount of binding buffer from 5X to 3X sample volume to facilitate removal of longer primer-dimers. Individual samples were quantified using the Quant-It high-sensitivity double stranded DNA kit (Invitrogen, Carlsbad CA USA) using the BioTek Synergy H1 microplate reader and combined at equimolar concentrations. The multiplexed library was cleaned using the MoBio UltraClean PCR clean up kit and eluted in 50 µl EB buffer. Dissimilar values from fluorometry and Illumina-specific qPCR indicated that the second PCR step did not add the Illumina-specific primers to all samples, and a final PCR, with conditions identical to the second PCR, was performed on the library, for a total of three cycles. Library length and concentration was verified using a bioanalyzer and qPCR, respectively. Amplicons were sequenced using paired end 250 bp reads on the Illumina MiSeq platform at Los Alamos National Laboratory. To offset the low diversity of nucleotides in the initial sequence common in amplicon sequences, genomic DNA from multiple bacterial isolates was added at approximately 30%.

Bacterial and fungal community sequence datasets were de-multiplexed individually using QIIME (Caporaso et al., 2010b), with quality filtering to remove any sequence with a mismatch to the barcode or primer sequence. Downstream sequence processing was conducted using UPARSE (Edgar, 2013). Sequences within an expected error greater than 0.5 and singleton sequences were removed. Putative chimeras were identified *de novo* using UCHIME (Edgar et al., 2011) and removed, and OTUs were delineated at 97% sequence similarity. The OTU table was exported, and all statistical analyses were conducted using the R statistical platform (r-sourceforge.org). Representative fungal and bacterial sequences were selected and classified against their respective Ribosomal Database Project (RDP) databases using the online tool. Any sequence identified as non-Fungal or non-Bacterial was removed from subsequent analyses. Sequences were deposited to the MG-RAST server (Meyer et al., 2008) under IDs 4635375.3 (Bacteria) and 4635376.3 (Fungi).

To construct phylogenetic trees, we utilized reference phylogenies constructed using partial to full-length ribosomal sequences. For the bacteria, we used the tree from Kembel et al. (2012), and for fungi we used a tree constructed from fungal sequences generated by the Assembling the Fungal Tree of Life Project (Celio et al., 2007). Representative OTU sequences were aligned to the reference taxa using PyNAST (Caporaso et al., 2010a) and placed on their respective reference phylogenies using pplacer (Matsen et al., 2010).

Statistical analyses were conducted using the R libraries picante (Kembel et al., 2010), vegan (Oksanen et al., 2013), and phyloseq (McMurdie and Holmes, 2013). Analyses were conducted by randomly selecting 5200 sequences for bacteria and 4500 sequences for fungi, with 99 trials for each analysis to limit bias resulting from random sampling of communities. Three bacterial and two fungal samples had insufficient

sequencing depth and were not included in the analyses. Richness measures, including OTU richness and phylogenetic diversity (PD) (Faith, 1992) were compared using Three-Way ANOVA with N treatment, microhabitat, and soil depth as fixed factors. Community composition based on OTU similarity (Bray-Curtis dissimilarity metric) and phylogenetic similarity using the weighted UniFrac distance (Lozupone and Knight, 2005) were compared using PERMANOVA with all three factors included in the model (Anderson, 2001). In addition, to examine responses at broader taxonomic scales, we grouped fungal and bacterial OTUs at class and phylum levels, respectively, and tested for significant differences in relative abundance across N treatment, soil depth, and microhabitat using Three-Way ANOVA.

Results

Following removal of low-quality sequences, a total of 1,340,717 fungal LSU sequences and 1,466,336 bacterial 16S sequences were included in the analysis. Across all samples, we observed 1990 fungal OTUs and 10167 bacterial OTUs. Bootstrap estimates of gamma diversity indicated that both communities were slightly under-sampled; the bootstrap estimate was 2226 (±45 SE) for fungi and 10884 (±160 SE) for bacteria (**Figure 2**).

Effects of Microhabitat and Soil Depth

Microhabitat and soil depth each had strong effects on fungal and bacterial community richness and PD. For the fungal community, soil depth was the strongest driver of diversity differences for both OTU richness ($F = 128$, $p < 0.001$) and PD ($F = 11.7$, $p = 0.001$). Surprisingly, richness and PD were higher in the bulk soil depth (**Figure 3**). OTU richness did not vary significantly between the shrub canopy and the interspace ($F = 3.34$, $p = 0.07$), although there was a trend for higher fungal richness in association with interspace compared to shrub canopy soils (**Figure 3**). However, PD of fungal communities associated with the interspaces was significantly higher than those found in the shrub canopy ($F = 4.42$, $p = 0.04$).

The bacterial community also showed strong differences in relation to soil depth. As with the fungal community, OTU richness ($F = 140$, $p < 0.001$) and PD ($F = 137$, $p < 0.001$) were significantly higher in the deeper bulk soils (**Figure 3**). Bacterial richness also differed significantly between the two microhabitats, with higher richness ($F = 0.24$, $p < 0.001$) and PD ($F = 58$, $p < 0.001$) observed in the interspaces than in the shrub canopy when analyzed across both soil depths.

Although for fungal and bacterial communities, the strongest richness differences were found between the two soil depths, the largest driver of variation in community composition was microhabitat (**Figures 4**, **5**). This pattern was observed using both OTU-based measures (Fungi: $F = 8.89$, R2 = 0.13, $p = 0.001$, Bacteria: $F = 6.10$, R2 = 0.10, $p = 0.001$) and phylogenetic-based measures (Fungi: $F = 11.6$, R2 = 0.15, $p = 0.001$, Bacteria: $F = 23.9$, R2 = 0.23, $p = 0.001$). Both communities were also delineated by soil depth, but this factor explained a lower proportion of community variation for both OTU-based (Fungi: $F = 2.50$, R2 = 0.04, $p = 0.001$, Bacteria: $F = 2.62$, R2 = 0.04, $p = 0.03$) (**Figure 4**) and phylogenetic measures (Fungi: $F = 9.49$, R2 = 0.12, $p = 0.001$, Bacteria: $F = 15.7$, R2 = 0.15, $p = 0.001$) of community similarity (**Figure 5**).

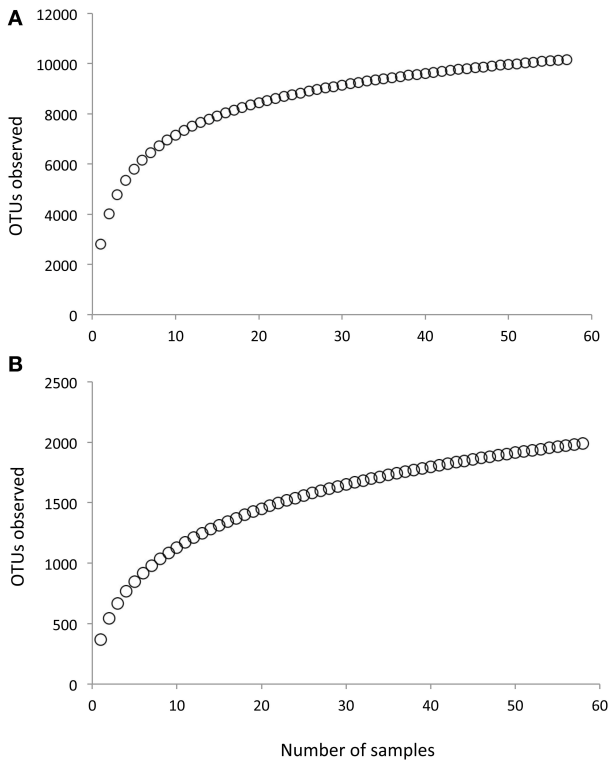

FIGURE 2 | Rarefaction curves of (A) Fungal communities and (B) Bacterial communities.

Nitrogen Effects on Microbial Communities

We found no evidence for fungal community responses to N addition. We observed no shifts in OTU richness in response to N addition ($F = 0.48$, $p = 0.62$) and no change in Faith's PD ($F = 0.11$, $p = 0.90$) (**Figures 4**, **5**). Neither OTU-based community composition ($F = 1.17$, R2 = 0.04, $p = 0.18$), nor phylogenetic composition ($F = 1.18$, R2 = 0.04, $p = 0.27$) shifted in response to experimental N additions (**Figures 4**, **5**).

In contrast to responses in the fungal community, we found consistent negative responses to N in the bacterial community diversity indices (**Figure 3**). Both richness ($F = 9.85$, $p < 0.001$) and PD ($F = 11.7$, $p < 0.001$) declined in N-amended plots. Tukey's Honestly Significant test indicated that for both measures, intermediate and high levels of N (N7 and N15) were not significantly different ($p > 0.2$ for each). Nitrogen addition was associated with community shifts using both OTU-based ($F = 1.34$, R2 = 0.04, $p = 0.02$) and phylogenetic measures ($F = 3.42$, R2 = 0.07, $p = 0.001$; **Figure 4**). Using the UniFrac distance measure, we also found a significant N X soil horizon depth interaction ($F = 1.87$, R2 = 0.04, $p = 0.04$), where community shifts were more pronounced at lower soil depths.

Broad Taxonomic Responses

The phylum Ascomycota was the dominant fungal phylum within this shrubland. At finer taxonomic scales, the most

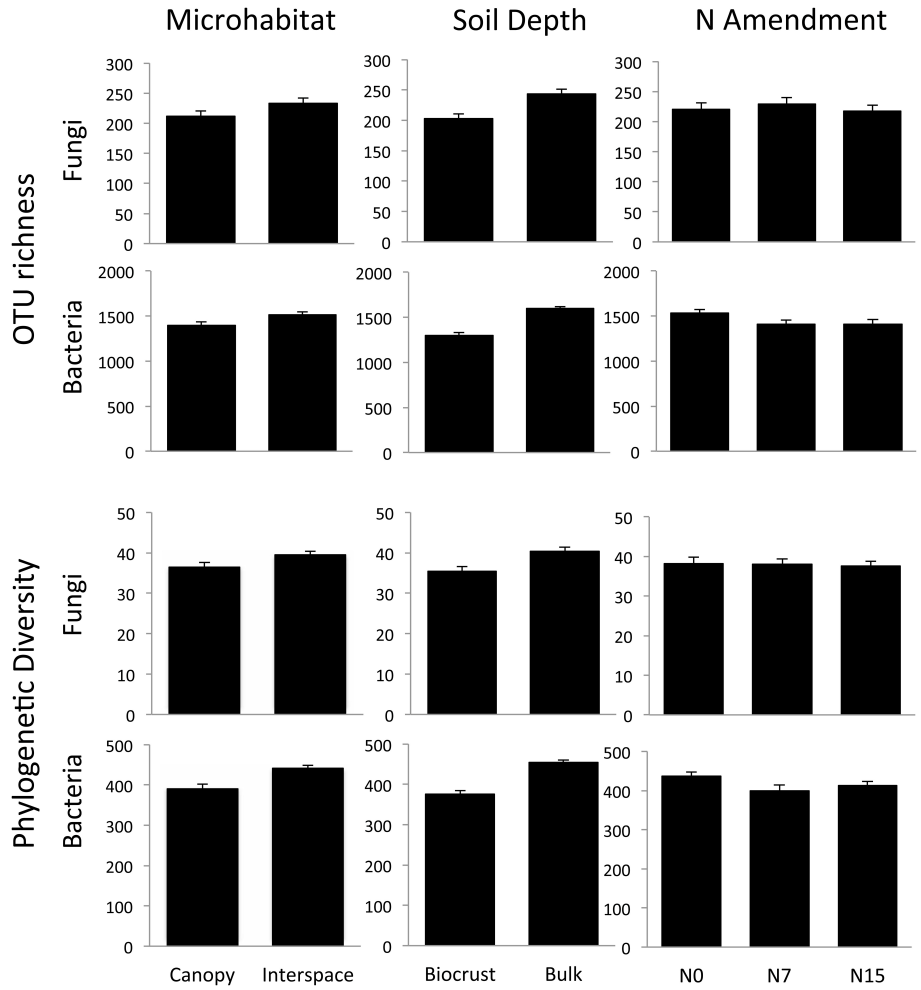

FIGURE 3 | OTU richness and phylogenetic diversity (Faith's PD) in the N-amended plots across different soil depths and microhabitats. Both communities showed significant differences across soil depths and microhabitats, but only bacteria showed significant shifts in response to N. While results are illustrated here by individual treatment, the results were analyzed using a Three-Way ANOVA framework. See Results text for statistical support.

abundant fungal classes included the Dothideomycetes, Pezizomycetes, and the Basidiomycete class Agaricomycetes. The bacterial community was dominated by the Proteobacteria, followed by the Actinobacteria and Acidobacteria (**Figure 6**).

When bacterial and fungal communities were examined by grouping OTUs into bacterial phyla or fungal classes, respectively, we found that only a single fungal class, the Sordariomycetes, showed a response to N addition, with a nearly linear increase in relative abundance in N amended plots ($F = 4.34$, $p = 0.02$). Three classes, the Leotiomycetes, Sordariomycetes, and Eurotiomycetes, were all significantly more abundant at deeper soil depths (for each, $p < 0.03$). No significant effect of microhabitat on the abundance of any fungal class was observed.

In contrast, numerous bacterial phyla showed significant shifts in relative abundance in response to N additions (**Table 1**). The two most abundant groups, the Actinobacteria and Proteobacteria, showed negative responses at the highest level of N input. Responses for the classes within the Proteobacteria were consistent, with declines observed for alpha, beta, delta, gamma, and epsilon classes. Four other bacterial phyla, the Acidobacteria, Chloroflexi, Firmicutes, and Verrucomicrobia, showed significant positive responses to N amendment. In addition, a number of bacterial phyla showed significant differences across soil depth and microhabitat type. The relative abundance of most phyla was higher at lower soil depths, with the exception of Proteobacteria and Verrucomicrobia, which exhibited higher relative abundance in the surface soils. Microhabitat also affected a large number of bacterial phyla. For example, the relative abundance of Acidobacteria was higher in the interspace soils, while the proportion of Bacteroidetes was higher in shrub-associated soils (**Table 1**).

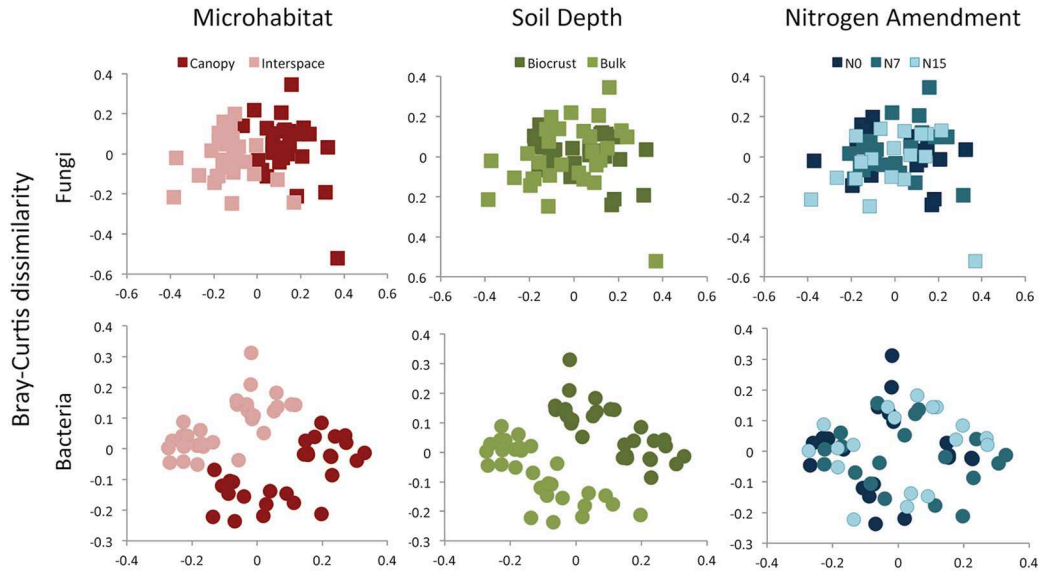

FIGURE 4 | Bray-Curtis dissimilarity among fungal or bacterial communities, displayed as 2D MDS plots. Plots are color coded to show microhabitat (red), soil depth (green), or N amendment (blue) differences. While results are presented for individual treatments, results were analyzed using a three-way framework with PERMANOVA. See Results text for statistical support.

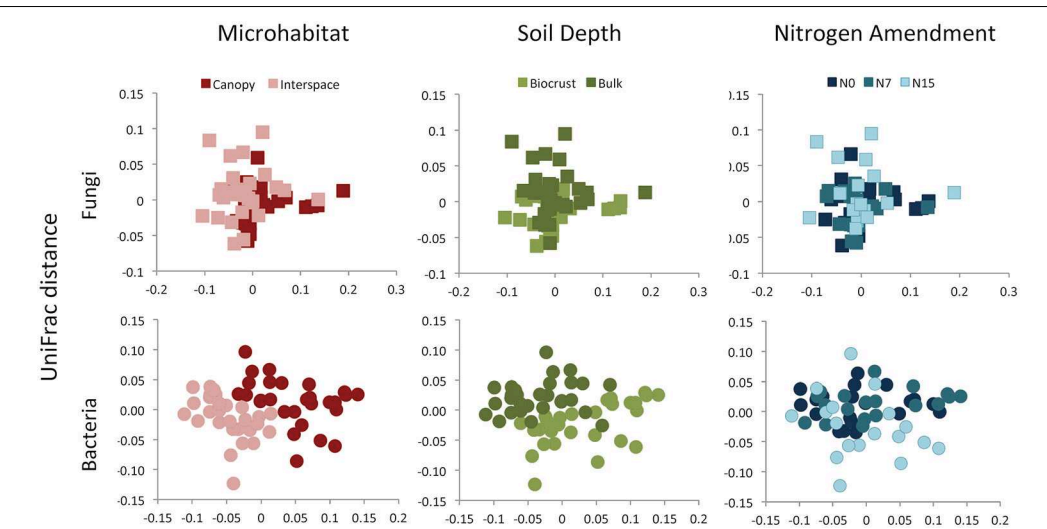

FIGURE 5 | Community composition (UniFrac distance) of fungal or bacterial communities, displayed as 2D MDS plots. Plots are color coded to show microhabitat (red), soil depth (green), or N amendment (blue) differences. See Results text for statistical support.

Discussion

Our study objective was to determine the cumulative impacts of N deposition, delivered every 2 months for 14 months, on the microbial communities within an arid shrubland ecosystem. Because of its mosaic nature, we partitioned the landscape into compartments and separately assessed the N amendment impacts on those compartments. This study represents the first high-depth-of-coverage, taxonomic survey of both the fungal and bacterial communities in four shrubland microhabitats—shrub canopy and interspace soils, each stratified at two shallow depths. We also examined the sensitivity of those fungal and bacterial communities to short-term (1 yr), relatively low concentrations of N amendment.

Fungal and Bacterial Community Differences with Soil Depth

In a companion report to this study, bacterial and fungal biomass, measured by qPCR, was significantly higher in the 0–0.5 cm biocrusts than in 0–10 cm depth bulk soil (>100% and >200% higher, respectively; Sinsabaugh et al., 2015). This is a typical pattern for arid shrublands and grasslands, where, by multiple

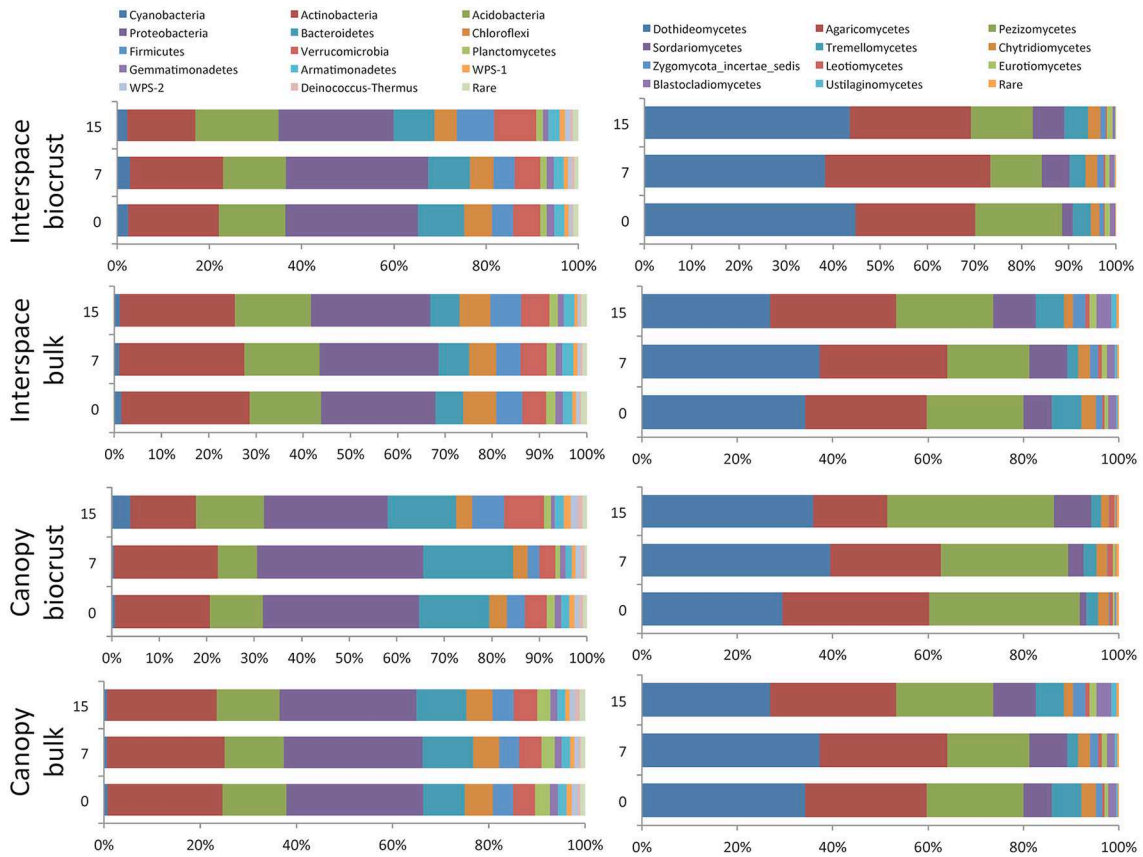

FIGURE 6 | Relative abundance of different bacterial phyla (left) and fungal classes (right). Only groups with a minimum relative abundance of 1% are shown.

measures, microbial biomass has been found to be concentrated in the upper few cm of soil (Dunbar et al., 2012; Pointing and Belnap, 2012; Steven et al., 2013). Biocrust biomass may differ substantially with soil type (Steven et al., 2012) and with biocrust developmental stage (Garcia-Pichel et al., 2003; Yeager et al., 2004), particularly in Cyanobacterial biocrusts where the proportion and density of the dominant cyanobacteria in the biocrust controls the overall biomass. Our study partitioned soil depth into intervals at the cm scale; however, biomass of biocrust soils is stratified even at the mm interval (Garcia-Pichel et al., 2003).

Although biomass (qPCR) was higher in the upper soil depths, the OTU-based richness for both fungal and bacterial communities was higher in the 0–10 cm depth interval compared to the 0–0.5 cm biocrust. We found no significant interaction between depth and microhabitat, indicating that this pattern was consistent for biocrusts and shrub canopy locations (**Figure 2**). This pattern has been found in prior studies of arid land biocrusts, where the dominance of a few cyanobacterial sequence types (often 40–60% of the total sequences) is associated with lower richness estimates (Johnson et al., 2012; Steven et al., 2013) relative to soils below the biocrust. A contrasting feature of this shrubland is that the relative abundance of Cyanobacteria within the biocrusts is very low (∼3%; **Figure 6**), indicating that the biocrusts here are poorly developed. As a result, it is possible that higher bacterial and fungal richness at lower soil depths is due to UV radiation stress coupled with drier soil conditions and wider temperature swings experienced by other soil organisms in the upper soil layers. For the fungal community, we also found evidence for selection of UV tolerant taxa in the upper soil depths relative to the deeper soils, with higher abundance of the orders Pleosporales and Dothidiales, which contain numerous species of melanized fungi (Sterflinger et al., 2012). Evaporation is likely greater in upper soil layers, and previous studies have shown that bacterial richness is strongly correlated with soil moisture in hyperarid sites, with decreasing diversity as moisture becomes increasingly limiting (Crits-Christoph et al., 2013).

For both the bacterial and fungal communities, broad taxonomic groups showed different patterns across soil depth. With the exception of Cyanobacteria noted above, the phylum level distribution of bacteria by depth is parallel to that reported by Steven et al. (2014), who showed that for three different soil types covered by well-developed biocrusts, the relative abundance of Acidobacteria, Actinobacteria, and Chloroflexi were higher below the surface biocrust than within the biocrust. Class level comparisons within the fungi showed higher abundance of the Leotiomycetes, Sordariomycetes, and Eurotiomycetes in the bulk soil when compared to the biocrust, with no significant shift in other classes (**Figure 6**). For example, the relative abundance of the Eurotiomycetes was double in

TABLE 1 | *F*-values from a Three-Way ANOVA comparing the abundance of different bacterial phyla in response to nitrogen(N) addition, soil depth, and microhabitat.

	N	N response	Soil depth	Depth response	Microhabitat	Micro- response
Acidobacteria§	13.7***	↑	6.0**	L	65.5***	I
Actinobacteria	10.1***	↓	57.2***	L	0.90	
Armatimonadetes±	1.14		0.11		46.0***	I
Bacteroidetes±	2.91		73.9***	U	92.3***	C
Chloroflexi	4.59*	↓	55.6***	L	45.8***	I
Firmicutes§	19.0***	↑	0.01		23.2***	I
Planctomycetes	1.52		58.2***	L	15.4***	C
Proteobacteria§	9.62***	↓	18.7***	U	29.4***	C
Verrucomicrobia§	12.1***	↑	6.80*	U	10.2**	I

*Significance levels are represented as *p < 0.05, **p < 0.01, ***p < 0.001. Nitrogen response indicates the overall trend of N response (positive or negative). Depth and microhabitat response indicates the factor where the highest abundance was observed, where U indicates upper biocrust soil layer, L indicates lower bulk soil layer, I indicates interspace soils, C indicates shrub canopy soils.*

§ Indicates a significant interaction between N and soil depth.

± Indicates a significant interaction between soil depth and microhabitat.

The arrows correspond to positive or negative responses to nitrogen.

the lower soil depths compared to the surface layers (1.25% and 0.61%, respectively). It is also notable that we observed higher abundance of the Agaricomycetes at this site compared to previous studies in drylands (Bates and Garcia-Pichel, 2009; Steven et al., 2014), although this class did not show significant responses to any of the three factors analyzed.

Fungal and Bacterial Community Differences between Shrub Canopies and Interspaces

Based on qPCR assays conducted at this site, fungal and bacterial biomass was higher in the shrub canopy soils than in the interspace soils (Sinsabaugh et al., 2015). In the current study, we found that the richness of both fungi and bacteria was higher in interspace soils compared to shrub-associated soils (**Figure 3**). This differs from the findings of Steven et al. (2014) conducted at a nearby site in the Mojave Desert in Nevada, in which fungal and bacterial richness was higher in shrub root zones than in the interspaces. This could be due to differences in biocrusts between this and the current study; the biological crusts examined in Steven et al. (2014) were well-developed and dominated by a high biomass of Cyanobacteria, whereas the relative abundance of cyanobacterial sequences in the biocrusts in the current study was relatively low, with greater dominance by Proteobacteria and Actinobacteria, thus more closely resembling the communities found at 0–10 cm soils in the Steven et al. (2014) study. Because the bacterial community is not dominated by a single phylum (Cyanobacteria) within the biocrusts at our site, it is possible that the different responses observed are linked with variability in competitive interactions between fungi and bacteria (Boer et al., 2005) within well-developed biocrusts compared to bulk soils, either directly via resource competition, or through the different types of carbon inputs in well-developed crusts (e.g., derived from Cyanobacteria).

