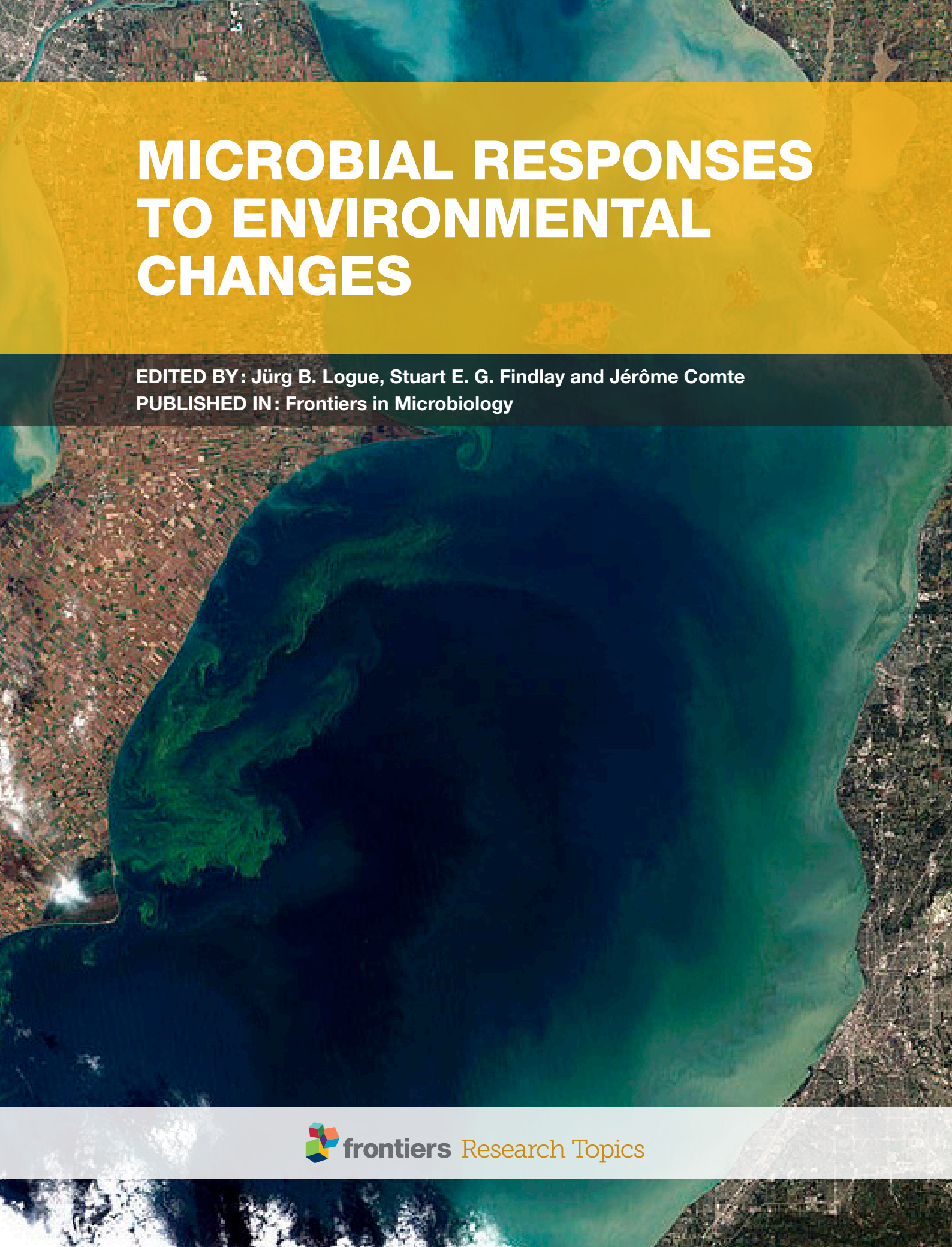

# MICROBIAL RESPONSES TO ENVIRONMENTAL CHANGES

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# MICROBIAL RESPONSES TO ENVIRONMENTAL CHANGES

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The green scum shown in this image is the worst algae bloom Lake Erie has experienced in decades. Vibrant green filaments extend out from the northern shore.
Image by NASA Earth Observatory: https://commons.wikimedia.org/wiki/File:Toxic_Algae_Bloom_in_Lake_Erie.jpg

Advances in next generation sequencing technologies, omics, and bioinformatics are revealing a tremendous and unsuspected diversity of microbes, both at a compositional and functional level. Moreover, the expansion of ecological concepts into microbial ecology has greatly advanced our comprehension of the role microbes play in the functioning of ecosystems across a wide range of biomes. Super-imposed on this new information about microbes, their functions and how they are organized, environmental gradients are changing rapidly, largely driven by direct and indirect human activities.

In the context of global change, understanding the mechanisms that shape microbial communities is pivotal to predict microbial responses to novel selective forces and their implications at the local as well as global scale. One of the main features of microbial communities is their ability to react to changes in the environment. Thus, many studies have reported changes in the performance and composition of communities along environmental gradients. However, the mechanisms underlying these responses remain unclear. It is assumed that the response of microbes to changes in the environment is mediated by a complex combination of shifts in the physiological properties, single-cell activities, or composition of communities: it may occur by means of physiological adjustments of the taxa present in a community or selecting towards more tolerant/better adapted phylotypes. Knowing whether certain factors trigger one, many, or all mechanisms would greatly increase confidence in predictions of future microbial composition and processes.

This Research Topic brings together studies that applied the latest molecular techniques for studying microbial composition and functioning and integrated ecological, biogeochemical and/or modeling approaches to provide a comprehensive and mechanistic perspective of the responses of micro-organisms to environmental changes. This Research Topic presents new findings on environmental parameters influencing microbial communities, the type and magnitude of response and differences in the response among microbial groups, and which collectively deepen our current understanding and knowledge of the underlying mechanisms of microbial structural and functional responses to environmental changes and gradients in both aquatic and terrestrial ecosystems. The body of work has, furthermore, identified many challenges and questions that yet remain to be addressed and new perspectives to follow up on.

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EDITORIAL
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# Editorial: Microbial Responses to Environmental Changes

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**Keywords: ecosystem functioning, environmental change, microbial community composition, microbial diversity, microbial ecology, micro-organism, next-generation sequencing**

Earth is teeming with taxonomically, phylogenetically, and metabolically highly diverse micro-organisms; organisms that are crucial for sustaining life on our planet in that they carry out processes of great biogeochemical significance. The processes and mechanisms underlying their diversity and distribution are, thus, of great interest, yet—despite their importance—only poorly understood.

Microbial communities may be structured by a large number of factors among which are environmental conditions: local, contemporary environmental conditions select and sort micro-organisms according to their ecological niche (Hutchinson, 1957). This corresponds to the so-called Baas-Becking hypothesis for microbial organisms "*everything is everywhere:* but *the environment selects*" (Baas Becking, 1934) that formed the basis of the later formulated species-sorting paradigm/perspective (Leibold et al., 2004; Holyoak et al., 2005). Species sorting has been repeatedly shown to be an important determinant for the assembly of microbial communities (e.g., Van der Gucht et al., 2007; Logue and Lindström, 2008). It has been suggested that the response of microbes to environmental conditions and changes thereof is mediated by a complex combination of adjustment (e.g., high adaptability and plasticity but also ability to horizontally transfer genetic material), replacement (e.g., high dispersal rates) and species interaction mechanisms, all facilitated by their fast population growth rates (Allison and Martiny, 2008; Comte and del Giorgio, 2011). Yet, the factors that determine the type and magnitude of the response of microbial communities remain unclear (de Vries and Shade, 2013). Given that many ecosystems are undergoing rapid and major environmental changes, obtaining a quantitative and process-level understanding of the mechanisms that affect microbes and microbial communities is pivotal for predicting the responses of microbial communities to novel or changing selective forces and their implications at the local, regional and global scale.

Advances in molecular biology have revolutionized our ability to describe microbial communities with regard to composition and biogeography, diversity and processing, but also the mechanisms of adaptation and evolution (Pedrós-Alió, 2006). In addition, the integration of theory into microbial ecology has greatly improved our understanding of the roles micro-organisms play across a wide range of biomes by providing researchers with organization, structure, mechanistic insight, and predictive power (Prosser et al., 2007). Technical advances and theoretical integration will advance our understanding of how microbial organisms may respond and evolve in a changing environment.

The aim of this Research Topic "Microbial responses to environmental changes" is to provide the reader with a selection of studies that have gone beyond a descriptive level and investigated the mechanisms by which microbial communities and associated processes respond to environmental gradients and changes. This Research Topic brings together studies that applied the latest molecular techniques for studying the composition and functioning of microbial communities and integrated ecological, biogeochemical and/or modeling approaches to provide a comprehensive

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and mechanistic perspective of the responses of micro-organisms to environmental changes. Contributions include original research (i.e., field surveys and laboratory or field experiments) and comprehensive reviews. They outline critical challenges that remain as well as new perspectives and ideas with regard to responses of micro-organisms to changes in the environment. Thus, patterns of response of microbial communities to environmental gradients and changes are documented for archaea, bacteria, fungi, and microbial eukaryotes, as well as a vast range of habitats including streams, ponds and lakes, ocean and seas, and sediments and soils, and varying spatial and temporal scales.

This Research Topic presents new findings on environmental variables influencing microbial communities, the type and magnitude of responses and differences in the response among microbial groups. As such, several studies have focused on how environmental drivers affected by climate change can structure microbial communities (Koyama et al., 2014; DeAngelis et al., 2015; Wu et al., 2015) and regulate microbial activities by, for example, influencing production (Aanderud et al., 2015; Adams et al., 2015), respiration (Amend et al., 2015), activity of enzymes (Berlemont et al., 2014; Penton et al., 2015), or carbon transformations and sequestration (Liang et al., 2015). Environmental field surveys, on the other hand, identified shifts in microbial community composition due to a range of environmental variables (Crevecoeur et al., 2015; Febria et al., 2015; Ling et al., 2015; Nguyen and Landfald, 2015; Sarmento et al., 2015).

To examine the type of response, experimental studies identified both adjustment and replacement effects, where microbial communities either adapted or shifted in composition as a result of environmental drivers. Aanderud et al. (2015) identified hundreds of rare bacterial taxa that increased in abundance within only a few days after rewetting of dry soil, some becoming even dominant and contributing to ecosystem functioning. DeAngelis et al. (2015), on the other hand, observed that soil microbial communities at first adapted to warming conditions before eventually (i.e., after 20 years) truly shifting in composition. In a short-term transplant experiment, Lindh et al. (2015) demonstrated that the mechanisms underlying the response of bacterial communities to changes in the environmental conditions varied depending on the successional state during which the change happened. This highlights the importance of considering time, when investigating the nature of response to changes in the environment.

A central question in ecology is how community composition influences ecosystem functioning (Loreau et al., 2002). Microbial ecologists, in particular, are faced with not fully comprehending whether changes in community composition lead to shifts in microbially mediated functions or whether shifts in these functions require changes in composition. Understanding is yet further hampered by the notion that environmental forces drive changes in both composition and function. Previous work has illustrated that exploring functional properties (i.e., traits) in this respect is highly promising (e.g., Martiny et al., 2015; Ruiz-González et al., 2015) and that the distribution of specific traits may govern the type of microbial response to environmental changes (e.g., Shade and Handelsman, 2012). A number of contributions explored how microbial assemblages responded to changes in environmental variables via trait variation. Mackey et al. (2014) analyzed the effects of changes in the iron-nitrogen ratio on phytoplankton and observed that nitrate additions favored slow-sinking single-celled over faster sinking chain-forming diatoms. Sarmento et al. (2015) showed that microbial life in aquatic surface microlayers was governed by different independent processes compared to that in the underlying waters. It is assumed that these surface microlayer communities directed most of their energy toward maintenance and repair processes due to the high UV-radiation levels rather than growth efficiency, which was higher in deeper layers. Changes have also been reported at the cell level, where heterotrophic bacteria featuring a range of homeostatic regulation mechanisms were able to accommodate changes in carbon and phosphorus concentrations (Godwin and Cotner, 2015). Crowther et al. (2014) have, in this respect, proposed a conceptual trait-based framework for studying how niche processes, such as stress tolerance or combative dominance, structure fungal assemblages across time and space, which in turn may provide insight into the relationship between community composition and functioning.

A major challenge in microbial ecology is to determine whether micro-organisms show unique features or have patterns in common with macro-organisms (i.e., plants and animals). Yet, whether distinct patterns can be distinguished between the different micro-organisms has received far less attention. In other words, do bacteria, for example, respond in the same way to environmental cues as archaea, fungi or microbial eukaryotes? Given that intrinsic properties but also key variables and processes involved in structuring the respective communities are different from each other, it is highly unlikely that responses follow the same patterns among these groups of micro-organisms. Indeed, several studies revealed distinct patterns by which, for instance, archaea and bacteria or bacteria and fungi responded to changes in their environment or particular environmental stressors. Nguyen and Landfald (2015), having examined variation in archaeal and bacterial community composition over a moderate environmental gradient naturally existing in the Barents Sea, showed that archaeal assemblages appeared to be structured by one specific variable (i.e., level of freshly sedimented phytopigments), while bacterial communities were significantly influenced by a broader set of environmental variables. Contributions focusing on bacteria and fungi highlighted differences in the sensitivity to drought (Berlemont et al., 2014), fertilization (Koyama et al., 2014; Liang et al., 2015) and warming (DeAngelis et al., 2015; Liang et al., 2015), which in turn impacted measured functional processes or traits (Berlemont et al., 2014; Liang et al., 2015). Finally, the sensitivity or resilience of diverse aquatic microbiota to environmental variation assessed in a review and synthesis analysis by Zeglin (2015) demonstrated that bacterial communities reacted differently to various environmental drivers compared to other microbial groups. Thus, assemblages may tend to converge or diverge as particular drivers wax or wane.

In summary, with ongoing climate change and alteration of habitats' integrity and structure, a mechanistic understanding

of the implications in terms of ecosystem functioning is greatly needed. The broad-range of articles presented here deepen our current understanding and knowledge of the underlying mechanisms of microbial structural and functional responses to environmental changes and gradients in both aquatic and terrestrial ecosystems. The body of work has, furthermore, identified many challenges and questions that remain to be addressed and new perspectives to follow up on. We are hopeful that this collection of studies will stimulate discussions and pave the way for future prospects far beyond this Research Topic.

## AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the editorial.

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ORIGINAL RESEARCH ARTICLE
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# Resuscitation of the rare biosphere contributes to pulses of ecosystem activity

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Dormancy is a life history trait that may have important implications for linking microbial communities to the functioning of natural and managed ecosystems. Rapid changes in environmental cues may resuscitate dormant bacteria and create pulses of ecosystem activity. In this study, we used heavy-water ($H_2^{18}O$) stable isotope probing (SIP) to identify fast-growing bacteria that were associated with pulses of trace gasses ($CO_2$, $CH_4$, and $N_2O$) from different ecosystems [agricultural site, grassland, deciduous forest, and coniferous forest (CF)] following a soil-rewetting event. Irrespective of ecosystem type, a large fraction (69–74%) of the bacteria that responded to rewetting were below detection limits in the dry soils. Based on the recovery of sequences, in just a few days, hundreds of rare taxa increased in abundance and in some cases became dominant members of the rewetted communities, especially bacteria belonging to the Sphingomonadaceae, Comamonadaceae, and Oxalobacteraceae. Resuscitation led to dynamic shifts in the rank abundance of taxa that caused previously rare bacteria to comprise nearly 60% of the sequences that were recovered in rewetted communities. This rapid turnover of the bacterial community corresponded with a 5–20-fold increase in the net production of $CO_2$ and up to a 150% reduction in the net production of $CH_4$ from rewetted soils. Results from our study demonstrate that the rare biosphere may account for a large and dynamic fraction of a community that is important for the maintenance of bacterial biodiversity. Moreover, our findings suggest that the resuscitation of rare taxa from seed banks contribute to ecosystem functioning.

**Keywords: $CO_2$ pulses, dormancy, desiccation, dominance, stable isotope probing (SIP), soil rewetting, seed bank, rarity**

## INTRODUCTION

In nature, most microorganisms live in unpredictable environments and experience conditions that are suboptimal for growth and reproduction. Some organisms attempt to maximize their long-term fitness by dispersing offspring into new and hopefully better habitats. Other organisms hedge their bets by entering a reversible state of reduced metabolic activity in a process known as dormancy. Dormancy builds seed banks, which are reservoirs of inactive individuals that can potentially be resuscitated in the future under a different set of environmental conditions (Lennon and Jones, 2011). Dormancy can protect taxa from extinction by buffering against demographic and environmental stochasticity (Kalisz and McPeek, 1992; Honnay et al., 2008). It can also reduce the strength of species interactions and allow taxa to coexist via the storage effect (Chesson and Warner, 1981). Recently, efforts have been made to integrate dormancy into ecosystem models by accounting for the physiological processes and energetic requirements associated with the active and inactive members of a microbial community (Stolpovsky et al., 2011; Wang et al., 2014a,b). These studies suggest that, in addition to being an important diversity-maintaining mechanism, dormancy may have important implications for understanding and predicting ecosystem processes.

Soil microorganisms play an essential role in regulating critical ecosystem processes, such as carbon sequestration, nutrient cycling, and the flux of trace gasses. Growing evidence suggests, however, that only a small fraction of the bacterial community may be responsible for soil processes occurring at any given point in time. In some cases, it is estimated that >90% of the microbial biomass is inactive, 50% of all bacterial taxa are dormant, and at least 25% of all soil genomes contain genes that enable individuals to be resuscitated from a dormant state (Alvarez et al., 1998; Lennon and Jones, 2011; Wang et al., 2014a). In particular, microbial activity can be extremely low in dry soils owing to a combination of desiccation stress and the reduced diffusion of substrates (Schimel et al., 2007). Under these relatively inactive conditions, precipitation events serve as an environmental cue that terminates microbial dormancy in dry soils (Saetre and Stark, 2005; Placella et al., 2012). As soils are rewetted, there is an increase in microbial metabolism (Iovieno and Baath, 2008;

Blazewicz et al., 2013) that corresponds with pulses of ecosytem activity. Within hours of a precipitation event, $CO_2$ production can be 500% higher than pre-wetting conditions (Fierer and Schimel, 2003) and when scaled over longer time periods, moisture-mediated pulses of ecosystem activity contribute up to 25% of the carbon budget in some terrestrial ecosystems (Schimel et al., 2007). Furthermore, recent studies suggest that historical exposure to soil moisture regimes may select for bacteria with functional traits that confer tolerance to drying and rewetting events (Evans and Wallenstein, 2014). Taken together, drying and rewetting events offer an ideal situation to evaluate the interactions between dormancy, microbial diversity, and ecosystem processes.

Resuscitation of dormant microbes may also provide an opportunity to explore the functional importance of the "rare biosphere." The rare biosphere is a term that was coined to describe the observation that most microbial taxa are extremely uncommon (Sogin et al., 2006). If microbial taxa contribute to ecosystem processes in proportion to their abundance then it may not be critical to focus on the rare biosphere (see Grime, 1998). However, it is well established that some rare groups of bacteria contribute disproportionately to certain biogeochemical processes. For example, a specific subset of rare methane oxidizing bacteria regulated methane emissions from riparian floodplains (Bodelier et al., 2013), while sulfate reduction in a peatland ecosystem was attributed to a single genus of bacteria that comprised less than 0.006% of the total microbial community (Pester et al., 2010). It is also important to consider that the relative abundance of bacterial populations can be highly variable through time due to fluctuations in environmental conditions (Pedrós-Alió, 2012; Hugoni et al., 2013; Shade et al., 2014). For instance, over half of the bacterial taxa in the Chesapeake Bay cycled between being abundant and rare over a 3 year period (Campbell et al., 2011). Previous work has suggested that shifts in the commonness and rareness of bacterial taxa may be due to transitions between active and inactive metabolic states (Jones and Lennon, 2010), but few studies have linked these dormancy dynamics to environmental change and pulses of ecosystem activity.

In this study, we explore the effects of soil moisture variability on bacterial resuscitation and ecosystem processes. After documenting pulses of trace gasses ($CO_2$, $CH_4$, and $N_2O$) in both field experiments and laboratory microcosms, we used heavy-water stable isotope probing (SIP) to identify bacteria that were resuscitated from a state of low metabolic activity based on the incorporation of $^{18}O$ into their DNA following the rewetting of dry soils. We demonstrate that a large number of rare taxa rapidly responded to shifts in soils moisture and contributed to pulses of ecosystem activity. Our findings suggest that shifts in environmental cues can affect the dormancy of bacterial communities in ways that maintain biodiversity and influence ecosystem processes.

## MATERIALS AND METHODS

### STUDY SITE

Our study took place at the W. K. Kellogg Biological Station (KBS) Long Term Ecological Research (LTER) site in southwestern Michigan, USA. We conducted field and laboratory experiments using land-use treatments that simulate some of the major ecosystem types found in the Upper Great Lakes region of North America, specifically agricultural crop rotation (T1), successional grassland (T7), deciduous forest (DF), and coniferous forest (CF) (Robertson et al., 2000). Average annual precipitation at the KBS LTER is 890 mm (±148.0 SD, $n = 21$) with half falling as snow, and the mean annual temperature is 9.0°C (±0.81 SD, $n = 21$, http://lter.kbs.msu.edu). All soils are fine-loamy, mixed, mesic Typic Hapludalfs with an average pH of 6.0 and a cation exchange capacity of approximately 5.5 cmol $kg^{-1}$.

### PULSES OF ECOSYSTEM ACTIVITY: FIELD EXPERIMENT

Prior to pursuing more mechanistic experiments, we conducted a field experiment to assess the water-limitation of microbial processes in our relatively mesic habitat. Over a 17-day period (June 15–July 2 2007), we manipulated rainfall by evenly dispensing 5 mm of distilled water onto a 3 × 3 m plot in one of the replicate agricultural field sites (T1) on days 4, 7, and 14 of the experiment. Before initiating the experiment, we deployed environmental sensors at 2 cm depth to quantify the temporal dynamics of soil $CO_2$, soil moisture, and soil temperature. The placement of the sensor near the soil surface allowed us to capture an integrated $CO_2$ response to moisture before the gas was released to the atmosphere (Riveros-Iregui et al., 2007; Aanderud et al., 2011). We measured $CO_2$ concentrations (ppmv) using non-dispersive infrared absorption with a 3% $CO_2$ GMT222 sensors (Vaisala, Helsinki, Finland), while monitoring soil moisture ($m^3$ $H_2O$ $m^{-3}$ soil) and temperature (°C) with ECH2O-TE sensors (Decagon Devices, Pullman WA, USA). Data from the sensors were generated every 10 s, averaged on a 30 min time-interval, and stored on field data loggers (CR1000, Campbell Scientific, Inc., Logan UT, USA). We analyzed the resulting data on a 12-h time-step using a time series multiple regression model:

$$CO_2(t) = CO_2(t-1) + moisture(t) + moisture(t-1) + \varepsilon_t \quad (1)$$

where $t$ is the current time step, $t - 1$ is the previous time step, and $\varepsilon_t$ is the residual error. We corrected for non-random distributions of the residuals using methods described elsewhere (Aanderud et al., 2011, 2013).

### PULSES OF ECOSYSTEM ACTIVITY: MICROCOSM EXPERIMENT

To gain insight into the microbiological contributions to pulsed ecosystem activity observed in the field, we performed a more controlled rewetting experiment in the laboratory. The microcosm approach was also used for the stable isotope probing (SIP) experiments, which we describe in the next section. Following the summer dry-down of the soils in July 2008, we sampled soils from three of the replicated plots from the four ecosystem types (agricultural crop rotation, successional grasslands, deciduous forests, and CFs). We removed 10 soil cores (0–5 cm soil depth) from randomly selected locations in each the three replicate plots with a soil corer (5 cm length × 2 cm width) and homogenized the soils to create 12 composite samples (4 ecosystem types × 3 replicates). The soils were immediately brought back to the laboratory

and passed through a 2 mm sieve. In triplicate, we dispensed 3 g of field-dry soil (≈0.05 g $H_2O$ g $soil^{-1}$) into 40 mL borosilicate glass vials with septated screw caps. For each ecosystem type, we randomly assigned three microcosms to a dry treatment (no water added). The remaining microcosms belonged to the rewetting treatment and received 0.6 mL of $H_2O$. We then incubated all 24 microcosms for 96 h at 25°C in a temperature-controlled incubator. During the experiment, we collected 1 mL of gas from the headspace of each microcosm every 12 h. With these gas samples, we quantified $CO_2$ using a LI-820 infrared gas analyzer (Lennon et al., 2012). In addition, we measured $CH_4$ and $N_2O$ using gas chromatography [Hewlett Packard 5890 Series II, Rolling Meadows, IL, USA, Ruan and Robertson (2013)]. We calculated the net production of trace gasses (μg C-$CO_2$, C-$CH_4$, or N-$N_2O$ g $soil^{-1}$) by summing the amount of gas generated during each of the eight 12-h increments and tested for the effect of rewetting on the gas production using Two-Way ANOVA and Tukey's HSD tests.

#### STABLE ISOTOPE PROBING (SIP)

Using the microcosm approach described above, we identified bacteria that were resuscitated by rewetting using $H_2^{18}O$-DNA SIP. We initiated SIP by adding 0.6 mL of $H_2^{18}O$ (97 atom% $^{18}O$; Isotech, Sigma-Aldrich, St. Louis, MO, USA) to a dry soil sample and incubating it for 72 h at 25°C. This rewetting created a five-fold increase in gravimetric moisture for all soil samples (dry soil ≈0.05 g $H_2O$ g $soil^{-1}$, rewetted soil ≈0.25 g $H_2O$ g $soil^{-1}$). As a control, we also used SIP to characterize the bacterial composition of the dry soils. This was done by adding 0.6 mL of $H_2^{18}O$ to a soil sample and immediately stopping bacterial activity by transferring the microcosm to −80°C. We then followed the ultracentrifugation, gradient fractionation, and DNA recovery procedures of SIP described in detail elsewhere (Schwartz, 2007; Aanderud and Lennon, 2011). Briefly, at least 1 μg of genomic DNA was extracted from soils using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA, USA), and was loaded into 4.7 mL OptiSeal polyallomer tubes (#361621, Beckman Coulter Inc., Brea, CA, USA) containing cesium trifluoroacetate (CsTFA, #17-0847-02, GE Healthcare, Salt Lake City, UT, USA) with a buoyant density 1.61 g $mL^{-1}$ (Leigh et al., 2007). Each 4.7 mL tube received approximately 2.9 mL of CsTFA and 1.75 mL of nuclease-free $H_2O$. The tubes were placed into a TLA 110 rotor and spun at 178,000 rcf (64,000 rpm) for 48 h at 20°C. After centrifugation, we collected 20 fractions (235 μL each) from each tube using a digitally controlled fractionator. We identified the unlabeled bacterial DNA in the dry treatment and $^{18}O$-labeled bacterial DNA in the rewetted treatment by performing qPCR on all gradient fractions via amplification of the 16S rRNA gene (Aanderud and Lennon, 2011). All of the above was done for a total of 24 samples (4 ecosystems × 2 watering treatments [dry vs. rewetted] × 3 replicates).

#### BACTERIAL COMMUNITY RESPONSES TO REWETTING

We characterized the bacterial communities in the dry and rewetted soils from the SIP samples using bar-coded sequencing of the 16S rRNA gene. We PCR-amplified the V1–V2 hypervariable region of the 16S rDNA gene using the bacterial primers 27F and 338R with unique 12-nt error correcting Golay barcodes (Fierer et al., 2008). The thermal cycle conditions were as follows: an initial denaturation step at 94°C for 3 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 30 s, and an extension at 72°C for 90 s. After pooling PCR amplicons at approximately equimolar concentrations, samples were sequenced at the Environmental Genomics Core Facility at the University of South Carolina in a 454 Life Sciences genome sequence FLX (Roche, Branford, CT, USA) instrument. All sequences were analyzed using mothur (v.1.29.2) an open-source, expandable software pipeline for microbial community analysis (Schloss et al., 2009). After removing barcodes and primers, we eliminated sequences that were <250 bp in length and sequences that had homopolymers longer than 8 bp. In addition, we denoised the sequences with AmpliconNoise (Quince et al., 2011). Finally, we removed chimeras using UCHIME (Edgar et al., 2011), along with chloroplast, mitochondria, archaeal, eukaryotic, and unknown rRNA gene sequences according to the Ribosomal Database Project (Cole et al., 2009). We then aligned our sequences against the SILVA database (Pruesse et al., 2007) with the SEED aligner, created operational taxonomic units (OTUs) based on uncorrected pairwise distances at the 97% sequence similarity level, and determined the phylogenetic identity of OTUs using the SILVA database.

To assess the effects of soil rewetting on bacterial communities from different ecosystems, first, we used multi-level partial least squares discriminant analysis (PLS-DA) and permutational multivariate analyses of variance (PERMANOVA). PLS-DA is an ordination technique that is especially suited to deal with datasets where there are a larger number of predictors (e.g., OTUs) than observations (samples), while alleviating problems arising from multicollinearity (Barker and Rayens, 2003; Pérez-Enciso and Tenenhaus, 2003). Importantly, PLS-DA allowed us to accommodate the paired nature of our experimental design (i.e., the non-independence between a dry and rewetted sample). PLS-DA was implemented with the mixOmics package in R (Dejean et al., 2013). While the PLS-DA aided in the visualization of our data, we tested for the main effects and interaction between the rewetting treatment and ecosystem type using PERMANOVA (Anderson, 2001), which was performed with the *adonis* function in the vegan package in R (Oksanen et al., 2013). Second, we quantified the compositional turnover that occurred for each experimental unit between the dry and rewetted time points using the Bray-Curtis dissimilarity index. Last, we quantified bacterial richness in our samples as the observed number of OTUs after rarefaction. The effects of rewetting and ecosystem type on richness were evaluated using Two-Way ANOVA with Tukey's HSD tests.

#### RESUSCITATION OF RARE BACTERIA

Unlike other properties of biological diversity (i.e., richness and evenness), there are few widely accepted ways to quantify rarity (Gaston, 1994). Often, somewhat arbitrary cutoffs are used (e.g., <0.1% of total recovery) to determine whether or not a taxon is considered rare. In this study, we made inferences about the putative contributions of rare bacteria to ecosystem activity by characterizing shifts in the rank abundance of taxa in response to rewetting. First, we determined the number of OTUs that were

present in both dry and rewetted soils (i.e., "shared"), along with the number of OTUs that were present in either the dry soils or rewetted soils (i.e., "unshared"). With this information, we defined a rare responder as a taxon that was below our detection limits in the dry sample, but recovered in the same experimental unit after rewetting. In addition to visualizing changes in relative recovery of OTUs with rank abundance curves, we tested for differences in the recovery of rare bacteria in the four ecosystems using One-Way ANOVA and Tukey's HSD tests. Last, taxonomic trends of rare responders in some of the major phyla and classes were shown in a heat map with hierarchal clustering using the *heatmap* function in the gplot package in R (Warnes et al., 2014).

## RESULTS

### PULSES OF ECOSYSTEM ACTIVITY

Microbial communities responded rapidly to soil rewetting and this resuscitation corresponded with pulses of ecosystem activity. In our field experiment, soil moisture increased in the agricultural site by at least 2.5-fold following each of the three simulated rainfalls. These rewetting events generated pulses of $CO_2$ that lasted more than 2 days (**Figure 1**). The autoregressive soil moisture model explained the majority of the observed variation in soil $CO_2$ concentrations ($R^2 = 0.83$). We found that $CO_2(t)$ was positively correlated with *moisture(t)* (23,292 ± 18.9 [mean ± SE], ppmv $CO_2$/cm$^3$ $H_2O$ cm$^{-3}$ soil, $t_{5.3} = 1232$, $P < 0.0001$), but was negatively correlated with *moisture(t − 1)* (−3949 ± 25.7 [mean ± SE], ppmv $CO_2$/cm$^3$ $H_2O$ cm$^{-3}$ soil, $t_{5.3} = -153.7$, $P < 0.0001$).

Similarly, rewetting altered trace gas production in the soil microcosm experiments conducted in the laboratory. Rewetting increased gravimetric soil moisture by a factor of five (≈0.05–0.25 g $H_2O$ g soil$^{-1}$). As a result, we observed up to a 20-fold increase in $CO_2$ production in rewetted soils compared to dry soils, irrespective of ecosystem type (Two-Way ANOVA, ecosystems × water treatment, $df = 3$, $F = 202$, $P = 0.001$, **Figure 2**). With the exception of soils from the agricultural site, $CH_4$ production was lower in rewetted soils than dry soils (Two-Way ANOVA, ecosystems × water treatment, $df = 3$, $F = 26.6$, $P = 0.002$, Supplemental Figure 1). Last, rewetting increased $N_2O$ production in grassland soils but decreased production in deciduous forest soils (Two-Way ANOVA ecosystems × water treatment, $df = 3$, $F = 10.3$, $P = 0.022$).

### STABLE ISOTOPE PROBING (SIP)

SIP was effective at distinguishing bacteria that resuscitated following soil rewetting. Based on the qPCR results, the dry treatment contained unlabeled DNA in fractions 12 and 13 with a buoyant density in CsTFA ranging from 1.531 to 1.548 g mL$^{-1}$, and the rewetted treatment contained $^{18}$O-labeled DNA in fractions 9 and 10 with buoyant density in CsTFA ranging from 1.574 to 1.585 g mL$^{-1}$ (Supplemental Figure 2). Thus, rewetting led to a 0.026–0.054 g mL$^{-1}$ increase in the buoyant density of $^{18}$O-labeled bacterial DNA. We used the DNA in fractions 12 and 13 to represent the bacterial communities in dry soil conditions and the DNA in fractions 9 and 10 to represent the bacterial communities in the rewetted soils.

### BACTERIAL COMMUNITY RESPONSES TO SOIL REWETTING

Across all ecosystems, rewetting had strong effects on bacterial community composition. This inference was based on the recovery of 29,931 quality sequences and 9256 unique OTUs (BioProject ID: PRJNA269181, http://www.ncbi.nlm.nih.gov/bioproject/). One of the 24 samples (a replicate from the deciduous forest) was not included in our analyses due to a large proportion of low quality sequences. Prior to rewetting, PLS-DA results demonstrated that bacterial communities from

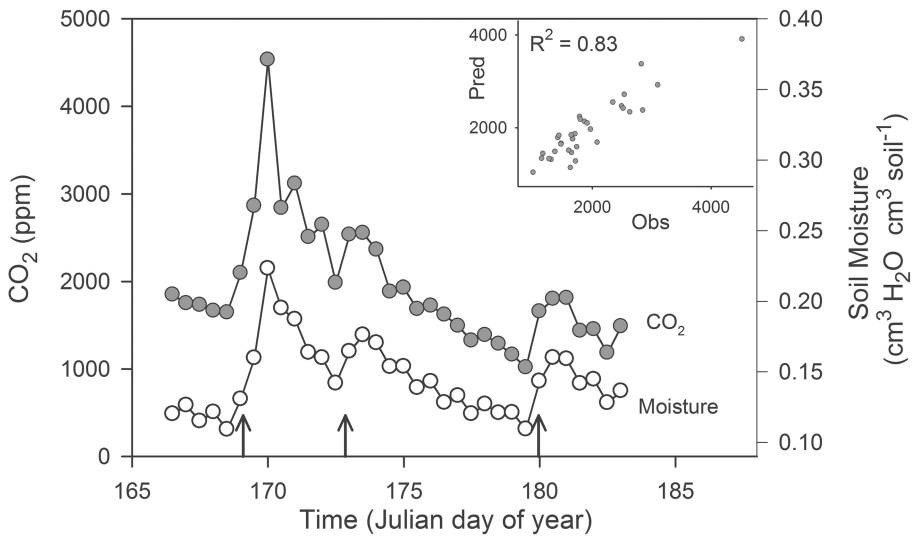


**FIGURE 1 | Pulses of ecosystem activity in an agricultural ecosystem stimulated by experimental rain events (upward pointing arrows).** The inset panel is a plot of the observed (Obs) and predicted (Pred) $CO_2$ generated from a multiple regression model. We estimated soil $CO_2$ concentrations (2 cm depth) and soil moisture (0–5 cm) using real-time sensor data averaged on a 12 h time-step from 15 June 2007 (day 166) through 3 July 2007 (day 184).