In addition to contributing to differences in richness, microhabitat was the strongest driver of shifts in community composition for both fungi and bacteria (**Figures 4**, **5**). The role of plants in altering microbial communities has been documented in numerous dryland systems, where communities associated with the rhizosphere were distinct from those found in un-vegetated soils (Andrew et al., 2012; Ramond et al., 2014; Steven et al., 2014). Plants have often been considered to be "islands of fertility" in arid ecosystems, and while biomass is often higher in the rhizosphere (e.g., Pointing and Belnap, 2012), Ben-David et al. (2011) found no significant differences in bacterial biomass between shrubs and interspace soils in the arid portion of the Negev desert. In the current study, we found that bulk soils can harbor diverse bacterial and fungal communities, but plants appear to select for a specific group of microbial taxa.

Soil Community Responses to N Amendment

Fungal and bacterial communities exhibited differential responses to N amendment. Fungal community biomass as measured by rRNA gene qPCR (Sinsabaugh et al., 2015) and richness did not change significantly with N amendment (**Figure 3**). Compositionally, members of the fourth most abundant fungal class, the Sordariomycetes, showed significant positive responses to N addition (**Figure 6**). Lauber et al. (2008) showed that members of this class residing in forest and pasture soils were higher in soils with higher P content and with higher C:N ratio. In our study site, N amendment significantly increased available N and P by 30 and 25% respectively, over the ambient condition (Sinsabaugh et al., 2015), and it is possible that the observed increase in Sordariomycete is a result of N or P no longer being limiting for these fungi.

In contrast to the limited responses exhibited by the fungal community, the bacterial community responded with changes in richness and composition. Bacterial richness declined with increasing levels of N (**Figure 3**), and the relative abundance of multiple bacterial phyla shifted with N amendment (**Table 1**). However, the bacterial composition response patterns to N amendment we observed are not consistent with previously hypothesized ecological strategies of soil bacteria (Fierer et al., 2007) or taxonomic response patterns for these phyla under N amendment in two mesic grasslands (Fierer et al., 2012;

Ramirez et al., 2012). Where culturable, the Acidobacteria, and Verrucomicrobia have been typified by small cells, slow growth, and ability to use a wide variety of complex carbon substrates; thus, they could be considered oligotrophs (Fierer et al., 2007). On the other hand, the Firmicutes may exhibit rapid growth followed by abundant spore formation and are generally considered copiotrophs. All three phyla contain species that can persist for extended periods of time in the soil in dormant states, becoming active under favorable conditions (Lennon and Jones, 2011). We found significant increases in the relative abundance of the Acidobacteria, Firmicutes, and Verrucomicrobia phyla despite their different ecological strategies. In contrast, we found that the relative abundance of Actinobacteria, Chloroflexi, and Proteobacteria decreased in soils due to increased soil N. There are few commonalities among and within these three phyla with regard to growth rates or known carbon use lifestyles (oligotrophs vs. copiotrophs) in the soil, indicating that in this arid ecosystem, responses cannot be readily predicted based on *a priori* estimates of ecological strategies. This could be due to differences among ecosystems (e.g., mesic vs. arid) or as a result of greater variability in ecological strategy within bacterial phyla than has been previously observed.

One of the most striking patterns observed was that N amendment resulted in a decrease in the relative abundance of two dominant phyla, the Proteobacteria and Actinobacteria. Previous studies on plant communities have found that rare taxa tend to be susceptible to N deposition (Shaw et al., 2002; Clark and Tilman, 2008). Here, we show the opposite response pattern in bacterial communities. The decreased abundance of the Proteobacteria may be of particular importance in drylands, as members of the phylum have been shown to promote plant growth and facilitate horizontal transfer to genes involved in photosynthesis (Makhalanyane et al., 2015). The driver of this unexpected response could be complex interactions between resource availability and microbial responses, or could be due to phenotypic plasticity under variable environmental conditions, such that closely related organisms in different environments exhibit different traits. Alternatively, N deposition could shift competitive interactions within the bacterial community, leading to taxonomic losses in abundant groups, such as the Proteobacteria. Different responses to N based on broad functional groups have been observed across previous studies. A recent meta-analysis found that N deposition increased autotrophic respiration by 22%, with a concurrent 13% decrease in heterotrophic respiration (Zhou et al., 2014).

The physiological underpinnings of the different, yet dramatic response of bacteria among experimental N-amendment studies remain unknown, and are difficult to infer or generalize at the phylum level. Possibilities include different within-phylum species composition among the different soils tested, different responses to N amendment in the alkaline soil of the arid shrubland compared to the acidic soils in more mesic settings, and *differences* due to inherent organic matter or elemental concentrations in the soils. Indeed, Liu and Greaver (2009) noted in a review article that soil microbial respiration increased in grassland biomes but tended to decrease in forest biomes with N additions. Similarly, Janssens et al. (2010), found that although soil respiration decreased an average of 15% across many forests with an N amendment, the response range was huge, ranging from −57% to +63%. Clearly, studies incorporating both community and process analyses are required to determine key responsive populations and soil process responses to altered N deposition.

Implications for Ecosystem Processes

Based on a companion study conducted at the same site, aspects of the N cycle (N mineralization, nitrification, and ammonification) were all higher in the biocrust compared to the deeper bulk soils, and only the upper biocrust layer showed a significant response to N addition (Sinsabaugh et al., 2015). Diversity has been linked with increased nutrient cycling rates (Philippot et al., 2013), but particular functional groups, such as denitrifying bacteria, are often studied in isolation. In natural communities with multiple co-existing functional groups, nutrient cycling rates are likely due not only to species richness, but also to interacting factors, such as the strength of competitive interactions or species traits such as community niche (Salles et al., 2009). For example, *Fusarium*, a fungal genus in the class Sordariomycetes, is known to function in denitrification. The Sordariomycetes was the only fungal class that responded to N addition; however, we also found higher richness in the lower soil depths with added N. In addition, fungal isolates capable of denitrification are widely distributed across the fungal phylogeny in classes such as the Eurotiomycetes and Leotiomycetes (Jasrotia et al., 2014).

Despite limited direct links between community measures and nutrient cycling, our findings have potential implications for pathways of N cycling within this system. The lack of response in the fungal community with strong shifts in the bacterial community suggests that increased N availability will likely shift toward fungal-dominated pathways in N-amended soils. Fungi and bacteria have different functional roles and patterns of resource utilization related to decomposition (Schneider et al., 2012), and shifts in the relative abundance of these two groups will likely alter biogeochemical cycles (Litchman et al., 2015). Whether community shifts observed will alter decomposition in this system is less clear. Nitrogen addition has been shown to increase decomposition rates, but this is not mediated by the initial phylogenetic relatedness of the fungal community (Amend et al., 2015). However, the realized composition of the fungal community was not measured in this study, and so the links between fungal communities and decomposition could not be compared directly. Fungi can operate at much lower soil water potentials (Chen et al., 2015), and so the functional roles of fungi in dryland ecosystems differ from the well-studied roles of fungi within mesic areas, particularly in terms of carbon utilization (Makhalanyane et al., 2015), denitrification (Laughlin and Stevens, 2002; Crenshaw et al., 2007; Chen et al., 2015), and potential lateral nutrient transfer among biocrusts and plants (Green et al., 2008). The findings of the current study illustrate that nitrogen effects on microbial communities are complex, and responses observed in one ecosystem may not be apparent in others. The impacts of nitrogen in terrestrial ecosystems are likely to occur across large portions of the globe, suggesting that increased efforts to quantify effects on microbial communities in less understood ecosystems will provide important insights into

how nitrogen inputs will alter ecosystem functions at landscape scales.

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Microsite Differentiation Drives the Abundance of Soil Ammonia Oxidizing Bacteria along Aridity Gradients

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Soil ammonia oxidizing bacteria (AOB) and archaea (AOA) are responsible for nitrification in terrestrial ecosystems, and play important roles in ecosystem functioning by modulating the rates of N losses to ground water and the atmosphere. Vascular plants have been shown to modulate the abundance of AOA and AOB in drylands, the largest biome on Earth. Like plants, biotic and abiotic features such as insect nests and biological soil crusts (biocrusts) have unique biogeochemical attributes (e.g., nutrient availability) that may modify the local abundance of AOA and AOB. However, little is known about how these biotic and abiotic features and their interactions modulate the abundance of AOA and AOB in drylands. Here, we evaluate the abundance of *amoA* genes from AOB and AOA within six microsites commonly found in drylands (open areas, biocrusts, ant nests, grasses, nitrogen-fixing shrubs, and trees) at 21 sites from eastern Australia, including arid and mesic ecosystems that are threatened by predicted increases in aridity. Our results from structural equation modeling suggest that soil microsite differentiation alters the abundance of AOB (but not AOA) in both arid and mesic ecosystems. While the abundance of AOA sharply increased with increasing aridity in all microsites, the response of AOB abundance was microsite-dependent, with increases (nitrogen-fixing shrubs, ant nests), decreases (open areas) or no changes (grasses, biocrusts, trees) in abundance with increasing aridity. Microsites supporting the highest abundance of AOB were trees, nitrogen-fixing shrubs, and ant nests. These results are linked to particular soil characteristics (e.g., total carbon and ammonium) under these microsites. Our findings advance our understanding of key drivers of functionally important microbial communities and N availability in highly heterogeneous ecosystems such as drylands, which may be obscured when different soil microsites are not explicitly considered.

Keywords: nitrogen cycle, drylands, biocrusts, nitrifiers, nitrification

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INTRODUCTION

Arid, semi-arid, and dry-sub humid ecosystems (drylands) constitute the planet's largest biome, and support over 38% of the global human population (Reynolds et al., 2007; Schimel, 2010). A major feature of dryland ecosystems is their spatial heterogeneity (Tongway et al., 2001; Maestre and Cortina, 2002). Drylands are characterized by a sparse coverage of plants, which are separated by open areas devoid of perennial vegetation. Plant patches include a wide variety of vegetation types such as grasses, nitrogen (N)-fixing shrubs, and trees, while open areas are often covered by biocrusts (soil communities dominated by mosses, lichens, and cyanobacteria) and support the nests and burrows of soil arthropods (Belnap et al., 2001; Bonachela et al., 2015). Recent studies suggest that each of these soil surface attributes has unique effects on microbial communities and ecosystem processes in drylands (e.g., mineralization; Castillo-Monroy et al., 2010; Hortal et al., 2013; Bonachela et al., 2015; Delgado-Baquerizo et al., 2015). However, until now, their effects have largely been evaluated separately. To date, no previous research has simultaneously evaluated how multiple soil surface features affect both microbial communities and soil variables in drylands, nor have any studies explored the likely mechanisms behind the observed microsite-specific effects on these ecosystem attributes.

Dryland ecosystems are highly vulnerable to climate change and desertification processes (Reynolds et al., 2007; Maestre et al., 2012). Recent studies suggest that the increase in aridity for the late 21st century forecasted for most drylands (Dai, 2013; Feng and Fu, 2013; Huang et al., 2016) will have negative impacts on the cover and richness of vascular vegetation (Maestre et al., 2012; Vicente-Serrano et al., 2012). Conversely, such declines in plant cover could expand the area occupied by open areas and biocrusts by increasing the surface available for colonization and growth of their constituent organisms (Belnap et al., 2001; Thomas et al., 2011). In addition to climate change, human activities such as overgrazing are also expected to have substantial negative effects on plants and biocrusts in drylands (Fuhlendorf and Engle, 2001; Eldridge et al., 2013). These global change drivers will shift the relative abundance of different microsites in these areas (Whitford, 2002; Escolar et al., 2012; Vicente-Serrano et al., 2012), likely altering their microsite-specific effects on microbial communities and ecosystem functioning. Thus, understanding how different soil microsites affect particular microbial communities and associated ecosystem functions is of paramount importance if we are to predict how dryland ecosystems will respond to global change.

Nitrogen is one of the most important factors limiting net primary productivity and organic matter decomposition in drylands (Schlesinger and Bernhardt, 2013). The availability of N for plants and microbes is regulated mainly by particular microbial guilds (see Robertson and Groffman, 2007 for a review). For example, the critical process of autotrophic nitrification, which converts ammonium to nitrite, is driven principally by the abundance of ammonia-oxidizing bacteria (AOB) and archaea (AOA; Nicol et al., 2008; Verhamme et al., 2011). Understanding the mechanisms that control the abundance of these microorganisms and their effects on soil N availability is thus critical for understanding and managing soil fertility and ecosystem productivity (Robertson and Groffman, 2007). The abundance of AOA and AOB in soils is known to be differentially affected by factors such as pH, organic matter quality and substrate availability (He et al., 2007; Nicol et al., 2008; Wessén et al., 2010; Rasche et al., 2011; Verhamme et al., 2011). Different dryland microsites (vascular plants, open areas, biocrusts, and insect nests), which have unique effects on soil attributes such as nutrient availability, organic matter quality and content (Castillo-Monroy et al., 2010; Hortal et al., 2013; Bonachela et al., 2015; Delgado-Baquerizo et al., 2015), could provide potentially different niches for AOA and AOB. Recent studies have shown that vascular plants can modulate the abundance of soil AOA and AOB along aridity gradients (Delgado-Baquerizo et al., 2013b), and that variations in the abundance of AOA and AOB drive variations in nitrification rates (Adair and Schwartz, 2008; Delgado-Baquerizo et al., 2013b; Hortal et al., 2013; Marusenko et al., 2013) in drylands. However, little is known about the role of plants and other biotic and abiotic features, such as insect nests and biocrusts, as modulators of the abundance of AOA and AOB in response to increasing aridity.

Here, we explore the effects of six markedly different microsites (open areas, biocrusts, ant nests, grasses, shrubs, and trees) on the abundance of *amoA* genes from AOB and AOA and on nitrate availability, and evaluate the mechanisms underlying microsite-specific effects on these variables at 21 sites along an aridity gradient in eastern Australia. As AOB have been previously reported to prefer high substrate concentration under plant canopies (Martens-Habbena et al., 2009; Verhamme et al., 2011; Delgado-Baquerizo et al., 2013b), we hypothesized that their abundance will peak in microsites with the highest ammonium availability. Conversely, because AOA abundance has been previously reported to increase with aridity in both open areas and under vascular plant canopies (Delgado-Baquerizo et al., 2013b), we predicted that AOA abundance would be microsite-independent and be driven by particular changes in soil properties derived from increasing aridity.

MATERIALS AND METHODS

Study Area

This study was carried out in 21 sites from eastern Australia (**Figure 1**; Supplementary Table S1). Locations for this study were chosen to represent a wide range of aridity conditions, including arid (arid and semiarid; $n = 12$; aridity index < 0.50) and mesic (dry-subhumid and humid; $n = 9$; aridity index > 0.50) ecosystems. Total annual precipitation and mean temperature ranged from 280 to 1167 mm and from 12.8 to 19.5°C, respectively. The sites surveyed encompass a wide variety of vegetation types, including grasslands, shrublands, savannas, dry seasonal forests, and open woodlands dominated by trees. Perennial vegetation cover ranged between 18 and 98%, and was dominated by *Eucalyptus* spp., *Acacia* spp., and *Austrodanthonia* spp. All our sites had a well-developed biocrust community

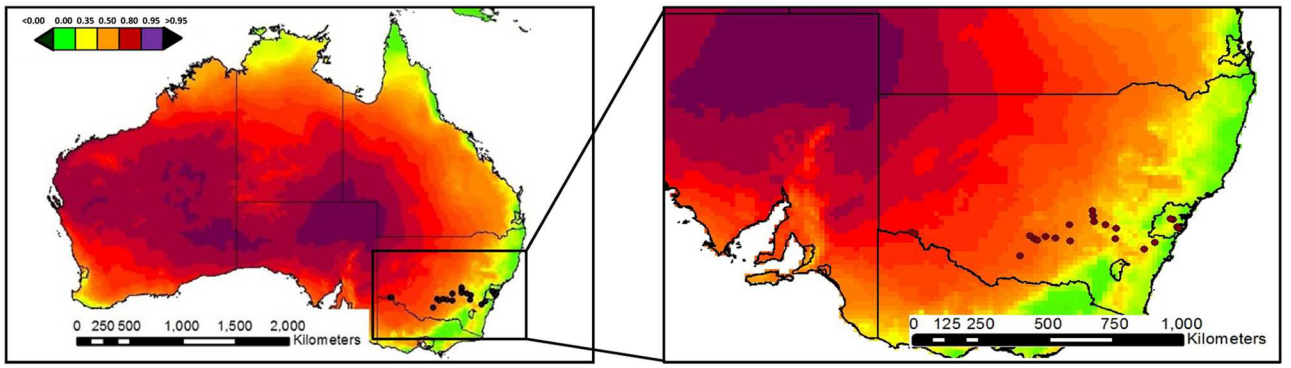

FIGURE 1 | Locations of the study sites. Color patterns indicate aridity (1 – aridity index) gradients. Aridity increases from green to purple in the graphs.

dominated by mosses (*Desmatodon convolutus*, *Barbula calycina*, *Didymodon torquatus,* and *Gemmabryum* spp.).

Sampling Design and Measurements

Soil sampling was carried out in March 2014 according to a standardized sampling protocol. A 30 m × 30 m plot representative of the dominant vegetation was established. The cover of the most abundant microsites (open areas, biocrust, ant nest entrances, grasses, N-fixing shrubs, and trees) at each site was measured using four 30-m transects and the line-intercept method, as described in Maestre et al. (2012). Aridity was determined as 1-aridity index [AI], where AI = precipitation/potential evapotranspiration (UNEP, 2012). Data of the aridity index were obtained from the global aridity map of the FAO (2014).

At each site, three soil cores (0–5 cm depth) were collected under the six most common microsites: open areas, biocrusts, ant nests, grasses (*Austrodanthonia* spp.), N-fixing shrubs (*Acacia* spp.), and trees (*Eucalyptus* spp.). Soil cores were then mixed to get a composite soil sample per microsite (six samples) in each of the sites. A minimum separation distance of 1 m between samples, and between these and plant patches was maintained to remove potential sources of non-independence between samples (Delgado-Baquerizo et al., 2013a). Following field sampling, the soil was sieved (2 mm mesh) and separated into two fractions. A fraction was immediately frozen at −20°C for quantifying the abundance of *amoA* genes from AOB and AOA (hereafter, AOA and AOB abundance). The other fraction was air-dried and stored for 1 month before physicochemical analyses.

We measured organic C, total N, pH, ammonia, and nitrate availability in all the soil samples. We selected these soil properties and nutrient variables because they are largely known to be important drivers of the abundance of AOA and AOB in terrestrial ecosystems (He et al., 2007; Nicol et al., 2008; Verhamme et al., 2011). The concentration of soil organic C was determined as described in Anderson and Ingram (1993). Soil total N was measured with a CN analyzer (Leco CHN628 Series, LECO Corporation, St Joseph, MI, USA). Soil pH was measured for all of the soil samples with a pH meter in soil and water suspension. Ammonium and nitrate were colorimetrically analyzed (Sims et al., 1995) from K_2SO_4 0.5 M soil extracts using a 1:5 soil: extract ratio as described in Jones and Willett (2006). The main soil properties for the different microsites and aridity conditions used in this study are shown in Supplementary Table S1.

Molecular Analyses

We measured the abundance of ammonia oxidizing bacteria (AOB) and archaea (AOA) at each microsite and location using quantitative PCR (qPCR). Soil DNA was extracted from 0.5 g of defrosted soil samples using the Powersoil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the instructions provided by the manufacturer. qPCR reactions were conducted in triplicate using 96-well plates on an CFX96 Touch™ Real-Time PCR Detection System (Foster city, CA, USA). The *amoA* genes of AOB and AOA were amplified using the primers *amoA1F* (GGGGTTTCTACTGGTGGT)/*amoA2R* (CCCCTCKGSAAAGCCTTCTTC) and *Arch-amoAF* (STAAT GGTCTGGCTTAGACG)/*Arch amoAR* (GCGGCCATCCATC TGTATGT), respectively, as described previously by Rotthauwe et al. (1997) and Francis et al. (2005). Efficiencies for all quantification reactions were higher than 90%, with R^2 values ranging from 0.90 to 0.99. Standards were run in triplicate in each assay, and our standard calibration curve was developed using a serial 10^{-3} and 10^{-9} dilution from 30 ng μl^{-1}. Samples fell within the limits of our standard curve, hence within the detection limit. We generated melting curves for each run to verify product specificity by increasing the temperature from 55 to 95°C. Melting curve analyses resulted in a single peak, confirming the specificity of all amplicons. Actual values of AOA and AOB abundances for the different microsites and aridity conditions used in this study are available in Supplementary Table S1.

qPCR Standard Curve Preparation

The AOA and AOB primers described above were used to amplify *amoA* genes from DNA extracted from composite soil samples. In parallel, both PCR products were cloned into *Escherichia coli* using a TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. One specific clone was selected

for AOA and AOB cultures in order to generate the standard curves. Plasmid DNA was extracted with a Plasmid Mini Kit (Invitrogen), and the insert was sequenced using M13F and M13R primers to check that AOA and AOB were correctly inserted into their respective plasmids (sequences from selected AOA and AOB clones are available in Supplementary Table S1). These sequences were compared to known *amoA* genes in the GenBank database (http://www.ncbi.nlm.nih.gov) using BLAST. This analysis showed that the sequences were >99% similar to known AOA and AOB genes.

Statistical Analyses

We tested for differences across aridity conditions (arid vs. mesic) and microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees) for the abundance of *amoA* genes from AOB and AOA with a two-way ANOVA, with microsite and aridity conditions as fixed factors. Prior to analyses, abundance of *amoA* genes from AOB and AOA and nitrate were log-transformed to improve normality. We conducted *post hoc* analyses (Tukey test) to explore differences in AOB, AOA and nitrate among microsites for those analyses where microsite effect was significant. When interactions between aridity conditions and microsites were significant, we carried our separate *post hoc* analyses for mesic and arid ecosystems. Spearman correlations were used to evaluate the relationship between aridity and both the relative abundance of the different microsites and the abundance of *amoA* genes from AOB and AOA in each microsite. All these analyses were carried out using SPSS for Windows, version 15.0 (SPSS Inc., Chicago, IL, USA).

We used structural equation modeling (SEM; Grace, 2006) to evaluate direct and indirect relationships between the different microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees), aridity (1-aridity index), soil properties (pH, C:N ratio, and soil C) and substrate (ammonium) on the abundance of *amoA* genes from AOB and AOA and nitrate availability. Unlike regression or ANOVA, SEM offers the ability to separate multiple pathways of influence and view them as a system, and thus is useful for investigating the complex networks of relationships found in ecosystems (Grace, 2006; Eisenhauer et al., 2015). Thus, this approach was the appropriate tool to evaluate our hypotheses because it allowed us to assess whether microsite effects on AOA and AOB abundance were directly or indirectly driven via nutrient availability and soil properties. The first step in SEM requires establishing an *a priori* model based on the known effects and relationships among the drivers of AOB and AOA abundance and nitrification process. Some data manipulation was required prior to modeling. We examined the distributions of all of our endogenous variables, and tested their normality. Soil C, C:N ratio, pH, ammonium, AOA and AOB abundances were log-transformed to improve normality. In these models, the different microsites (open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees) are categorical exogenous variables (Grace, 2006) with two levels: 0 (specific microsite) and 1 (remaining microsites). This approach allowed us to compare the effect of a particular microsite (e.g., ant nests) on the abundance of AOA and AOB compared with the average of the remaining microsites. Categorical exogenous variables can be used in SEM because distributional assumptions do not apply to them (Grace, 2006). The combined effects of nitrifier (AOA and AOB) abundances on nitrate availability were included in our model as a composite variable (Grace, 2006). With a good model fit (see below), we could confidently interpret the path coefficients of the model and their associated *P*-values. A path coefficient is analogous to the partial correlation coefficient, and describes the strength and direction of the relationship between two variables (Grace, 2006). We parameterized our models after data manipulation and used our datasets to test them for their overall goodness of fit. There is no single universally accepted test of overall goodness of fit for SEM that is applicable in all situations regardless of sample size or data distribution. Here, we used the chi-squared test (χ^2; the model has a good fit when $0 \leq \chi^2 \leq 2$ and $0.05 < P \leq 1.00$) and the root mean square error of approximation (RMSEA; the model has a good fit when $RMSEA\ 0 \leq RMSEA \leq 0.05$ and $0.10 < P \leq 1.00$). Additionally, and because some variables were not normal, we confirmed the fit of the model using the Bollen-Stine bootstrap test (the model has a good fit when 0.10 < bootstrap $P \leq 1.00$; Schermelleh-Engel et al., 2003).

We calculated the standardized total effects of microsite, aridity, soil properties and substrate on abundance of AOA and AOB and nitrate availability. The net influence that one variable has upon another is calculated by summing all direct and indirect pathways between the two variables. If the model fits the data well, the total effect should approximately be equivalent to the bivariate correlation coefficient for that pair of variables (Grace, 2006). All the SEM analyses were conducted using AMOS 20.0 (IBM SPSS, Chicago, IL, USA).