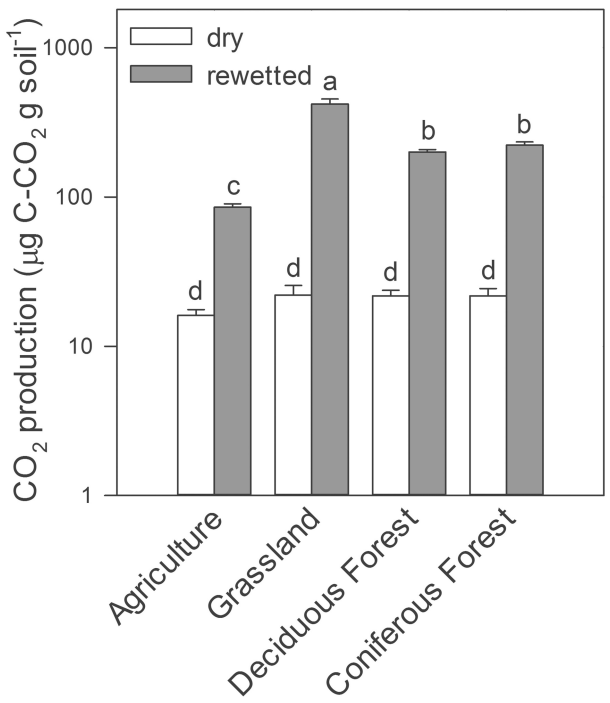


**FIGURE 2 | $CO_2$ production from laboratory microcosms with dry and rewetted soils obtained from four ecosystem types.** Values are means ± SEM ($n = 3$) with letters indicating differences ($P < 0.05$) based on a Two-Way ANOVA and Tukey's HSD test.

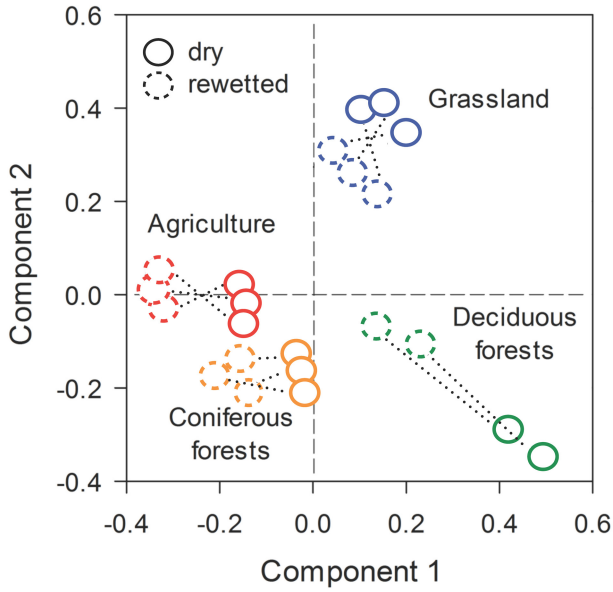


**FIGURE 3 | Stable isotope probing (SIP) revealed rapid shifts in bacterial community composition in soils that were rewetted with heavy water ($H_2^{18}O$).** The multivariate ordination was generated using Partial Least Squares Discriminant Analysis (PLS-DA) on a sample × OTU matrix that took into account ecosystem type and pairs of dry-rewetted samples (indicated by dashed lines).

the dry soils separated in ordination space based on ecosystem type. After rewetting, bacterial communities retained a signature of the ecosystem from which they were derived, but were separated in ordination space relative to the dry conditions (**Figure 3**). PERMANOVA results supported these interpretations: both ecosystem type ($P = 0.008$) and rewetting ($P = 0.002$) had a strong effect on bacterial composition. There was a marginally significant ecosystem × rewetting interaction on composition ($P = 0.08$), suggesting that deciduous forest communities may have been more responsive than bacterial communities from the other ecosystems (see **Figure 3**).

The significant effects of rewetting were associated with rapid turnover in bacterial composition. Based on Bray-Curtis pairwise comparisons, bacterial composition diverged by 65–74% during the rewetting period. Turnover was slightly higher in the deciduous forest and grassland sites than soil bacteria from the CF (One-Way ANOVA, $df = 7$, $F = 5.20$, $P = 0.03$, **Figure 3**). Despite large and rapid shifts in composition, rewetting did not affect bacterial richness within an ecosystem (Supplemental Figure 3).

## RESUSCITATION OF RARE BACTERIA

Our results indicate that the rewetting of dry soil resuscitated rare bacterial taxa. Irrespective of ecosystem type, a large fraction (69–74%) of the OTUs recovered after rewetting was not detected from the paired sample under dry conditions (**Figure 4**). Some of the taxa that responded to rewetting were also recovered in the initial, dry samples (26–31%). Within this shared pool, 45–55% of the OTUs were comprised of singletons and doubletons in dry soils, lending further support to the view that pulses of ecosystem activity were associated with the resuscitation of rare taxa.

The resuscitation of rare taxa suggests that the dominance structure of soil bacterial communities may be highly dynamic. This view is supported by large shifts in the rank abundance distributions of rare taxa across ecosystem types (**Figure 5**). In each ecosystem, hundreds of rare taxa, which were below detection limits in the dry soils, increased in recovery and rank after rewetting. Together, these rare OTUs comprised 48–59% of the sequences that were recovered in the rewetted samples. The contribution of rare OTUs in the rewetted samples varied among ecosystems. There was higher recovery of rare responders in grassland and deciduous forests than agriculture sites and CF communities (One-Way ANOVA, $df = 3, F = 7.66, P = 0.01$). Across all ecosystems, 13 rare OTUs became dominant members (≥1% recovery) of rewetted communities and were repeatedly ranked in the top 11 taxa of the different communities. Of these rare responders, most (85%) were Proteobacteria, with 38% belonging to the Alphaproteobacteria family Sphingomonadaceae; 23% belonging to the Betaproteobacteria family Comamonadaceae; and 15% belonging to the Betaproteobacteria family Oxalobacteraceae. Despite larger responses of the aforementioned taxa, rare bacteria were recovered in all of the major phyla and classes found in our samples. However, the response of these coarse taxonomic groups to rewetting was ecosystem-specific. The recovery of rare Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, and Gemmatimonadetes varied among the four ecosystems

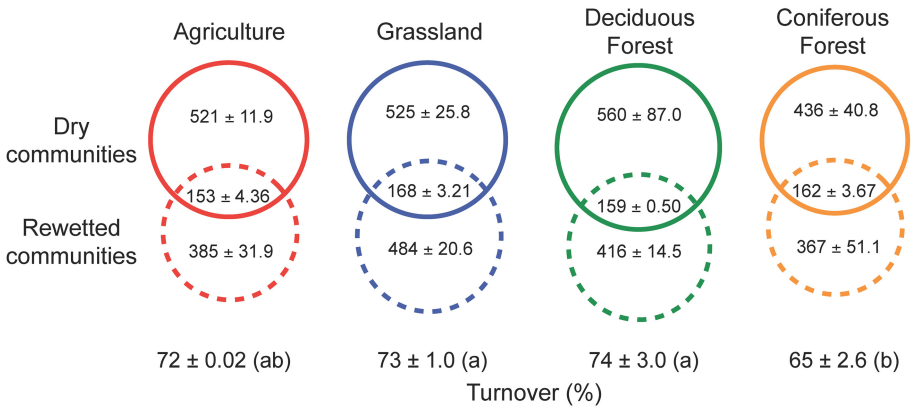


**FIGURE 4 | Resuscitation of rare bacteria contributed to shifts in bacterial composition following the rewetting of soils with heavy water ($H_2^{18}O$).** High rates of turnover (estimated using Bray-Curtis dissimilarity) were due in large part to the recovery of rare OTUs that were below detection limits in the sampling of dry soils. The numbers of rare OTUs (means ± SEM) are presented in dashed solid circles, while the shared OTUs occurring in both dry and rewetted soils are present in the intersection of the dashed and solid circles. OTU numbers are based on the observed number of OTUs present after rarefaction by sequence number. Different letters indicate significant differences ($P < 0.05$) based on a One-Way ANOVA and Tukey's HSD test.

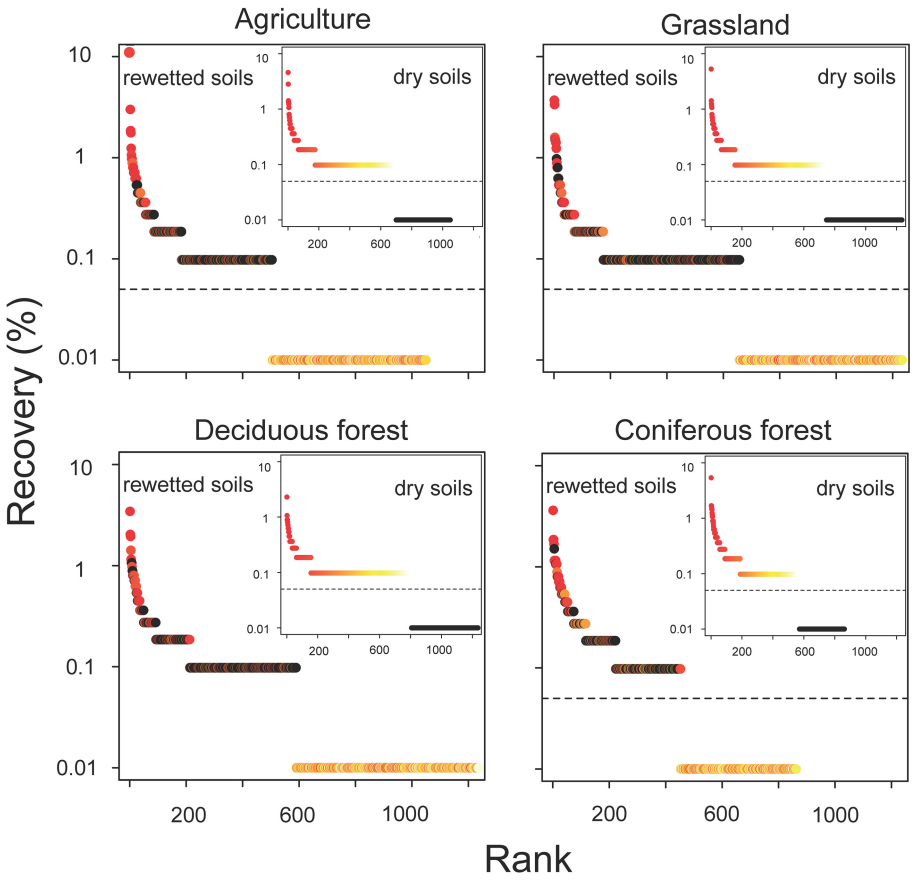


**FIGURE 5 | Resuscitation resulted in dynamic shifts in the rank abundance of rare bacteria in rewetted soils.** OTUs are color-coded based on their ranked recovery in the dry soils (inset panel): red taxa had the highest recovery, yellow the lowest, and black were below detection in the initial (dry) sample. OTUs with ranks falling below the dashed horizontal line were below detection limits.

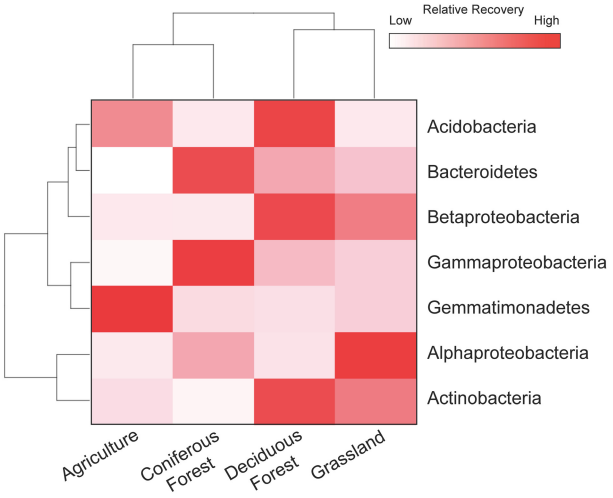


**FIGURE 6 | Rare bacteria demonstrated ecosystem-specific responses to rewetting.** Heat map showing the distribution of rare OTUs for four phyla and three Proteobacteria subclasses that contributed ≥ 1% to the total recovery of rewetted communities. Values are based on means with hierarchal clustering of ecosystem (bottom) and phylum (left).

(**Figure 6**). For example, the recovery of rare Betaproteobacteria was at least two-fold higher in grasslands and deciduous forests than the two other ecosystems and Gemmatimonadetes were 2.2-times higher in agricultural sites than the three other ecosystems.

## DISCUSSION

Results from our stable isotope probing (SIP) allowed us to identify a diverse array of fast-growing soil bacteria that were associated with pulses of ecosystem activity. A large fraction of these bacteria (69–74%) consisted of rare taxa, which accounted for 60% of the 16S rRNA reads in rewetted soil samples. Our findings suggest that rare taxa are important for the maintenance of soil bacterial diversity and that the resuscitation of these taxa from seed banks contributes significantly to soil processes like $CO_2$, $CH_4$, and $N_2O$ production.

### REWETTING AND PULSES OF ECOSYSTEM ACTIVITY

Using field manipulations and laboratory microcosm experiments, we observed that dry soils generated large pulses of ecosystem activity when they were rewetted. The $CO_2$ pulses in our field study were ephemeral and closely tracked soil moisture dynamics during drying and rewetting (**Figure 1**), while microcosm experiments revealed that $CO_2$, $CH_4$, and $N_2O$ production were strongly influenced by rewetting (Supplemental Figure 1). Moisture-mediated pulses of ecosystem activity have been observed in a variety of habitats and are a well-recognized phenomenon in soil science (Birch, 1964; Fierer and Schimel, 2003; Lee et al., 2004; Jenerette et al., 2008). Rewetting is thought to stimulate microbial activity via two primary mechanisms. First, increases in soil moisture release microorganisms from desiccation stress (Lennon et al., 2012). Second, microorganisms encounter high concentrations of resources during rewetting events. Although rewetting may enhance the availability of substrates within the soil matrix and make protected soil organic matter more accessible, many studies suggest that resuscitated microorganisms are consuming the cellular constituents of other microorganisms (Fierer and Schimel, 2003; Xiang et al., 2008). For example, it is well documented from work with isolates that some microorganisms produce and accumulate osmolytes as an adaptive response to desiccation stress (Csonka, 1989). In nature where soil moisture is dynamic, compatible solutes (e.g., proline, glycine, betaine) need to be disposed of to maintain osmotic equilibrium. It has been argued that the release of microbial osmolytes during rewetting may be responsible, at least in part, for pulses of ecosystem activity observed terrestrial ecosystems (Schimel et al., 2007, but see Boot et al., 2013). Regardless of the exact mechanisms, it is well estalished that at least some microorganisms undergo rapid transitions from low to high activity when dry soils are rewetted, and that these changes in metabolism have consequences for ecosystem proceses.

### RARE BACTERIA CONTRIBUTED TO PULSES OF ECOSYSTEM ACTIVITY

Our results revealed that rare bacterial taxa contributed to pulses of ecosystem activity following rewetting. In this study, we conservatively classified rare taxa based on the detection limits of our sequencing. Specifically, if a taxon was recovered in a rewetted sample, but not in the paired dry sample then we considered it rare. Based on this logic, we found that rare bacteria comprised 69–74% of taxa and nearly 60% of the 16S rRNA gene sequences in rewetted communities, irrespective of the ecosystem sampled. Many of the sequences recovered from our soil samples likely came from heterotrophic microorganisms. We assume that when these bacteria became labeled with $^{18}O$ they generated $CO_2$ as a byproduct of both anabolic and catabolic processes. A much smaller fraction of the bacteria that responded to rewetting were recovered in the initial dry sample (26–31%). Of these shared taxa, approximately 50% were represented in the dry sample by either singletons or doubletons, which lends further support to the view that pulses of ecosystem activity were associated with the resuscitation of rare taxa.

Over the past decade, there has been considerable interest in the "rare biosphere" (Sogin et al., 2006; Hugoni et al., 2013; Logares et al., 2014). Most scientists agree that rare taxa are important for cataloging biodiversity, but it is less clear whether or not they are important for ecosystem processes. For example, the core microbiome refers to a collection of organisms that are consistently encountered among similar habitats, and therefore is thought to be essential for carrying out vital processes (Shade and Handelsman, 2012). Rare taxa have a lower probability of being considered part of the core microbiome, and as a result, it is hypothesized that these taxa may contribute minimally to ecosystem processes (Pedrós-Alió, 2012). For example, a taxon may be rare if by chance it disperses into a local community from a large pool of regional species (Pedrós-Alió, 2006). In this case, an organism may find itself in an environment for which it is not particularly well adapted. It is predicted that these transient microbes will have low rates of metabolism, and thus contribute minimally to ecosystem functioning (Pedrós-Alió, 2012). There are also "resident" rare taxa, which may have different ecological strategies and metabolic profiles. Some rare taxa may be consistently active,

but have very slow growth rates (Hugoni et al., 2013). Other groups of taxa may be conditionally rare with the potential to rapidly respond to environmental change through shifts in physiology (Shade et al., 2014), including resuscitation from dormancy (Lennon and Jones, 2011).

Our findings are consistent with the view that rare species perform essential functions in an ecosystem. It has long been recognized that the removal of some rare taxa can have a large effect on ecosystem processes (Paine, 1966). For example, in a recent meta-analysis of macroscopic organisms (i.e., coral reef fishes, alpine plants, and tropical trees), it was shown that functional trait diversity could largely be attributed to rare species (Mouillot et al., 2013). In microbial systems, the direct manipulation of rare taxa via dilution has been shown to affect soil processes, including the establishment of pathogens (van Elsas et al., 2012) and rates of nitrogen cycling (Philippot et al., 2013).

#### THE IDENTITY OF RARE RESPONDERS

Based on the design of our SIP experiment, the bacteria responding to rewetting can be viewed as fast-growing taxa. Many of these bacteria were initially rare but became dominant members of the community following rewetting. Taxa belonging to the Sphingomonadaceae (Alphaproteobacteria) were one such group of bacteria that responded to rewetting. Many representatives of the Sphingomonadaceae are aerobic, heterotrophs (Reddy and Garcia-Pichel, 2007; Kyselková et al., 2009) that exhibit extreme metabolic versatility as evidenced by their ability to use organic substrates ranging from glucose to aromatic hydrocarbons (Alonso-Gutiérrez et al., 2009; Xie et al., 2011; Regonne et al., 2013). Previous studies in a Mediterranean grassland also documented that the sphingomonads are responsive to soil rewetting events (Placella et al., 2012). In addition, some members of the Betaproteobacteria responded to rewetting. For example, the Comamonadaceae and Oxalobacteraceae are root- and rhizosphere-associated bacteria (Ofek et al., 2012; Dibbern et al., 2014) that are generally recognized as fast-growing organisms. Together, representatives of these families accounted for 38% of the $^{18}$O-lableed taxa in our study. Last, our results suggest that a few fast-growing taxa were potentially stimulated by microbial byproducts generated during soil rewetting (i.e., methane). For example, as methane declined in rewetted soils (Supplementary Figure 1), we observed an increase in the recovery of taxa belonging to the Methylocystaceae (Alphaproteobacteria), which are known methanotrophs (Gulledge et al., 2001).

Several phyla and classes of bacteria exhibited ecosystem-specific responses to soil rewetting. For example, the recovery of rare Gemmatimonadetes was higher in agricultural sites than the other ecosystems investigated in this study. Gemmatimonadetes are abundant in ecosystems that experience low levels of moisture and frequent soil drying (DeBruyn et al., 2011). The lack of irrigation in our agricultural sites combined with the high rates of evapotranspiration from the fields may have increased the relative recovery of these taxa. Also, the recovery of rare Betaproteobacteria was higher in grasslands and deciduous forests. These two ecosystems support diverse plant communities and high levels of primary productivity and Betaproteobacteria was possibly stimulated by the flush of photosynthate or the variety of root exudates accompanying rewetting (Fierer et al., 2007; Eilers et al., 2010).

Recent studies have demonstrated that $H_2^{18}$ O-DNA SIP can be an effective tool for linking microbial taxa to ecosystem processes that are influenced by moisture availability (Aanderud and Lennon, 2011; Adair and Schwartz, 2011; Woods et al., 2011). We assume that while bacteria were growing and incorporating $^{18}$O into their DNA, they were also contributing to the pulses of ecosystem activity that resulted from the rewetting. However, there are a number of important caveats that should be highlighted. First, some bacteria may have responded to rewetting but used $H_2^{18}O$ to meet catabolic maintenance energy demands to sustain existing cells (van Bodegom, 2007) or for the upregulation of cellular machinery for growth, such as RNA, ribosomes, and amino acids (Blazewicz et al., 2013). We would not expect to recover these taxa in our DNA-based analyses. Second, other soil organisms (e.g., archaea, fungi, and nematodes) could have contributed to the observed pulses of ecosystem activity, but our PCR primers did not capture the response of these taxa to rewetting. Last, not all bacteria that were recovered with our sequencing contributed to the pulses of ecosystem activity that we measured. For example, chemolithoautotrophic bacteria do not produce $CO_2$ as a byproduct of their metabolism. Therefore, our $H_2^{18}O$ DNA-SIP captured some of the rare bacteria that *did not* contribute to processes, but also missed other microorganisms that *did* contribute to processes.

#### DYNAMIC RANK ABUNDANCE DISTRIBUTIONS

In addition to linking rare bacteria to pulses of ecosystem activity, our results provide insight into how these taxa might contribute to the maintenance of biodiversity. All else being equal, rare species have a higher probability of going extinct (locally or globally) than common species (Lawton et al., 1994). However, there are some advantages to being rare, such as reduced risk of predation and parasitism, especially for asexually reproducing organisms like most bacteria (Pedrós-Alió, 2006). Previous work has shown that common bacteria were comprised largely of dormant individuals, while rare taxa were disproportionately more active (Jones and Lennon, 2010). Although based on a snapshot in time, these findings suggest that transitions into and out of dormancy could lead to dynamic rank abundance distributions (Lennon and Jones, 2011). The rare biosphere most likely contains both dormant taxa, as well as active but slow-growing taxa. As such, it is important to emphasize that the rare biosphere is not synonymous with a seed bank. Being rare does not necessarily imply dormancy, just as being abundant does not necessarily imply high metabolic rates. Our results revealed that many (but not all; see **Figure 4**) rare taxa with relatively low levels of metabolic activity were capable of responding to an environmental cue (e.g., moisture). This view is consistent with recent findings, which report that many habitats (e.g., air, skin, oceans, gut, etc.) are comprised of conditionally rare taxa (Shade et al., 2014). In other words, stochastic or predictable changes in the environment may cause large changes in the relative abundance of microbial taxa in space or time.

The results from the current study provide support for the notion of a dynamic microbial rank distribution. Dry soils were

comprised of bacteria that on an aggregate level had low levels of metabolic activity. We observed a high degree of compositional turnover (65–74%) in just a few days. This pattern could be attributed to the resuscitation of rare OTUs that were below detection in the largely dormant dry soil. By this definition, approximately 60% of all sequences from the rewetted soils could be attributed to rare taxa. Hundreds of bacterial taxa were not only metabolically resuscitated, but also reproduced fast enough to become dominant members of the community (**Figure 5**). However, the temporal resolution of our data only allows us to speculate about the persistent effects of moisture-mediated resuscitation on bacterial community composition. For example, it is possible that rewetting only created ephemeral "blooms" of fast growing bacteria. Tracking taxa through repeated drying and rewetting cycles would provide a test of whether or not resuscitated bacteria retain a high rank or if they fall back into the tail of the rank abundance distribution. In addition, future studies could evaluate the importance of deterministic vs. stochastic processes that influence bacterial responses to rewetting. Our findings suggest that bacteria within an ecosystem type responded similarly to changes in soil moisture (**Figure 3**), but it is unclear whether or not there is long-term coherence in the relative abundance of microbial taxa following environmental change. In sum, our study suggests that rare bacteria may not be just transient members of the community; at least in some cases, these taxa are recruited into dominant roles due to environmental fluctuations as they exit dormancy (see Shade et al., 2014).

## CONCLUSION

Shifts in environmental cues can affect the dormancy of microbial communities, specifically member of the rare biosphere, in ways that maintain biodiversity and influence ecosystem processes. Rewetting stimulated the growth of rare bacteria, which increased their rank abundance, and contributed to ecosystem processes disproportionately to their recovery in dry soils. Thus, rewetting provides evidence that rapid changes in environmental conditions may cause dynamic shifts in rank abundance among bacteria and helps maintain the high levels of biodiversity in soils. Owing to contributions of rare species to essential ecosystem processes, more attention needs to be directed toward understanding microbial seed banks and the functional importance of the rare biosphere.

## AUTHOR CONTRIBUTIONS

Zachary Aanderud and Jay Lennon designed the study. Zachary Aanderud, Jay Lennon and Noah Fierer conducted the experiments. Zachary Aanderud, Jay Lennon, Noah Fierer, and Stuart Jones analyzed and interpreted the data, helped write and review the manuscript. Both Zachary Aanderud and Jay Lennon agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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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.2015.00024/abstract

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# Isolating the effects of storm events on arctic aquatic bacteria: temperature, nutrients, and community composition as controls on bacterial productivity

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Storm events can pulse nutrients and carbon from soils and provide an important subsidy to food webs in oligotrophic streams and lakes. Bacterial nutrient limitation and the potential response of stream aquatic bacteria to storm events was investigated in arctic tundra environments by manipulating both water temperature and inorganic nutrient concentrations in short (up to 4 days) and long duration (up to 2 weeks) laboratory mesocosm experiments. Inorganic N and P additions increased bacterial production ($^{14}$C-labeled leucine uptake) up to seven times over controls, and warmer incubation temperatures increased the speed of this response to added nutrients. Bacterial cell numbers also increased in response to temperature and nutrient additions with cell-specific carbon uptake initially increasing and then declining after 2 days. Bacterial community composition (BCC; determined by means of 16S denaturing gradient gel electrophoresis fingerprinting) shifted rapidly in response to changes in incubation temperature and the addition of nutrients, within 2 days in some cases. While the bacteria in these habitats responded to nutrient additions with rapid changes in productivity and community composition, water temperature controlled the speed of the metabolic response and affected the resultant change in bacterial community structure, constraining the potential responses to pulsed nutrient subsidies associated with storm events. In all cases, at higher nutrient levels and temperatures the effect of initial BCC on bacterial activity was muted, suggesting a consistent, robust interaction of temperature, and nutrients controlling activity in these aquatic systems.

**Keywords: aquatic, arctic, bacterial production, diversity, experiment, nutrients, 16S rRNA, temperature**

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## Introduction

Nutrient limitation of bacteria occurs in a wide variety of aquatic habitats including wetlands, rivers, lakes, and marine habitats (Morris and Lewis, 1992; Mohamed et al., 1998; Waiser, 2001; Castillo et al., 2003; Kuosa and Kaartokallio, 2003; Granéli et al., 2004). Bacterial growth in freshwater habitats of arctic Alaska is likely to be nutrient limited because of low nutrient supply, but bacteria in these environments must also contend with low temperatures that may limit bacterial growth (White et al., 1991; Panzenbock et al., 2000) and interact with nutrient

limitation. For example, the bacterial response to nutrients has been linked to seasonal variations in temperature, and the degree of nutrient limitation can vary with season and water temperature (Hall et al., 2009; Hoikkala et al., 2009). Additionally, direct testing of temperature and nutrient effects often indicates co-limitation by these factors (Wiebe et al., 1992; Pomeroy and Wiebe, 2001; Vrede, 2005; Mindl et al., 2007; Säwström et al., 2007).

In the Arctic, pulses of nutrients flushed from soils during storm events act as important subsidies to oligotrophic lakes and streams (Stieglitz et al., 2003). Because bacteria are limited in their ability to retain nutrients (Vadstein, 2000), pulsed nutrient supply can suspend bacterial nutrient limitation at least for the duration of the pulse. Rapid changes in temperature, nutrients, and the quality and quantity of organic matter associated with storm pulses may limit the ability of bacterial communities to shift to an optimal activity for a given resource supply when environmental variability is on the same time scale as their growth rate. Therefore, examining the effects of these pulses on bacteria on the time scale of storm events may provide a mechanistic understanding of the interaction of temperature and nutrient limitation in any aquatic habitat that experiences pulsed nutrient supply (e.g., storm events). In addition, separating the individual influences of temperature and nutrients from their interactive effect is required to fully examine the impacts of these drivers on bacterial activity and composition in natural habitats.

Bacterial communities contain populations with different metabolic capabilities and thus different potential responses to changing temperature and nutrients. Shifts in community composition occur as populations change in dominance in response to different optimal conditions or differential mortality. For example, several investigators have found correlations between bacterial community composition (BCC) and resource supply in natural habitats (Pearce, 2005; Yannarell and Triplett, 2005; Xing and Kong, 2007). Previously rare populations can increase in abundance in response to a new substrate (Szabo et al., 2007; Nelson and Carlson, 2011; Crump et al., 2012) and the now altered community may be able to access different substrates and may have different nutrient requirements, affecting both community structure and function. What has not yet been determined is the interaction of temperature and nutrients with bacterial community structure in natural habitats. Individually, warmer temperatures and increased nutrient concentrations can increase bacterial productivity (White et al., 1991; Ram and Sime-Ngando, 2008) and, potentially, select for communities that can reproduce fastest under those conditions. However, in highly variable environments, bacterial communities may be constrained to a short-term physiological response, particularly if temperature and nutrients select for different bacterial populations, resulting in a relatively static community because populations lack the time to respond.

An initial observational study of bacterial production (BP) during storm events indicated covariance of several potential drivers of activity such as temperature and nutrients. Thus, in this study, we conducted experiments with natural bacterial communities to isolate the influence of temperature and nutrient supply on bacterial activity, growth rate, and community structure. We hypothesized that productivity of bacterial communities in ultra-oligotrophic arctic streams and lakes would be elevated mainly by temperature or nutrients based on the natural environmental characteristics (e.g., DOM, temperature, and nutrients) the communities usually experience. We anticipated that communities from sites with high quality algal organic matter and more constant temperature, such as lake outlets, would be less nutrient limited and more strongly affected by temperature. Conversely, communities at sites with low quality terrestrial organic matter and more variable temperatures, such as headwater streams, would respond more strongly to nutrients than temperature. In all cases we found that nutrient treatments approximating maximum natural concentrations had a larger impact on BP than did elevated temperature, although warmer incubation temperatures increased the speed of this response to added nutrients; this suggests a robust interaction of temperature and nutrients controlling bacterial activity in these aquatic systems.

## Materials and Methods

### Study Site

Sites are located on the north slope of the Brooks Range, Alaska, at the Toolik Field Station (68°38′N, 149°36′W). Samples were collected from the inlet and outlet of lakes I-8 and Toolik. Toolik Lake is a multi-basin lake, draining a catchment of 66.9 $km^2$, and has a single outlet. Two kilometers upstream of the main Toolik inlet stream is an 18-ha lake, Lake I-8, which has a large headwater stream inlet, I-8 inlet, and a single outlet, I-8 outlet (**Figure 1**).

For summers 2003–2007, average water temperatures were 9.4 and 12.3°C for Lake I-8 inlet and outlet, respectively, and 11.3 and 13.8°C for Toolik inlet and outlet, respectively (Adams et al., 2010). All of the lakes in the Toolik Lake catchment are oligotrophic, with mean primary productivity of ∼3.2 μmol C/L/day and mean chlorophyll *a* (chl *a*) concentrations of ∼1.0 μg/L (Kling et al., 2000). I-8 outlet had consistently greater summer concentrations of chl *a* than the I-8 inlet (average of 1.0 μg/L versus 0.31 μg/L, respectively; Supplementary Table S1). Similarly, Toolik Lake, and thus Toolik outlet, had a greater chl *a* concentration than Toolik inlet (average of 1.36 μg/L versus 0.55 μg/L; Kling et al., 2000). All sites had low average concentrations of $NH_4$ (<0.8 μM) and $PO_4$ (<0.08 μM); however, storm-related pulses of higher concentration and loading did occur (Supplementary Table S1). Both I-8 inlet and Toolik inlet had higher mean concentrations of $NO_3$ than I-8 outlet and Toolik outlet (Supplementary Table S1). There are frequently 2–3 storm events during the summer season, which begins after snow-melt runoff in May.

### Field Measurements

In order to detect patterns of bacterial response to natural variations in temperature and nutrient concentrations, temperature, dissolved organic carbon (DOC), inorganic nutrients, and BP were measured weekly in Lake I-8 inlet, I-8 outlet, and Toolik inlet from approximately June 15 to August 20, 2003–2007, and

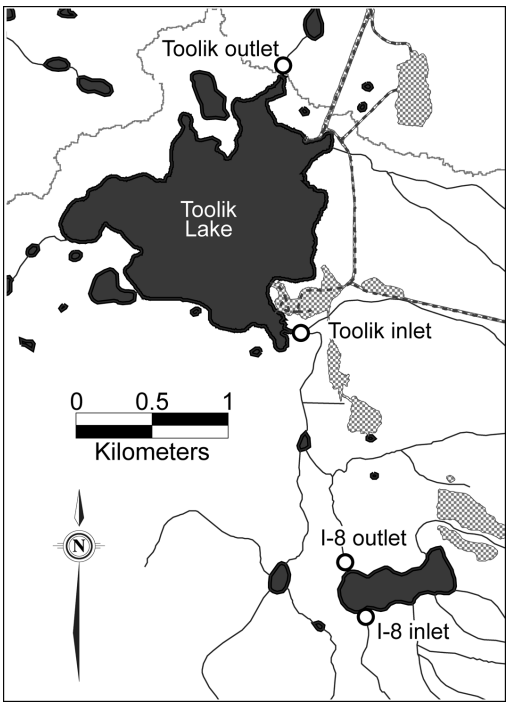


**FIGURE 1 | Sampling locations at Lake I-8 and Toolik Lake, Alaska.** Hatched areas are gravel pads, dashed lines are roads, black lines are streams, and the gray line is the northern edge of the watershed of Toolik Lake.

was measured five times in Toolik outlet in summer 2004. Chl *a* was sampled 3–21 times at each site (2003–2007) and DOM measurements of Ultraviolet (UV) absorbance, protein, and phenolics were measured at all sites starting in 2004 (through 2007). Temperature at I-8 inlet (2005–2006) and I-8 outlet (2004–2006) was measured continuously during summer with Onset HOBO temperature loggers (Bourne, MA, USA). Stream discharge and temperature were monitored in Toolik inlet using a Stevens PGIII Pulse Generator (Portland, OR, USA) and a Campbell Scientific Model 247 conductivity and temperature probe (Logan, UT, USA) connected to a Campbell Scientific CR510 datalogger. Temperature at all sites, including Toolik outlet, was also measured during sample collection with a digital thermometer (Fisherbrand Traceable; Thermo Fisher Scientific, Waltham, MA, USA).

Dissolved organic carbon concentration was measured in water collected in the field and immediately filtered through GF/F filters (Whatman, GE Healthcare Life Sciences, Pittsburgh, PA, USA) and acidified to pH ∼3.5 and kept cold and dark until analysis on a Shimadzu TOC-5000 instrument (Columbia, SC, USA) using high-temperature, platinum-catalyzed combustion to $CO_2$ and infrared detection. UV absorbance of DOM was measured on unfiltered samples using a quartz cell with a 5 cm path length on a Shimadzu 1601-UV scanning spectrophotometer in the wavelength range of 220 to 400 nm, total proteins were measured using a modified Bradford Reagent method (Bradford, 1976), and total phenolics were measured using the Folin Ciocalteu assay (Waterman and Mole, 1994) and comparing samples to humic acid standards. Chl *a* concentration was determined on GF/F filters and corrected for phaeophytin following Kling et al. (2000).

Inorganic nutrient concentrations were measured in water samples filtered through ashed (450°C, 4 h) GF/F filters (Whatman) upon collection and stored in the dark at 4°C ($NH_4$ and $PO_4$) or frozen ($NO_3$) until analysis. Ammonium concentrations were determined within 48 h using a fluorometric OPA method modified from Holmes et al. (1999), and phosphate concentrations were determined within 48 h spectrophotometrically using the molybdenum ascorbic acid assay (Murphy and Riley, 1962). Frozen nitrate samples were analyzed on an Alpkem Flow system 3000 Autoanalyzer (Alpkem, Saskatoon, SK, Canada, now OI Analytical, College Station, TX, USA) using flow injection with a cadmium reduction coil method modified from Armstrong et al. (1967).

Bacterial production was measured using $^{14}C$ labeled-leucine uptake following Kirchman (1994) assuming an isotopic dilution of 1 resulting in a conversion factor of 1.55 kg C (mol leu)$^{-1}$. Each measure was calculated from the incubation with $^{14}C$ leucine of three unfiltered 10 mL subsamples, and one 10 mL control killed with trichloroacetic acid (TCA), for ∼3 h before ending with 5% TCA (final concentration). Samples were filtered onto 0.2 μm nitro-cellulose filters, extracted using ice-cold 5% TCA, placed in scintillation vials, dissolved using ethylene glycol monoethyl ether, flooded with Scintisafe scintillation cocktail and counted on a liquid scintillation counter (Packard Tri-Carb 2100TR; Perkin Elmer, Waltham, MA, USA).

## Mesocosm Experiments

Experiments were conducted to test the response of BP and community composition to enhanced nutrient concentrations typical of storm events under different temperature conditions. All experiments used temperature treatments that matched summer mean (12°C) and high (17°C) water temperatures. One experiment tested the response of bacteria to low-level nutrient additions over a 4-day period using nutrient concentrations similar to average natural concentrations measured in Toolik inlet during storm events. A second experiment tested the response of bacteria to higher levels of nutrients for up to 2 weeks using nutrient concentrations similar to the maximum natural concentrations measured in Toolik inlet (Supplementary Tables S2 and S3). This high-level nutrient experiment was repeated six more times. In four of these experiments the source of bacterial communities and incubation water was varied. In two experiments the source of bacterial communities was varied.