RESULTS

The cover of open areas, biocrusts, ant nests, grasses, N-fixing shrubs, and trees ranged from 0–43%, 0–73%, 0–1%, 0–25%, 0–10%, and 0–70%, respectively. The cover of biocrusts increased ($\rho = 0.67$, $P = 0.001$), while that of ant nests and trees decreased along the aridity gradient studied ($\rho_{\text{ant nests}} = -0.55$, $P = 0.010$; $\rho_{\text{trees}} = -0.74$, $P < 0.001$). The cover of the remaining microsites did not vary significantly with aridity, though clear trends were observed in some cases ($\rho_{\text{bare ground areas}} = 0.35$, $P = 0.120$; $\rho_{\text{grasses}} = -0.41$, $P = 0.064$; $\rho_{\text{N-fixing shrubs}} = -0.40$, $P = 0.069$). Our results revealed important differences in the abundance of *amoA* genes from AOB (but not AOA) among microsites in both arid and mesic ecosystems (**Figure 2**). Ant nests, N-fixing shrubs and trees showed the highest AOB abundance (**Figure 2A**). This was particularly evident for ant nests and N-fixing shrubs under the most arid conditions, as indicated by the significant aridity conditions × microsite interaction ($P < 0.001$; **Figure 2A**). Thus, this interaction provides evidence that the size effect of microsite on AOB differ between xeric and mesic ecosystems. Biocrusts and open areas had the lowest AOB abundance (**Figure 2A**), particularly in the arid sites (aridity conditions × microsite interaction: $P < 0.001$; **Figure 2A**). We did not find significant differences between microsites for AOA abundance, which consistently showed the highest abundance in the most arid

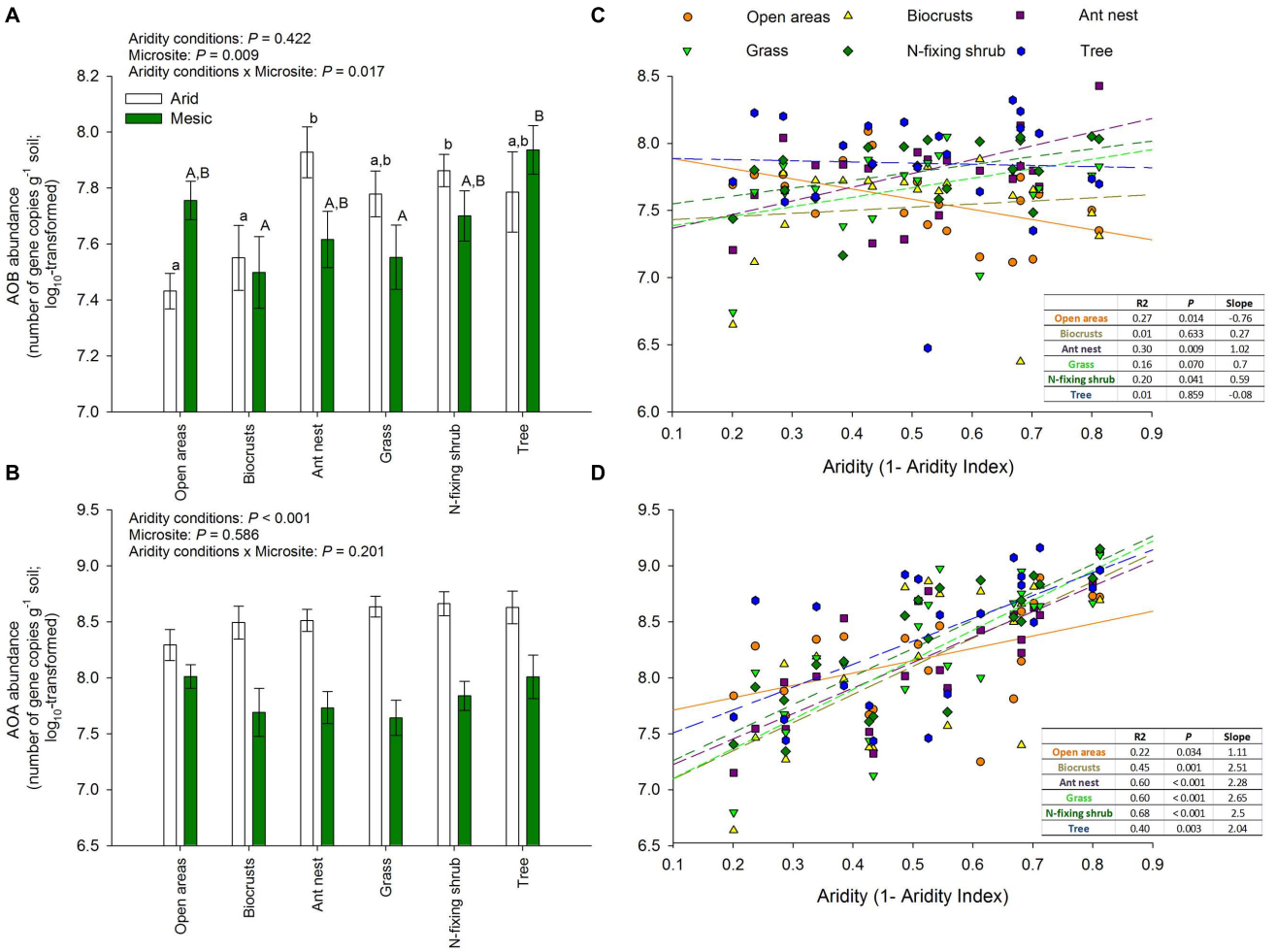

FIGURE 2 | Changes in the abundance of *amoA* genes from AOB and AOA across different levels of aridity. (A,B) Show mean ± SE, $n = 12$ and 9 for arid and mesic ecosystems, respectively. Upper case letters indicate differences between microsites (Tukey *post hoc* test after ANOVA). When interactions between aridity conditions and microsite were found, we conducted *post hoc* analyses independently for arid and mesic ecosystems. In this case, lower and upper case letters are used to show microsite differences separately for arid and mesic ecosystems. **(C,D)** Represent linear regressions between aridity and of the abundance of *amoA* genes from AOB and AOA across different microsites. The solid and dashed lines in **(C,D)** represent the fitted linear regressions.

parts of the gradient ($P < 0.001$; **Figure 2B**). Interestingly, the abundance of *amoA* genes from AOA increased with increasing aridity in all microsites (**Figure 2D**), but the response of AOB to aridity was microsite dependent, with increases (grasses, N-fixing shrubs and ant nests), decreases (open areas) or no changes (biocrusts and trees) in their abundance with increasing aridity (**Figure 2C**). We found the highest nitrate availability under ant nests, followed by trees and N-fixing shrubs ($P < 0.001$; **Figure 3**). Differences between arid and mesic ecosystems were not observed for this variable ($P > 0.05$; **Figure 3**).

Our *a priori* SEM model was satisfactorily fitted to our data, as suggested by non-significant χ^2 values ($\chi^2 = 0.6$–4.2; $P = 0.37$–0.98; $df = 4$), non-parametric Bootstrap *P*-values ranging from 0.46 to 0.97 and by values of RMSEA ranging from 0.00 to 0.03 ($0.53 < P < 0.98$; **Figure 4**). Most microsite effects (i.e., open areas, ant nests, grasses, and trees) on the abundance of *amoA* genes from AOB were indirectly driven by variations in soil C and ammonium availability, suggesting that these predictors adequately explained the abundance of AOB beneath these microsites. Contrary to this, we found a predominant direct positive and negative effect of N-fixing shrubs (**Figure 4E**) and biocrusts (**Figure 4B**) on the abundance of AOB, respectively, indicating that other unmeasured factors may have driven the indirect effects of these microsites on the abundance of *amoA* genes from AOB. Open areas and biocrusts showed a negative direct effect on soil C and ammonium compared with the other microsites, promoting an indirect negative effect on the abundance of AOB and on nitrate availability (via soil C and ammonium; **Figures 4A,B**). Conversely, ant nest and grasses had an indirect positive effect on AOB abundance via their influence on that of ammonium (**Figures 4C,D**), while trees had an indirect positive effect on AOB abundance by affecting soil C, hence the availability of ammonium in soil (**Figure 4F**). For AOA, our SEM results revealed that aridity had the highest

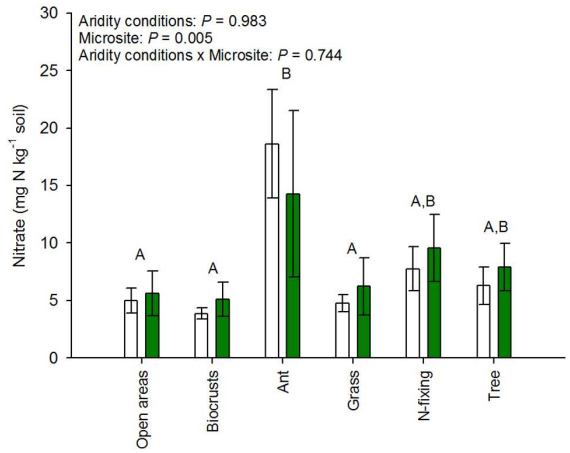

FIGURE 3 | Nitrate availability in the different microsites studied. Data are mean ± SE, $n = 12$ and 9 for arid and mesic ecosystems, respectively. Upper case letters indicate differences between microsites (Tukey *post hoc* test after ANOVA).

direct positive effect on the abundance of *amoA* genes from AOA (**Figure 4**); aridity also had an indirect positive effect on these microorganisms by reducing both the C:N ratio (negatively related to the abundance of AOA), and the amount of soil C (positively related to ammonium; **Figure 4**) and by enhancing soil pH (positively related to AOA abundance; **Figure 4**).

Our SEM models supported the microsite dependence of AOB (but not AOA) abundance in the ecosystems studied, and indicated that both aridity and microsite differentiation were the most important factors predicting the abundance of AOA and AOB, respectively (**Figure 5**). Ant nests, N-fixing shrubs and trees had a total positive effect (sum of direct and indirect effects from SEM) on the abundance of AOB, while open areas and biocrusts showed the highest negative total effect compared to other microsites (**Figure 5A**). Nitrogen-fixing shrubs and trees were the only microsites showing positive total effects on the abundance of AOA, but the magnitude of these effects was about seven-times lower than the total positive effect from aridity on such abundance (**Figure 5B**).

The abundance of both *amoA* genes from AOA and AOB had positive total effects on nitrate availability (**Figure 5C**). Ant nests (+), soil C (+), C:N ratio (−), ammonium (+) and both AOA (+) and AOB (+) abundance were the main predictors of nitrate availability (**Figures 4** and **5**). Aridity had a negative, but weak (i.e., close to 0), effect on nitrate concentration, while the positive effect of AOA abundance on nitrate was higher than that of AOB abundance (**Figure 5**).

DISCUSSION

Microsite-Specific Effects on the Abundance of AOB and AOA

Our study provides empirical evidence that microsite differentiation modulates the abundance of particular groups of microorganisms, such as AOB, in response to increases in aridity. This pattern was not found for AOA, as their abundance was driven mainly by aridity, regardless of the microsite considered. Strikingly, ant nests were one of the microsites with the highest abundance of AOB. Ants are one of the most widespread insect groups globally (Pêtal, 1998; Lenoir et al., 2001), and their activity during nest construction has major effects on multiple soil properties, including clay mineralogy, water infiltration and retention and nutrient cycling (Lenoir et al., 2001; Bonachela et al., 2015; Wu et al., 2015). Ant nests have been reported to support higher concentrations of inorganic N (ammonium and nitrate) and phosphorus due to the accumulation of animal and plant litter (Lenoir et al., 2001; Bonachela et al., 2015; Wu et al., 2015), which can be used by the plants growing near them (Sternberg et al., 2007; Wagner and Nicklen, 2010). They have also been observed to promote mineralization and nitrification processes in soils (Pêtal, 1998; Lenoir et al., 2001). This is particularly important in drylands, where N is one of the most important factors limiting the growth of plants and microorganisms (Austin et al., 2004; Schlesinger and Bernhardt, 2013). Thus, ant nests may act as "resource islands" (*sensu* Reynolds et al., 1999), providing suitable habitat for particular groups of microbes such as AOB, which support the high nitrification rates and nitrate availability reported here and in other studies (Pêtal, 1998; Lenoir et al., 2001; Wagner and Jones, 2006).

We found that the effects of vascular plants on the abundance of AOB varied depending on their functional attributes. The observed differences in the abundance of AOB between microsites may have been due to their different effects on microclimate and soil properties. The surface of soils close to and under trees and N-fixing shrubs receives greater levels of litter than soils adjacent to bare ground, biocrusts or small grasses (Vesterdal et al., 2012; Travers and Eldridge, 2015), and has lower temperature and higher infiltration rates that may lead to improved soil moisture conditions (Breshears et al., 1997; Berdugo et al., 2014; Eldridge et al., 2015). Overall, these environmental changes may provide a refuge for AOB in drylands. Interestingly, although small grasses had a lower effect on AOB abundance than other vascular plants, they still provide a better refuge for AOB than open areas and biocrusts, a response likely linked to the higher ammonium content found under grasses. Our results build up those from Delgado-Baquerizo et al. (2013b), who found that vascular plants can modulate the abundance of AOB in drylands, by emphasizing the role of different plant functional types on the abundance of these microorganisms.

Contrary to the results for AOB abundance, which was highly microsite-dependent, abundance of AOA increased along the aridity gradient studied, and was not affected by any of the microsites considered. Our results mimic those from Delgado-Baquerizo et al. (2013b), who also found increases in the abundance of AOA along an aridity gradient in Mediterranean grasslands dominated by *Stipa tenacissima*. Other studies have also reported high abundance of AOA in drylands (Adair and Schwartz, 2008; Marusenko et al., 2013). Remarkably, our results further suggest that this effect is microsite-independent, and that

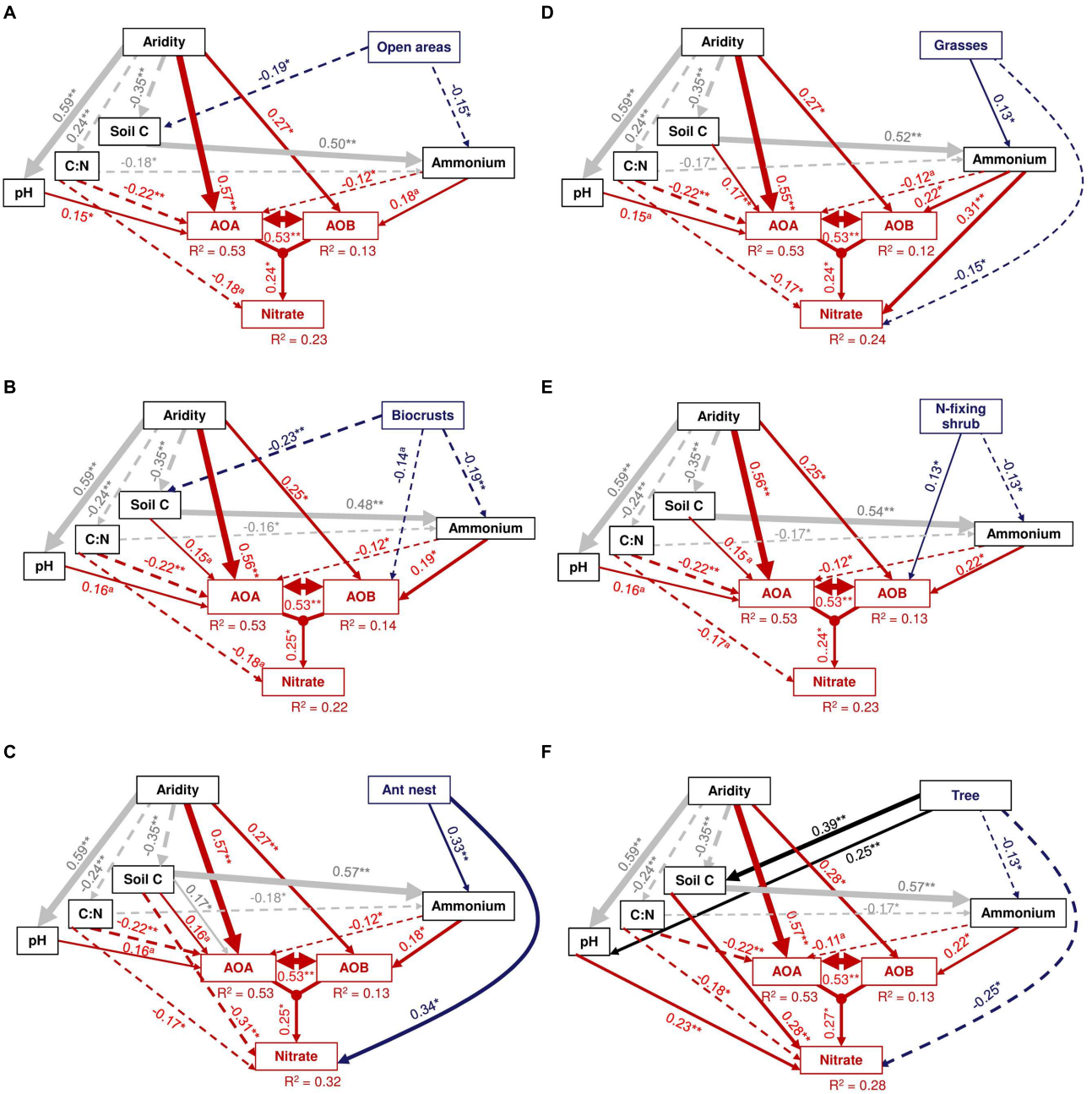

FIGURE 4 | Results from structural equation modeling showing the direct and indirect effects of aridity and different microsites on the abundance of AOB and AOA and on nitrate availability. Each panel represents the model used for each microsite (indicated in the upper right box of each model). Numbers adjacent to arrows are standardized path coefficients, analogous to relative regression weights, and indicative of the effect size of the relationship. Continuous and dashed arrows indicate positive and negative relationships, respectively. The width of arrows is proportional to the strength of path coefficients. Double-headed arrows between AOA and AOB indicate that the abundance of AOA can influence that one from AOB and *viseversa*. The proportion of variance explained (R^2) appears above every response variable in the model. Significance levels are as follows: $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

aridity can be the best predictor for AOA abundance in the studied ecosystems. These results further support the notion that AOA often occupy those niches with more extreme conditions (i.e., low water and nutrient availability), where they usually outcompete AOB (Valentine, 2007; Adair and Schwartz, 2008; Moin et al., 2009; You et al., 2009).

Microsite as a Modulator of the Abundance of AOB and AOA in Response to Aridity

Our results indicate that microsite differentiation not only modified the abundance of AOB, but also their responses

to aridity. While the abundance of AOA increased with increasing aridity, irrespective of the particular microsite, the response of AOB abundance was microsite dependent, with increases (grasses, N-fixing shrubs, and ant nests), decreases (open areas) or no changes (biocrusts and trees) in abundance with increasing aridity (**Figures 2C,D**). Predicted increases in aridity for the late 21st century in most drylands (Dai, 2013; Feng and Fu, 2013) will negatively impact upon vascular vegetation cover in drylands (Maestre et al., 2012; Vicente-Serrano et al., 2012), and this may increase the proportion of suitable habitat for biocrusts, and thus their cover (Belnap et al., 2001; Thomas et al., 2011). Accordingly, we found a significant decrease in the cover of trees (and clear trends in the same direction in the cover of N-fixing shrubs and grasses), and an increase in the cover of biocrusts, with aridity. The decrease in plant cover and the increase of open areas will likely increase the abundance of AOA at the expenses of AOB due to the high resistance to water and nutrient stresses of the former (Adair and Schwartz, 2008; Verhamme et al., 2011).

Overall, all of our models indicate that the abundance of AOA and AOB is positively related to nitrate availability in our study sites, suggesting that autotrophic ammonia oxidation may be extremely important for N cycling in dryland ecosystems. This is particularly true for AOA abundance, which showed the highest total positive effect on nitrate availability in our models (**Figure 3**). Thus, considering the positive effect of aridity on AOA abundance and the importance of the positive effect of AOA on nitrate availability, our results suggest that AOA abundance could contribute to buffer the direct negative effects of aridity increasing on nitrate availability in drylands (**Figure 5C**; Delgado-Baquerizo et al., 2016). The role of these microorganisms in nitrification is largely supported in laboratory studies (e.g., Verhamme et al., 2011), but our study confirms the importance of the relationship between ammonia-oxidizing microbe abundance and the availability of nitrate under field-based conditions in drylands. Indeed, these results are consistent with reports showing that the relative dominance of nitrate will increase with increasing aridity (Schlesinger et al., 1990; Delgado-Baquerizo and Gallardo, 2011), and suggest that any increase in aridity resulting from climate change (Dai, 2013; Feng and Fu, 2013) may promote losses in nitrate to both the atmosphere and underground water (Robertson and Groffman, 2007; Delgado-Baquerizo et al., 2016).

Mechanisms that Account for Microsite-Specific and Aridity Effects on the Abundance of AOB and AOA

Our SEM approach allowed us to identify the most likely mechanisms controlling microsite and aridity effects on the abundance of AOB and AOA. For example, microsite differentiation indirectly alters the abundance of AOB via organic C and ammonium availability. Thus, the positive effects of ant nests and grasses on the abundance of AOB were indirectly

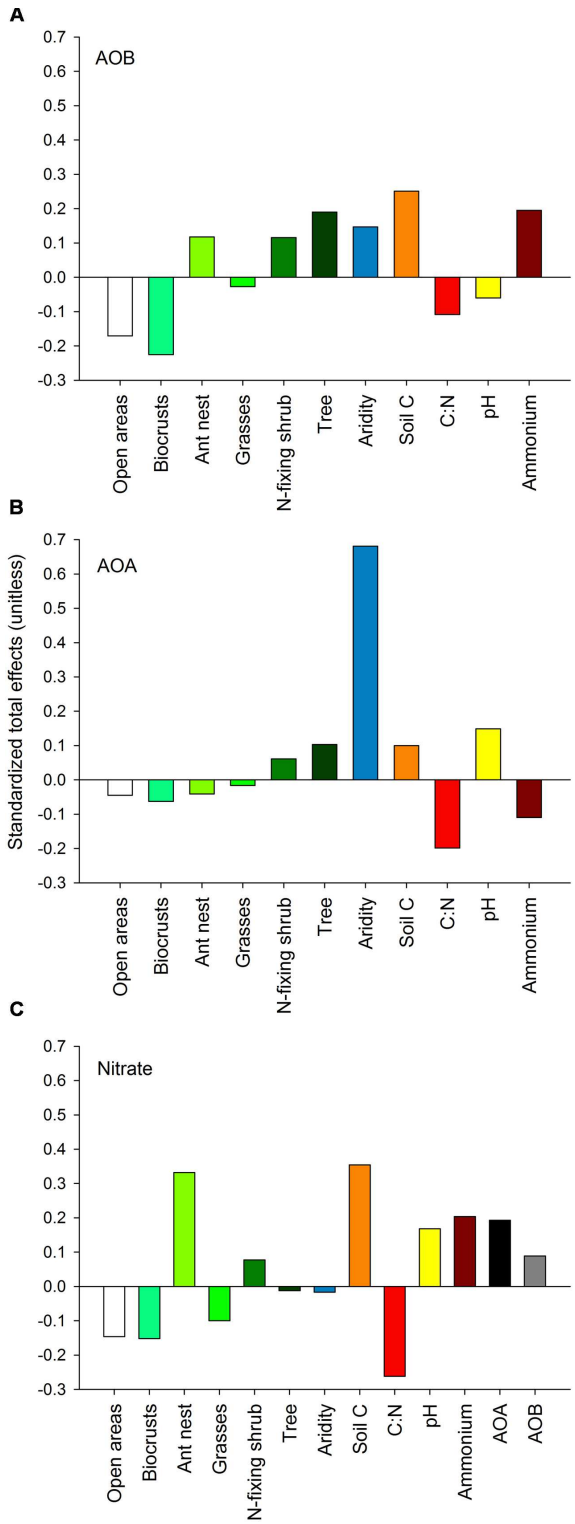

FIGURE 5 | Standardized total effects (direct plus indirect effects) derived from the structural equation modeling, including the effects of aridity and the different microsites evaluated on the abundance of AOB (A) and AOA (B) and on nitrate availability (C). The total effects of aridity, soil C, pH, C:N and ammonium on AOB and AOA abundance and nitrate availability were averaged from all six models in **Figure 4**.

driven by changes in ammonium availability. Ammonium is the main substrate for ammonia-oxidizing organisms (He et al., 2007; Nicol et al., 2008; Verhamme et al., 2011). Thus, compared with the other microsites, the positive effect of ant nests and grasses on ammonium contents observed here and in previous studies (Pêtal, 1998; Lenoir et al., 2001; Wu et al., 2015) may help to explain the positive effect of these microsites on the abundance of AOB. Similarly, the positive effect of trees on AOB may be indirectly mediated by the highest soil organic matter content found in this microsite (vs. other microsites evaluated), which, in turn, increases the availability of ammonium. Conversely, the negative impact of open areas on soil organic matter and ammonium availability may explain the low abundance of these microorganisms found in this microsite.

Interestingly, N-fixing shrubs and biocrusts were the only two microsites showing direct effects on AOB abundance in our SEMs (**Figures 4B,E**). One of these mechanisms could be synergistic effects between microbial communities involved in N cycling that thrive under N-fixing shrubs microsites (Zhang et al., 2015). For example, N-fixing shrubs are well-known to increase the abundance of microorganisms related to N fixing such as *Rhizobium* spp., which may promote the abundance and diversity of AOB by providing higher concentrations of ammonia to these microorganisms (Zhang et al., 2015). The observed indirect positive effect of N-fixing shrubs on nitrate availability via abundance of AOB (**Figure 4**) further suggests that all the N fixed under N-fixing shrubs (i.e., ammonium) may be transformed directly to nitrate, as suggested by the negative direct effect of this microsite on ammonium availability and by the typical high nitrification rates reported for N-fixing shrubs in drylands (e.g., Delgado-Baquerizo and Gallardo, 2011; Delgado-Baquerizo et al., 2011). Conversely, biocrust was the only microsite negatively affecting the abundance of AOB. Thus, while all the effects of open areas on AOB abundance are indirectly driven via the lowest soil C and ammonium availability found in this microsite, other unmeasured factors may indirectly drive the low AOB abundance under biocrusts. For instance, biocrusts often had a higher amount of soil phenols than open areas (Delgado-Baquerizo et al., 2015), which known allelopathic effects on particular soil bacterial communities (Herbert and Bertsch, 1995; Zsolnay, 1996; Saenz et al., 2006), and this could explain, at least in part, the direct negative effect of biocrusts on the abundance of AOB.

Indirect effects of aridity on soil properties such as pH may explain the highest abundance of AOA in the most arid locations. For example, we found a positive indirect effect of aridity on the abundance of AOA mediated by soil pH, which is supported by previous studies suggesting links between soil pH and the dominance of AOA (Nicol et al., 2008; Moin et al., 2009; Gubry-Rangin et al., 2011; Delgado-Baquerizo et al., 2013b). In addition, and contrary to what we found with AOB, the availability of ammonium had a negative direct effect on the abundance of AOA. Negative effect of ammonium availability on the abundance of AOA has been previously reported under laboratory conditions (from ∼155 ug N/g of soil; Verhamme et al., 2011). Both the large positive and direct effects of aridity and the negative direct effect of ammonium availability on the abundance of amoA genes from AOA support the notion that these microorganisms may outcompete AOB under oligotrophic conditions due to their high resistance to water and nutrient stress (Adair and Schwartz, 2008; Verhamme et al., 2011). This would allow AOA to carry out nitrification under the most unfavorable environmental conditions.

Taken together, our results indicate that soil microsite differentiation results in altered abundance of AOB along aridity gradients. The abundance of amoA genes from AOA is mainly driven by aridity in the drylands studied. These results are linked to indirect effects of microsite differentiation on amoA genes from AOB mediated by changes in soil properties such as soil carbon and ammonium availability, and to direct effects of aridity on amoA genes from AOA. These findings advance our understanding about how biotic and abiotic features control small-scale variations in microbial abundance and associated ecosystem processes in highly heterogeneous ecosystems such as drylands. They also indicate that identifying the main microsites promoting the abundance of particular microbial communities is particularly relevant for understanding how ongoing climate change may affect ecosystem functioning in a warmer and drier world.

AUTHOR CONTRIBUTIONS

MD-B designed this study in consultation with DJE, FTM, and BKS. Field data was collected by MD-B and DJE. Laboratory and statistical analyses were done by MD-B. The first draft of this paper was written by MD-B and all co-authors significantly contributed to improve it.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.00505

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Isolation of a significant fraction of non-phototroph diversity from a desert Biological Soil Crust

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Biological Soil Crusts (BSCs) are organosedimentary assemblages comprised of microbes and minerals in topsoil of terrestrial environments. BSCs strongly impact soil quality in dryland ecosystems (e.g., soil structure and nutrient yields) due to pioneer species such as *Microcoleus vaginatus*; phototrophs that produce filaments that bind the soil together, and support an array of heterotrophic microorganisms. These microorganisms in turn contribute to soil stability and biogeochemistry of BSCs. Non-cyanobacterial populations of BSCs are less well known than cyanobacterial populations. Therefore, we attempted to isolate a broad range of numerically significant and phylogenetically representative BSC aerobic heterotrophs. Combining simple pre-treatments (hydration of BSCs under dark and light) and isolation strategies (media with varying nutrient availability and protection from oxidative stress) we recovered 402 bacterial and one fungal isolate in axenic culture, which comprised 116 phylotypes (at 97% 16S rRNA gene sequence homology), 115 bacterial and one fungal. Each medium enriched a mostly distinct subset of phylotypes, and cultivated phylotypes varied due to the BSC pre-treatment. The fraction of the total phylotype diversity isolated, weighted by relative abundance in the community, was determined by the overlap between isolate sequences and OTUs reconstructed from metagenome or metatranscriptome reads. Together, more than 8% of relative abundance of OTUs in the metagenome was represented by our isolates, a cultivation efficiency much larger than typically expected from most soils. We conclude that simple cultivation procedures combined with specific pre-treatment of samples afford a significant reduction in the culturability gap, enabling physiological and metabolic assays that rely on ecologically relevant axenic cultures.

Keywords: biological soil crusts, culturability, isolation, dryland microbiology, microbial diversity

Introduction

Dryland regions, including hyperarid, semiarid, arid, alpine and polar regions, cover approximately one third of the Earth's land surface (Belnap, 2006). Large expanses of these drylands are covered by Biological Soil Crusts (BSCs) (Bowker et al., 2002). BSCs are photosynthetic, diazotrophic communities of bacteria, fungi, algae, lichens and mosses (Belnap, 2006) that have adapted to the infrequent rainfall, high evaporation rates and prolonged UV exposure that limits habitability in these environments. BSCs provide significant ecosystem services within drylands through the aggregation of soil particles that reduces wind and water erosion (Mazor et al., 1996), altering the balance between water run-off and infiltration, thus impacting local hydrologic redistribution (Brotherson and Rushforth, 1983; Kidron and Yair, 1997), and importantly increasing the fertility of soil through nitrogen (N) and carbon (C) fixation (Lange et al., 1994).