Each experiment was a factorial design of manipulated temperature (12 and 17°C) and nutrients with an inoculum of natural bacterial communities (Supplementary Tables S2 and S3). For all experiments, triplicate incubations of each of the four treatments (12 incubations per experiment) were initiated within 4 h of water collection, and all contained by volume 10% of 1.0 μm filtered water (bacterial inoculum) and 90% of 0.2 μm filtered water collected concurrently. All mesocosms were incubated in the dark (to exclude photosynthesis) in incubators or water baths set to treatment temperature ±1°C. Starting volumes for each experiment varied from 1 to 3 L (Supplementary Table S3) due to the

logistical constraints of transporting large volumes of water to the field station. Experiments were conducted in plastic containers that were acid-washed and rinsed with 0.2 μm filtered sample water. Most experiments were conducted in 4 L LDPE cubitainers (Thermo Fisher Scientific) except Experiments 4a,b, which were conducted in 1 L HDPE bottles (Nalgene, Rochester, NY, USA).

### Experiment 1

The experiment conducted with low-level nutrient addition used water and bacteria from Toolik inlet collected on June 22, 2007. This experiment, which was conducted last in our sequence of experiments, was used to determine if there was a threshold of response to added nutrients, and to track the responses of bacterial activity and BCC to temperature and nutrient treatments. Bacterial communities in Toolik inlet represent mixed communities of bacteria from headwater streams and lakes in the Toolik watershed, including nearby Lake I-8 (Crump et al., 2012). Inorganic nutrients were added to nutrient treatments to achieve the average concentration typically observed in Toolik inlet during a storm event (1.5 μM $NH_4NO_3$ and 0.25 μM $KH_2PO_4$; **Figure 2**; Supplementary Table S1). BP was measured in all replicates and treatments at approximately 0, 2, 4, 6, 8, 10, 14, 21, 26, 32, 39, and 49 h, and BP was also measured in the 12°C treatments at 60, 72, 83, and 98 h. Samples for DNA and cell counts were collected at 26 and 49 h and the 12°C treatments were also sampled at 72 and 98 h. Sampling of the 17°C treatments was discontinued after BP stopped increasing.

### Experiment 2

One experiment with high-level nutrient addition used water and bacteria from Lake I-8 inlet starting on June 27, 2006. This experiment was conducted to track the response of bacterial activity and community composition at different temperatures to the maximum nutrient concentrations typically observed in Toolik inlet during storm events. Inorganic nutrients were added to nutrient treatments to achieve these concentrations (6.4 μM $NH_4NO_3$ and 0.45 μM $KH_2PO_4$). BP was measured at 2, 4, 6, 9, 11, and 14 days, and samples for DNA and cell abundance were collected at 2, 4, 9, and 14 days.

### Experiments 3a–d

Four high-level nutrient experiments were conducted varying the source of bacterial communities and incubation water starting on July 12, 2005 to examine community-specific responses (that is, the effect of different initial community compositions). These experiments were performed with bacteria and water from Lake I-8 inlet and Lake I-8 outlet in factorial combination. Bacterial communities from Lake I-8 inlet are headwater stream communities with no contribution of bacteria from lakes, whereas communities from Lake I-8 outlet are lake communities (Crump et al., 2007). BP was measured at 2, 4, 6, 9, 12, and 14 days, and samples for DNA and cell abundance were collected at 14 days.

### Experiments 4a,b

Two high-level nutrient experiments were conducted varying the source of bacterial communities starting on July 18, 2006 to confirm the changes in BCC identified in Experiments 3a–d. One experiment was performed with bacteria and water from Lake I-8 inlet, and the other with bacteria and water from Lake I-8 outlet. Samples for DNA and cell counts were collected at 6 and 11 days. BP was not measured.

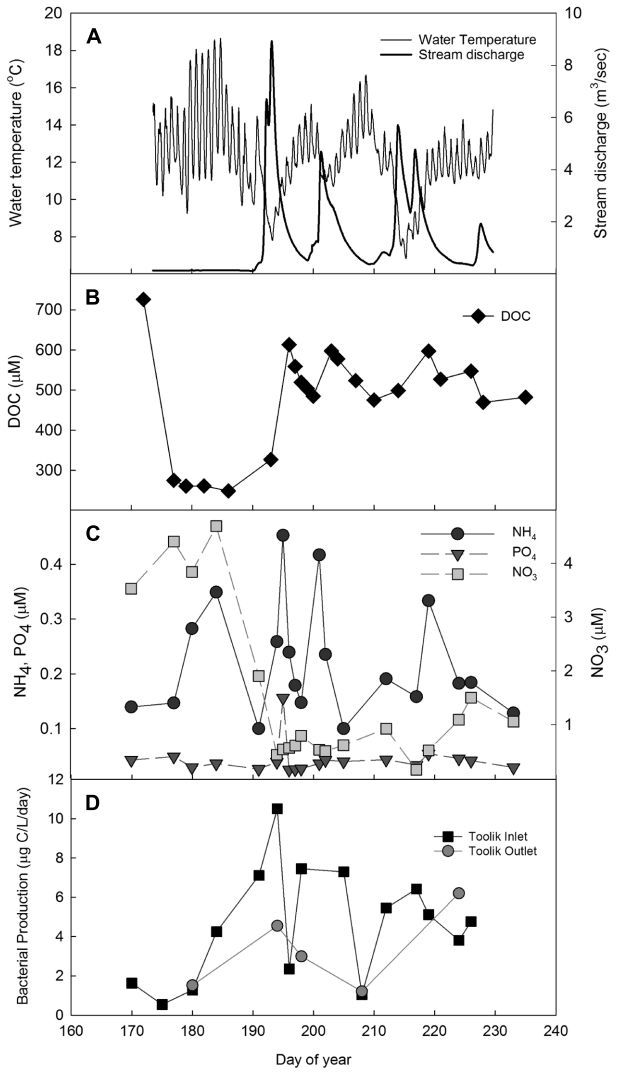


**FIGURE 2 | Toolik inlet stream during summer 2004 from June 8 to August 27. (A)** Temperature (thin line) and stream discharge (thick line); **(B)** DOC (♦); **(C)** $NH_4$ (●), $PO_4$ (▼), and $NO_3$ (■); **(D)** BP at Toolik inlet (■) and outlet (●).

## Bacterial Abundance and Community Composition Analyses

Samples for cell counts were preserved with 2.5% of glutaraldehyde (final concentration) and stored at 4°C until analysis. Samples from 2005 were counted on a FACSCalibur (BD Biosciences, San Jose, CA, USA) flow cytometer following del Giorgio et al. (1996). Sub-samples were stained with SYBR green (Life Technologies, Grand Island, NY, USA) in the dark for a minimum of 15 min (Marie et al., 1997; Lebaron et al., 1998). The concentration of beads in the standard 1 μm bead solution

and concentration of cells in multiple confirmatory samples were measured by epifluorescence microscopy. Samples from 2006 and 2007 were counted on a LSR II flow cytometer (BD Biosciences) as described by Ewart et al. (2008) with data acquired in log mode for at least 60 s and until 20,000 events were recorded, with the minimum green fluorescence (channel 200) set as the threshold.

DNA samples were collected from laboratory mesocosms by filtering ∼500 mL of sample through a Sterivex-GP 0.2 μm filter (EMD Millipore, Billerica, MA, USA). Filters were preserved using a DNA extraction buffer as described by Crump et al. (2003) and stored at −80°C until extraction. DNA was extracted using phenol–chloroform (Crump et al., 2003, 2007) and PCR amplified using 357f with a G-C clamp and 519r universal 16S rDNA bacterial primers on a Bio-Rad thermocycler (Hercules, CA, USA) following (Crump et al., 2003, 2007). DNA was then separated using denaturing gradient gel electrophoresis (DGGE) with an 8% acrylamide gel cast with either a 40 to 60% or 35 to 55% gradient of urea and formamide (Crump et al., 2003, 2007). Gels were run on a CBS scientific DGGE system (Del Mar, CA, USA) for 18 to 24 h at 75 volts and 65°C. A DGGE ladder, previously constructed from PCR-amplified clones of 16S rRNA genes from Toolik Lake (Crump et al., 2003) was run every six lanes in order to accurately assess the vertical position of bands across each gel.

Imaging of DGGE banding patterns was performed with Quantity One software (Bio-Rad) on a Chemi-Doc gel documentation system (Bio-Rad), gel bands were identified using GelCompar II software (Bionumerics, Applied Maths, Austin, TX, USA) to create a presence–absence matrix as described by Crump and Hobbie (2005). DGGE is capable of detecting bacterioplankton populations that make up at least 0.1 to 0.4% of bacterioplankton in a sample, depending on copy number of rRNA operons per cell and PCR primer specificity (Muyzer et al., 1993; Kan et al., 2006). Each band represents an operational taxonomic unit (OTU) of bacteria, although occasionally multiple sequences may be present within a band (Crump et al., 2003, 2004) or bacteria may differ in a more variable region of the 16S gene; therefore, changes detected here are considered to be a conservative index of shifts of community composition.

## Statistical Analyses

Pairwise similarity values of the DGGE bands were calculated using the Dice equation in order to condense presence–absence data into percent community similarities between samples. PROXCAL was used to create non-metric multidimensional scaling (NMDS) graphs of sample similarities. Two-way, between-subjects ANOVA were performed in which percent similarity between samples was designated as the dependent variable with categorical dummy variables indicating the same or different treatment types of incubation temperature or nutrient addition as predictors. Both normal distribution of data and homogeneity of variance were verified using a Shapiro–Wilk test with data log-transformed where necessary. On the two datasets that did not meet ANOVA assumptions following data transformations (temperature and nutrients on day 1), the non-parametric Kruskal–Wallace test was performed to verify the significance of ANOVA results (**Table 1**). All statistical analyses were performed with SPSS (version 17, IBM, Armonk, NY, USA).

# Results

## Field Measurements: Storm Events, Nutrients, and Bacterial Production

During the summer of 2004, there were three large storm events characterized by rain and subsequent increases in stream discharge ($>4$ m$^3$/s at Toolik inlet; **Figure 2A**). The events occurred on 9–15 July, 18–24 July, and 30 July – 7 August (two combined events). Ammonium concentrations at Toolik inlet spiked either at the initiation of or immediately after each of the three storm events, while phosphate concentrations increased only immediately after the first event; nitrate concentrations were diluted during these events (**Figure 2C**). At Toolik inlet, peaks in BP corresponded with the occurrence of the three storm events (**Figure 2D**). There was also a small increase of BP at the outlet of the lake after the first storm event.

## Mesocosm Experiments – Bacterial Activity

BP was elevated by increased temperature and nutrients in all mesocosm experiments, but the timing and magnitude of these treatment responses varied with the concentration of nutrients and, when tested, the initial composition of the bacterial community. When a relatively low level of nutrients was added to water from Toolik inlet (Experiment 1), the bacteria grew more rapidly in the high temperature (17°C) incubations than the

**TABLE 1 | Experiment 1.**

| Dependent variable: community similarity | | | | | |
|---|---|---|---|---|---|
| | | Significance (*p*-value) | | | |
| | df | 1 day | 2 days | 3 days | 4 days |
| Corrected model | 3 | 0.051 | 0.000 | 0.001 | 0.020 |
| Intercept | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| Temperature | 1 | 0.437* | 0.002 | | |
| Nutrients | 1 | 0.747* | 0.001 | 0.001 | 0.020 |
| Temperature * nutrients | 1 | 0.006 | 0.163 | | |
| | | Degrees of freedom | | | |
| Error | | 62 | 62 | 13 | 13 |
| Total | | 66 | 66 | 15 | 15 |
| Corrected total | | 65 | 65 | 14 | 14 |

*R Squared = 0.12 (Adjusted R Squared = 0.08) 1 day*
*R Squared = 0.25 (Adjusted R Squared = 0.21) 2 days*
*R Squared = 0.59 (Adjusted R Squared = 0.56) 3 days*
*R Squared = 0.35 (Adjusted R Squared = 0.30) 4 days*
*Results of the tests of significance (ANOVA) for the impact of incubation temperature and low-level nutrient addition on the % similarity of bacterial community composition (BCC) between samples in Experiment 1 at Toolik inlet. The p-value for the main effects (temperature and nutrients) and the interaction term is given for each day of the experiment; days 3 and 4 are for low-temperature treatments only. A Kruskal–Wallace test for day 1 data (* in Table) confirmed non-significance with $p = 0.907$ for temperature and $p = 0.969$ for nutrients. The overall model R-squared is given for each day of the experiment.*

low temperature (12°C) incubations regardless of nutrient treatment. For example, the 17°C treatments had ∼10-fold higher BP (**Figure 3A**) and cell-specific carbon uptake (**Figure 3C**) after 1 day in both nutrient treatment and control incubations. At 17°C, nutrient treatment increased BP by 1.8 times over the control by day 2, but at 12°C this treatment effect was much greater, increasing BP by 6.1 times over the control by day 3. Similarly, at 12°C, the cell-specific carbon uptake continued to increase with the nutrient addition when allowed to respond longer than 2 days compared to the 17°C incubation response at days 1 and 2. Peak BP was similar at both temperatures in the fertilized treatments, but was elevated at 17°C in the unfertilized treatment. The number of bacterial cells increased roughly exponentially in all treatments after an initial decrease (**Figure 3B**).

When a higher level of nutrients was added to water from Lake I-8 inlet (Experiment 2), the treatments with added nutrients grew more rapidly than unfertilized controls regardless of temperature (**Figure 4**), showing elevated BP and cell-specific carbon uptake after 2 days at both temperatures (**Figures 4A,C**). At both temperatures, high-level nutrient treatment increased BP by 7 times over controls, although this treatment effect was observed more rapidly at 17°C (2 days) than at 12°C (4 days). Cell-specific carbon uptake peaked after 2 days in all treatments, similar to Experiment 1 with low-level nutrient treatment, and

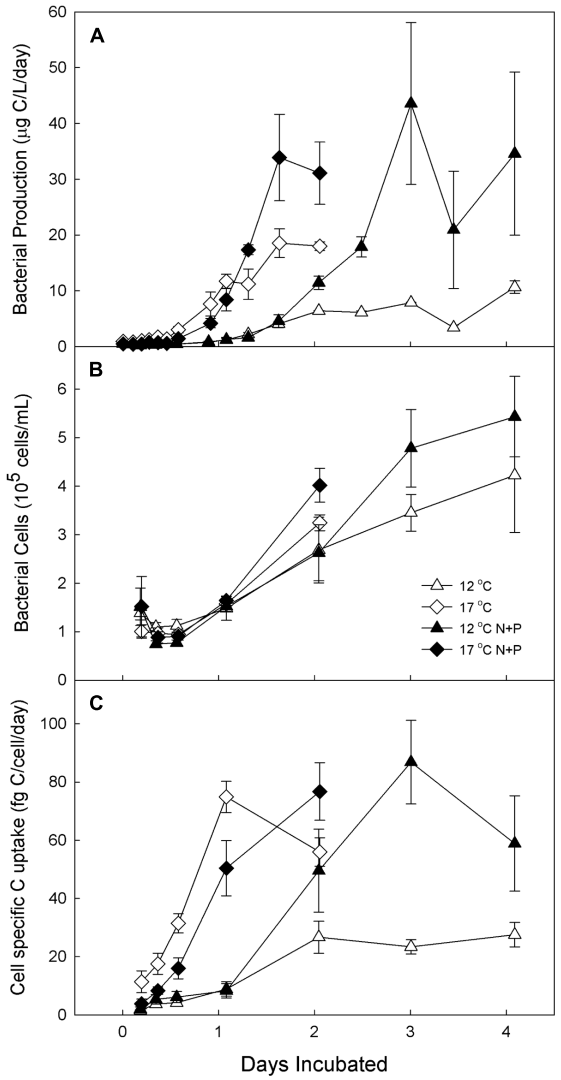


**FIGURE 3 | Experiment 1.** Bacterial production **(A)**, cell abundance **(B)**, and cell-specific carbon uptake **(C)** in water from Toolik inlet incubated for 4 days at 12 and 17°C with and without low-level nutrient amendments. Error bars are SE of the mean of experimental replicates ($n = 3$). Samples are designated by incubation temperature (▲ = 12°C and ♦ = 17°C) with open symbols for no nutrients added and closed symbols for nutrients added.

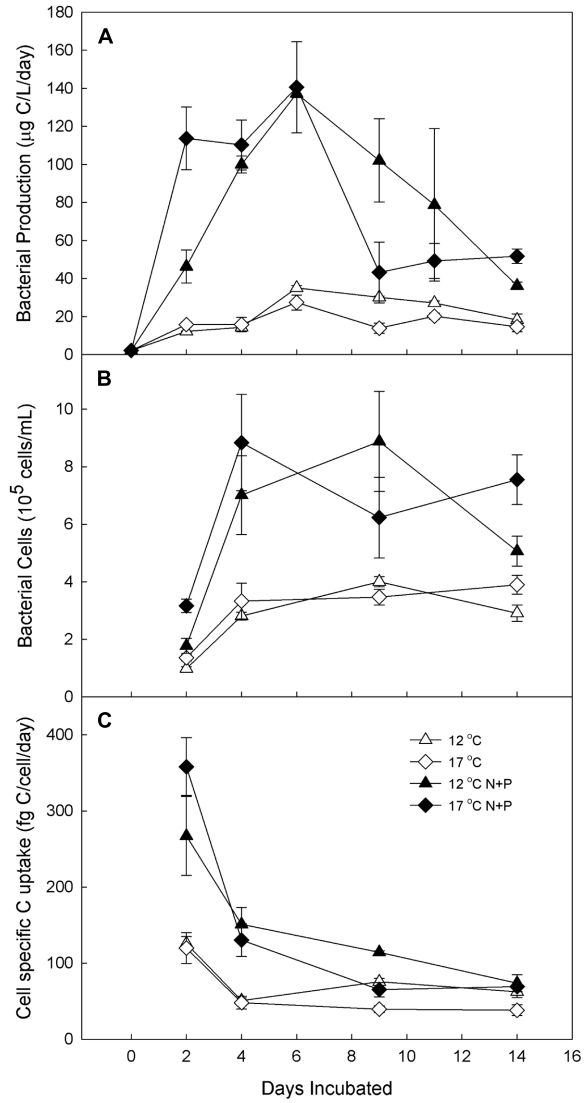


**FIGURE 4 | Experiment 2.** Bacterial production **(A)**, cell abundance **(B)**, and cell-specific carbon uptake **(C)** in water from Lake I-8 inlet incubated for 2 weeks at 12 and 17°C with and without high-level nutrient amendments. Error bars are SE of the mean of experimental replicates ($n = 3$). Samples are designated by incubation temperature (▲ = 12°C and ♦ = 17°C) with open symbols for no nutrients added and closed symbols for nutrients added.

then decreased during the extended incubation period due to increased cell abundances and decreased BP. Peak BP was the same for both temperatures in the fertilized treatment, but was elevated at 17°C in the unfertilized controls. Cell abundance, BP, and cell-specific carbon uptake in the fertilized treatments were greater than unfertilized controls and were greater in this experiment compared to those in the low-level nutrient treatments described above. In both experiments (high-level and low-level nutrients), elevated temperatures increased the speed of the BP response to nutrient additions (**Figures 3** and **4**) and increased the maximum rate of BP in unfertilized controls, but did not greatly change the maximum rate of BP in fertilized treatments.

This high-level nutrient experiment was repeated with two sources of water and two sources of bacterial communities (Experiments 3a–d) to test the influence of initial BCC and initial water chemistry on treatment responses to temperature and fertilization. Water chemistry was different at the two sites used for these experiments, Lake I-8 inlet and Lake I-8 outlet. Water at Lake I-8 inlet contained 706 μM DOC, 0.76 mg protein $L^{-1}$, 1.16 μM total phenolics, and 0.09 μg chl *a* $L^{-1}$, and had a UV absorbance of 154.3 (scanning from 220 to 400 nm, 5 cm quartz cell). Water at Lake I-8 outlet had lower concentrations of dissolved organics and a much higher concentration of chl *a* (514 μM DOC, 0.57 mg protein $L^{-1}$, 0.65 μM total phenolics, 0.77 μg chl *a* $L^{-1}$, UV absorbance of 98.9). BCC at the two sites also differed, with 50% community similarity at the time of initial collection. The treatment response of BP to fertilization was similar regardless of the source of incubation water or the source of bacterial inocula, increasing more rapidly in the 17°C treatment but reaching approximately the same maximum productivity at both temperatures in all experiments after 4 days (**Figure 5**). However, in the unfertilized experiments, the response of BP to source waters and temperature was different for the two bacterial communities. The bacterial community from the inlet had elevated BP after 2 days, but the bacterial community from the outlet had lower BP after 2 days; this rate remained low, particularly when the outlet community was incubated in "unfamiliar" water from the inlet stream.

## Mesocosm Experiments – Communities

Bacterial community composition shifted quickly during regrowth following initial dilution in the experimental mesocosms, and the composition of these communities varied with treatment. After 1 day bacterial communities in the experiment with low-level nutrient additions (Experiment 1) were not significantly different among treatments, but by day 2 a two-way ANOVA of percent similarity between bacterial communities in different treatments indicated that both temperature

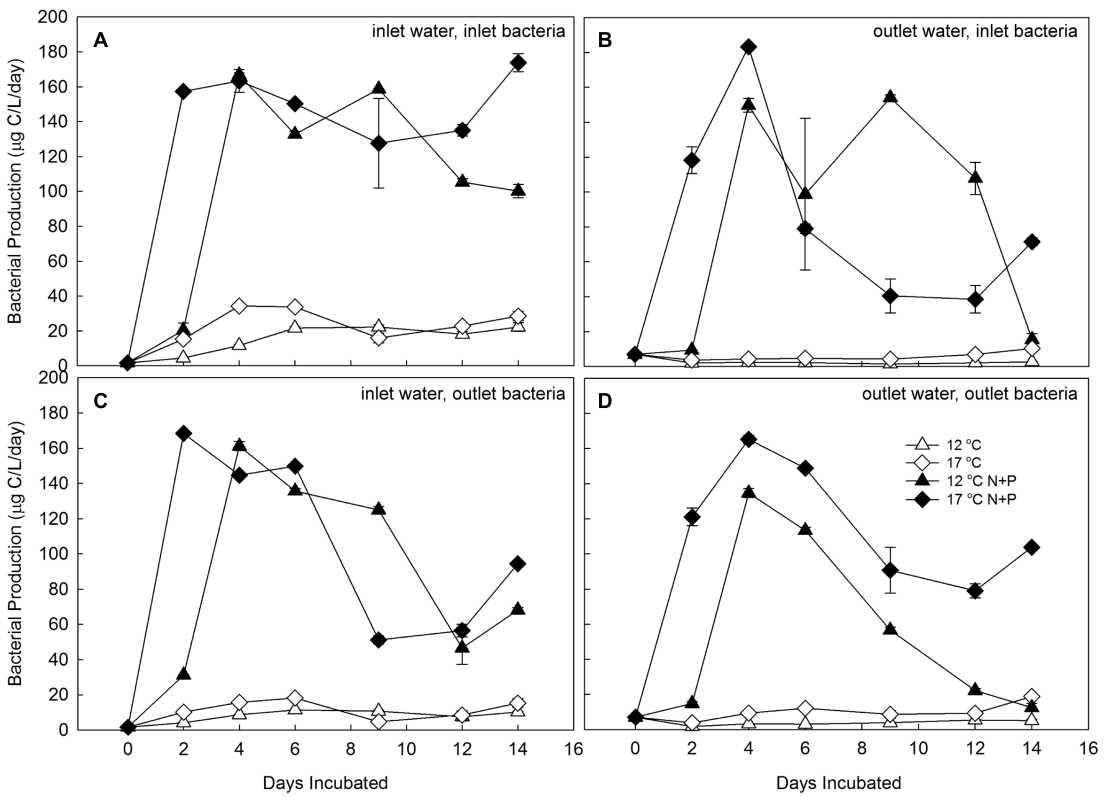


**FIGURE 5 | Experiment 3.** Bacterial production in water from Lake I-8 inlet **(A,C)** and Lake I-8 outlet **(B,D)** inoculated with bacterial communities from Lake I-8 inlet **(A,B)** and Lake I-8 outlet **(C,D)** and incubated for 2 weeks at 12° and 17°C with and without high-level nutrient amendments. Error bars are SE of the mean calculated from analytical replicates ($n = 3$). Samples are designated by incubation temperature (▲ = 12°C and ♦ = 17°C) with open symbols for no nutrients added and closed symbols for nutrients added.

and nutrient addition were statistically significant indicators of percent similarity (**Table 1**). The pairwise similarities between fertilized and unfertilized treatments at 12°C declined steadily from 85–76 to 68–64% over the 4 days (**Figure 6**; Supplementary Figure S1), compared to the relatively constant and high pairwise similarities within replicates of the same treatment over the same time period (95, 82, 92, 93%, days 1–4).

Similar patterns in BCC were found in the experiment with high-level nutrient additions to water from Lake I-8 inlet (Experiment 2). BCC clustered by nutrients and temperature in the NMDS analysis for days 2, 4, 9, and 14 (**Figure 7**; Supplementary Figure S1). Nutrients and temperature were statistically significant predictors of community similarity for all time points during the incubation, but the interaction term between temperature and nutrients ceased to be significant on day 14 (**Table 2**). As in the experiment with low level nutrient additions, the average pairwise similarities between fertilized and unfertilized treatments at 12°C declined over time from 79–71 to 54–59% at 2, 4, 9, and 14 days, respectively, while pairwise similarities within replicates of each treatment remained similar (92, 82, 88, 81% over the same time period). At 17°C there was also a substantial change in BCC when nutrients were added (mean difference between controls and nutrient additions of 18 ± 3.6%, SE), but unlike at 12°C there was little change over time. The shift in BCC at 17°C occurred very rapidly, and within 2 days the pairwise similarity between fertilized and unfertilized treatments was only 69%, and this value remained steady over the 14 days experiment (69, 67, 64, 67% at 2, 4, 9, and 14 days, respectively; **Figure 7**; Supplementary Figure S1).

Replicate high-level nutrient experiments tested the influence of initial BCC and initial water chemistry on BCC response to nutrient and temperature treatments. BCC was assessed after 14 days during the four experiments (Experiments 3a–d) conducted with bacteria and water from Lake I-8 inlet and Lake I-8 outlet in factorial combination. In these experiments, the initial bacterial community was the only statistically significant predictor of community similarity at the end of the experiment (**Table 3**), but nutrient addition was nearly significant ($p = 0.058$) and the interaction term between initial bacterial community and nutrients was significant ($p = 0.007$). For the inlet community (Experiments 4a,b), a two-way ANOVA indicated that temperature and nutrients were both statistically significant predictors

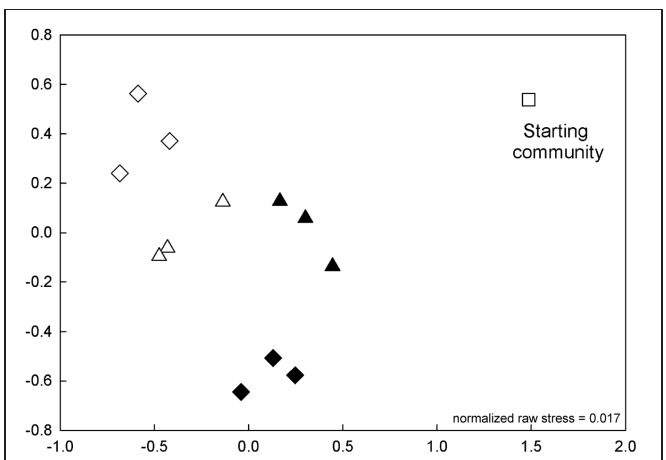


**FIGURE 6 | Experiment 1.** Non-metric multi-dimensional scaling (NMDS) plot of community similarity on collection (starting community, day 0) and day 2 of the low-level nutrient experiment. The bacterial community collected from collection site is designated by □. Samples are designated by incubation temperature (▲ = 12°C and ♦ = 17°C) with open symbols for no nutrients added and closed symbols for nutrients added.

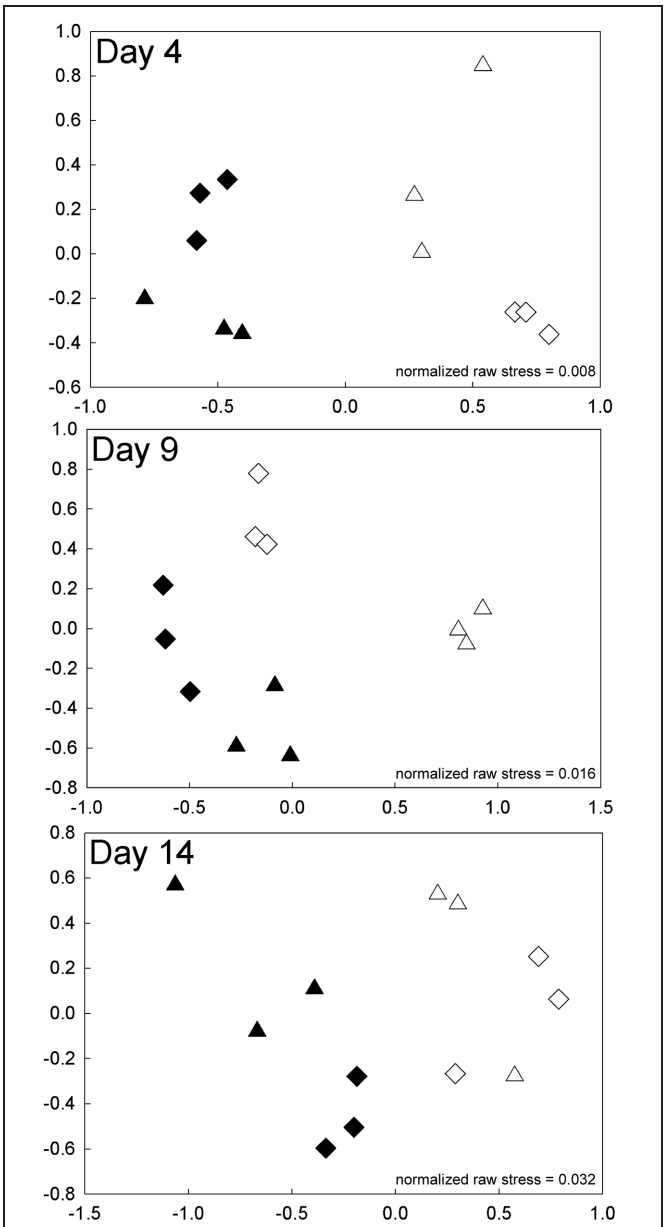


**FIGURE 7 | Experiment 2.** NMDS plots of bacterial community composition on days 4, 9, and 14 of the high-level nutrient experiment. Samples are designated by incubation temperature (▲ = 12°C and ♦ = 17°C) with open symbols for no nutrients added and closed symbols for nutrients added.

**TABLE 2 | Experiment 2.**

| Dependent variable: community similarity | | | | | |
|---|---|---|---|---|---|
| | | Significance (*p*-value) | | | |
| | df | 2 days | 4 days | 9 days | 14 days |
| Corrected model | 3 | 0.000 | 0.000 | 0.000 | 0.000 |
| Intercept | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| Temperature | 1 | 0.000 | 0.022 | 0.000 | 0.002 |
| Nutrients | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| Temperature * nutrients | 1 | 0.001 | 0.001 | 0.003 | 0.210 |
| Error | 62 | | | | |
| Total | 66 | | | | |
| Corrected total | 65 | | | | |

*R Squared = 0.63 (Adjusted R Squared = 0.61) 2 days*
*R Squared = 0.62 (Adjusted R Squared = 0.60) 4 days*
*R Squared = 0.53 (Adjusted R Squared = 0.51) 9 days*
*R Squared = 0.41 (Adjusted R Squared = 0.38) 14 days*
*Results of the tests of significance (ANOVA) for the impact of incubation temperature and high-level nutrient addition on the % similarity of BCC between samples in Experiment 2 at Lake I-8 inlet. The p-value for the main effects (temperature and nutrients) and the interaction term is given for each day of the experiment. The overall model R-squared is given for each day of the experiment.*

**TABLE 3 | Experiments 3a–d.**

| Dependent variable: community similarity | | |
|---|---|---|
| | df | Significance (*p*-value) |
| Corrected model | 14 | 0.008 |
| Intercept | 1 | 0.000 |
| Bacteria source | 1 | 0.000 |
| Incubation water source | 1 | 0.813 |
| Temperature | 1 | 0.182 |
| Nutrients | 1 | 0.058 |
| Starting community * nutrients | 1 | 0.007 |
| Error | 105 | |
| Total | 120 | |
| Corrected total | 119 | |

*R Squared = 0.24 (Adjusted R Squared = 0.14)*
*Results of the tests of significance (ANOVA) for similarity of bacterial community composition on day 14 of four replicate high-level nutrient experiments in which the source of bacteria and incubation water was varied. The p-value for the main effects (bacteria source, water source, temperature, and nutrients) and the interaction term is given, along with the overall model R-squared.*

**TABLE 4 | Experiments 4a,b.**

| Dependent variable: community similarity | | | | | |
|---|---|---|---|---|---|
| | | Significance (*p*-value) | | | |
| | df | inlet, 6 days | inlet, 11 days | outlet, 6 days | outlet, 11 days |
| Corrected model | 3 | 0.000 | 0.000 | 0.000 | 0.049 |
| Intercept | 1 | 0.000 | 0.000 | 0.000 | 0.000 |
| Temperature | 1 | 0.000 | 0.001 | 0.217* | 0.088 |
| Nutrients | 1 | 0.000 | 0.000 | 0.000* | 0.037 |
| Temperature * nutrients | 1 | 0.070 | 0.163 | 0.622 | 0.097 |
| | | Degrees of freedom | | | |
| Error df | | 51 | 42 | 62 | 41 |
| Total df | | 55 | 46 | 66 | 45 |
| Corrected total df | | 54 | 45 | 65 | 44 |

*R Squared = 0.44 (Adjusted R Squared = 0.41) inlet 6 days*
*R Squared = 0.66 (Adjusted R Squared = 0.64) outlet 6 days*
*R Squared = 0.49 (Adjusted R Squared = 0.46) inlet 11 days*
*R Squared = 0.18 (Adjusted R Squared = 0.12) outlet 11 days*
*Results of the tests of significance (ANOVA) for similarity of bacterial community composition after 6 and 11 days in two replicate high-level nutrient experiments conducted with bacteria and water from Lake I-8 inlet and Lake I-8 outlet. A Kruskal–Wallace test for outlet day 6 data (* in Table) confirmed non-significance with p = 0.619 for temperature and significance of p = 0.000 for nutrients. The p-value for the main effects (temperature and nutrients) and the interaction term is given for day 6 and day 11, along with the overall model R-squared for these days.*

of the similarity of community composition between treatments, with no significant interaction terms (**Table 4**). A comparable analysis of the outlet BCC similarities indicated that only nutrient addition was a significant predictor of BCC similarity between samples.

## Discussion

Storm events can cause dramatic changes in arctic stream temperature and flushing of soil nutrients and DOC into streams (e.g., Kling et al., 2014), and these changes directly impact the growth and composition of bacterial communities (e.g., Judd et al., 2006). In this study we demonstrate that temperature affects the speed of response to nutrient subsidies, and show how bacterial communities respond to the individual and combined effect of these two drivers. Observations of BP at Toolik inlet showed that stream discharge, DOC, nutrient concentrations, and BP all changed in response to summer storm events (**Figure 2**). Water temperature was inversely related to stream discharge but did not appear to strongly constrain bacterial response to substrate additions of DOC and nutrients washed in from soil water at these temperatures, as has been previously observed (Adams et al., 2010). DOC also increased with peaks in discharge, but after an initial threefold increase with the first event, concentrations later in the summer varied by only ∼20% (**Figure 2**). Although the increases in DOC with storm events were lower later in the summer, bacteria could be sensitive to small variations in labile DOC supply particularly if storms also brought in pulses of ammonium from soil waters (**Figure 2**, day ∼220) that enable the bacteria to access previously unavailable carbon (Harder and Dijkhuizen, 1983; Kirchman, 1994; Gasol et al., 2009). However, the general covariance of discharge, nutrients, and DOC along with an inverse relationship with water temperature during the storm events makes it difficult to identify the main control of bacterial activity in natural systems, requiring the isolation of these factors in experiments.

### Mesocosms – Bacterial Activity

Bacterial activity responded rapidly to added nutrients in all experiments indicating strong nutrient limitation of bacterial communities in the Toolik Lake region. High-level nutrient treatments approximating maximum natural levels had a larger

impact on BP than did elevated temperature. Low-level nutrient treatments also boosted BP, but the effect was limited compared to the effect of elevated temperature, which increased BP more quickly than did nutrients. While the high-level nutrient effect was observed across different bacterial communities collected at different times, it is possible that some of the response seen with the lower level of nutrients could be attributed to community-specific responses (as discussed below). This suggests that nutrient concentrations at the upper range of those found in the environment are required to overcome the restriction of low temperature on bacterial activity. For example, BP in high-level nutrient treatments reached similar magnitudes of activity regardless of temperature. This is in contrast with Vrede's (2005) finding that low temperatures superseded any other control, including P limitation. Temperature did control the cellular response to added nutrients in all of our experiments, with higher temperatures increasing the speed at which bacterial activity increased. This is likely due to the increased speed of biochemical reactions and higher affinity for substrates at warmer temperatures (Nedwell, 1999), and increased response to nutrients at higher temperatures has been observed in other studies (Mindl et al., 2007; Säwström et al., 2007). The faster response of BP to nutrients at higher temperature occurred regardless of sampling location, initial community composition, or DOM concentration and source, indicating that this temperature–nutrient interaction may be a robust feature controlling bacterial activity in many aquatic environments.