BSCs strongly regulate soil quality in dryland ecosystems, are major contributors to soil C-cycling, and represent the significant source of net primary productivity introducing nutrients into soil in many of these ecosystems (Housman et al., 2006). In early successional stages of BSCs, *Microcoleus vaginatus*, a filamentous cyanobacterium, forms a polysaccharide mesh derived from fixed atmospheric C (Mazor et al., 1996). This bed helps further colonization of BSCs by diazotrophic cyanobacteria, other bacteria, fungi, lichens and mosses in a temporal succession (Mazor et al., 1996; Kuske et al., 2012). Hence, *M. vaginatus* is not only the most dominant organism in early successional stages of BSCs (Büdel et al., 2008; Zaady et al., 2010), but a pioneer in the development of this ecosystem.

Better understanding of carbon transformation and flux in early successional stages of BSCs requires knowledge of the direction of carbon flow from the primary producer, *M. vaginatus,* to the non-phototrophic components of the community. Early successional stages of BSCs can harbor a rich community of bacteria (Csotonyi et al., 2010; Steven et al., 2012a; Angel and Conrad, 2013), fungi (Bates and Garcia-Pichel, 2009; Bates et al., 2011; Abed et al., 2013), archaea (Nagy et al., 2005; Soule et al., 2009), protozoa (Bamforth, 2008), and nematodes (Pen-Mouratov et al., 2011). Although it is well known that BSCs can be diverse in composition, the roles of and interactions between community members have not been elucidated. Currently, functional roles of cryptic members of microbial communities can be inferred with different degrees of success using culture-independent approaches (e.g., metagenomics, metatranscriptomics) from soils (Van Elsas et al., 2008), oceans (DeLong et al., 2006), extreme environments (Aliaga Goltsman et al., 2009), and also BSCs (Rajeev et al., 2013). Further, assembly of metagenome short reads mapped to reference databases has helped to reconstruct small and large subunits of the rRNA gene (Miller et al., 2011; Bengtsson et al., 2012). This novel approach has helped to recover the community structure of known and unknown rRNA genes across different domains of life (Nagy et al., 2012; Wrighton et al., 2012) while eliminating biases associated with PCR priming.

Pure cultures of microorganisms are invaluable to validate metagenome-derived hypotheses using simplified well-controlled systems. The highest cultivation efficiency of heterotrophic aerobic bacteria in a dryland ecosystem was achieved in samples from the Atacama Desert. In this ecosystem, up to 0.1% of the bacterial community (by comparison of colony forming units to microscopic cell counts per gram of soil) was cultured in a complex rich medium (Garcia-Pichel et al., 2003; Connon et al., 2007; Lester et al., 2007). However, traditional cultivation techniques often lack key environmental components, such as appropriate concentrations of nutrients, micronutrients and suitable growth matrices, which influence bacterial fitness and culturability. Recent work has demonstrated that improved bacterial culturability can be achieved through the use of more environmentally relevant conditions appropriate to the habitat in question. For example, this was achieved in bulk soil (Janssen et al., 2002; Sait et al., 2002; Schoenborn et al., 2004; Davis et al., 2005; Rocha et al., 2009) and freshwater ecosystems (Bruns et al., 2002, 2003) by reducing nutrient availability, prolonging incubation times and mitigating oxidative stress by the addition of protective agents. Recovery percentages from soils and other environments can thus be increased by simple modifications to existing protocols. However, despite such improvements the overwhelming majority of the bacterial diversity in many ecosystems has remained cryptic so far (Rothschild, 2006) Furthermore, it is not clear what fraction of this diversity is indeed capable of growth and replication. DNA may be extracellular (thus inflating diversity estimates), cells may be permanently injured, and dormant cells require specific conditions and co-factors to induce resuscitation (Dewi Puspita et al., 2012).

Previous attempts to isolate bacteria from BSC, using traditional culture techniques and media, have yielded an important collection of BSC isolates (Gundlapally and Garcia-Pichel, 2006). Different isolates of this library have been described as novel species. They affiliated to Fungi (Bates et al., 2006), Bacteroidetes (Reddy and Garcia-Pichel, 2005, 2013), Actinobacteria (Reddy et al., 2007; Reddy and Garcia-Pichel, 2009), and Proteobacteria (Reddy et al., 2006; Reddy and Garcia-Pichel, 2007, 2015). Phenotyping of these isolates revealed cryptic information regarding their ecology. For example, it was possible to infer presence of pigments (Reddy and Garcia-Pichel, 2005; Bates et al., 2006; Reddy et al., 2006), facultative fermentation strategies (Reddy et al., 2006), psychro- (Reddy and Garcia-Pichel, 2005, 2009), and dessication tolerance (Reddy and Garcia-Pichel, 2007). These examples demonstrate how a larger collection of isolates would improve our understanding of non-photosynthetic organisms in BSC ecosystem functioning.

To attempt to culture a broad range of heterotrophic aerobic bacteria from BSCs we used a polyphasic approach based on: (1) the use of low carbon availability media; (2) the modification of growth media with addition of micronutrients and oxidative stress protective agents; (3) the use of gellan gum as an alternative solidifying agent; and (4) the incubation of samples for prolonged periods (up to 50 days) under an elevated CO_2 (5%) atmosphere. In addition, prior to isolation, we attempted to resuscitate from stasis different members of the microbial community by pre-incubating BSCs under dark or light conditions to mimic the distinct physiological conditions that occur during a diurnal cycle.

To address which microbes are present and gain information on when they might be activated, we used an amplification- and primer- independent approach, reconstructing the small subunit (SSU) rRNA genes from metagenomic and metatranscriptomic sequence reads using the EMIRGE software (Miller et al., 2011). Finally, we relate the phylogeny of the isolates recovered in this study to those of the reconstructed SSU rRNA gene sequences to determine what fraction of the bacterial diversity was recovered by isolation using these simple modifications to existing cultivation methods.

Materials and Methods

Sample Collection

Petri dishes (6 × 1 cm) were used to core and transport samples of BSCs from the Green Butte Site near Canyonlands National Park (38°42′53.9″N, 109°41′34.6″W, Moab, Utah, USA). All samples were taken in July 2011 from an area of approximately 3 m radius and were 1 cm deep. Details on the sampling site, sampling methodology and storage of the samples prior to the experiment have been previously described (Strauss et al., 2011). Samples were transported air-dry, and stored in dark, under an atmosphere in equilibrium with LiCl desiccant, until experimentation.

Bacterial Isolations

Activation of Different Fractions of the BSC Microbial Community

Prior to bacterial extraction for bacterial isolation, we attempted to activate two different fractions of the microbial community present in the BSC. To stimulate the microbial community active in day light we added 10 mL of ultrapure sterile water to six Petri-dishes containing BSCs and placed them for 4 h in direct sun light under greenhouse conditions (School of Life Sciences, Arizona State University, Phoenix, AZ, USA). To activate the microbial community stimulated under dark conditions, we added 10 mL of ultrapure water to six other Petri-dishes containing BSC and incubated them in the dark for 5.5 h. These experiments were performed in the greenhouse to mimic conditions from where the BSCs were derived at Moab, UT, (26–28°C) as closely as possible.

BSC Microbial Suspension

After their respective incubation times, we mixed 10 g (wet weight) of the six BSC samples for each of the pre-incubation condition with 190 mL of saline solution (0.95% NaCl) and blended for 1 min at 12,000 rpm (blending was repeated 2 times with a 30 s interval) to release as many bacterial cells as possible. These suspensions were used for dilution plating.

Media Preparation and Plating

For each of the six different BSC samples and both pre-incubation conditions, we used five different solid media to inoculate the BSC suspensions. R2A, a widely used standard medium for cultivation, was modified to different C concentrations from Eaton et al. (2005) to prepare 1/10 R2A, and to prepare 1/20 R2G and 1/100 R2G in which we used gellan gum (Gelrite, Research Products International, Illinois, USA) as an alternative solidifying agent. To prepare 1/10 R2A the following ingredients were dissolved in 1 L of ultrapure water: proteose peptone, 0.05 g; starch, 0.05 g; glucose, 0.05 g; yeast extract, 0.05 g; casein hydrolysate, 0.05 g; dipotassium phosphate, 0.3 g; sodium pyruvate, 0.3 g; magnesium sulfate anhydrous, 0.024 g; agar, 14 g. To prepare 1/20 R2G the following ingredients were dissolved in 1 L of ultrapure water: NaCl, 4.5 g; proteose peptone, 0.025 g; starch, 0.025 g; glucose, 0.025 g; yeast extract, 0.025 g; casein hydrolysate, 0.025 g; dipotassium phosphate, 0.3 g; sodium pyruvate, 0.15 g; magnesium sulfate anhydrous, 0.024 g; gelrite, 12 g. To prepare 1/100 R2G the following ingredients were dissolved in 1 L of ultrapure water: proteose peptone, 0.005 g; starch, 0.005 g; glucose, 0.005 g; yeast extract, 0.00 5g; casein hydrolysate, 0.005 g; dipotassium phosphate, 0.3 g; sodium pyruvate, 0.3 g; magnesium sulfate anhydrous, 0.024 g; gelrite, 12 g. CAT, an oligotrophic medium amended with catalase as oxidative protective agent (Rocha et al., 2009), was modified by using 12 g/L of gelrite as solidifying agent. VXylG, containing many micronutrients and having low carbon availability was prepared as described by Davis et al. (2005). 1/10 R2A, 1/20 R2G, 1/100 R2G, CAT prior to amendment with catalase, and VXylG prior to the addition of the vitamin solutions, were sterilized by autoclaving at 121°C for 20 min. The catalase and vitamin solutions were sterilized by filtration through a 0.2 μm filter (MILLEX GP, Millipore, USA) and added to CAT and VXylG respectively when the medium was cooled to 53°C.

Colony Forming Unit (CFU) Enumeration, Colony Selection, and Storage of Isolates

For CFU enumeration, each of the six BSC suspensions and pre-incubation conditions was serially diluted tenfold in sterile saline (0.95% NaCl), after which (per dilution) 0.1 mL was plated onto the different media. Petri-dishes were kept in their original plastic bags sealed with tape and incubated at 25°C, 18% O_2 and 5% CO_2 for 50 days. The increased CO_2 was used to mimic an aerobic microbial community under high metabolic activity. Colony formation was followed in time by counting the number of CFUs emerging between 3 and 50 days. Comparisons were made between log of CFU counts per gram of dry soil from the different media inoculated with BSCs incubated in the dark or in the light. Differences were calculated by analysis of variance using the "aov" function in the R statistical programming environment, v2.15.1 (R Core Team, 2013). Pairwise comparisons were made using Fisher's Least Significant Difference (LSD) test using the "agricolae" package.

During purification of axenic cultures, we pooled BSC samples per medium and incubation conditions. After 23 days for 1/10 R2A, 35 days for 1/20 R2A, 50 days for 1/100 R2G, and VXylG, and 80 days for CAT, a total of 700 CFUs (∼25–50 from each medium per BSC incubation condition) were randomly picked from plates that had received the two highest dilutions of the BSC suspensions. CFUs were streaked to purity three consecutive times on the same medium and allowed to grow to new colonies for up to 4 months. For storage of isolates, cells from axenic cultures were suspended in saline amended with glycerol (final concentration 40%) and stored at −80°C. The experimental

design for bacterial isolation is further outlined in Supplementary Figure 1.

Identification of Isolates from BSC by SSU rRNA Gene Sequencing

For PCR amplification of the SSU rRNA gene, DNA from each of the axenic isolates was extracted using the Bacterial Genomic DNA Isolation Kit (Norgen Biotek Corp., Thorold, ON, Canada) according to the protocol provided by the manufacturer. Later, duplicate 50 μL PCR reactions containing 10 ng of genomic DNA were prepared as follows: 1X Takara ExTaq Buffer; 2.5 mM; each deoxyribonucleoside triphosphate; 400 nM of each primer, 27F (Lane et al., 1985) and 1492R (Rochelle et al., 1992); and 2.5 U of Takara ExTaq DNA polymerase (Takara Inc, Mountain View, CA, USA). PCR amplifications were performed in a T100 Thermal Cycler (BioRad, USA) with the following thermocycling conditions: one cycle of 94°C for 5 min; 30 cycles at 94°C for 60 s, 55°C for 60 s, 72°C for 90 s; and one cycle at 72°C for 10 min. The PCR products obtained were purified with Agencourt AMPure XP - PCR Purification beads (Brea, CA, USA), and later used for sequencing.

To obtain SSU rRNA gene sequences, PCR products were sequenced from both ends using primers 27F and 1492R at the UC Berkeley DNA Sequencing Facility (Berkeley, CA, USA). The forward and reverse reads for each isolate were assembled using the Geneious® 6.1.2 software (Biomatters Ltd., Auckland, New Zealand). The isolate sequences were then classified using the assignment_taxonomy.py script of the QIIME software package (Caporaso et al., 2010) with the RDP classifier mapping to release 108 of the SILVA database (Quast et al., 2012).

Metagenomic Library Construction and Sequencing

The DNA used to construct the metagenomic library was extracted from samples collected in the same area as was used for microbial isolation. DNA extraction was performed using bead beating (1.4-mm ceramic spheres, 0.1-mm silica spheres, and one 4-mm glass bead) for cell lysis and phenol chloroform based extraction followed by an AllPrep kit (Qiagen, CA) for nucleic acid purification (Rajeev et al., 2013). Illumina library preparation and sequencing were performed in accordance with the standard protocols of the DOE Joint Genome Institute (Walnut Creek, CA, USA). Briefly, 2 μg of DNA was sheared in 100 μl using the Covaris E210 with the setting of 10% duty cycle, intensity 5, and 200 cycle per burst for 3 min per sample and the fragmented DNA was purified using QIAquick column (Qiagen) according to the manufacturer's instructions. The sheared DNA was end-repaired and A-tailed according to the Illumina standard PE protocol and purified using the MinElute PCR Purification Kit (Qiagen) with a final elution in 12 μl of Buffer EB. After quantification using a Bioanalyzer DNA 1000 chip (Agilent), the fragments were ligated to the Illumina adaptors according to the Illumina standard PE protocol, followed by a purification step of the ligation product using AMPure SPRI beads. The Illumina libraries were quantified using a Bioanalyzer DNA High Sensitivity chip (Agilent) and sequenced on an Illumina HiSeq 2500.

Metatranscriptomic Library Construction and Sequencing

Previously, we had performed a 3-day wet-up experiment to study the response of *M. vaginatus* to hydration and desiccation in BSCs (Rajeev et al., 2013). The samples used in this study were collected on the same day and from a location within a 3 m radius. To construct the metatranscriptomic libraries we used RNA from the samples 6A (18 h after wet-up, biological replicate A, termed Night time) and 9B (25.5 h after wet-up, biological replicate B, 2.5 h after full lights were on, termed Day time) from the Rajeev et al. (2013) study.

After preparing total RNA as described previously (Rajeev et al., 2013), libraries for Illumina sequencing were prepared using the TruSeq RNA kit (Illumina, USA) according to the instructions of the manufacturer. We used total RNA as our template because our target was rRNA gene sequences. Briefly, the total RNA was chemically fragmented and a first and second strand of cDNA were made. The ends of the cDNA were repaired and the 3′ ends were adenylated. The adenylated cDNA was ligated to the Illumina adapters and library fragments were enriched with 12 cycles of PCR before library purification, quantification and validation. PCR purification was performed using the Agencourt AMPure XP—PCR Purification beads (Brea, CA, USA) according to the procedures described by the manufacturer. To reduce the influence of the dominant community member (*M. vaginatus*) and increase the representation of rRNA from the low abundance species, we treated an aliquot of the total RNA Illumina libraries with a Duplex-Specific thermostable Nuclease (DSN) enzyme (Evrogen, Moscow, Russia) during template re-annealing according to instructions provided by the manufacturers. The DSN treated libraries (hereafter termed DSN-RNA) were PCR enriched for 12 cycles, quantified and validated. The four metatranscriptomic libraries (Day time total-RNA, Day time DSN-RNA, Night time total-RNA, Night time DSN-RNA) were pooled and paired-end sequenced for 151 cycles using an Illumina GAIIx sequencer in the Earth Sciences Division at Lawrence Berkeley National Lab.

Reconstruction of SSU of rRNA Genes from Metagenome and Metatranscriptome Reads

Near-full-length SSU rRNA sequences were reconstructed from Illumina reads with EMIRGE (Miller et al., 2011) using sequence reads from the metagenomic, total-RNA and DSN-RNA metatranscriptomic libraries. We used Trimmomatic (Bolger et al., 2014) as sequence quality trimmer. When below quality level 3, bases were trimmed from the end of the sequences. Paired-end reads where both reads were at least 60 nucleotides in length after trimming were used as inputs. Sixty iterations were run when using emirge.py (metagenome) or emirge_amplicon.py (metatranscriptomes), the maximum length of the sequences was 151 bp, the average insert length was 212 and their standard deviation ranged from 61 to 93 bp. We used all reconstructed sequences for the analysis. The reads were mapped to the release 114NR of the SILVA rRNA gene database (Quast et al., 2012), clustered at 94% similarity, using the tool cluster_fast from the USEARCH software (Edgar, 2010). To the SILVA database 114NR, we added the SSU rRNA gene sequences of the isolates recovered in this

study and that of *Microcoleus vaginatus* PCC 9802 (Garcia-Pichel et al., 2001) isolated from the same geographic area as the isolates in this study. Subsequently, we fixed the non-standard characters and constructed a Bowtie index as described by Miller et al. (2011). Chimeric sequences were removed using the reference database mode of the UCHIME algorithm (Edgar et al., 2011); the SILVA database 114NR clustered at 94%, as described above, was used as reference database.

Comparisons of SSU rRNA Genes from Isolates and SSU rRNA Genes Reconstructed from Metagenomes and Metatranscriptomes

We attempted to determine the fraction of the metagenome and metatranscriptome libraries that were also recovered by cultivation. We binned sequences with more than 97% similarity into the same taxonomic unit. To determine which sequences were more than 97% similar, we used the tool UCLUST from the USEARCH package (Edgar, 2010) where we clustered the SSU rRNA gene sequences of all isolates with those of the SSU rRNA genes reconstructed from metagenomes and metatranscriptomes. Briefly, the fasta files containing the sequences of all isolates were concatenated into the fasta files containing the ribosomal sequences reconstructed by EMIRGE. Sequences were then sorted by length prior to OTU clustering using UCLUST.

We used the R software environment and the package "VennDiagram" version 1.6.0 to determine the intersection of the OTUs isolated across the different media and pre-incubation conditions, and also the intersection between isolate OTUs and OTUs reconstructed from metagenomes and metatranscriptomes. The fractional abundance of the microbial diversity in the metagenomic and metatranscriptomic libraries that also was represented in our isolate pool was denoted as the sum of the relative normalized abundance of the different reconstructed OTUs that were more than 97% similar to the SSU rRNA genes of isolates.

SSU rRNA genes reconstructed from metagenomes and metatranscriptomes were classified using the assign_taxonomy.py script (QIIME, Caporaso et al., 2010). To be able to assign the phylogeny of both Prokaryotes and Eukaryotes we used SILVA database release 108 as a non-default reference database (described in the QIIME tutorials, qiime.org/tutorials/processing_18S_data.html). The isolate with the longest sequence was chosen as cluster representative when comparing only isolates across media or pre-incubation conditions. Phylogenetic trees of SSU rRNA gene sequences of isolates and those reconstructed from metagenomes and metatranscriptomes were made using representatives of these sequences clustered at 97% similarity. The sequences were aligned using the SINA software version 1.2.7 (Pruesse et al., 2012). Jukes-Cantor phylogenetic trees were calculated using the FastTree software (Price et al., 2010) using default parameters. Circular tree layouts were prepared using the online software Interactive Tree of Life (Letunic and Bork, 2011).

Accession Numbers of Nucleotide Sequences, Metagenome, and Metatranscriptomes

Sequences of isolate cluster representatives were deposited at EBI under accession numbers LN614590–LN614705 (http://www.ebi.ac.uk/ena/data/view/LN614590-LN614705). The metagenome library used in this study (total of 134,295,345 paired end reads) is deposited as JGI Project ID 404128 [http://genome.jgi.doe.gov/pages/dynamicOrganismDownload.jsf?organism=LigCruGreenButte]. The four metatranscriptome libraries used in this study are deposited at the EBI European Nucleotide Archive under the project accession number PRJEB7437. The number of paired end reads per library were as follows: Crust_6A_Total, 6,343,659; Crust_6A_DSN, 30,725,320; Crust_9B_Total, 773,360; and Crust_9B_DSN, 10,054,801. As EMIRGE reconstructed SSU rRNA genes are hypothetical sequences, they were not submitted to a public database but are provided as supplementary material (Supplementary_Material_BSC_EMIRGE_outputs.zip).

Results

Impact of Pre-Incubation Conditions on Isolate Recovery

CFUs from the BSCs pre-incubated under dark or light conditions appeared within 2–3 days of incubation (Supplementary Figure 2). Between inoculation and 50 days of incubation, the rate of colony appearance varied by media type and BSC pre-incubation condition. In samples pre-incubated under light, isolate recovery on 1/10 R2A, 1/10 and 1/100 R2G showed similar rates and reached a maximum after 14–21 days, while CAT and VXylG media showed similar initial slopes but extended isolate recovery at a slower rate from days 14–50. Isolate recovery trajectories from BSCs pre-incubated under dark conditions showed divergent patterns from those under light conditions with 1/100 R2G showing a slower rate of accumulation.

After 50 days of incubation, there was a general trend of more CFUs per gram of dry soil being obtained in samples pre-incubated in the dark relative to those pre-incubated in the light (**Figure 1**). Fisher's LSD test showed CAT plates incubated both under dark and light conditions had the highest CFU number (**Figure 1**). In 1/10 R2A and 1/20 R2G, we observed no significant difference between media inoculated with BSCs pre-incubated in the dark or in the light in samples incubated ($p > 0.05$). Both 1/100 R2G and VXylG showed higher CFU numbers in plates incubated in the dark ($p < 0.05$). The lowest averages of CFUs were observed in 1/20 R2G, 1/100 R2G, and VXylG incubated under light conditions.

We attempted to purify 700 colonies from all media. From those, 580 were successfully purified to single colonies, and 524 isolates were successfully streaked to purity after three continuous passes. For media inoculated with BSC samples pre-incubated in the light, 34, 31, 55, 12, and 21 isolates were recovered on 1/10 R2A, 1/20 R2G, 1/100 R2G, CAT, and VXylG respectively ($n = 153$). For media inoculated with BSC samples pre-incubated in the dark, 73, 52, 91, 5, 28 isolates were recovered on 1/10 R2A, 1/20 R2G, 1/100 R2G, CAT, and VXylG respectively ($n = 249$).

Different Media Recover Phylogeneticaly Distinct Organisms

The sequences of the SSU rRNA gene of 401 isolates from all five media were affiliated with four bacterial phyla. Isolate D2B05, recovered from 1/20 R2G, was classified as a fungal eukaryote

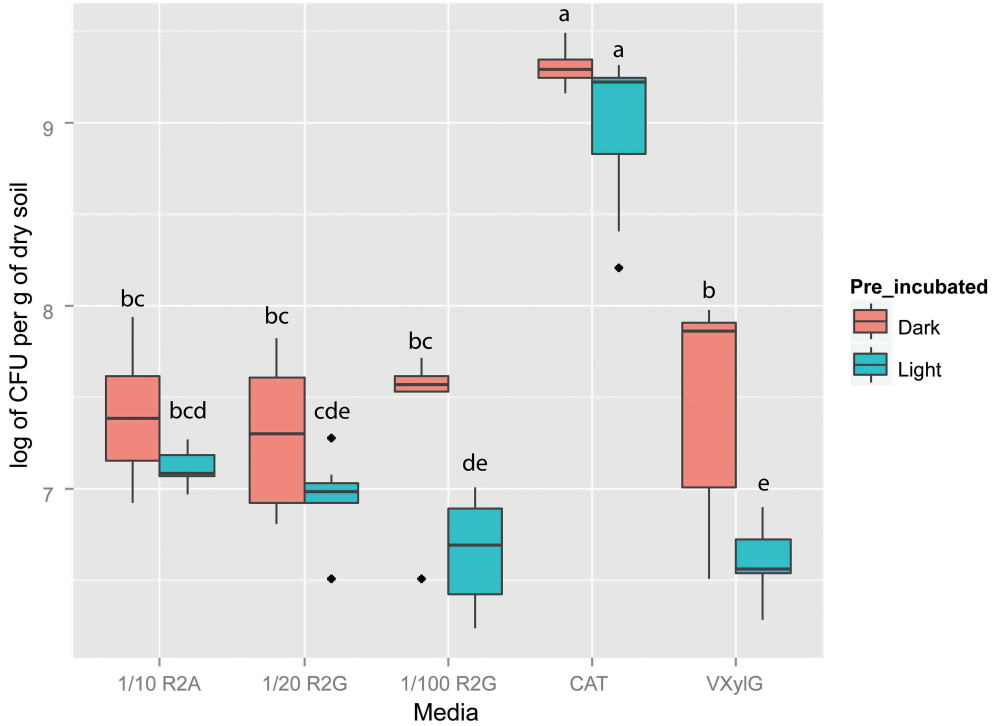

FIGURE 1 | Boxplot of colony forming units on different media (i.e., 1/10 R2A, 1/20 R2G, 1/100 R2G CAT, and VXylG) after 50 days incubation. Vertical bars represent standard deviations of the mean. Top and lower lines of boxes represent, respectively, 75th and 25th percentiles. Horizontal bars represent the medians. Points outside the boxes represent outliers. Light/Dark indicate plates counted from samples incubated, respectively, in the light and in the dark. Boxes with same letters are not statistically different according to *t*-test ($p > 0.05$).

within the Agaricomycotina subdivision (**Table 1**). Representatives of Actinobacteria and Proteobacteria (mostly Alphaproteobacteria) were obtained from all media. Firmicutes were recovered from 1/20 and 1/100 R2G only and Bacteroidetes from VXylG only. Overall the majority of isolates were affiliated with the Actinomycetales order (**Table 1**).

Clustering of the isolates at 97% 16S rRNA gene sequence homology yielded a total of 116 clusters (115 bacterial and one fungal) among the 402 isolates (**Figure 2**). Notably, only two clusters were detected across all five media (**Figure 2**). These clusters were represented by isolates L1C52A and D1A18 (**Figure 2**) and were affiliated with the genera *Williamsia* and *Microbacterium* respectively. The cluster with the largest number of isolates was affiliated with *Arthrobacter globiformis* (accession number JF439618, 99% similar to representative isolate D1A04).

The majority of the bacterial clusters (77 out of 115) were recovered from a single medium (**Figure 3A**), while the remaining were distributed over 2–5 media, indicating that each medium selected for distinct fractions of the BSC microbial community. Similarly, pre-incubation conditions selected for a subset of the microbial community with only about 27% (31 of 115) being derived from both light and dark pre-incubations (**Figure 3B**). For the complete distribution of isolate clusters according to their media and pre-incubation conditions, refer to Supplementary Table 1.

Only *M. vaginatus* Was Detected across All Metagenomic and Metatranscriptomic Libraries

In order to compare the diversity of the recovered isolates to the original BSC population, we analyzed rRNA genes using a PCR-independent approach to eliminate PCR primer bias. After removal of chimeric sequences (23.1% of the total), reconstruction of SSU rRNA genes using EMIRGE analysis of shotgun metagenomic reads from this BSC resulted in 251 full-length sequence clusters at 97% similarity. Estimates of sequence abundances using coverage information suggested dominance of Bacteria with a minor fraction of Archaea (99.4 and 0.6% respectively, Supplementary Table 2). As expected, the dominant taxon was classified as *M. vaginatus* at over 41% abundance. The most abundant non-cyanobacterial phylotypes were represented by members of the Actinomycetales (18.2%) and the Alphaproteobacteria (13.3%).