Several studies have identified interacting effects of temperature and substrate on heterotrophic bacterial growth (reviewed in Pomeroy and Wiebe, 2001), and in many of these studies the effect of substrate addition on growth rate was enhanced at low-temperature and minimal at high temperatures, presumably because of reduced substrate affinity at low temperatures (e.g., Wiebe et al., 1992). This pattern was detectable in our low-level nutrient experiment in which increases in BP and cell-specific carbon uptake due to nutrient addition were greater at 12°C than at 17°C. However, these patterns were not detectable in the high-level nutrient experiments (**Figures 4** and **5**), suggesting that storm-related nutrient pulses in arctic freshwaters must be of sufficient magnitude to overcome temperature limitation on bacterial growth and substrate affinity.

Cell numbers mirrored the corresponding BP measurements for all the experiments. For example, in the experiment with low-level nutrient addition, the cell-specific carbon uptake was more rapid at warmer temperatures, as found in other studies (White et al., 1991; Adams et al., 2010). In contrast, when greater amounts of nutrients were added, cell-specific carbon uptake was greater in fertilized treatments regardless of temperature. In the high-level nutrient experiments, cell-specific uptake was particularly high after 2 days and declined afterward once cell numbers reached a maximum. The same was not observed for the low-level nutrient experiment, possibly because the experiment was not extended until cell numbers reached a maximum or perhaps because of differences in initial community composition. Nevertheless, during the early phases of the experiments when cell numbers were still increasing, cell-specific carbon uptake reached a peak in all treatments and that peak was higher in incubations amended with nutrients.

Elevated cell-specific carbon uptake in nutrient treatments suggests elongation or growth in size of cells. Bacteria differ in size and shape by community and by growth stage (Lebaron et al., 2002). When nutrients are present, bacteria can delay cell division to take advantage of the resources and increase in size (Shiomi and Margolin, 2007). This interpretation is supported by observations in our experiments indicating a large number of long, filamentous bacteria appearing in the nutrient addition treatments. Apparently when large amounts of inorganic nutrients were added, both growth and reproduction of the filamentous portion of the bacterial community were stimulated, regardless of incubation temperature.

## Mesocosms – Communities

The fast response of bacteria to nutrient inputs was also observed in community dynamics. Both incubation temperature and nutrient addition changed community structure in as little as 2 days (**Tables 1** and **2**; **Figure 6**). The composition of communities created by nutrient addition steadily diverged from the controls in all experiments, especially in the 12°C treatments (Supplementary Figure S1). However, in the 17°C treatment BCC changed rapidly when nutrients were added, but then stayed similar over time. We interpret this to be a function of the higher rates of bacterial activity at warmer temperatures, which could lead to faster shifts in population or species dominance by the superior competitors under nutrient-enhanced conditions. This interpretation is supported by the elevated BP rate observed within 1–2 days in all experiments at 17°C compared to 12°C (**Figures 3** and **4**).

Despite the observed role of temperature in controlling the rate of change in BCC, under some conditions nutrients may be stronger drivers of community structure than temperature. This was demonstrated in the experiments using water and bacteria from I-8 inlet. This stream is not buffered by upstream lakes, and it had variable temperature and slightly higher nutrient concentrations than did I-8 outlet, which had more stable and higher temperatures than did I-8 inlet (Supplementary Tables S1 and S2). Communities from I-8 inlet responded to both temperature and nutrient additions (**Tables 2** and **4**), while communities from I-8 outlet responded to nutrients but not to temperature (**Table 4**). Bertoni et al. (2008) also found that shifts in BCC in response to nutrient additions were dependent on initial community composition, and this dependence may have reflected the *in situ* temperatures of different seasons. The different response of communities at the I-8 inlet and outlet to nutrient addition supports the hypothesis of community-specific nutrient limitation; in other words, communities that develop in separate, different habitats can respond uniquely to nutrient enrichment over time. These varied responses also suggest that natural site variability of temperature and nutrient concentrations are poor predictors of the stability of community composition in the face of rapid environmental change. Thus we suggest that community-specific responses to temperature and nutrients are not limited to BP and cell counts, but they also influence the stability of the community composition itself through competition between populations within the community.

Water temperature also impacts bacterial response to nutrients, particularly when these two factors are decoupled during storm events. As observed in the mesocosm experiments, colder temperatures can delay or diminish the response of bacterial activity to pulsed inorganic nutrients, and changing water temperature can shift bacterial communities to different populations than those stimulated by inorganic nutrients. Hall et al. (2009) also suggest that the bacterial response to temperature and nutrients changes seasonally, with summer and winter communities having different nutrient efficiencies relative to water temperature. According to this model, warm-adapted summer communities use nutrients more efficiently than winter communities. Here we show that the overall bacterial response to the interaction between temperature and nutrients is constrained by low temperatures, but that temperature constraints can be overcome by high levels of nutrients typical of storm-water pulses. We also show that population shifts resulting from differential responses to temperature or nutrients play an important role in the rapid shifts in community composition we observed.

## Conclusion

We demonstrate that aquatic bacteria in an arctic tundra environment can be nutrient limited, as predicted by theory, given that the processing of allochthonous, terrestrial carbon entering oligotrophic lakes, and streams requires additional nutrients for enzyme formation beyond regular cellular function. Experiments showed that inorganic nutrient additions and raised temperature increased bacterial productivity and growth rates rapidly, but nutrients above certain levels moderated the restrictive influence of low temperature on bacterial growth. Similarly, temperatures above certain levels drove very rapid shifts in BCC through the mechanism of enhanced activity accelerating the outcome of competition between species under new environmental conditions, such as altered nutrient concentrations during storm events. In addition, it appears that DOM characteristics (terrestrial versus algal, DOC concentrations) and initial environmental temperature and nutrient concentrations were poor predictors of the bacterial response to increased temperature and nutrients. We suggest that the resulting complex response to shifting temperature and nutrients occurs because different members of these communities are limited by different environmental factors. Consistent with this suggestion is our observation that shifts in community composition can occur very rapidly (∼2 days), and the resulting community can vary depending on the temperature and nutrient conditions during the pulse. A fast response of BP was also observed in the field following storm events during which discharge, ammonium, and BP peaked together. Bacterial communities in these habitats can respond rapidly to nutrient pulses through increased growth and shifting community composition, particularly at higher temperatures. Overall, these results suggest that under steady or slightly changing environmental conditions (e.g., temperature and nutrients), the initial BCC has a strong effect on the function of the community as measured by BP. As temperature or nutrient concentrations increase, BCC shifts rapidly but the influence of the initial community composition as a driver of community function diminishes, and the environmental controls on bacterial activity dominate. Thus the interaction between community composition (diversity) and function can shift rapidly as the environment changes, as exemplified by the often dramatic storm events that commonly affect aquatic ecosystems.

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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.2015.00250/abstract

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# Nitrogen addition, not initial phylogenetic diversity, increases litter decomposition by fungal communities

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Fungi play a critical role in the degradation of organic matter. Because different combinations of fungi result in different rates of decomposition, determining how climate change will affect microbial composition and function is fundamental to predicting future environments. Fungal response to global change is patterned by genetic relatedness, resulting in communities with comparatively low phylogenetic diversity (PD). This may have important implications for the functional capacity of disturbed communities if lineages sensitive to disturbance also contain unique traits important for litter decomposition. Here we tested the relationship between PD and decomposition rates. Leaf litter fungi were isolated from the field and deployed in microcosms as mock communities along a gradient of initial PD, while species richness was held constant. Replicate communities were subject to nitrogen fertilization comparable to anthropogenic deposition levels. Carbon mineralization rates were measured over the course of 66 days. We found that nitrogen fertilization increased cumulative respiration by 24.8%, and that differences in respiration between fertilized and ambient communities diminished over the course of the experiment. Initial PD failed to predict respiration rates or their change in response to nitrogen fertilization, and there was no correlation between community similarity and respiration rates. Last, we detected no phylogenetic signal in the contributions of individual isolates to respiration rates. Our results suggest that the degree to which PD predicts ecosystem function will depend on environmental context.

**Keywords: climate change, phylogenetic diversity, ecosystem function, fungi, leaf litter decomposition, microcosm, nitrogen fertilization**

## INTRODUCTION

The ubiquity and abundance of terrestrial fungi is indicative of their pivotal role in providing ecosystem services. It has been estimated that 1 g of soil contains as much as 200 m of fungal hyphae (Leake et al., 2004). In particular, fungi are key players in the degradation of dead plant material (litter), and are capable of breaking down complex carbon sources such as lignin, hemicellulose, and chitin (Lindahl et al., 2007; Allison et al., 2009). Soils contain roughly twice the carbon of either the atmospheric or vegetation pools (Batjes, 1996), and nutrients in the litter layer are presumably the most labile and rapidly cycled. Therefore, changes in litter decomposition rates are likely to have the most immediate impacts on carbon cycling (Cornelissen et al., 2007).

Recent empirical evidence suggests that differences in microbial community composition correlate with differences in community functioning. A handful of studies have examined this relationship using reciprocal transplants (Balser and Firestone, 2005; Strickland et al., 2009; Cleveland et al., 2013) or community filtering methods (Griffiths et al., 2000; Austin et al., 2006) and have found significant differences among community responses and process rates (but see Wertz et al., 2007). A few other studies have demonstrated a positive relationship between microbial species richness and community functioning by creating *de novo* assemblages of isolated microorganisms (Naeem et al., 2000; Bell et al., 2005). Presumably, the basis of this relationship is the positive correlation between the number of species and the variety of different, perhaps complementary, traits that contribute to a functional process.

In theory, a community spanning greater evolutionary history – i.e., encompassing higher phylogenetic diversity (PD) – ought to contain a greater number of non-redundant traits. Indeed, recent work suggests that phylogenetic relatedness among plant species is correlated with their trait similarity, leading to a positive relationship between a plant community's PD and its productivity (Cadotte et al., 2008; Flynn et al., 2011). Many microbial traits are phylogenetically patterned as well (McGuire et al., 2010; Treseder et al., 2011; Lennon et al., 2012; Martiny et al., 2013). In fact, a comparative genomic analysis demonstrated some phylogenetic conservatism for extracellular enzymes (Zimmerman et al., 2013) and glycoside hydrolases (Berlemont and Martiny, 2013), examples of traits that could directly influence litter decomposition rates. Similarly, the ability of leaf-decomposer fungi to metabolize various organic nitrogen compounds seems to be genetically correlated (McGuire et al., 2010). These results suggest that not only are leaf litter fungi functionally distinct, but that PD might be a better predictor of decomposition rate than taxonomic diversity in and of itself.

The relationship between microbial PD and ecosystem functioning is particularly important in light of global change. Many studies demonstrate that fungal communities are sensitive to

global change (Avis et al., 2008; Andrew and Lilleskov, 2009; Dang et al., 2009; Edwards et al., 2011; Edwards and Zak, 2011; Kerekes et al., 2013), and that once disturbed, microbial communities do not often rapidly recover to their original state (Allison and Martiny, 2008). Moreover, microbial response tends to be patterned by phylogeny, such that perturbed communities consist of more closely related species than would be expected by chance (Placella et al., 2012; Evans and Wallenstein, 2014). Specifically, drought and thermal tolerance appears to be phylogenetically patterned at the phylum level (Treseder et al., 2014).

Overall, fungal traits, including those involved in decomposition and in the response to changing environments, appear to be phylogenetically conserved. Thus, we first test the hypothesis that PD of the fungal species pool is positively correlated with community functioning, measured here as litter respiration. We combine 42 fungal species isolated from a natural litter ecosystem, spanning approximately 600 million years of evolutionary history, into a series of communities along a PD gradient. We hold initial species richness constant to control for portfolio effects as richer communities are more likely to contain better competitors (Tilman, 1999). We further hypothesize that PD, by increasing the breadth of a community's traits, will also buffer a community's sensitivity to environmental change. To test this hypothesis, we fertilized a subset of the microcosm communities with nitrogen, one aspect of ongoing environmental change in the grassland ecosystem from which the fungi were sampled (Fenn et al., 2010). We predicted that differences in decomposition rates between fertilized and ambient microcosms would be inversely proportional to the PD of its community.

## MATERIALS AND METHODS

### SAMPLING AND FUNGAL ISOLATION

Leaf litter was collected from a grassland savannah located near Irvine, CA, USA (33.74 N, 117.70 W), described in detail elsewhere (Allison et al., 2013). The site is dominated by invasive grasses and forbs. The same leaf litter was divided into two portions for fungal isolations and the microcosm experiment. For isolations, leaf litter was homogenized in a sterile coffee grinder, and filtered through sequential 2 mm, 212 μm, and 106 μm prefilters. The 106–212 μm size fraction was transferred to a sterile 100 μm nylon vacuum filter, washed twice in 200 mL sterile $H_20$, and transferred to 30 ml 0.6 carboxymethylcellulose solution (an emulsifier). The filtrate was sequentially diluted until 50% of 10 μl aliquots yielded either 0 or 1 fungal colony after 1 week of incubation. Ten microliter aliquots of filtrate were added to 800, 1 ml titer tubes containing 500 μl of solid MEA, water, MNM, or Thorn's medium (Thorn et al., 1996) amended with Kanamycin and Ampicillin (200 tubes per medium). Tubes were incubated at room temperature until growth was detected.

### FUNGAL IDENTIFICATION, SEQUENCING, AND COMPARISON WITH CULTIVATION INDEPENDENT FIELD DATA

Isolates were sorted into visually distinct morphotypes, and a representative of each was PCR amplified using the primers ITS1f-TW13 (White et al., 1990; Gardes and Bruns, 1993), which spans ∼1,400 bp, including both ITS spacers and the D1 and D2 regions of the gene encoding for the large ribosomal RNA subunit. Amplicons were sequenced in two directions using the sequencing services of Beckman Coulter, using the same PCR primers. For taxon circumscription and identification, the ITS spacers were excised from adjacent 18s, 5.8s, and 28s gene regions using an algorithm based on Hidden Markov Models (ITSx; Bengtsson-Palme et al., 2013) and concatenated and clustered into groups containing 97% sequence identity or greater using Sequencer's (version 4.7; Gene Codes) "contig" function. A single isolate from each contig was selected for subsequent analysis. Sequences are deposited in Genbank under accession numbers KF733341–KF733375.

Taxa were compared to a distribution of fungal communities enumerated using 454 sequencing technology from the field site over a 2-years sampling period (as described in Matulich and Martiny, 2014). Portions of the 28s encoding gene were matched to environmental DNA sequences at 97% sequence identity using the nearest neighbor clustering algorithm of the UCLUST package (Edgar, 2010). Taxonomic assignments of the environmental sequences were determined using the RDP fungal LSU classifier (Liu et al., 2012).

### PHYLOGENETIC TREE

The 28s portions of the sequences (and outgroups *Spizellomyces punctatus* and *Rozella allomycis*) were aligned using MAFFT's L-INS-i algorithm (Katoh et al., 2009), and a maximum likelihood tree was calculated in RaXML (Stamatakis et al., 2008) on the CIPRES server using default settings (**Figure 1**).

### COMMUNITY ASSEMBLY AND MICROCOSM CONSTRUCTION

Fifty distinct species pools were assembled along a PD gradient such that each microcosm contained seven taxa, each taxon was present in 7–10 communities, and no community shared more than three taxa. Isolates were selected to enable phylogenetically nested species pools containing both closely related congeners as well as distinct phyla (**Figure 1**).

Leaf litter for microcosms was homogenized in a Wiley mill, and sterilized via gamma irradiation for 48 h. Sterility was verified by plating litter on nutrient media. Selected fungal isolates were transferred to petri dishes containing growth medium with 5 mm cellophane disks on top, over which fungal colonies grew. Ten cellophane disks per isolate were transferred to a tube containing sterile water and one 3 mm silica bead and were briefly vortexed to suspend fungal cells. Microcosms were established in 40 ml sterile vials with gas-tight septum caps filled with 2 g sterile sand, 200 mg leaf litter substrate and 40 μl of fungal slurry for each species (280 μl total). Each community was replicated six times, and half of the replicates received a supplement of 71.4 μg $NH_4NO_3$. We estimate that this amount represents a litter C: fertilizer N ratio of approximately 5,000, equivalent to roughly 0.2 kg/ha. This is an ecologically relevant amount of Nitrogen that is lower than typical field deposition trials (Allison et al., 2009), and represents less than 10% annual deposition in this location (Fenn et al., 2010). Fungus free control microcosms were also run with and without nitrogen addition, replacing sterile $H_2O$ for fungal slurry volume.