Shotgun metatranscriptomes were prepared using RNA obtained from samples taken during light and dark sampling time points from a BSC wet-up experiment that has been previously described (Rajeev et al., 2013). As we expected the dominance of *M. vaginatus* to inhibit the detection of less abundant bacterial taxa, we used a library normalization approach (duplex-specific thermostable nuclease enzymatic digestion, termed DSN-RNA) typically employed to reduce the influence of abundant housekeeping transcripts in mRNA analysis (Bogdanova et al.,

TABLE 1 | Taxonomic distribution of isolates from the early successional stage desert Biological Soil Crust from Moab, UT.

Domain	Phylum	Class	Order	Family	Dark[a]	Light[b]
Bacteria	Actinobacteria	Actinobacteria	Actinobacteridae	Actinomycetales	126	192
			Rubrobacteridae	Solirubrobacterales	5	4
	Bacteroidetes	Sphingobacteria	Sphingobacteriales	Cytophagaceae	-	**4**
	Firmicutes	Bacilli	Bacillales	Bacillaceae	8	9
	Proteobacteria	Alphaproteobacteria	Rhizobiales	Beijerinchiaceae	-	**1**
				Bradyrhizobiaceae	4	12
				Methylobacteriaceae	1	1
			Rhodospirillales	Acetobacteraceae	7	25
		Betaproteobacteria	Burkholderiales	Comamonadaceae	-	**1**
		Gammaproteobacteria	Pseudomonadales	Moraxellaceae	**1**	-
Eukaryota	Fungi	Dikarya	Basidiomycota	Agaricomycotina	**1**	-
Total					153	249

[a] *Isolates recovered from Biological Soil Crust samples incubated in the Dark.*
[b] *Isolates recovered from Biological Soil Crust samples incubated in the Light.*
Numbers in bold indicate taxa found in only one pre-incubation condition.

2009) and we compared this to a standard shotgun RNA library derived from the same material (total-RNA). After removal of chimeric sequences (approximately 9.3% of the total), Metatranscriptomes obtained from light (day time) samples had a strong dominance of *M. vaginatus* (69%) and total sequences for cyanobacteria comprised more than 82% of the predicted rRNA gene abundances. This contrasts with the dark (night time) sample where cyanobacteria abundances were approximately 50%. As the proportion of cyanobacteria RNA phylotypes declined at night, numerous bacterial groups became detectable (Supplementary Table 2). After removal of chimeric sequences (approximately 13.7% of the total), over 4700 and 3800 reconstructed SSU rRNA gene OTUs were derived from the dark (night) sampling total-RNA and DSN-RNA respectively (Supplementary Table 2). These OTUs were distributed between Archaea, Bacteria and Eukarya, with again most sequences being affiliated with the Bacteria, although the abundance of both Archaea and Eukarya were proportionally greater in the night time DSN-RNA library compared to the total-RNA library. The libraries derived from day-time sampling yielded substantially fewer numbers of OTUs, however DSN treatment resulted in a four-fold increase in OTU numbers suggesting the low estimate of diversity was due to cyanobacterial dominance. Analysis of the *M. vaginatus* proportion of the sequences shows an abundance of approximately 25% in the total-RNA library declining to less than 10% after DSN treatment and the proportion of non-phototrophic bacteria increasing from 17% to over 70% (**Table 2**).

Comparisons between the different sequences reconstructed by EMIRGE from the metagenome and metatranscriptome libraries demonstrated that only *M. vaginatus* was detected across all sequencing libraries (Supplementary Figure 3). From the 251 OTUs detected in the metagenome, 38 were found in at least one of the metatranscriptomic libraries (Supplementary Figure 3).

Representation of Isolates within the BSC Microbial Community: Comparison to Metagenome and Contrasting Metatranscriptomes

To determine what fraction of the total phylotype diversity (OTUs weighted by relative abundance in the community) in our BSC metagenome and metatranscriptomes was recovered as isolates, we determined the overlap between isolate sequences and OTUs derived from metagenome or metatranscriptome sequences (Supplementary Figure 4). Not including *M. vaginatus* which was previously isolated from these crusts, 7 OTUs were shared between the isolates and the metagenome reconstructed sequences. When considering the proportional abundances of these metagenome OTUs (excluding *M. vaginatus*), at 97% homology these isolates represented approximately 8% of the bacterial relative abundance (**Table 2**). Similarly, 16 and 12 isolates overlapped with sequences from the dark (night time) total-RNA and DSN-RNA metatranscriptomes respectively, representing approximately 3.2% of the community relative abundance. The 16S rRNA gene of two isolates clustered at 97% homology with OTUs reconstructed from the light (day time) total-RNA metatranscriptome. These two OTUs comprised 4.4% of the relative abundance observed for that metatranscriptomic library. Supplementary Table 3 shows the phylogenetic affiliation and relative abundance of OTUs with isolated representatives.

Discussion

Here, we report the first attempt to assess what fraction of the aerobic heterotrophic bacterial diversity of an early successional stage biological soil crust could be cultivated by simple plating under aerobic conditions. To this end, we isolated over 400 bacteria using five different media formulations with varying nutrient concentration, solidifying agents, presence of

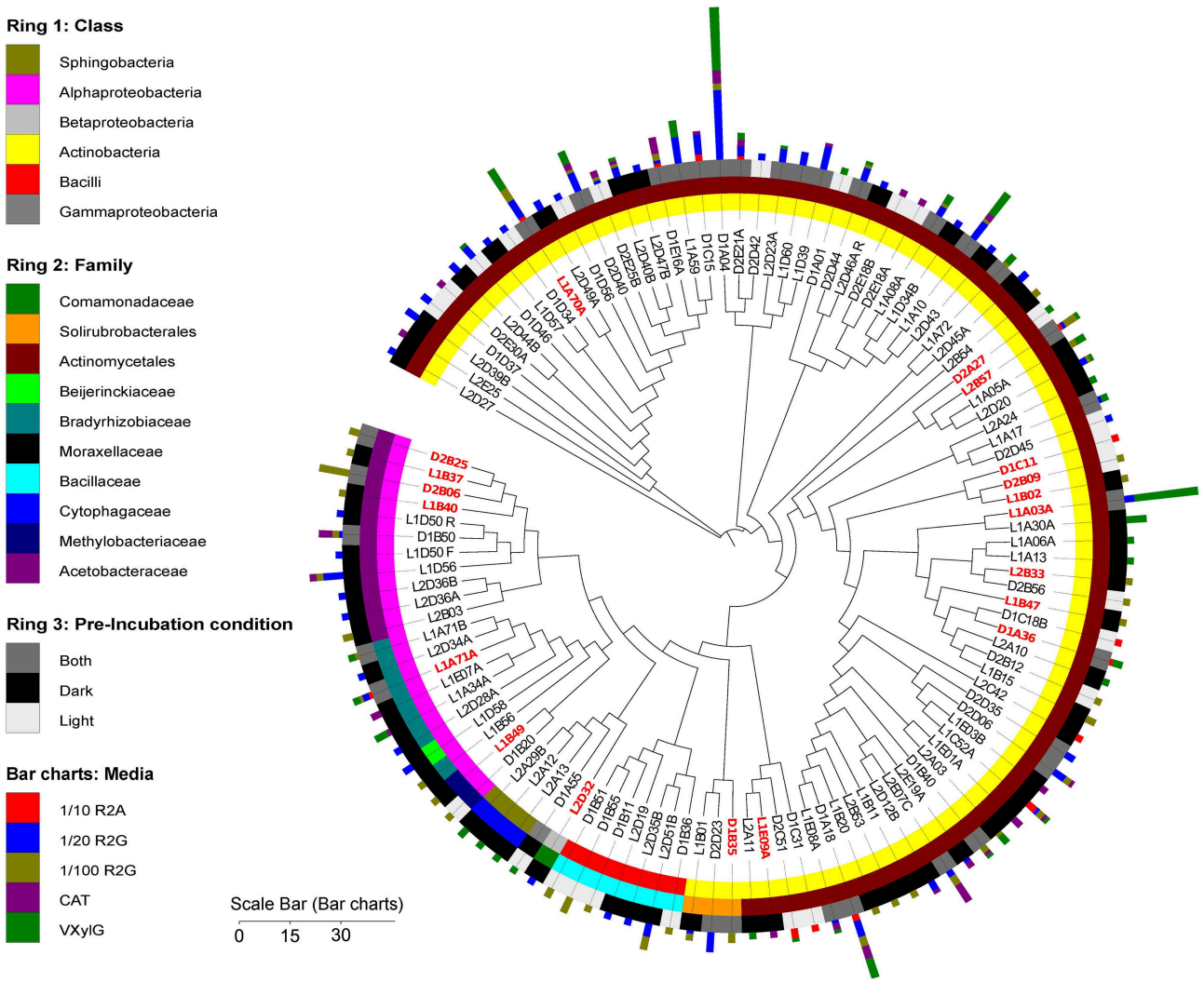

FIGURE 2 | Phylogenetic tree with representative sequences of the bacterial isolates clustered at 97% similarity. Leaf labels indicate representative sequence. Rings, from the inner to the outside circles, represent: Ring 1, taxonomy (class level) of cluster representative isolate; Ring 2, taxonomy (family level) of cluster representative isolate; Ring 3, pre-incubation condition of Biological Soil Crust samples prior bacterial isolation procedures. Bar charts, the different media from where the isolates were recovered. Size of the bars indicates the number of isolates in each of the clusters. Red and bold leaf labels indicate which representative sequences clustered at 97% similarity with OTUs reconstructed from the metagenome or metatranscriptome libraries. Scale bar indicates the number of isolates per representative sequence.

oxidative-protective agents and micronutrients. We developed a number of reference sequence datasets to place the isolate diversity in the context of BSC diversity under distinct physiological conditions (dry, wet, dark, light) by using a primer- and amplification-independent method (EMIRGE), to reconstruct rRNA gene sequences from shotgun metagenomes and metatranscriptomes.

Over the past 15 years, numerous strategies have been developed to obtain axenic or mixed cultures of many soil microorganisms previously classified as unculturable (Janssen et al., 2002; Sait et al., 2002; Bruns et al., 2003; Schoenborn et al., 2004; Davis et al., 2005; Rocha et al., 2009). Once isolated, it is challenging to place organisms into a useful environmental context and most studies have compared the yield of isolates in terms of numbers relative to direct counts of soil microorganisms; it is from these types of comparisons that the classical descriptions of the proportion of culturable organisms and the "great plate count anomaly" are derived (Suzuki et al., 1997; Bruns et al., 2002, 2003; Connon and Giovannoni, 2002; Janssen et al., 2002; Sait et al., 2002; Ellis et al., 2003; Tamaki et al., 2005; Connon et al., 2007; Lester et al., 2007; Shivaji et al., 2011). In contrast, few attempts have been made to assess the fraction of microbial diversity that can be cultured (Kämpfer et al., 1996; Suzuki et al., 1997; Tamaki et al., 2005; Rocha et al., 2009; Shivaji et al., 2011; Colin et al., 2013) and their numerical importance and most of these studies have characterized diversity using cloning and sequencing to a limited extent.

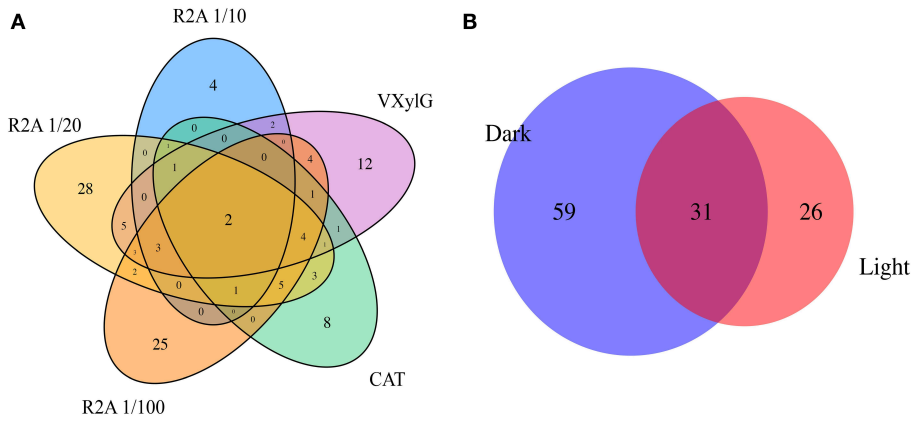

FIGURE 3 | Venn diagrams showing the intersection of isolate clusters found in (A) the different media (i.e., 1/10 R2A, 1/20 R2G, 1/100 R2G, CAT, and VXylG) and (B) pre-incubation conditions (Dark and Light).

Here, our use of multiple media, different pre-incubation conditions and extended incubation times was designed to improve the recovery of a more diverse range of bacterial heterotrophs from BSCs. Similarly, our use of BSC samples taken under distinct physiological conditions (dry, wet, dark, light) to reconstruct their phylogenetic composition using non-amplification based methods, was designed to improve our knowledge of the true diversity in these systems and to provide an appropriate context to place cultivated taxa.

As expected, the appearance of visible CFUs differed by both media type and pre-incubation condition. In general, media with lower nutrient concentrations and oxidative stress protectants selected for more organisms with slower growth rates as evidenced by the extended recovery of isolates out to 50 days. Similarly, Davis et al. (2005) demonstrated that in media with high nutrient concentrations, isolates ceased to appear 2–4 weeks after inoculation. When comparing the recovery of isolates across media types, it was significant that only the CAT medium, containing low nutrient concentrations and amended with catalase as a protectant against oxidative stress, showed significantly more recovered CFUs than other media. Previous efforts to retrieve culturable isolates from BSCs obtained CFUs of approximately 6.8 log units per g of BSC (Garcia-Pichel et al., 2003) which was similar to the range reported here, with the exception of CFUs recovered on CAT media that were approximately 1.5 log units greater. Although in a rhizosphere system, the use of the CAT medium also resulted in higher CFU counts compared with 1/10 R2A (Rocha et al., 2009), for bacteria inhabiting a BSC this was somewhat surprising as given its predominantly oxidizing nature (with the exception of dark active periods) one might expect traits associated with oxidative stress protection to be selected for in many individuals (Wang and Sheng, 2011; Steven et al., 2012b; Rajeev et al., 2013; Reddy and Garcia-Pichel, 2013).

Although here we have reported the appearance of visible colonies, it has been observed that micro-colonies (below 250 μm diameter) may comprise a large fraction (on average 48%) of CFUs from rhizosphere soils (Rocha et al., 2009) and over 75% from gardening soil (Watve et al., 2000) and that micro-colonies may continue to emerge over 12–24 weeks post-inoculation (Davis et al., 2011). Therefore, we acknowledge that our recovered isolates likely missed organisms with this growth property. However, as our overall goal was the recovery of organisms to enable hypothesis testing related to physiological roles and functions inferred from metagenomic approaches, obtaining a broad diversity of organisms that were also numerically abundant in our BSC was the objective.

The rate of isolate recovery for a specific medium also varied somewhat based on the pre-incubation condition used, suggesting that a different subset of the bacterial population may have been activated due to this treatment. In fact, of the 116 (115 bacterial and one fungal) OTUs represented by the isolates, only 31 were in common between the dark and light pre-incubated samples (**Figure 3B**). One significant example of this differential recovery is the Bacteroidetes members (four *Hymenobacter* species) that were only recovered from the dark incubated samples. Relatives of these isolates have been recovered from similar crusts in the Colorado Plateau previously (Reddy and Garcia-Pichel, 2013). We only observed differences in CFU numbers between plates pre-incubated under light and those pre-incubated in the dark in 1/100 R2G and VXylG. The current study was designed to yield higher diversity of aerobic heterotrophs, however anaerobes and chemoautotrophic physiologies are also likely important in these systems and further studies should target their isolation and pre-activation.

Analysis of the overlap in isolate recovery across all media (at 97% homology, **Figure 3A**) shows the majority of the clusters (>66%) were recovered from only a single type of media. The only two isolates recovered in all five different media were affiliated with species belonging to the Actinomycetes *Williamsia* spp. and *Microbacterium* sp.. Representatives of *Williamsia* are known to inhabit arid environments such as Antarctic desert soils (Guerrero et al., 2014) and have been previously isolated from BSCs (Gundlapally and Garcia-Pichel, 2006) as have *Microbacterium* sp. (Gundlapally and Garcia-Pichel, 2006). The diversity of isolates recovered in our study expanded that observed by Garcia-Pichel et al. (2003) by isolating not

TABLE 2 | Relative abundance (percentage) of rRNA OTUs and overlap at 97% similarity between metagenome (MG) and metatranscriptome (MT) reconstructed OTUs and isolated bacteria.

Sequence type	Sample	Treatment[a]	Library prep.[b]	Number of EMIRGE OTUs	Relative Abundance[c] (Domains)			Relative Abundance (Bacteria)			Relative abundance of isolates with >97 similarity to MG/MT reconstructed sequences		
					Bacteria (%)	Archaea (%)	Eukaryota (%)	*M. vaginatus* (%)	Cyanobacteria (non *M. vaginatus*) (%)	Non-cyanobacteria (%)	Number	Total Bacteria (%)	Non-phototrophic Bacteria (%)
Metagenome	MTG	Prior wet-up	Total DNA	251	99.43	0.57	BDL	41.34	6.69	51.97	8	4.28%	8.23%
Metatranscriptome	6	Night time (18 h)	Total RNA	4708	98.03	0.26	1.71%	16.96	34.80	48.25	16	1.55%	3.21%
			DSN treated	3829	92.82	0.58	6.60%	3.23	6.41	90.36	12	2.98%	3.30%
	9	Day time (25.5 h)	Total RNA	50	99.75	0.25	BDL	24.59	58.15	17.26	2	0.75%	4.37%
			DSN treated	213	99.92	0.08	BDL	9.67	19.54	70.79	1	BDL	BDL

[a] *The metagenomic library was prepared with DNA extracted from samples Prior wet-up, and the metatranscriptomic libraries were prepared with RNA extracted 18 h (night time) or 25.5 h (day time) after wet-up.*

[b] *RNA used for library preparation. The libraries were prepared with total cDNA (Total RNA) or Double Strand Specific Nuclease (DSN) treated cDNA to reduce the influence of dominant organisms (DSN treated).*

[c] *BDL, below detection limit.*

only members of Methylobacteriaceae, Bacillaceae, and Actinomycetales, but also Comamonadaceae, Solirubrobacterales, Beijerinckiaceae, Bradyrhizobiaceae, Moraxellaceae, Cytophagaceae and Acetobacteraceae. The only group isolated by Garcia-Pichel et al. (2003) not found in this study was Caulobacteraceae. Another attempt to isolate a broad range of isolates from the crust managed to isolate 34 different species (grouped at 97% similarity) from the same area where our samples were collected (Gundlapally and Garcia-Pichel, 2006). None of these isolates showed more than 97% similarity to the isolates recovered in the current study (data not shown), supporting the hypothesis that each medium/incubation condition or even different isolation attempts will yield a novel set of species. Alternatives to this hypothesis would be that: due to the limited number of isolates, each sample has not reached saturation in isolates recovered in each medium and hence phylogeny in each medium and pre-incubation condition look different; or, every piece is indeed different and just the major players are the same.

A total of 251 different SSU rRNA gene OTUs were obtained by EMIRGE (Miller et al., 2011) reconstruction from metagenome reads. A previous study including a shotgun metagenome of dryland soil microbial communities detected the presence of Cyanobacteria, Proteobacteria, Actinobacteria, Chloroflexi Acidobacteria, Planctomycetes, OP10 and Firmicutes in both early and late successional BSC developmental stages in the Nevada Desert (Steven et al., 2012a). In our study, with the exception of OP10, we detected all phyla observed by Steven et al. (2012a). Further, we also detected Archaea, Gemmatimonadetes, Verrucomicrobia, and Fungi. Another study in arid and hyperarid BSC (Angel and Conrad, 2013) did not detect the presence of Chloroflexi, Gemmatimonadetes or Verrucomicrobia among the detected bacterial groups in dry crust. Differences observed between our study and that of Steven et al. (2012a) and Angel and Conrad (2013) may be related to the different geographical positions of the BSC tested in both studies, as well as differences caused by barcoding, PCR amplification and high-throughput sequencing.

We constructed four metatranscriptomic libraries from this BSC to determine the sequences of organisms with intact ribosomes under two contrasting physiological conditions (dark and light). Two of these libraries were constructed with total RNA (Total-RNA) and these libraries were split and then treated with a duplex-specific nuclease (DSN) to enrich the detection of low abundance transcripts (Yi et al., 2011), generating a second set of libraries termed DSN-RNA. We hypothesized that DSN treatment would improve our representation of the true diversity of BSCs by reducing the masking effect of dominant cyanobacteria. EMIRGE reconstruction of SSU rRNA genes from the total-RNA library sampled under light conditions confirmed this hypothesis where *M. vaginatus* sequence relative abundance was reduced from ∼70 to ∼24% and Alphaproteobacteria increased from 5% to about 40% (Supplementary Table 1). Concurrently, detected OTUs increased from 56 to 213 with certain broad groups such as the Actinomycetales going from undetectable to prominent. In contrast to this, DSN treatment of the BSC metatranscriptomes sampled under dark conditions also showed a shift in dominance away from *M. vaginatus* (44 to 6%) to Actinomycetales

(∼8 to 19%). Further, the impact of sampling in the dark alone (*M. vaginatus* rRNA abundance declined from ∼70 to 44%) was sufficient to uncover substantially greater diversity compared to that found in samples taken under light conditions. Although changing the detectable species diversity, DSN treatment did not always result in an increase in OTUs detected. This highlights the value of using natural shifts in metabolic activity, such as those observed between light and dark conditions in these samples (Rajeev et al., 2013) to improve representation of microbial groups in these systems. While shifts in rRNA relative abundance may not be directly correlated with changes in activity (Blazewicz et al., 2013), we observed shifts in relative rRNA abundance between the light and dark samples (separated by only 3 h) with a sharp decline in cyanobacteria. These abrupt changes clearly have biological relevance and suggest that organisms in BSCs rapidly shift between metabolic states and alter cellular ribosomal content within hours.

After establishing an improved view of the true diversity of these BSCs, we calculated the relative cultivation efficiency relative to OTUs uncovered using the metagenome and metatranscriptome analyses. Overall, our isolates encompassed just over 8% of the non-phototrophic bacterial abundance in the metagenome. When accounting for the presence of *M. vaginatus* (Garcia-Pichel et al., 2001), as estimated using EMIRGE reconstruction (Supplementary Table 3), more than 45% of the OTUs in this BSC metagenome have been represented by culture-dependent techniques. We also hypothesized that the best concordance between our cultured isolates and the various windows of microbial diversity estimated here would be achieved when comparing to metatranscriptomes rather than metagenomes as organisms detected due to their rRNA are more likely to be metabolically active. Overall, 33% of our isolated OTUs matched closely (97%) to OTUs detected by sequence reconstruction. The majority of matches corresponded to sequences from the dark metatranscriptome, and only few matching those from the light metatranscriptome.

The cultivation efficiency in desert soils is reported between 0.0007 and 0.1%, calculated by comparing CFU counts with those visualized by microscopy (Connon et al., 2007; Lester et al., 2007). A previous attempt to isolate aerobic copiotrophs from BSC samples from the same area where we acquired our samples, resulted in 0.51% cultivation efficiency, again based on comparison of CFUs to microscopic counts (Garcia-Pichel et al., 2003). However, the units and parameters found in the previous studies may not be comparable to the ones reported here.

In the current study, we recovered up to 8% of the microbial diversity detected in these BSC communities. The detection of diversity, and thus estimates of recovery are clearly impacted by the structure (e.g., evenness) of the community sampled and also the depth of sequencing performed. Due to these interacting factors, EMIRGE reconstruction may not capture sequences that exist in a long tail of low abundance organisms. Additionally, in our study, only 15% of the ribosomal genes reconstructed from the metagenome were also found in the metatranscriptomic libraries, suggesting that many detected OTUs may originate from dormant or dead organisms or possibly extracellular DNA—making a true estimate of the composition of a soil microbiome problematic. Nevertheless, to the best of our knowledge, ours is the first study to attempt cultivation efficiency calculation in terms of OTU recovery without the influence of PCR amplification bias.

Conclusions

In the age of genomics, the value of isolated microorganisms is frequently overlooked, yet all observations from genomics remain hypotheses until validated via biochemical or physiological assays either *in vitro* or *in vivo*. We combined amplification independent analysis of microbial populations with media of varying composition to recover bacteria that were differentially resuscitated from stasis through pre-incubation. We recovered 402 bacterial and one fungal isolate in axenic culture, comprising 116 OTUs (115 bacterial and one fungal). We found that each medium recovered mostly distinct OTUs and that our view of the true diversity was significantly impacted by the physiological state of the BSC when sampled, with dominant organisms frequently obscuring our detection of less abundant members. We related the recovered isolates to the fraction of biomass (estimated abundance) of common OTUs in the BSC and found that, not including the dominant cultured phototroph, more than 8% of the relative abundance was represented by our isolates. Although not completely closing the culturability gap in BSCs, our study indicates that it is possible to reduce this gap using simple cultivation procedures.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://www.frontiersin.org/journal/10.3389/fmicb.2015.00277/abstract

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Typical soils from a cold desert, Taylor Valley, Antarctica. Photo by Thulani P. Makhalanyane

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REVIEW
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Emerging spatial patterns in Antarctic prokaryotes

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Recent advances in knowledge of patterns of biogeography in terrestrial eukaryotic organisms have led to a fundamental paradigm shift in understanding of the controls and history of life on land in Antarctica, and its interactions over the long term with the glaciological and geological processes that have shaped the continent. However, while it has long been recognized that the terrestrial ecosystems of Antarctica are dominated by microbes and their processes, knowledge of microbial diversity and distributions has lagged far behind that of the macroscopic eukaryote organisms. Increasing human contact with and activity in the continent is leading to risks of biological contamination and change in a region whose isolation has protected it for millions of years at least; these risks may be particularly acute for microbial communities which have, as yet, received scant recognition and attention. Even a matter apparently as straightforward as Protected Area designation in Antarctica requires robust biodiversity data which, in most parts of the continent, remain almost completely unavailable. A range of important contributing factors mean that it is now timely to reconsider the state of knowledge of Antarctic terrestrial prokaryotes. Rapid advances in molecular biological approaches are increasingly demonstrating that bacterial diversity in Antarctica may be far greater than previously thought, and that there is overlap in the environmental controls affecting both Antarctic prokaryotic and eukaryotic communities. Bacterial dispersal mechanisms and colonization patterns remain largely unaddressed, although evidence for regional evolutionary differentiation is rapidly accruing and, with this, there is increasing appreciation of patterns in regional bacterial biogeography in this large part of the globe. In this review, we set out to describe the state of knowledge of Antarctic prokaryote diversity patterns, drawing analogy with those of eukaryote groups where appropriate. Based on our synthesis, it is clear that spatial patterns of Antarctic prokaryotes can be unique at local scales, while the limited evidence available to date supports the group exhibiting overall regional biogeographical patterns similar to the eukaryotes. We further consider the applicability of the concept of "functional redundancy" for the Antarctic microbial community and highlight the requirements for proper consideration of their important and distinctive roles in Antarctic terrestrial ecosystems.

Keywords: spatial pattern, Antarctica, prokaryotes, functional redundancy, biogeography

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Introduction

Due to their importance to the fundamental assembly of ecosystems, considerable effort has been devoted to study of the interactions of spatial scale, external physicochemical parameters and species distributions (e.g., King et al., 2010; Nemergut et al., 2011; Westgate et al., 2014). Spatial patterns of species distribution arise from the interactions between physical, chemical, and biological drivers (Legendre and Fortin, 1989; Prosser et al., 2007), placed in the context of the past regional colonization and evolutionary history of any given region (Convey et al., 2014). From the physical environment perspective, environmental gradients clearly influence the establishment and maintenance of viable populations; however, the spatial scale considered is also important in defining these environmental gradients (Wiens, 1989). For instance, in soils, environmental parameters at micro-scale are strongly correlated with the soil texture, pore space, and local topography (e.g., Tromp-Van Meerveld and Mcdonnell, 2006). Nevertheless, climatic features such as precipitation, solar radiation and temperature, acting at far larger spatial scale, also have an important influence (Grundmann, 2004; Griffiths et al., 2011; Convey et al., 2014). In addition to physical and chemical environmental influences, community assembly is also controlled by biological features such as dispersal, interaction (e.g., competition, predation), motility and reproduction (Ettema and Wardle, 2002; Webb et al., 2002).

Among exceptional ecosystems of the planet, Antarctic terrestrial environments are characterized by high winds, intense UV radiation, desiccation, and low temperatures. These physical stressors challenge Antarctic life (Kennedy, 1993; Convey, 1996; Wall and Virginia, 1999; Hogg et al., 2006; Cary et al., 2010) and, combined with physical isolation and geographical barriers (e.g., circumpolar oceanic and atmospheric currents), limit inter- and intra-continental connectivity and underlie the level of endemicity present in Antarctica today (Franzmann, 1996; Clarke et al., 2005; Adams et al., 2006; Barnes et al., 2006; Taton et al., 2006; Convey et al., 2008, 2009, 2014; Vyverman et al., 2010). Given the many differences in physical setting and adaptive requirements, as well as the scales of biological organization involved (e.g., Figure 2 in Peck, 2011), researchers have sought to understand the links between spatial diversity and functioning of Antarctic communities and the differences in comparison to other ecosystems (see Convey et al., 2014 for discussion). Detailed and spatially explicit knowledge of Antarctic biodiversity is essential to enable construction of a comprehensive framework for conservation planning (Hughes and Convey, 2010, 2012; Terauds et al., 2012; Convey et al., 2014; Chown et al., 2015), and to provide baseline data for ecological modeling and prediction (Gutt et al., 2012); however, our knowledge of microbial systems and functions is, at best, fragmented, both globally and in the Antarctic specifically (Tindall, 2004; Cary et al., 2010; Chong et al., 2013).

In this review, we collate current knowledge of Antarctic microbial diversity and biogeography. Adopting a similar approach to that of Martiny et al. (2006), we focus our discussion primarily on Antarctic prokaryotic spatial patterning, making reference to patterns inferred in Antarctic eukaryotic studies where appropriate. We do not assume that the prokaryotes exhibit the same ecological patterns as the eukaryotes, however, the latter have been relatively well-studied and provide a useful comparison. We identify gaps in current knowledge, along with limitations in the methodologies available. Our synthesis leads to the proposition of a new conceptual model to explain the mechanisms underlying species-function relationships in Antarctica, and the experimental framework required to provide such mechanistic insight based on empirical data.

Macroecological Patterns in Antarctica

Antarctica has traditionally and pragmatically been divided into three biogeographic zones, the sub-Antarctic, maritime Antarctic, and continental Antarctic (Convey, 2013). The sub-Antarctic includes a ring of oceanic islands located between c. 45° and 55°S, close to the Antarctic Polar Frontal Zone (Convey, 2007b; Selkirk, 2007). These experience relatively higher precipitation and milder and much less variable temperatures in comparison to the maritime and continental zones, and host the most complex Antarctic terrestrial ecosystems. The maritime Antarctic includes the Scotia Arc archipelagos of the South Sandwich, South Orkney and South Shetland Islands and the majority of the Antarctic Peninsula southward to Alexander Island. Crytogamic fellfield is the most typical vegetated habitat along the coastline and associated low lying islands. In addition, vegetation "hotspots" can be found on the nitrogen-rich ornithogenic gelisols formed near seabird colonies or seal haul-out areas (Michel et al., 2006; Bokhorst et al., 2007). Finally, continental Antarctica comprises the eastern and southern parts of the Antarctic Peninsula, and the remainder of Antarctic continent. Terrestrial ecosystems within this region are restricted to small isolated "islands" of ice-free ground located mainly either in the low-lying coastal zones, or in the form of isolated nunataks and the higher altitudes of inland mountain ranges, with the striking exception of the McMurdo Dry Valleys in Victoria Land which cover an area of approximately 40,000 km^2.

In recent years, large-scale spatial comparisons have refined our understanding and revealed a far greater complexity in the patterns of biogeography present in the terrestrial ecosystems of Antarctica than previously appreciated (Chown and Convey, 2007; Convey et al., 2008; Terauds et al., 2012). For instance, studies across a range of terrestrial macro- and micro- eukaryotic organisms (plants, algae, insects, springtails, mites, nematodes, tardigrades, rotifers) have revealed a strong division between the Antarctic Peninsula and the remainder of the continent (e.g., Maslen and Convey, 2006; Peat et al., 2007; Pugh and Convey, 2008; De Wever et al., 2009; Iakovenko et al., in press). Chown and Convey (2007) proposed that this distinction represented an ancient boundary analogous to the Wallace Line of south-east Asia, reflecting Antarctic historical contingency (the "Gressitt Line"). Separately, a strong localized diversity was also detected when comparing the genetic lineages of Antarctic microbial

eukaryotic organisms across different locations (Lawley et al., 2004; Namsaraev et al., 2010). More recently, a spatial analysis of 38,854 entries and 1823 eukaryote taxa recorded in the Antarctic Biodiveristy Database (ABD)[1] revealed 15 distinct 'Antarctic Conservation Biogeographic Regions' across Antarctic terrestrial environments (five within the classical maritime Antarctic region and 10 from the continental Antarctic; Terauds et al., 2012).

Spatial Patterns of Prokaryotic Diversity

The elucidation of spatial patterns of organization in Antarctic eukaryotes provides an excellent opportunity for microbiologists to evaluate the degree to which prokaryotic biogeography in the Antarctic mirrors or differs from that of the eukaryotes, and to shed new light onto the functioning of Antarctic terrestrial ecosystems. If biogeographic processes in both major groups operate at similar spatial scales, then a homogenous set of mechanisms can be hypothesized to govern these processes, and a consistent response to environmental changes can be predicted. In contrast, the finding of distinct spatial patterns would be indicative of fundamental differences in, for instance, life history, survival strategies, or dispersal limitation. The latter would, further, have important implications for the planning of biosecurity and biodiversity management in Antarctica, including in the application of guidelines and protocols developed under the Environmental Protocol to the Antarctic Treaty and the definition of Antarctic Specially Protected Areas (ASPAs), as current practice has almost completely been built upon knowledge of macro-organisms such as vertebrates, invertebrates, and plants (Hughes et al., 2015).

Over the last decade, encouraged by improved technical and methodological capabilities, knowledge of the spatial scaling and the functional capabilities of Antarctic prokaryotic communities has started to increase. It is thus timely to review our knowledge of bacterial biogeography in Antarctica and to ask how spatial patterns influence ecological functions in the microbial communities of Antarctica.

Site-specific Bacterial Diversity

Airborne Diversity

Antarctica is an extremely windy place. Long distance inter-continental air mass movement has been shown to be a viable route for non-native propagules from Australia, South America, and South Africa to reach and potentially establish in Antarctica (Linskens et al., 1993; Marshall and Convey, 1997; Greenslade et al., 1999; Convey, 2005; Pearce et al., 2009). Locally, the magnitude and direction of air movement vary widely across Antarctica. However, strong and complex networks of aeolian exchange and interaction are apparent. For instance, the low-lying coastal regions of the Antarctic continent and Antarctic Peninsula periodically experience high velocity katabatic winds which may bring mineral dust from the continental interior (Turner et al., 2009; Pearce et al., 2010). It is not clear if this enables the transfer of viable propagules from the polar plateau to the coastal region, however, similar air movements have been documented in back trajectory analyses of air parcels studied microbiologically (Marshall, 1996; Hughes et al., 2004; Pearce et al., 2010; Bottos et al., 2014b). Additionally, the circumpolar coastal winds (circulating west to east) increase the mixing of air masses between the interior and coastal areas, and further facilitate inter-regional aeolian movement between different ice-free regions in Antarctica (Wynn-Williams, 1991; Reijmer et al., 2002; Parish and Bromwich, 2007).

The very limited aerobiological survey data currently available from the Antarctic Peninsula and continental Antarctic generally suggested low airborne bacterial diversity and a minimal contribution of local propagules into the aerosol (Hughes et al., 2004; Pearce et al., 2010; Bottos et al., 2014b). For instance, marine-related sequences constituted <10% of the airborne bacterial diversity detected at Halley V Research station on the Brunt Ice Shelf at the base of the Weddell Sea and at Rothera Point, to the west of the Antarctic Peninsula, despite substantial sea-spray influence in both locations. Separately, Bottos et al. (2014b) observed little overlap between the aerosol and soil bacterial diversity in the McMurdo Dry Valleys.

Overall, there was little similarity in bacterial diversity in the studies reported by Hughes et al. (2004), Pearce et al. (2010), and Bottos et al. (2014b). Although this might relate to differences in methodologies employed in each study, the differences might also be underlain by the environmental stresses faced in long duration airborne dispersal (e.g., Hughes et al., 2004; see also review by Pearce et al., 2009). High community variation was also detected when comparing the microbiota of aerosols collected in close proximity (e.g., ∼2 km apart, Bottos et al., 2014b), further supporting strong spatial variation in Antarctic aerosols. However, a number of cyst forming and desiccation resistant genera such as *Frankia*, *Rubrobacter*, *Sphingomonas,* and *Paenibacillus* were found. These genera might form the core of an airborne bacterial community that is universal across Antarctica (Pearce et al., 2010; Bottos et al., 2014b).

Soil Microbial Diversity

Recent Antarctic terrestrial microbiological studies using molecular approaches generally support the occurrence of highly specific community membership across space. For instance, in bacterial culture collections developed from nine distinct sites in the Antarctic Peninsula, and the Ronne, Maud, and Enderby sectors of continental Antarctica (Peeters et al., 2012), only 3.4% of the total isolates were common to more than one site. More generally, it has been estimated that <1% of total bacterial diversity is culturable in temperate environments (Hugenholtz, 2002), so these common isolates may represent an even smaller percentage of the overall diversity. In a similar report of highly localized bacterial distribution patterns derived using a culture-independent technique, Lee et al. (2012) reported that, in four cold desert habitats located within an 80 km radius in the McMurdo Dry Valleys, the proportion

[1]http://data.aad.gov.au/aadc/biodiversity/

of rare phylotypes specific to only one site ranged between 48 and 72%.

At higher phylogenetic levels, such as phylum or class, the dominant membership of Antarctic soil bacterial communities is relatively consistent (e.g., Yergeau et al., 2007b; Pointing et al., 2009; Chong et al., 2012b), including common groups found in soil ecosystems globally such as Acidobacteria, Proteobacteria, Firmicutes, and Bacteroidetes (Janssen, 2006; Youssef and Elshahed, 2008). Nevertheless, in comparisons across different Antarctic regions, strong compositional differences become apparent. For example, soil from Antarctic Peninsula sites was dominated by taxa affiliated with Alpha-proteobacteria and Actinobacteria and had low representation of Bacteriodetes, while the reverse pattern was apparent in soil from the Ellsworth Mountains (Yergeau et al., 2007b). Separately, Actinobacteria contributed the largest proportion of the overall soil bacterial community in Victoria Land, more than double that detected in the former two locations (Bottos et al., 2014a, and references therein). Again, methodological differences may contribute to such observations, although it is notable that diversity variations are also apparent in comparisons of regional samples using standardized methodology (Yergeau et al., 2007b; Sokol et al., 2013).

Even greater variation was apparent in the 'rare' members of the community – those which make up less than 0.05% of the community composition. For instance, members of *Verrucomicrobia* and *Spirochaetes* were detected rarely in rhizosphere soil in the Antarctic Peninsula but were completely absent from mineral soils in the Antarctic Dry Valleys (Teixeira et al., 2010; Lee et al., 2012). Both these studies employed massively parallel next generation sequencing (NGS) techniques targeting similar 16S regions (V4–V5 vs. V3–V5) and reported high average sequence coverage at 90%. Assuming that the disparity in the community assembly between locations is not due to methodological variation, it might be a reflection of the different requirements and life history strategies of various microbial lineages.

Environmental Selection vs. Geographical Isolation

Syntheses of studies of physiological adaptation and life history strategies of Antarctic organisms have suggested that the distribution of Antarctic terrestrial life is generally driven by abiotic environmental gradients in variables such as the availability of water or specific nutrients (Kennedy, 1993; Convey, 1996; Barrett et al., 2006a; Hogg et al., 2006; Convey et al., 2014). For example, the water gradient at Mars Oasis (Alexander Island, Antarctic Peninsula) leads to a clear separation between populations of *Mortierella* and *Serendipita*-like *Sebacinales*, *Tetracladium*, *Helotialian* fungi and black yeasts (Bridge and Newsham, 2009). Similar trends have also been observed in studies of soil arthropods, for instance with some mite species such as *Gamasellus racovitzai* and *Alaskozetes antarcticus* showing a stronger resistance to desiccation stress than others such as *Stereotydeus villosus*, while the length of the active season appears to be more strongly influenced by the moisture available in the environment for some species than others (Convey et al., 2003). Green algae including *Nostoc* spp. and *Gloeocapsa* spp. are sensitive to salinity and hence are usually absent from areas subjected to frequent windblown sea-spray (Broady, 1996). In addition, heavy metals including copper are detrimental to the growth and the cell wall structure of cyanobacteria and might thereby inhibit the distribution of the photosynthetic microbes in the Dry Valleys (Wood et al., 2008).

Although most such syntheses have been based on studies of Antarctic invertebrates and plants, similar findings are apparent in recent molecular studies of Antarctic soil bacterial communities (**Table 1**). For instance, in the Ross Sea region of continental Antarctica, Aislabie et al. (2008) found strong positive correlation between bacterial community diversity and soil pH and nutrient content. In the Dry Valleys of the same region, Lee et al. (2012) proposed that salt and copper content in the soil, along with altitude, were the major drivers of microbial community composition. Over a spatial gradient of a few kilometers in a coastal area of maritime Antarctica, Chong et al. (2012a) similarly reported that community structure was largely determined by pH and altitude. Magalhães et al. (2012) working near Darwin Mountain (Transantarctic Mountains) found different ion concentrations were the main driver of diversity. It is striking that none of these studies established strong distance decay or occupancy-distance relationships in bacterial community composition, consistent with the findings of a recent large-scale spatial study within the Transantarctic Mountains (Sokol et al., 2013). Based on spatially stratified sampling that spanned seven degrees of latitude, Sokol et al. (2013) showed that local edaphic gradients (e.g., pH and moisture) exerted stronger control over the bacterial community composition than was explained by spatial scaling alone. In comparison, however, spatial partitioning was prominent in the cyanobacterial community, potentially indicating differences in dispersal controls between cyanobacteria and the soil bacterial community.

A large-scale compilation of bacterial 16S rRNA gene sequence data retrieved from Antarctic soil habitats ranging from 45 to 78°S revealed that majority of the Antarctic soil habitats included were phylogenetically clustered (genetically closely related, see Webb et al., 2002), implying strong habitat filtering in the Antarctic terrestrial environment (Chong et al., 2012b). Souza et al. (2008) hypothesized that bacterial community homogenization in nutrient-depleted environments might be obstructed by low cell density, which could reduce the likelihood of horizontal gene transfer across the community. Additionally, environmental stress might further exert sympatric selective pressure in different micro-niches in the soil, promoting the prevalence of specialists in each ecotype. Such factors might underlie the detection of the highly specialized communities reported in various studies (Lee et al., 2012; Peeters et al., 2012). In a separate large-scale latitudinal survey in the Antarctic Peninsula/Scotia Arc region, Yergeau et al. (2007b) showed a significant latitudinal influence on the bacterial community composition of bare ground sites. However, for locations with moss/lichen cover, the effect of local

TABLE 1 | Major environmental parameters influencing terrestrial bacterial community composition.

Major environmental parameters[a]	Correlate with[b]	Microbiological approach	Region	Spatial range	Reference
pH	BCS	DGGE	Signy Island	<10 km	Chong et al., 2010
pH	BCS	NGS	Windmill Island	<100 km	Siciliano et al., 2014
pH	BCS	NGS	McMurdo Dry Valleys	<100 km	Van Horn et al., 2013
pH	BR and CS	TRFLP	Antarctic Peninsula	<10 km	Chong et al., 2012b
pH and EC	BCS	TRFLP	Scott Base	<1 km	O'Neill et al., 2013
pH and EC	BCS	Cloning	Ross Sea region	<100 km	Aislabie et al., 2008
pH and EC	BCS	TRFLP	McMurdo Dry Valleys	<100 km	Geyer et al., 2013
pH and copper	BCS	DGGE, TRFLP and Cloning	Alexander Island	<10 km	Chong et al., 2012a
pH and moisture	BCS	ARISA	Victoria Land	>100 km	Smith et al., 2010
pH, nitrate, temperature	BCS	DGGE	Cross regional study	>100 km	Yergeau et al., 2007c
Altitude and EC	BCS	ARISA	McMurdo Dry Valleys	<100 km	Lee et al., 2012
Carbon content	BR	TRFLP	McMurdo Dry Valleys	<100 km	Geyer et al., 2013
Carbon, nitrogen, and EC	BR	ARISA	Darwin Mountain	<5 km	Magalhães et al., 2012
Carbon, nitrogen, and moisture	BCS	DGGE	South Shetland Archipelago	<5 km	Ganzert et al., 2011
Carbon, nitrogen, and moisture	Microbial abundance	CFU counts	Cross regional study	>100 km	Yergeau et al., 2007c
Carbon, nitrogen, and chloride	BR	NGS	Windmill Island, Eastern Antarctica	<100 km	Siciliano et al., 2014

[a]*EC, electrical conductivity;* [b]*BCS, bacterial community structure; BR, bacterial richness.*

vegetation cover far outweighed any influence of geographical isolation.

If a combination of soil edaphic parameters and nutrient availability is the main driving force for prokaryotic community assembly in harsh Antarctic environments, it is perhaps justifiable to postulate that taxonomic diversity in Antarctica should be lower in comparison to those of temperate and tropical regions. Additionally, the Antarctic bacterial community might resemble those of other cold desert habitats such as parts of the Arctic and high altitude montane regions. Detailed molecular microbial assessments of Antarctic terrestrial ecosystems have, in contrast, demonstrated that Antarctic soil environments, including those from true frigid desert soils, harbor broad lineages with flexible functions that are comparable to other ecosystems globally (Cowan et al., 2002, 2014; Cary et al., 2010). In comparison, strong regional variation in Cyanobacteria and Archaea distribution was observed when comparing the distributions of these taxa across different desert habitats (Bahl et al., 2011; Bates et al., 2011). Separately, examination of the global distribution of cold-adapted genera including *Polaromonas, Psychrobacter,* and *Exiguobacterium* suggested that the Antarctic species formed distinct mono- and/or paraphyletic clusters specific to Antarctica when compared with close representatives from other regions (Rodrigues et al., 2009; Darcy et al., 2011).

At a regional scale, geographical isolation clearly contributes to Antarctic microbial community diversification (Papke and Ward, 2004; Bahl et al., 2011). Indeed, simply by using the pragmatic and non-scientifically established geographical sectors of Antarctica outlined by Pugh and Convey (2008), Chong et al. (2012b) showed significant genetic separation in 16S rRNA gene sequences between soil bacterial communities obtained from the different sectors, a separation that was particularly apparent in *Flavobacterium* and *Arthrobacter* (**Figure 1**) although, again, such conclusions may be influenced by the application of inconsistent methodologies. However, the pattern found was also consistent with the Gressitt Line of Chown and Convey (2007), potentially suggesting the presence of a "universal" spatial constraint for both Antarctic higher and lower organisms.

Overall, we suggest that the spatial organization of Antarctic prokaryotic communities is highly dependent on the spatial scale studied. At small to moderate spatial scales (100 m–1000 km), community assembly is highly sensitive to the heterogeneity in local physicochemical parameters. At regional scale (>1000 km), however, the disparity in membership may reflect stronger influence of historical contingency (sympatric speciation) and dispersal limitations than geomorphological variation *per se*.

Issues and Limitations of Antarctic Prokaryotic Biogeography

Various limitations currently hamper the interpretation of spatial patterns in Antarctic prokaryotic communities. We highlight some of the major hurdles faced here.

Species Conundrum

Clear definition of species or taxonomic unit is a major prerequisite of efforts to characterize spatial patterns of distribution. As prokaryotic microorganisms, along with many algae and fungi, are generally cryptic (morphologically indistinguishable) and metabolically flexible, the distinction between different "species" is commonly based on variation in a phylogenetic marker (e.g., the 16S rRNA gene). The use of the phylogenetic markers has several advantages (e.g., they are evolutionarily conserved in all prokaryotes, lateral transfers of the genes are rarely reported, large databases are available, and

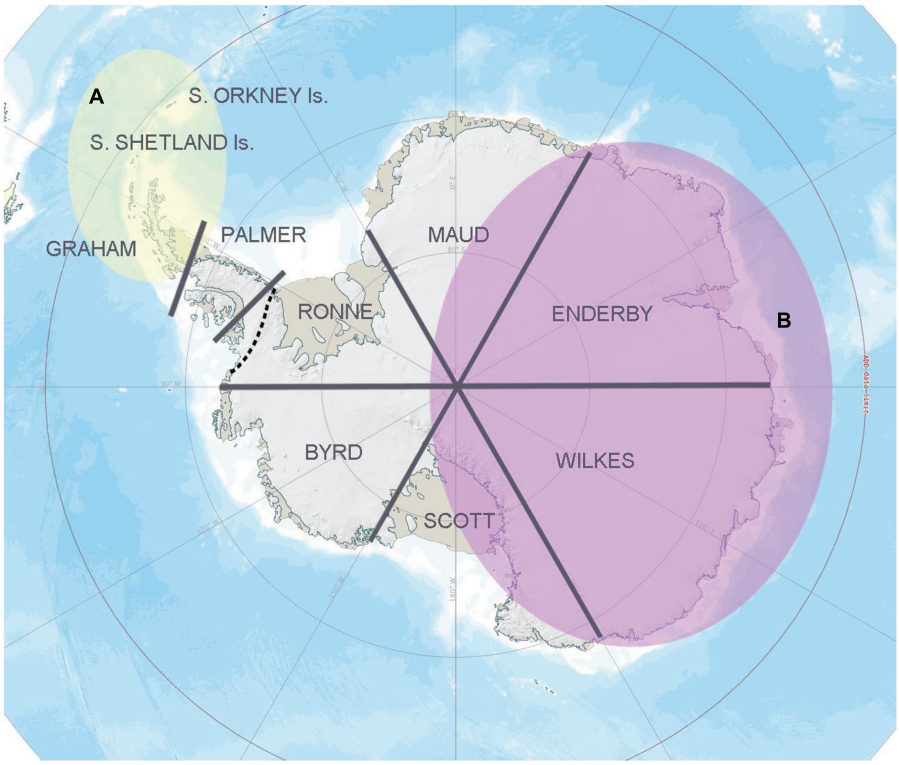

FIGURE 1 | Regional bacterial biogeography pattern based on the 16S rRNA gene data information. A strong genetic separation was detected in the overall soil bacterial community composition and Bacteroidetes assemblages retrieved between zone A (yellow) and zone B (purple; Chong et al., 2012b), representing different sides of the "Gressitt Line" (dotted line). A similar pattern was observed in bacterial isolates affiliated with the genera *Flavobacterium* and *Arthrobacter* (Chong et al., 2013).

the need for pure isolates is removed; Hugenholtz, 2002; Cole et al., 2009; Fierer and Lennon, 2011), although the inference of ecological role using phylogenetic markers alone is not always straightforward. For instance, variability between bacterial genotype and phenotype is well-documented (Fuhrman, 2009; Priest et al., 2012). Indeed, the level of variability in phylogenetic markers is itself variable across taxa (De Wever et al., 2009; Fraser et al., 2009), raising the often ignored problem that there is no clear or universally accepted level of variation required for the definition of a distinct species (Green and Bohannan, 2006), either within a particular lineage or across groups more generally. In Antarctic bacterial studies to date, a range of 97–99% cut-off points in sequence homology in the 16S rRNA gene has been applied (Aislabie et al., 2008; Newsham et al., 2010; Pearce et al., 2010; Peeters et al., 2011a). One alternative approach to overcome this problem is to define the phylogenetic relationship using the "metagenomics binning" strategy of Sharon et al. (2013). However, the assembly of short metagenomic fragments can itself be erroneous as it is sensitive to the occurrence of dispersed repeats. This is further exacerbated by the presence of closely related but heterogeneous genomes common in natural microbial populations. Nevertheless, these issues are being addressed through improvement in sequencing platforms and chemistry (e.g., Illumina TruSeq, Pacific Biosciences sequencing) that permit the generation of long and structurally explicit reads (Quail et al., 2012; Sharon et al., 2015).

Technical Limitations

Over the past century, considerable progress has been made in the understanding of prokaryotic diversity in Antarctica. In the early 1900s the isolation of microorganisms quickly disproved the general perception that Antarctica is "sterile and devoid of life," and it was already observed that the isolates were phenotypically similar to those from tropical and temperate regions. In the 1990s, by comparing Antarctic isolates with their closest relatives from elsewhere, a few studies started to suggest that the former were genetically distinct (Franzmann and Dobson, 1993; Franzmann, 1996). However, the true spectrum of prokaryotic life in Antarctica still lay beyond the reach of scientific study owing to the lack of isolates and ability to develop cultures.

This started to change when molecular microbiological profiling and cloning techniques came into play (Nocker et al., 2007). Antarctic soil profiling is now typically revealing a high diversity of microbial life, including in less studied habitats such as hypolithic and endolithic environments (Pointing et al., 2009; Cowan et al., 2010). Relatively recently, the advent of massively parallel NGS is further improving our knowledge of the functionality and diversity of Antarctic prokaryotic communities

(Bates et al., 2011; Pearce et al., 2012; Tytgat et al., 2014; Kim et al., 2015). It is important to highlight that interpretation of NGS data is highly dependent on the quality of sequence assembly, OTU assignment and annotation. As suggested earlier, the key issue is to produce high quality long reads for downstream bioinformatics analysis.

The wealth of new data has improved the interpretation of ecological dynamics and diversity in Antarctic ecosystems (Cary et al., 2010; Cowan et al., 2014). It is, however, important to realize that diversity patterns have commonly been inferred by comparing preceding reports from similar habitats, or the collation of a series of local data for regional interpretation. Such approaches have usually involved studies with inconsistent methodologies and hence need to be handled with care.

Our understanding of the distribution of the rare members (contributing <0.05% overall diversity – for the purposes of this paper we consider 0.05% as 'rare,' although there appears to be no accepted definition in microbiological studies) of the Antarctic biosphere remains particularly weak. Although high-throughput NGS approaches provide a better option for capturing these rare community members than clone library and profiling methods, their short-read length is only suitable for informing on the presence of rare species and provides little information about their ecological role and functions (Sharon et al., 2015; Youssef et al., 2015).

Further, it is known that DNA/RNA extraction techniques may be selective toward purifying the genetic signature of taxa with weak cell walls (Hirsch et al., 2010). It is also unclear how representative the extracted DNA/RNA is, as the mechanism of interaction between soil, DNA and RNA is poorly understood (Lombard et al., 2011). For example, legacy DNA and RNA may contribute a substantial fraction of the detected gene signature in Antarctic soil due to enhanced preservation under the cold and arid environmental conditions (Chong et al., 2013; Cowan et al., 2014). The requirement for application of PCR, especially in cloning, DNA profiling and targeted metagenomics approaches, also introduces potential bias into the downstream interpretation, as sequences with high affinity to the primer sequences may be preferentially amplified in this process (Taberlet et al., 2012).

It is intuitively obvious that the application of one approach will not be sufficient to provide a complete picture of the prokaryotic community in Antarctica (or elsewhere). As the available technology advances, detailed systems biology approaches linking the diversity, RNA transcript (metatranscriptomics), metabolite (metabolomics), and protein (metaproteomics) signatures will be required to examine the contribution of richness and diversity to the ecological services provided by Antarctic prokaryote communities (Zengler and Palsson, 2012).

Lack of Spatial Coverage

Microbiological studies in Antarctica have taken place since the earliest expeditions exploring the continent (Ekelöf, 1908). Until recent decades, studies have been culture-based and focused on describing the novelty of isolated strains, and to relating apparent diversity to local environmental features (e.g., Holdgate, 1977; Franzmann and Dobson, 1993). Historically such studies, which generally do not require elaborate systematic spatial sampling methodologies, have often been opportunistic in nature, depending on the presence of particular researchers with appropriate specialist skills at any given location and season (Chown and Convey, 2007). Consequently, historical microbiological work has been heavily spatially biased to areas accessible from particular research stations and, in particular, to a few relatively well-sampled regions in the Scotia Arc, west Antarctic Peninsula, McMurdo Dry Valleys and the coastal region of Wilkes Land (Smith et al., 2006; Aislabie et al., 2008; Chong et al., 2012b, 2013; Dennis et al., 2013).

The global ubiquity theory postulates that the dispersal potential of microbes (including prokaryotes) is less confined by geographical barriers than is the case for larger organisms (Baas Becking, 1934; Finlay, 2002). While the universal applicability of this theory is increasingly questioned (Martiny et al., 2006; Woodcock et al., 2007), studies such as De Wever et al. (2009) and Bahl et al. (2011) do appear to suggest strongly that the Antarctic microbiota is more distinct than that of the other continents globally, supporting the effectiveness of the barriers isolating the Antarctic continent.

There is a general consensus that the influence of abiotic factors in population selection is expected to be amplified under harsh Antarctic conditions (Barrett et al., 2006a; Hogg et al., 2006). Perhaps as a result, most microbial biogeographical studies to date in Antarctica have given strong emphasis to the role of local environmental drivers in defining community composition and structure (Barrett et al., 2006b; Chong et al., 2010; Ganzert et al., 2011; Magalhães et al., 2012), and few have considered the spatial patterns, controls and functions that might be apparent at a larger sampling scale in Antarctica.

While lack of spatial coverage is a limitation that has been identified as being common to all other major Antarctic taxonomic groups (Chown and Convey, 2007, 2012; Peat et al., 2007; Convey et al., 2012; Terauds et al., 2012), the limitation is more severe in the prokaryotes than in eukaryotic groups. Placed this in context, at present bacteria and archaea together contribute less than 6% of the total records available in the ABD[2] (accessed 9 August, 2015). However, spatial issues are now gaining increasing attention, and have formed an integral part of recent scientific initiatives of several national operators such as the United Kingdom (Ecosystems Programme[3]), Australia (Terrestrial and Nearshore Ecosystem programme[4]) and New Zealand (New Zealand Terrestrial Antarctic Biocomplexity Survey[5]). The need for increasingly close cooperation in the form data of sharing, sampling coordination and field support has been identified clearly in the recent Scientific Committee on Antarctic Research 'Antarctic and Southern Ocean Horizon Scan' (Kennicutt et al., 2014a,b). With the ever-increasing data becoming available, more light will be shed on the effect of spatial scaling on Antarctic biotas.

[2]https://www1.data.antarctica.gov.au/aadc/biodiversity/taxon_drilldown.cfm

[3]http://www.antarctica.ac.uk/bas_research/our_research/current/programmes/ecosystems/

[4]http://www.antarctica.gov.au/science/terrestrial-and-nearshore-ecosystems-environmental-change-and-conservation

[5]http://www.ictar.aq/nztabs.cfm

Ecological Functions and Biogeography of Antarctic Bacterial Communities

There is general agreement on there being a positive correlation between species diversity and functional richness: the greater the number of species, the greater the functional richness of a community, or alternatively, fewer species being present leads to a lack of functional redundancy (Peterson et al., 1998). In a highly diverse ecosystem, the likelihood of overlapping ecological function between species increases, creating communities that may be functionally similar despite involving different combinations and proportions of individual species.

Due largely to the absence of the major soil eukaryotic groups and the lack of biotic interactions (Hogg et al., 2006), functional redundancy is often assumed and predicted to be low in Antarctic soil (Convey, 2007a). If so, then each species in a given Antarctic community might be responsible for the provision of a distinct and irreplaceable ecological function. This idea is in congruent with the observation of low nematode species count and low cross-biome functional diversity in Antarctic Dry Valley soils (Wall and Virginia, 1999; Fierer et al., 2012). As ecological resilience is built upon the functional diversity of the ecosystem, habitats hosting extremely low biodiversity, as has been suggested for some inland dry valley ecosystems in Antarctica (Wall and Virginia, 1999; Hodgson et al., 2010; Fernandez-Carazo et al., 2011; Peeters et al., 2011b), might be particularly vulnerable to environmental disturbance (Tiao et al., 2012). Combining the concepts of low biodiversity and limited function, the detection of regional bacterial biogeography within Antarctica may also imply the presence of regional-specific variation in functional capability in the continent's soils.

Yergeau et al. (2007a, 2009) provided evidence of a close relationship between phylogenetic diversity and functional gene distribution in Antarctic soil. Using a combination of Geochip microarray and real-time PCR approaches, they suggested that a significant proportion of the variation in functional diversity observed along a latitudinal transect in fellfield soils between the Falkland Islands (51°S), Signy Island (60°S), and Anchorage Island (67°S) could be explained by geographical location, with the three locations harboring phylogenetically distinct soil bacterial communities (Yergeau et al., 2007b).

Chan et al. (2013) assessed the functional diversity of the McKelvey Valley in the McMurdo Dry Valleys, using a much updated Geochip microarray. They established, in contrast to the previous study, that Antarctic hypoliths, chasmoendoliths and bare soil hosted significantly different functional diversity, with the former including a greater range of stress-response related genes, and the latter including specific genes affiliated with hydrocarbon transformation and lignin-like degradation pathways. However, little functional variation was detected between the five bare soil samples examined, despite the samples having previously been shown to support heterogeneous phylogenetic diversity (Pointing et al., 2009). Similarly, Yergeau et al. (2012) showed that the majority of members of the Antarctic Peninsula soil community were functionally similar (functional generalists) despite apparent differences in microbial diversity particularly between vegetated and non-vegetated sites (Yergeau et al., 2007b), potentially indicating some level of redundancy in the Antarctic soil system. The number of functional genes detected in these soils was also surprisingly high in absolute terms, with some sites in the McMurdo Dry Valleys harboring functional richness comparable to temperate and tropical forests (Fierer et al., 2012).

Several recent studies applying newly available molecular approaches have drawn conclusions relating to microbial diversity that are contrary to the common belief that reduced biodiversity in Antarctica equates to a functionally challenged ecosystem (Cowan et al., 2002; Pearce et al., 2012; Stomeo et al., 2012). This highlights the need to develop studies examining microbial interactions, such as communication (e.g., quorum sensing and quenching) and competition in these systems. For instance, *Clostridium* and *Flavobacterium*, which usually dominate nutrient-rich habitats such as penguin rookeries (Aislabie et al., 2009), penguin guano (Zdanowski et al., 2005) and the rhizosphere (Teixeira et al., 2010) were also part of the core phyla detected in extremely arid mineral soils (Tiao et al., 2012). These lineages may play a pivotal role in nutrient release in the event of chance deposition of nutrients (e.g., in the form bird perches or seal carcasses) in the Dry Valleys (Cary et al., 2010; Tiao et al., 2012). In parallel, Hughes and Lawley (2003) detected the fungal genus *Verticillium*, rarely found in saline habitats, in gypsum encrusting rocks on Alexander Island in the maritime Antarctic. One explanation for the detection of such "unusual" taxa might be that the low competition in these less diverse environments facilitated greater success of "chance colonization" for rare species, allowing them to develop greater flexibility and occupy niches that would typically be occupied by other specialists in more diverse systems (Chase and Myers, 2011). In addition, Székely et al. (2013) suggested that species sorting is more prominent in competitive environments.

A meta-analysis of studies examining diversity–function relationships (Nielsen et al., 2011) concluded that species diversity and functional properties in soil systems did not have a simple linear relationship, rather often showing idiosyncratic patterns. They further concluded that species traits were more important in controlling functionality in the ecosystem than richness *per se*. This would suggest both that loss of an individual species may not always translate into a detrimental effect on ecological function, and that the absence of a species with an important trait will be catastrophic to the maintenance of the ecosystem. This is consistent with the argument of Konopka et al. (2014) that, while microbial community composition is in constant flux, functionality can remain steady as long as the function is maintained by populations within the community.

Developing this concept further, and integrating the increasing reports of bacterial regionalization within the Antarctic (Yergeau et al., 2007b; Chong et al., 2013; Sokol et al., 2013), we propose here a new conceptual model to explain the mechanism underlying species-function relationships in Antarctica. The Antarctic soil ecosystem is supported by a highly diverse but region-specific bacterial community. For instance, nutrient-rich (e.g., penguin rookeries) and nutrient-poor (e.g., barren soil) environments from different Antarctic regions contain both copiotrophs (high nutrient requirement, e.g.,

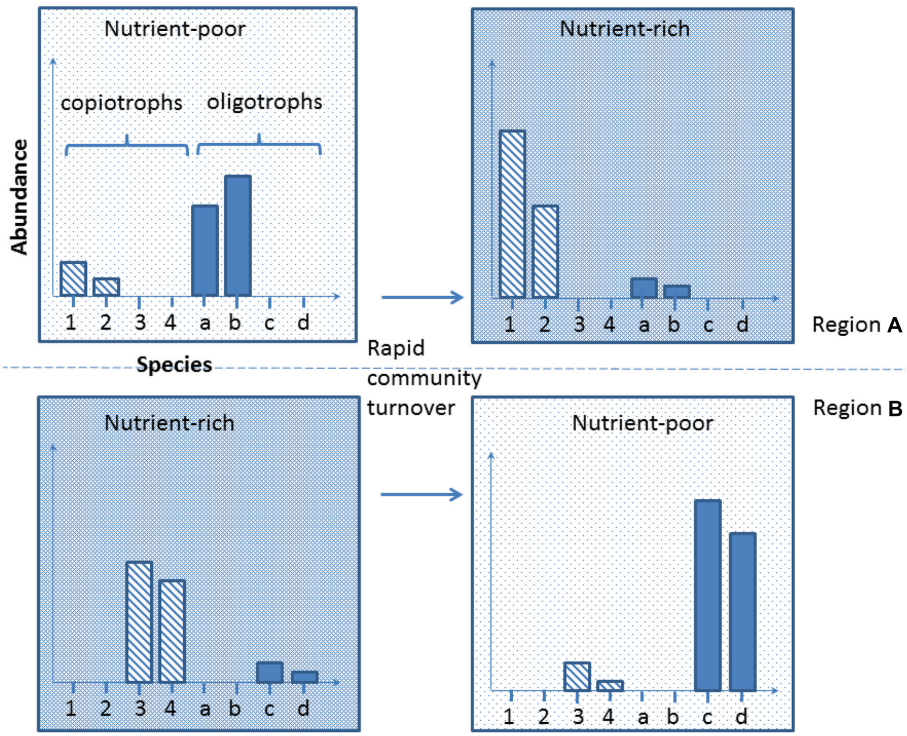

FIGURE 2 | A representation of Antarctic bacterial community dynamics in response to external environmental perturbation. We believe that the Antarctic soil system harbors diverse functional traits that are preferentially selected based on suitability for the contemporary environmental conditions. Major environmental alteration may result in currently rare species being selected for and a major community compositional shift occurring. Note that habitats from different Antarctic regions may harbor different species with similar traits (upper vs. lower row).

Flavobacterium spp.) and oligotrophs (low nutrient requirement, e.g., *Acidobacterium* spp.; Fierer et al., 2007; Aislabie et al., 2008; Chong et al., 2010; Bottos et al., 2014a). Soil samples obtained across different regions exhibit distinct community memberships with reference to these groups, but the phylogenetic similarity of their members is greater within the same biogeographic region than it is between regions (**Figure 2**, comparing upper and lower panels). In any particular system, the biomass of the copiotrophs and oligotrophs is dependent on the ecological characteristics of the habitat present. Nutrient-poor habitats host a greater percentage of oligotrophs such as Acidobacteria that convert recalcitrant carbon such as xylan (from autotrophs) and pectin (from wind-blown plant materials) into labile carbon (Bokhorst et al., 2007; Ward et al., 2009), while copiotrophs such as some Bacteroidetes dominate nutrient-rich sites, degrading the available high molecular weight organic carbon (Zdanowski et al., 2005; Aislabie et al., 2008; Chong et al., 2010). Changes in local environmental conditions, such as deposition of nutrients through aeolian transfer, or loss through leaching, can trigger rapid community turnover to match the new functional requirement (Saul et al., 2005; Barrett et al., 2006a; Tiao et al., 2012; Dennis et al., 2013; **Figure 2**). If such community compositional shifts involve specialists (rare species with unique traits) being lost or reduced below a critical biomass level, this may become a limiting factor in responding to subsequent changes (**Figure 3**).

We acknowledge that this hypothesis could be difficult to test under normal field conditions due to the technical limitations applying to currently available molecular microbiology approaches, such as detection limits (for rare biosphere <0.05%) for both diversity and function and problems in discriminating the functions of individuals from various populations of the same community. Nevertheless, it was evident from a field study by Tiao et al. (2012) that rapid compositional shift in in response to nutrient enrichment by a seal carcass was detectable in the McMurdo Dry Valleys. One practicable approach to testing this would be to conduct detailed functional quantification in a series of microcosm experiments (cf. Newsham and Garstecki, 2007), analyzing the outcomes using long metagenomic reads (Sharon et al., 2015). Each microcosm would encompass different combinations of phylogenetically distinct microbial isolates with known function in order to represent a diversity gradient. Ecological thresholds could then be determined by comparing the minimum biomass of any given specialist required before a drop ('step change') in any ecological function is detected when growth conditions are altered.

Conclusion

Over the last decade, rapid advances in molecular methodologies and progressive improvement in sampling strategies have started

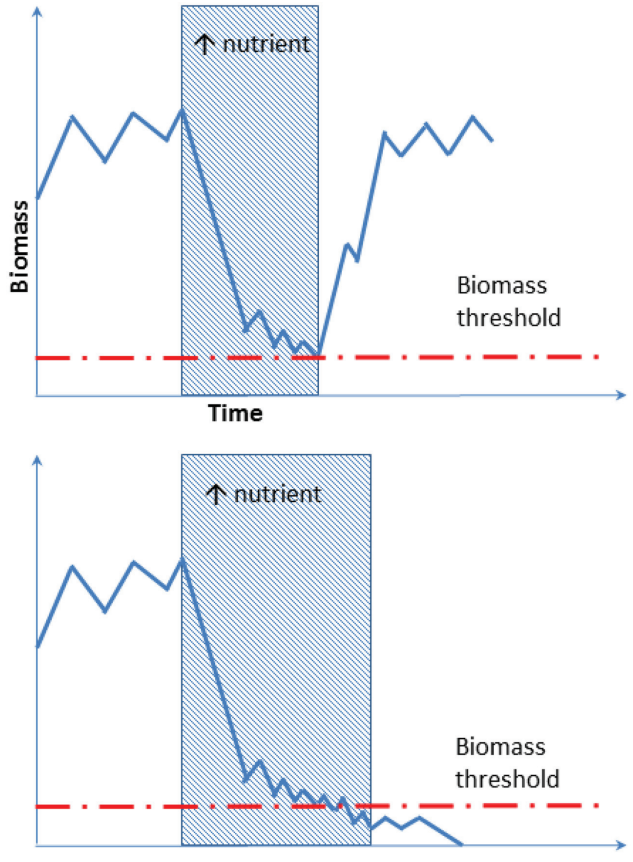

FIGURE 3 | Schematic illustration of an oligotroph's response to alteration in local nutrient content. The oligotroph is suppressed periodically when large amounts of nutrients are available. Biomass then returns to the original level when the nutrients become depleted by the copiotroph, promoted by environmental change. In the event of prolonged nutrient alteration oligotrophs may drop below the biomass threshold (lower graph), and it will not recover even if nutrient levels returns to the original state.

to realize some of the vast potential of Antarctic microbiology. Despite continuing restrictions in spatial coverage, Antarctic microbiologists are now increasingly confident that Antarctic soil ecosystems harbor a rich bacterial community performing versatile ecological functions (Cowan et al., 2002; Pearce et al., 2012; Chan et al., 2013). Based on recent molecular studies, it is clear that the functional capability of Antarctic soil communities is not simply linearly related to species richness, and considerable functional overlap has been observed between species (Yergeau et al., 2012; Chan et al., 2013). This is an important paradigm shift from the long-held view of simple ecosystems with low functional redundancy typifying Antarctica (Wall and Virginia, 1999).

Recent studies also demonstrate that the Antarctic soil microbial ecosystem is flexible and capable of rapid community adjustment in response to external environmental fluctuation (Tiao et al., 2012; Dennis et al., 2013). Such functional resilience may be a result of phenotypic plasticity of Antarctic biota and millions of years of adaptive selection. Nevertheless, we propose that community organizational shifts in response to perturbation are limited by the threshold biomass of the often rarer species that provide important functions required under contemporary environmental conditions (**Figure 3**). This, however, does not mean that the generalists forming the dominant biosphere are unresponsive to the environmental changes. For instance, rapid ecological drift was found to affect both prevalent and rare phyla in a multi-year mummified seal transplantation experiment conducted in the McMurdo Dry Valleys (Tiao et al., 2012).

Building on the observation of highly specific and localized patterns of bacterial biodiversity in community membership, and the presence of bacterial zonation or regionalization within Antarctica we suggest that, under comparable environmental conditions, the "limiting species" for ecological function will not be the same across different Antarctic regions.

Our model has important implications both to the direction of future research and to biosecurity management of Antarctic microbial ecosystems. First, it is important to understand how cross-trophic interactions are maintained under relevant spatial scales for both the prokaryotic and eukaryotic elements of the Antarctic terrestrial ecosystem. For instance, we now understand that, at a superficial scale, the Gressitt Line boundary may be applicable to both Antarctic macro- and microbiotas, but it is not clear whether parallel ecosystems across this boundary display similar or different trophic networks.

Second, acknowledging that each biogeographical region comprises phylogenetically distinct communities, it is imperative to identify the different key limiting species that determine functional resilience at different scales of spatial organization. However, given that functionally limiting species are often minority community elements, it can be challenging to detect their presence. As a further complication, the molecular signature of target species can potentially be masked by legacy DNA or RNA preserved under cold and arid Antarctic conditions. There is also currently a lack of knowledge of biomass or abundance thresholds required to sustain "specialist" populations. In order to generate greater understanding, there is a pressing need to extend the spatial coverage of microbial research across Antarctica, and the temporal sampling of field manipulation studies similar to those performed by Yergeau et al. (2012) and Dennis et al. (2013). Additionally, research should also focus on the evaluation of varying responses of communities in each distinct Antarctic biogeographic region to environmental variability and change, the introduction of non-native microbiota, and other anthropogenic impacts (Tin et al., 2009; Cowan et al., 2011; Chown et al., 2012). In conclusion, currently available evidence generally supports the proposition that Antarctic prokaryotes display large-scale regional biogeography similar to the patterns detected in eukaryotic groups. This allows a pragmatic comparison of the prokaryote and eukaryote spatial scaling and spatial patterns. Current functional assessments also point to the likelihood of functional redundancy existing in Antarctic prokaryotic communities.

Nevertheless, it is clear that several key pieces of the puzzle are still missing, including the lack of spatially explicit information, and data on the genetics and functions of the rarer members of the Antarctic microbial communities. These gaps can be addressed in part through developing coordinated fundamental microbiology surveys across Antarctica, and complementary functional assessments through mesocosm studies.

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Carbon-Fixation Rates and Associated Microbial Communities Residing in Arid and Ephemerally Wet Antarctic Dry Valley Soils

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Carbon-fixation is a critical process in severely oligotrophic Antarctic Dry Valley (DV) soils and may represent the major source of carbon in these arid environments. However, rates of C-fixation in DVs are currently unknown and the microorganisms responsible for these activities unidentified. In this study, C-fixation rates measured in the bulk arid soils (<5% moisture) ranged from below detection limits to ~12 nmol C/cc/h. Rates in ephemerally wet soils ranged from ~20 to 750 nmol C/cc/h, equating to turnover rates of ~7–140 days, with lower rates in stream-associated soils as compared to lake-associated soils. Sequencing of the large subunit of RuBisCO (*cbbL)* in these soils identified green-type sequences dominated by the 1B cyanobacterial phylotype in both arid and wet soils including the RNA fraction of the wet soil. Red-type *cbbL* genes were dominated by 1C actinobacterial phylotypes in arid soils, with wetted soils containing nearly equal proportions of 1C (actinobacterial and proteobacterial signatures) and 1D (algal) phylotypes. Complementary 16S rRNA and 18S rRNA gene sequencing also revealed distinct differences in community structure between biotopes. This study is the first of its kind to examine C-fixation rates in DV soils and the microorganisms potentially responsible for these activities.

Keywords: CO_2 fixation, Antarctic soils, primary production, Dry Valleys, microbial communities

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INTRODUCTION

The McMurdo Dry Valleys (DV) of Antarctica represents one of the coldest, driest and most oligotrophic desert systems on Earth (Cary et al., 2010). Due to the lack of higher trophic levels, microorganisms dominate the arid DV soils (Cary et al., 2010), and as a result, community dynamics and ecological function are independent of other biological processes and are most likely directly coupled to the chemical and physical environment. This ecosystem therefore provides an extraordinary opportunity to examine metabolic adaptations that allow communities to function in extreme environments.

With the recent discovery of high microbial cell concentrations (5×10^5 to 4×10^8 g wet weight^{-1}) and microbial diversity comparable to that of temperate soils(Cowan et al., 2002; Smith et al., 2006; Niederberger et al., 2008; Cary et al., 2010), there is great interest in resolving the sources, controls and turnover of carbon in these severely oligotrophic DV soils

(Burkins et al., 2000, 2001; Barrett et al., 2005, 2006b, 2007; Elberling et al., 2006; Hopkins et al., 2006b, 2009; Cary et al., 2010; Feng et al., 2010). Soil organic carbon (SOC) concentrations in the bulk arid DV soils do not typically exceed 1.0 mg g^{-1}, with concentrations being at least an order of magnitude higher in soils associated with ephemerally wetted lake and stream systems (Parsons et al., 2004; Barrett et al., 2006a; Elberling et al., 2006; Hopkins et al., 2006b, 2009; Ball et al., 2009; Cary et al., 2010; Feng et al., 2010). Wetted soils form in the DV during the summer months when temperatures become warm enough to melt lake ice edges and the surfaces of glaciers resulting in the formation of moats around the edges of lakes/ponds and short-lived (4–12 weeks) melt-water streams. Collectively, these form important hydrological links between glaciers and lakes (McKnight et al., 1999, 2007; Takacs-Vesbach et al., 2010). The wetted soils associated with these systems are well-documented hotspots of biogeochemical cycling and can contain dense microbial mat communities that bind the top 1–2 cm of soil together (Runkel et al., 1998; McKnight et al., 1999, 2004, 2007; Maurice et al., 2002; Gooseff et al., 2003). Mat communities in these ephemerally wet soils are typically cyanobacterial-, or moss-dominated and exhibit extremely patchy distribution (McKnight et al., 2004; Adams et al., 2006; Takacs-Vesbach et al., 2010). These communities survive the winter months in a desiccated state and, in some cases, are re-activated through hydration by summer melt-waters and over multiple wetting events can form large concentrations of responsive biomass (Vincent and Howard-Williams, 1986; McKnight et al., 1999).

In contrast to the high productivity wetted soils, SOC in bulk arid DV soils is hypothesized to originate from three major sources; (1) legacy deposits of ancient lake sediments, (2) allochthonous inputs from high productivity sites via wind transportation and (3) *in situ* CO_2-fixation. As supported by laboratory-based studies, SOC turnover in DV soils has proven to be surprisingly rapid given the conditions, in the range of decades to ∼150 years (Burkins et al., 2001; Barrett et al., 2006b; Elberling et al., 2006; Hopkins et al., 2009; Tiao et al., 2012). It therefore seems unlikely that legacy deposits have survived to the present day, and if so, may only represent a minor or recalcitrant fraction of current SOC pools with more contemporary sources of C sustaining C-cycling in DV soils (Hopkins et al., 2006b; Feng et al., 2010).

Aeolian transport of mat detritus from high productivity sites has also been hypothesized to be an important source of organic C to bulk arid DV soils (Elberling et al., 2006) and a proven facilitator of soil respiration (Hopkins et al., 2006a). However, aeolian transport has been estimated to be an insignificant method of carbon delivery (0.01–7 g C m^{-2} $year^{-1}$) and may only be relevant to regions in close proximity to lake systems (Lancaster, 2002; Barrett et al., 2006b). Independent studies have also shown that the natural stable isotopic (^{13}C and ^{15}N) abundances of soil organic matter (SOM) differ between high and low productivity sites, with SOC isotopic signatures from remote locations (i.e., at large distance from wet sources and at higher elevation) resembling endolithic sources, and sites closer to high productivity sites and at lower elevation resembling lacustrine signatures (Burkins et al., 2000, 2001; Hopkins et al., 2009). Most recently, through the use of both gas chromatography/mass spectrometry and nuclear magnetic resonance spectroscopy, Feng et al. (2010) have shown that certain SOM compounds differ between bulk arid soils and microbial mats associated with a nearby lake, suggesting either a fast turnover of lake derived material in nearby arid soils or insignificant aeolian distribution (Feng et al., 2010). *In situ* CO_2-fixation (primary productivity) has therefore been hypothesized to be the most consistent source of C, appearing to make the largest contribution to SOC and replenishing C stocks in DV soils (Burkins et al., 2000, 2001; Hopkins et al., 2009). However, C-fixation rates and the microorganisms responsible for these activities in DV soils remains largely unknown with a recent DNA-based study (Chan et al., 2013) of DV biotopes indicating that autotrophic functionalities are present in the endemic biota encompassing members of the Cyanobacteria, Archaea, Actinobacteria, and Proteobacteria.

The most common CO_2-fixation pathway for chemo- and phototrophs is the reductive pentose phosphate/Calvin–Benson–Bassham (CBB) cycle. Ribulose-bisphosphate carboxylase (RuBisCO; EC 4.1.1.39) is a key enzyme responsible for the fixation of CO_2 in this pathway (Tourova and Spiridonova, 2009) and exists as two major forms, I and II, that share ∼25–30% amino acid similarity. Form I RuBisCO has eight large subunits (encoded by the *cbbL* gene) and eight small subunits (encoded by the *cbbS* gene). Based on phylogenetic analyses the large subunit can be further divided into two independent 'green' and 'red' types as defined by amino acid sequence identities. The green-type has two variants: IA occurring in several proteobacteria and IB occurring in plants, green algae, and cyanobacteria. The red-type also has two variants: IC, specific to α- and β-proteobacteria and ID to non-green algae (Alfreider et al., 2009; Tourova and Spiridonova, 2009). Form II RuBisCO consists of two large subunits encoded by the *cbbM* gene (Shively et al., 1998). The *cbbL* and *cbbM* genes are routinely used as molecular markers for the identification of autotrophs in natural microbial communities (Watson and Tabita, 1997; Giri et al., 2004; van der Wielen, 2006; Alfreider et al., 2009; Videmšek et al., 2009), due to sequence conservation, its essential function in C-fixation and the large number of RuBisCO gene sequences in public databases. However, whilst RuBisCO sequences do provide valuable phylogenetic insight into the identity of C-fixers, RuBisCO taxonomic groupings do not always concur with 16S rRNA gene phylogeny, most likely the result of horizontal gene transfer and gene loss or duplication events (Spiridonova et al., 2004; Tourova and Spiridonova, 2009).

C-fixation is hypothesized to be a highly important process in DV soils, perhaps representing the major source of C in the bulk arid soils. However, rates of C-fixation in both arid and high productivity soils of the DV are unknown, and the microorganisms responsible for these activities remain unidentified. The objective of this study was therefore to: (1) estimate rates of C-fixation, as measured by $^{13}CO_2$ uptake in contrasting wet and dry DV soil habitats and (2) identify microbial community structure and identify C-fixers via complementation of 16S rRNA and 18S rRNA gene sequencing with RuBisCO gene sequencing. Results from this study revealed

distinct differences in community structure between both arid and wetted DV soils biotopes. Moreover, rates of C-fixation detected in both biotopes with low levels of SOC were higher than expected. Therefore, these collective results lend further credence to the hypothesized turnover rates of C in DV soils.

MATERIALS AND METHODS

Site Description and Sample Collection

Sites and sampling methods are as described previously (Niederberger et al., 2012). In short, a transect-based sampling approach was utilized consisting of three or four sampling points originating from site 1 defined as a "wet" zone (soils with overlying stream, lake or pond water) extending through a hyporheic zone containing obvious microbial mats to the final site (i.e., site 3 or 4) situated in a typical arid DV mineral soil. Gravimetric water content of soil samples were measured as described previously (Niederberger et al., 2012).

Measurement of CO_2-fixation Rates

CO_2 fixation was measured using a stable isotope enrichment method modeled after Finzi-Hart et al. (2009). Mats were sampled with a cut-off 5 ml plastic syringe and four 1 cm deep cores placed in a 27 ml glass serum bottle. Serum bottles were completely filled with water from the sampling site and gas bubbles removed before sealing with a rubber septa and crimp seal. A total of 10 μl of 0.233 M $NaH^{13}CO_3$ was then added and samples incubated for 24 h at *in situ* conditions. After incubation, bottles were opened and the contents poured into 50 ml centrifuge tubes. Samples were rinsed three times by washing with stream water, centrifuging at low speed and pouring off the supernatant, and then dried at 60°C. Samples were then homogenized in a mortar and pestle, weighed into aluminum foil cups and folded into small pellets using forceps. N and C content and stable isotope mass ratio ($d^{15}N$, and $d^{13}C$) were determined at the UC Davis Stable Isotope Facility Davis (Davis, CA 95616, USA). Values were corrected using internal standards and CO_2 fixation rates calculated as detailed previously (Montoya et al., 1996).

Nucleic Acid Isolation, cDNA Synthesis from mRNA, and Polymerase Chain Reaction (PCR)

Nucleic acid (DNA and RNA) isolation, cDNA synthesis and confirmation of DNA removal in RNA extracts and DNase treated extracts undertaken as described previously (Niederberger et al., 2012). RuBisCO form I *cbbL* green- and red-type genes were PCR amplified utilizing respective primer pairs RubIgF:RubIgR and RubIrF:RubIrR corresponding to positions 571–1382 and 196–1016 of the *cbbL* gene as outlined by Spiridonova et al. (2004). Commonly utilized primer pair, cbbL595F:cbbL1387R (Elsaied and Naganuma, 2001; Giri et al., 2004), was also tested to amplify the form I *cbbL* green-type gene; however, non-specific banding was observed within electrophoretic profiles (results not shown). The RuBisCO from II *cbbM* gene was PCR amplified utilizing a nested approach with primer pairs RuIIF1:RuIIR3 and RuIIF2:RuIIR2 as outlined by Spiridonova et al. (2004).

Gene Cloning and Restriction Fragment Length Polymorphism (RFLP) Analyses

RuBisCO PCR amplicons were excised from ethidium stained 2% agarose TAE gels and purified using the GenElute^{TM} gel extraction kit (SIGMA) and ligated (pCR4-TOPO vector; Invitrogen), transformed (One Shot TOP10 chemically competent *Escherichia coli*; Invitrogen) and clones selected as described previously (Niederberger et al., 2012). Inserts from ∼30 clones of each 96 clone library were sequenced and the remainder of the clones screened by RFLP using restriction enzymes (*Hae*III and *Rsa*I; New England BioLabs) as commonly used for partial length *cbbL* amplicons (Alfreider et al., 2009). Due to a high frequency of *Hae*III cut sites and the subsequent small DNA fragments (∼ <50 bp), visualization and comparison between samples within agarose gels was difficult (also confirmed through simulated *in silico* restriction endonuclease digestion of pre-sequenced clones). Therefore, RFLP was performed with *Rsa*I alone and representative clones of each RFLP type sequenced. *cbbL* amino-acid sequences are deposited as accession numbers KP836071 to KP836108 in the NCBI GenBank database *cbbL* gene sequences and 16S rRNA and 18S rRNA gene sequences are deposited in the Knowledge Network for Biocomplexity[1] under identifier ID: knb.756.1.

cbbL Gene Analyses

Sequences were aligned using ClustalW (Thompson et al., 1994) and a PHYLIP output file used to construct a Jukes-Cantor corrected distance matrix by the DNADIST program of PHYLIP[2] and operational taxonomic units (OTUs) defined and rarefaction analyses undertaken using the DOTUR program (Schloss and Handelsman, 2005). Representative *cbbL* gene sequences (90% sequence similarity) were aligned and translated within the Geneious software environment[3] and aligned to translated *cbbL* genes obtained from the NCBI GenBank database. The alignment was manually checked and a phylogenetic tree constructed using the Jukes-Cantor genetic distance model and the Neighbor-joining method with 1000 bootstrap re-samplings.

Amplicon Pyrosequencing, Processing, and Analyses

Tag-encoded FLX (Roche) amplicon pyrosequencing of the V1–V3 regions of the 16S and 18S rRNA gene was performed on DNA extracts by Research and Testing Laboratories (Lubbock, TX, USA[4]). Resulting data were then processed using the Quantitative Insights Into Microbial Ecology (QIIME) toolkit (Caporaso et al., 2010). In brief, rRNA gene sequences were

[1]https://knb.ecoinformatics.org
[2]www.phylip.com
[3]www.geneious.com
[4]www.researchandtesting.com

quality trimmed (QIIME defaults; >200 bp), split according to barcoded tags and sequences binned into operational taxonomic units (OTU) at 97 and 95% for bacteria and eukaryotes, respectively. Following quality trimming, a total of 7640 and 7163 partial length (>200 bp) 16S rRNA gene sequences were obtained for ML1–2 and ML1–4 respectively. Bacterial taxonomic assignment was undertaken on all quality trimmed 16S rRNA gene sequences using the online RDP classifier tool (at 80 confidence level) and associated RDP database (Cole et al., 2009). Eukaryotic taxonomic assignment was undertaken on a representative sequence from each OTU using the Basic Local Alignment Search Tool (BLAST) within the QIIME toolkit against the SILVA 18S rRNA gene database (Pruesse et al., 2007) as obtained from mothur (Schloss et al., 2009). Bacterial 16S rRNA gene rarefaction analyses and library comparisons (LIBCOMPARE) were performed using the tools within the online RDP pyrosequencing pipeline (Cole et al., 2009) and 18S rRNA gene rarefaction within mothur (Schloss et al., 2009).16S rRNA gene and 18S rRNA gene sequences are deposited within the Knowledge Network for Biocomplexity as stated above.

RESULTS

A total of four transects in the vicinity of Miers Valley were utilized as part of the study, including both lake- and stream-associated sites (Supplementary Table S1). Carbon-fixation rates were higher at lake-associated soils (2.15–751.29 nmol C/cc/h) as compared to stream systems (below detection limits to 15.83 nmol C/cc/h). Differences in C-fixation rates between dry and wetted soils was only apparent for lacustrine soils with rates for arid soils (<5% water content) ranging from undetected to 11.83 nmol C/cc/h as compared to wetted soils (12.52–751.29 nmol C/cc/h).

The ML1 transect on the northern shore of Miers Lake was chosen as a representative transect for the in-depth identification of C-fixers and microbial communities between contrasting wet (ML1–2, ∼23% moisture content) and dry soils (ML1–4, ∼2% moisture content). This transect was chosen due to the noticeable differences in C-fixation rates between wetted and arid soils, and because the N_2-fixing microorganisms and nitrogenase activities of the soils have previously been described (Niederberger et al., 2012).

Both form I green- and red-type *cbbL* genes were detected in total DNA extracts from wet (ML1–2) and dry (ML1–4) sites (detection of *cbbL* genes by PCR is summarized in Supplementary Table S1). Amplicons of the green-type *cbbL* gene were faintly detectable by electrophoresis from cDNA in ML1–2, with an additional 10 thermocycles providing sufficient amplicon concentrations for cloning purposes; however, the green-type *cbbL* gene was not detected in cDNA from the arid (ML1–4) site. The red-type *cbbL* gene was not detected in cDNA from either ML1–2 or ML1–4. The form II *cbbM* gene was also detectable in DNA extracts; but not in the corresponding cDNA preparations. Because the expressed *cbbM* gene was not detected in these samples, *cbbL* was utilized to identify the autotrophic microorganisms inhabiting these sites.

The total number of *cbbL* gene clones from each library ranged between 61 and 80 (Supplementary Table S1) with the exception of ML1–2 cDNA. This library contained only 44 sequences due to the presence of large number (49) of sequences closely related to 23S rRNA genes (results not shown), attributed to non-specific PCR amplification. Although low numbers of *cbbL* clones were sequenced, rarefaction (90%) analyses indicate that, for all samples, the green-type sequences were well-represented whilst red-type sequences were under-represented (Supplementary Information: Supplementary Figure S1).

For both the wet and dry samples, 100% of the green type *cbbL* phylotypes grouped within the 1B variant of cyanobacterial-related phylotypes (**Figure 1**), i.e., the green-type 1A variant was not detected. Diversity levels were similar for all sample types, 7, 6, and 7 OTUs for ML1–2, ML1–2 cDNA, and ML1–4, respectively and the majority of the sequences were most closely related to clones from the water column of Lake Bonney in the DV (**Figure 1**). Unexpectedly, the red-type clone libraries also contained 1B variant green-types (**Figure 2**). However, these 1B variant sequences were not added to the green-type phylogenetic tree as the red-type primers amplify a different region of the green-type *cbbL* gene than targeted by the green-type primers. In contrast to the green-type library, the wet and the dry samples differed considerably in the red-type library. For the dry ML1–4 soil, the red-type library was dominated by 1C variant phylotypes related to the actinobacteria (76%, **Figure 2**), with the remainder related to the proteobacterial 1C variant clade while the actinobacterial and proteobacterial 1C variants made up only 23 and 20% of the total phylotypes detected in the wet ML1–2 soil and 1D algal signatures were numerically dominant in this sample (32%, **Figure 2**).

Diversity plateaus within 16S rRNA gene based rarefaction plots indicate sufficient representation (Supplementary Information: Supplementary Figure S2), with a lower total diversity level for the arid ML1–4 site. Taxonomic affiliations of the 16S rRNA sequences are presented in **Figure 3**. Noticeable differences between the wet (ML1–2) and arid (ML1–4) sites include higher concentrations of cyanobacteria and alphaproteobacteria for ML1–2 (i.e., 47 and 14%, respectively and 7 and 4% for ML14), and higher concentrations of *Bacteroidetes* and gammaproteobacteria for ML1–4 (55 and 7%, respectively vs. 7 and 2% for ML1–2). Various significant (as supported by e-scores) differences in taxonomic representation were observed between the wet and dry sites using the online RDP LIBCOMPARE tool at an 80% confidence level. The most significant ($<1E^{-12}$) are listed in Supplementary Table S2 and include the presence of *Gillisia* in dry ML1–4 soil (∼27% of total *Bacteroidetes*) and the absence of this genus in wet ML1–2 soil, a high contribution (∼59%) of *Streptophyta* in the total cyanobacteria detected in the wet ML1–2 soil with this genus being unobserved in the arid ML1–4 site. The GpI group of cyanobacteria were also well-represented in the wet ML1–2 soil (∼26% of total detected cyanobacteria) with less than 1% representation in dry ML1–4 soil, and interestingly, the majority (∼91%) of the cyanobacteria detected in the dry ML1–4 soil

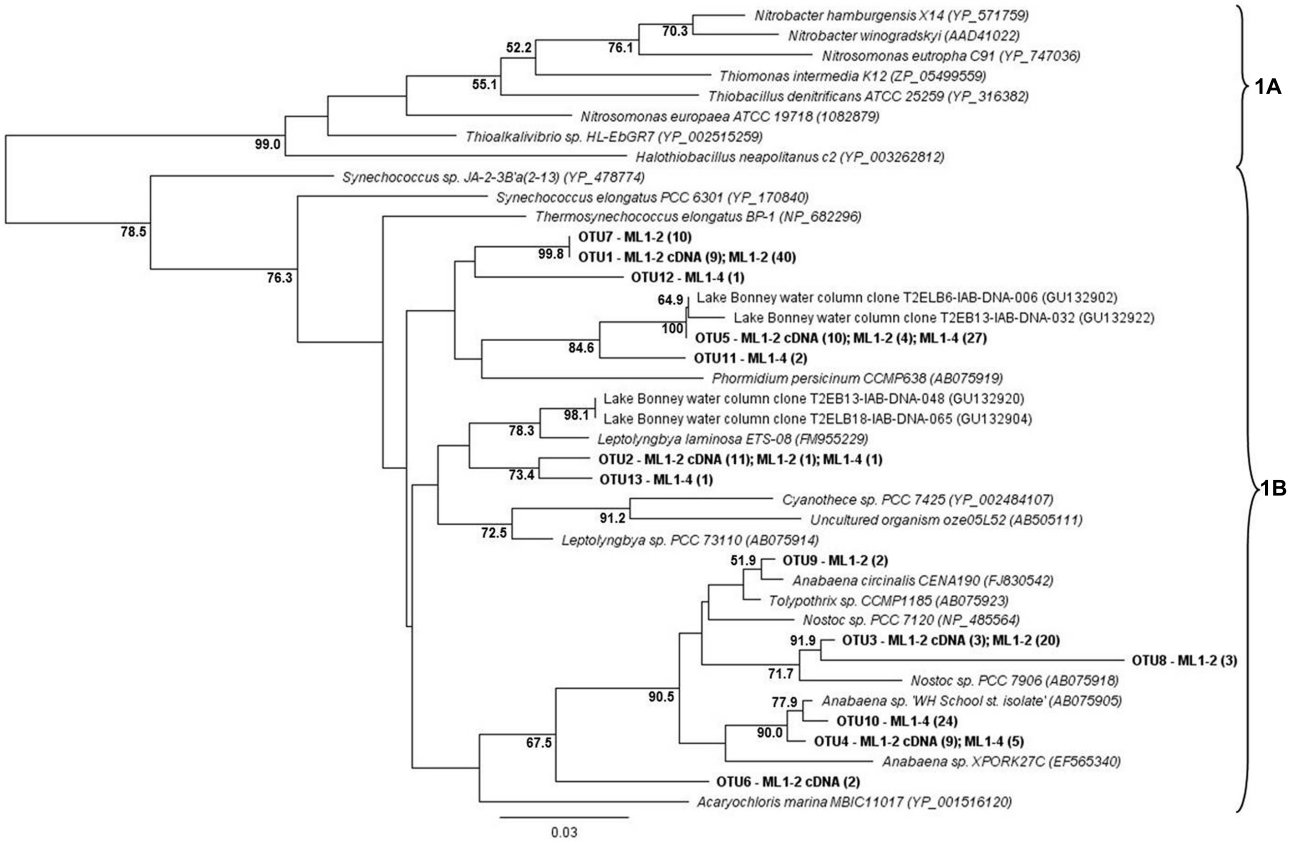

FIGURE 1 | Neighbor joining tree showing the phylogenetic relationships of translated green-type *cbbL* sequences as based on 127 amino-acid residues. The tree was constructed using the Jukes-Cantor distance model. *Sinorhizobium meliloti* 1021 (NP_436731) was used as an out-group and has been removed from the tree. Bootstrap supports are indicated as percentages (>50%) of 1000 replicates.

were related to the GpIV group, with this group only making up 6% of wet-associated cyanobacterial signatures. Although only minor components of the dry ML1–4 soil (<1%), members of the Deinococcus-Thermus group were not detected in the wet ML1–2 soil.

A total of 14,642 and 2,958 quality trimmed 18S rRNA gene sequences were obtained for ML1–2 and ML1–4 respectively. Rarefaction analyses at 90% sequence cut-off, indicate sufficient sampling for both ML1–2 and ML1–4 with higher diversity levels observed at the dry ML1–4 site (Supplementary Figure S3). Phylogenetic analyses indicate that wetted ML1–2 soil was almost completely dominated (>99%) by Virdiplantae, with 99.8% of these sequences most closely related to the moss Ephemerum (**Figure 3**). For the dry soil, a diverse assemblage of eukaryotes was observed including members of various phyla (**Figure 3**).

DISCUSSION AND CONCLUSION

Dry Valley soils are severely carbon-limited and can be considered some of the most oligotrophic on the planet (Cary et al., 2010). Current literature has highlighted the importance of C-fixation as a means of consistent replenishment of organic carbon in DV soils (Parsons et al., 2004; Hopkins et al., 2009; Feng et al., 2010); however, the extent that DV soil microbial communities undertake C-fixation or their reliance on external sources of C remains unknown. This study therefore provides important insights into this fundamental biogeochemical cycle and the understanding of carbon transformations in DV soils by focusing on communities found in the Miers Valley. Autotrophy is an energetically expensive process, which is usually slow and under strict control (Alfreider et al., 2009), especially in limited conditions such as soils of the DV. Therefore, complementary stable isotope- and mRNA-based methods were applied to resolve activities and to identify the associated active microbial component.

As expected, carbon fixation rates were typically higher wetter soils as compared to arid soils (>5% moisture) with rates being considerably lower in stream systems as compared to the lake-associated soils. This is most likely caused by the transient nature of the streams making the establishment of permanent microbial communities difficult as evidenced by the formation of thick mats on lake edges but not in the Miers Valley streams. The transects involved in this study have also been described in a previous study investigating nitrogen-fixation activities in these soils (Niederberger et al., 2012).

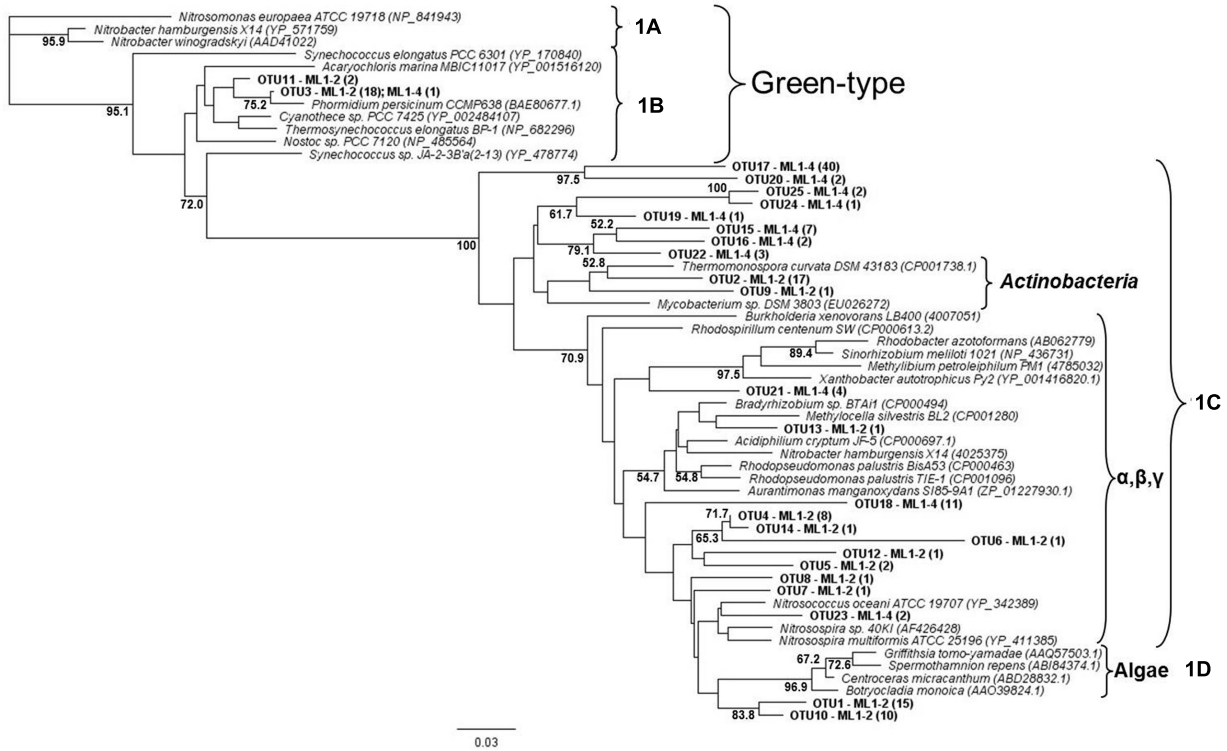

FIGURE 2 | Neighbor joining tree showing the phylogenetic relationships of translated red-type *cbbL* sequences as based on 120 amino-acid residues. The tree was constructed using the Jukes-Cantor distance model and green-type sequences was used as an out-group. Bootstrap supports are indicated as percentages (>50%) of 1000 replicates.

Few studies have reported carbon-fixation rates in the DV, with rates in the bulk arid soils being considered extremely low, ranging from 1 to 20 g C m^{-2} $year^{-1}$ (Friedmann, 1993; Novis et al., 2007; Cary et al., 2010). Rates of carbon addition to DV soils are also hypothesized to be less than or equal to respiration rates ($\sim$ <6.5 g C m^{-2} $year^{-1}$) otherwise soil carbon reservoirs would already be depleted (Burkins et al., 2001). In this study, C turnover rates in the ephemerally wet soils ranged between $\sim$7 and 140 days and equated to micrograms of C-fixed per cubic centimeter on a daily basis in both wetted and arid biotopes. These rates fall within the same range (3.22 μg C L^{-1} day^{-1}) as recently measured in the water column of Lake Bonney situated in the Antarctic DV (Kong et al., 2012a). C-fixation rates reported in this study are well-above previous estimates; however, it is important to note that rates were measured during the high productivity summer months associated with warmer temperatures, wetter soils and a longer photo-period, and focused specifically on microbial mats. Therefore, at least during the Polar summer period, autotrophs play a major role in carbon replenishment of DV soils and further corroborate suggestions that as opposed to the reliance of carbon from high productivity soils from aeolian redistribution (Burkins et al., 2000, 2001; Barrett et al., 2006c; Hopkins et al., 2009; Feng et al., 2010), *in situ* CO_2-fixation may be the largest contributor to SOC in DV soils (Burkins et al., 2000, 2001; Hopkins et al., 2009).

The cyanobacterial-related form 1B *cbbL* gene was the only phylotype detected in the green-type sequences for both the wet and dry soils, with the 1A proteobacterial-related phylotype remaining undetected. These cyanobacterial sequences from both the wet and dry sites were related to sequences from Lake Bonney in Taylor Valley, a larger and more northern Valley than the Miers (Kong et al., 2012b). The 1B green-type phylotype was the only *cbbL* gene expressed in the wet sample, indicating that at the time of sampling, cyanobacteria were the major contributors to the observed C-fixation there. Although C-fixation rates were measureable in some arid soil samples, expression of RuBisCO genes was not seen, and therefore, the members directly responsible for these activities remain unidentified. Expression of red-type sequences was also not seen in the wet or dry soils, but the presence of the genes in DNA isolated from these sites shows that there were other organism present besides caynobacteria that have the potential to fix carbon. The wet soil contained both 1C- (both actinobacterial and proteobacterial) and 1D-related sequences, with algal (1D) dominance (32%). Similarly 1D-related sequences have been proven to dominate the water column of Lake Bonney in the DV (Kong et al., 2012b). Proteobacterial red-type signatures in the wetted soil were most closely related to nitrifying bacteria, a group of chemolithoautotrophs that oxidize either ammonia or nitrite and are typically present in areas of high ammonia concentrations. While we did not attempt to measure nitrification in this study,

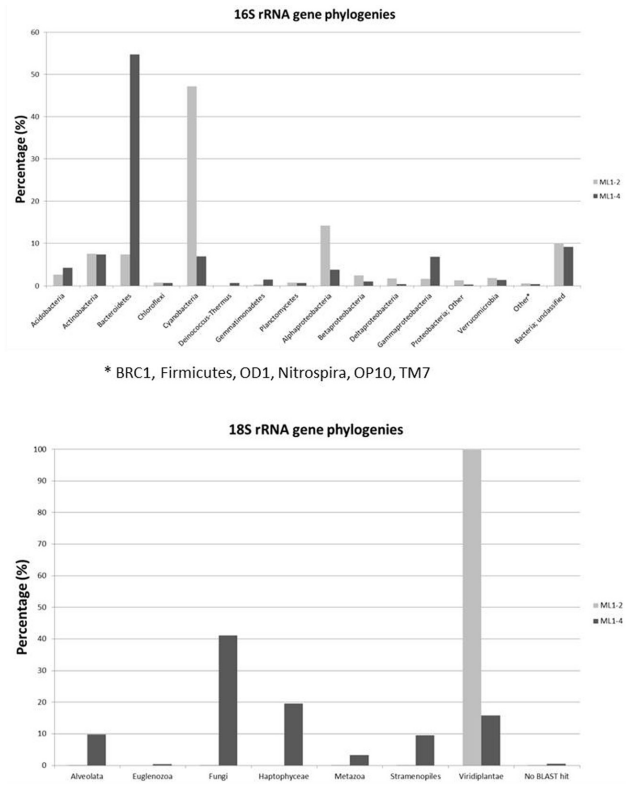

FIGURE 3 | Bacterial and Eukaryotic phylogeny of 16S rRNA and 18S rRNA genes from wet (ML1–2) and dry (ML1–4) Miers Lake soil sites.

it is known to occur in DV soils, with highest rates at wetted lake margins (Hopkins et al., 2006a), suggesting that some of the measured C-fixation could be based on chemical- rather light-based energy. Red-type gene sequences were not shared between wet and dry soils; the bulk arid soil was dominated by actinobacterial-related 1C signatures (76%) with algal (1D) phylotypes absent. 16S rRNA gene results did not reflect the difference in actinobacterial *cbbL* gene presence in dry vs. wet soils, with a similar percentage of actinobacterial signatures in both biotopes as was also noted in a previous study comparing high and low DV productivity soils (Niederberger et al., 2008) with 18S rRNA gene sequencing indicating an almost complete dominance of wetted soils by Virdiplantae. Babalola et al. (2009) investigate and describe in depth, Actinobacteria in DV soils with various molecular-based studies proving that both *Actinobacteria* and *Bacteroidetes* are commonly dominant members of arid soils including the DV (Fierer et al., 2007; Niederberger et al., 2008; Pointing et al., 2009; Lee et al., 2012; Tiao et al., 2012; Bottos et al., 2014); therefore, it is not surprising that this group of organisms have C-fixation capabilities thereby permitting their subsistence in these severely oligotrophic soils. In fact, an important study by Chan et al. (2013) has reported the presence of RuBisCo signatures in Antarctic DV habitats specifically form I from cyanobacteria and forms II and III indicated as being from Archaea, *Actinobacteria*, and Proteobacteria. These results also suggest a significant capability in chemoautotrophy in these habitats.

The rates of C-fixation in both arid and wetted DV soils reported in this study coupled with documented low levels of SOC (Parsons et al., 2004; Barrett et al., 2006a; Elberling et al., 2006; Hopkins et al., 2006b, 2009; Ball et al., 2009; Cary et al., 2010; Feng et al., 2010) lends further credence to the hypothesized high turnover rates of C in DV soils. Therefore, at least in the summer months, *in situ* autotrophic C-fixation can replenish soil SOC levels with arid soils most likely dominated by actinobacterial C-fixers with a more diverse microbial community in wetted soils dominated by cyanobacterial-related activity. The recent discovery of genetically localized communities between valleys also indicates that these communities maybe endemic and that inter-valley aeolian-based redistribution maybe negligible (Lee et al., 2012). If this holds true, communities in these distinct biotopes cannot rely on consistent external sources of carbon and must be adapted to exist under these extreme dry and nutrient-limited conditions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2015.01347

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