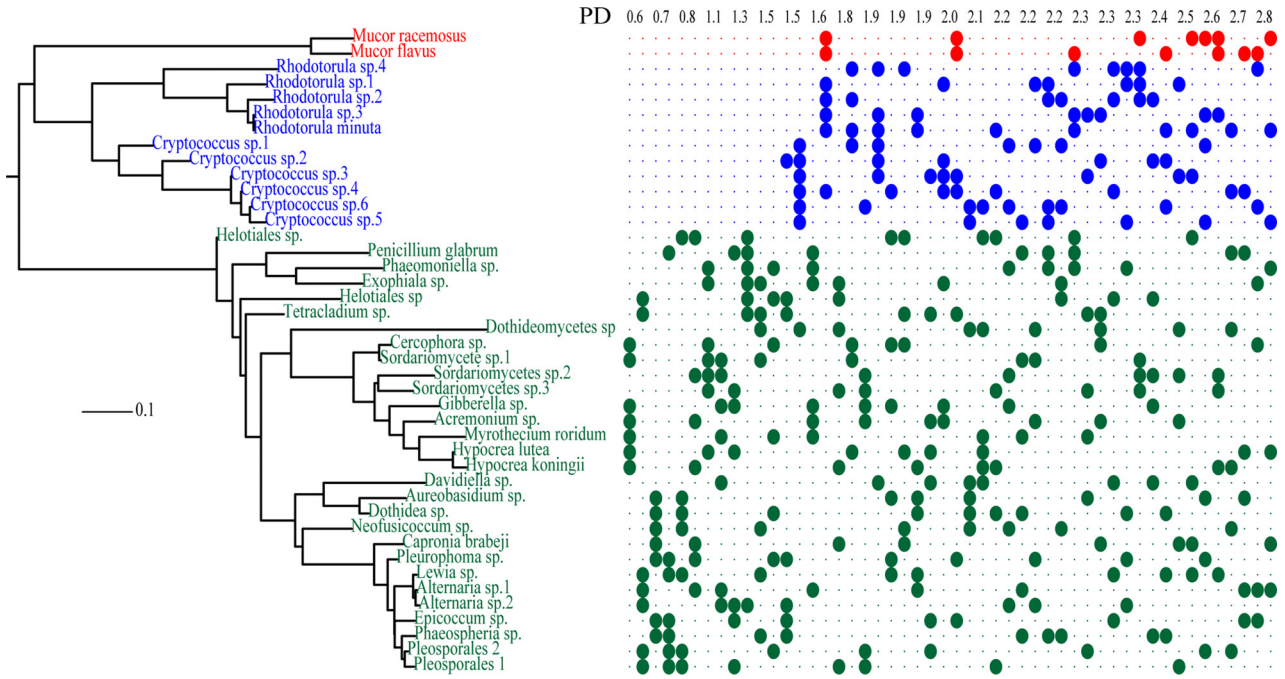


**FIGURE 1 | Maximum likelihood tree and community composition of microcosms.** Each column of the grid indicates the composition of one of 50 mock communities consisting of seven species in total. Species are colored by taxonomy as follows: Ascomycetes (green), Basidiomycetes (blue), and Mucoromycetes (red). Communities are ordered by increasing phylogenetic diversity (PD) as indicated by the scale at top.

### MEASUREMENT OF $CO_2$ PRODUCTION

$CO_2$ mineralization rate, our proxy metric for litter decomposition, was measured as the amount accumulated in the microcosm headspace over 24 h. Concentrations were measured after the first and third days and then weekly for a total of 66 days. The day prior to each measurement, microcosms were opened under sterile conditions, equilibrated with ambient air for 5 min, and then sealed. For each measurement, an 8 ml subsample of headspace gas was withdrawn by syringe and injected into an infrared gas analyzer (PP-Systems EGM-4). After measurement, vials were vented by rotating caps $\frac{1}{4}$ turn until 24 h prior to subsequent measure. A different syringe was used for each community to prevent cross-contamination.

### STATISTICAL ANALYSES

We assessed the interactions between decomposition rates and PD using a repeated measures ANCOVA model with sampling day (factor 14 levels) and nitrogen addition (factor 2 levels) as fixed effects, initial PD as a covariate, and community composition (factor 50 levels) as a random effect. PD was calculated three ways: (1) as a measure of the cumulative phylogenetic branch length (PD) contained amongst all community members, (2) as a measure of the nearest taxon index (NTI), which is the mean phylogenetic distance between all taxa and their closest relatives in a community, and (3) as the net relatedness index (NRI) which is the mean phylogenetic distance between all pairs of taxa within a community. All indices were calculated in the R package "picante" (Kembel et al., 2010), and the ANCOVA model was built using the stats package in the R programming environment (R Core Team, 2013).

To test whether there was a correlation between similarity of initial community composition and rates of respiration we used a Mantel test with 999 randomized permutations to assess significance levels. A pseudo cumulative respiration value was calculated by summing 24 h sample time points multiplied by the number of days preceding the last sample. Pairwise differences in cumulative measured $CO_2$ were tested for correlation with shared community membership (Jaccard's index) and shared phylogenetic branch length (Unifrac). We calculated correlations for nitrogen addition microcosms, ambient microcosms, and both together using the R package "vegan" (Oksanen et al., 2013).

To determine whether individual species were significantly associated with increased or decreased respiration rates (compared to average) we calculated a multiple linear regression model. Each species was considered a factor with two levels (present or absent). A dummy species representing mean $CO_2$ production across all communities was added as a reference.

To the determine whether individual species contributed to differences in community response with nitrogen addition, a multiple linear regression was calculated as above, substituting cumulative $CO_2$ with the proportional difference between treatments (ambient/nitrogen fertilization). Models were validated by plotting residuals vs. fitted values, and normal quantile–quantile plots. Models were made using the "stats" package in the R programming environment (R Core Team, 2013), and data was formatted using the package "reshape2" (Wickham, 2007).

Coefficients from these linear multiple regression tests were tested for phylogenetic signal using Blomberg's K statistic

(Blomberg et al., 2003) using the "picante" package (Kembel et al., 2010). This test measures whether variance among taxa differs from expectations given a Brownian motion evolutionary model. Values <1 indicate greater variance than expected whereas values >1 indicate phylogenetic signal, with significance determined by comparing the observed variance distribution with 999 randomizations.

## RESULTS

### EFFECTS OF PHYLOGENETIC DIVERSITY AND NITROGEN FERTILIZATION ON DECOMPOSITION RATES

We found that all three measures of PD were highly correlated (PD-NTI $R^2 = 0.86$; NTI-NRI $R^2 = 0.71$; NRI-PD $R^2 = 0.96$) and selection of one vs. another had no impact on the significance of any results. Therefore, only the results of PD are reported here.

Community PD was not correlated with respiration nor interacted with any other component of the experiment. Instead, nitrogen fertilization, and its interaction with time, appeared to drive respiration rates, with the earliest sampling dates of nitrogen-fertilized microcosms showing the highest levels of respired carbon (**Table 1**).

Phylogenetic diversity also did not correlate with difference in respiration rate between fertilized and ambient microcosms (ANCOVA; $F_1$ value: 0.071, $P = 0.791$). Differences did correlate with sampling date, however. Nitrogen addition had the greatest impact during the earliest sampling dates, with the differences in respiration between ambient and fertilized microcosms diminishing over the course of the experiment ($F_{10}$ value: 1.864, $P = < 0.048$). In nearly all cases, rates of decomposition peaked between days one and three and steadily declined throughout the duration of the experiment (**Figure 2**).

### COMMUNITY COMPOSITION AND RESPIRATION RATES

Community composition was not correlated with cumulative respiration, whether using either a measure of shared taxa ($\text{Mantel}_{\text{Jaccard}}$ $r = -0.017$, $P = 0.17$) or a measure of shared phylogenetic branch length ($\text{Mantel}_{\text{UniFrac}}$ $r = -0.025$, $P = 0.621$). This was the case whether we considered the ambient or nitrogen fertilized treatments together or individually.

### CONTRIBUTION OF INDIVIDUAL TAXA TO COMMUNITY DECOMPOSITION RATES

Individual contributions to community respiration rates were measured using a general linear model in which the coefficient associated with each of the species indicates its contribution to respiration or to the difference between decomposition in the fertilized and control microcosms. The coefficients are approximately normally distributed (**Figure 3**), indicating that each species was effectively equivalent with a few notable exceptions. Non-fertilized microcosms containing *Cryptococcus* sp. 6, *Rhodotorula* sp. 3, and *Hypocrea lutea* showed significantly slower rates of respiration compared to average, whereas none of these species appeared to impact fertilized microcosms (**Table 2**). Conversely, *Cercophora* sp. was correlated with significantly higher rates of respiration in ambient microcosms.

Blomberg's $K$ test for phylogenetic signal between correlation coefficients and phylogenetic relatedness were near zero for all groups of microcosm (ambient, fertilized, combined) and none were significantly different from random expectations (**Table 2**). In fact, species within the genus *Cryptococcus* contained both the lowest and fourth highest coefficient scores.

### ABUNDANCE OF ISOLATES IN THE FIELD SURVEY

Thirteen of 42 isolates were detected in the field survey, comprising 0.91% of the total sequence abundance (**Table 3**). The

**Table 1 | Results of the ANCOVA Model testing the effects of phylogenetic diversity (PD), nitrogen addition and sampling time on respiration (top), and on the affects of nitrogen addition on community respiration (cumulative difference; below).**

| Variable | Factor | Df | Sum squares | Mean squares | *F* | *P*-value |
|---|---|---|---|---|---|---|
| Respiration | PD | 1 | 89969 | 89969 | 0.029 | 0.866 |
| | Residuals | 48 | 150170078 | 3128543 | | |
| | Nitrogen | 1 | 5.76E + 07 | 57603071 | 99.018 | **<0.001** |
| | Time | 10 | 1.53E + 09 | 153271897 | 263.469 | **<0.001** |
| | PD:nitrogen | 1 | 1.14E + 05 | 114248 | 0.196 | 0.658 |
| | PD:time | 10 | 3.01E+06 | 300580 | 0.517 | 0.879 |
| | Nitrogen:time | 10 | 7.71E + 07 | 7707967 | 13.25 | **<0.001** |
| | PD:nitrogen:time | 10 | 1.43E + 06 | 142590 | 0.245 | 0.991 |
| | Residuals | 1008 | 5.86E + 08 | 581745 | | |
| Cumulative difference | PD | 1 | 0.37 | 0.369 | 0.071 | 0.791 |
| | Residuals | 48 | 248.77 | 5.183 | | |
| | Time | 10 | 1.00E-27 | 1.00E-28 | 1.864 | **0.048** |
| | PD:time | 10 | 1.04E-28 | 1.04E-29 | 0.193 | 0.997 |
| | Residuals | 480 | 2.58E-26 | 5.37E-29 | | |

*Bolded values indicate statistical significance at 0.05.*

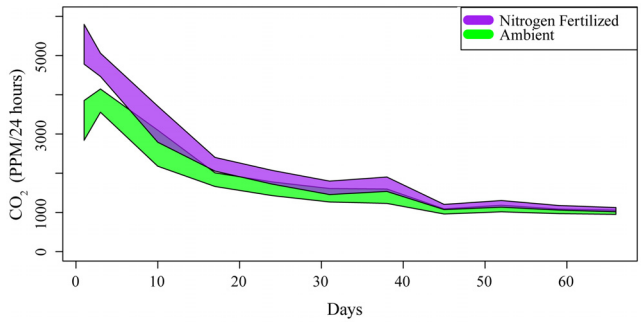


**FIGURE 2 | Time series of mineralized $CO_2$, a proxy for decomposition.** Each polygon contains two SE of the mean amongst replicates for each community under ambient and nitrogen fertilized conditions. Units are parts per million (PPM) of $CO_2$ accumulated in microcosms over 24 h.

single most abundant taxon isolated was *Cryptococcus* sp. 3, accounting for 0.669% of the total sequence abundance. Family level taxonomy was determined for 30 isolates, all of which were detected in the field survey. Cumulatively these families contained >88% of all sequence abundance, and were dominated by the Davidiellaceae and the Pleosporaceae (comprising 39.0 and 37.6% of the relative abundance, respectively).

## DISCUSSION

The species richness–function relationship presumes a linkage between a species and trait diversity. Under this model taxa are functionally variable and the sum of individual species contributes to combined community functioning. We hypothesized that, due to the tendency of close fungal relatives to contain a more similar suite of traits, PD of litter fungi would be a better predictor of functioning (respiration rate) than taxonomic diversity alone, as has been shown recently amongst communities of marine bacteria (Gravel et al., 2012; Venail and Vives, 2013).

Contrary to our prediction, we found no evidence, by any measure, for a relationship between PD and respiration rates. Initial PD of microcosm taxa pools did not correlate with

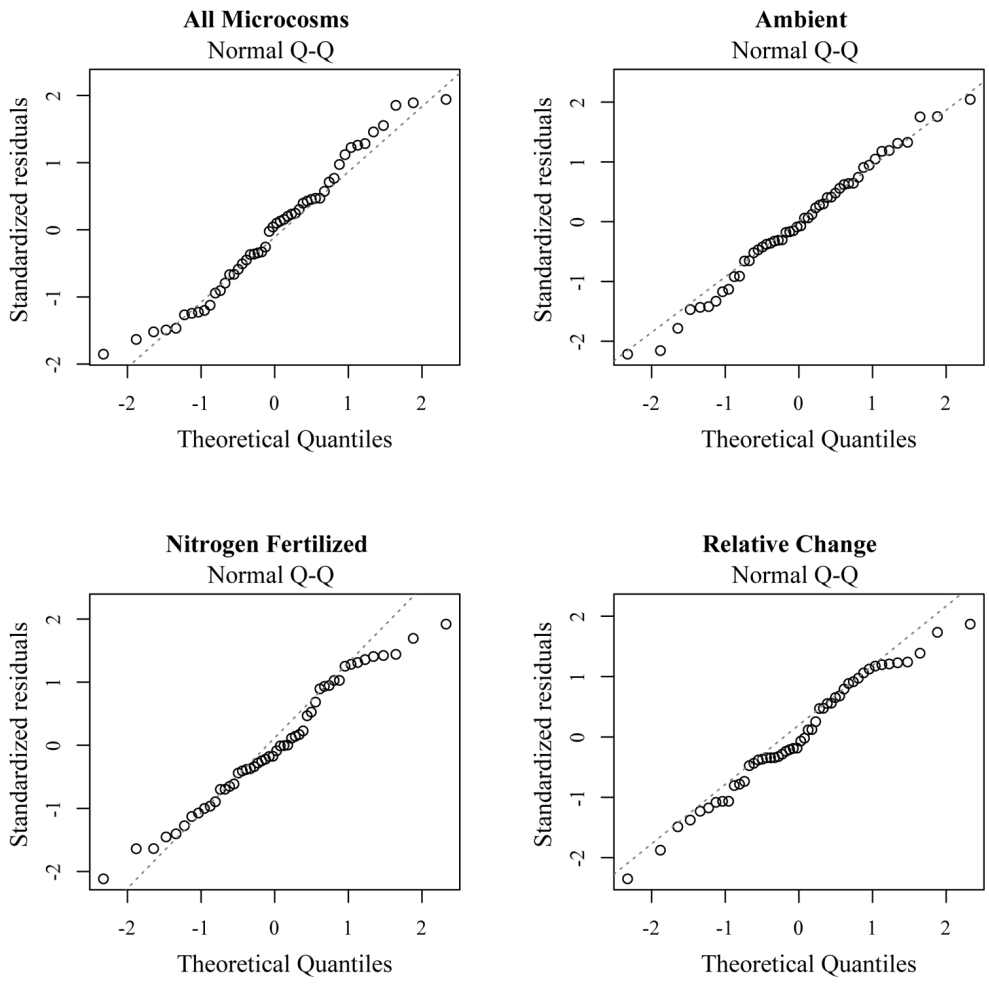


**FIGURE 3 | Quantile–quantile (Q–Q) normality plots indicating normal residuals for all four response variables.**

**Table 2 | Results from the multiple regression analyses of species effects on decomposition, from left to right: all microcosms combined, ambient, nitrogen fertilized, and the effect size of fertilization.**

| Species | Combined *T*-value | Ambient *T*-value | *N* + *T*-value | Difference *T*-value |
|---|---|---|---|---|
| *Rhodotorula*_sp.1 | 0.603 | 0.073 | 0.778 | 0.699 |
| *Rhodotorula*_sp.3 | −1.136 | **−2.391*** | −0.065 | **1.931** |
| *Rhodotorula_minuta* | −0.019 | 0.855 | −0.558 | −1.592 |
| *Rhodotorula*_sp.2 | 1.08 | 1.687 | 0.426 | −0.757 |
| *Rhodotorula*_sp.4 | −1.109 | −1.414 | −0.635 | 0.315 |
| *Cryptococcus*_sp.1 | 0.908 | 0.972 | 0.636 | −0.161 |
| *Cryptococcus*_sp.2 | 0.115 | 0.482 | −0.143 | −0.835 |
| *Cryptococcus*_sp.3 | 0.215 | 0.166 | 0.191 | 0.026 |
| *Cryptococcus*_sp.6 | −1.57 | **−3.552**** | 0.065 | **3.169*** |
| *Cryptococcus*_sp.5 | 0.601 | 1.136 | 0.114 | −1.398 |
| *Cryptococcus*_sp.4 | −1.413 | −1.66 | −0.898 | 0.331 |
| *Mucor_racemosus* | −0.39 | −0.656 | −0.125 | 0.438 |
| *Mucor_flavus* | 1.459 | 1.814 | 0.865 | −0.995 |
| Helotiales_sp. | 1.008 | 0.835 | 0.857 | 0.527 |
| *Penicillium_glabrum* | −0.766 | −1.723 | 0.026 | 0.687 |
| *Phaeomoniella*_sp. | −0.088 | 0.418 | −0.381 | −0.04 |
| *Exophiala*_sp. | −0.33 | −0.582 | −0.089 | −0.302 |
| Helotiales_sp | −0.898 | −1.481 | −0.305 | 0.325 |
| *Tetracladium*_sp. | 0.421 | 0.158 | 0.477 | 0.236 |
| Dothideomycetes_sp | −0.884 | −0.138 | −1.122 | −1.112 |
| *Cercophora*_sp. | **1.875** | **2.572*** | 0.961 | −1.434 |
| Sordariomycete_sp.1 | −0.631 | −1.166 | −0.136 | 1.016 |
| Sordariomycetes_sp.2 | −0.95 | −0.597 | −0.926 | −0.914 |
| Sordariomycetes_sp.3 | 1.75 | 1.465 | 1.48 | −0.38 |
| *Gibberella*_sp. | 0.207 | −0.639 | 0.681 | 1.254 |
| *Hypocrea_lutea* | −1.566 | **−2.512*** | −0.577 | **1.27** |
| *Hypocrea_koningii* | 0.214 | 0.069 | 0.25 | 0.509 |
| *Myrothecium_roridum* | −0.115 | 0.26 | −0.318 | −0.689 |
| *Acremonium*_sp. | −0.002 | 0.724 | −0.453 | −1.262 |
| *Davidiella*_sp. | 1.248 | 0.649 | 1.301 | 0.468 |
| Aureobasidium_sp. | −0.062 | 0.257 | −0.244 | −0.411 |
| *Dothidea*_sp. | 0.444 | 0.56 | 0.259 | −0.159 |
| *Lewia*_sp. | −0.662 | −0.807 | −0.402 | −0.414 |
| *Alternaria*_sp.1 | 0.988 | 0.375 | 1.117 | 0.876 |
| *Alternaria*_sp.2 | −0.088 | 0.005 | −0.123 | −0.229 |
| *Phaeosphaeria*_sp. | −0.43 | −0.619 | −0.202 | 0.327 |
| Pleosporales_2 | 0.746 | 0.937 | 0.436 | −0.084 |
| Pleosporales_1 | −0.009 | 0.646 | −0.414 | −0.634 |
| *Epicoccum*_sp. | −0.007 | 0.506 | −0.325 | −0.778 |
| *Pleurophoma*_sp. | −0.262 | −1.211 | 0.395 | 1.173 |
| *Capronia_brabeji* | −0.736 | −1.609 | −0.004 | 0.725 |
| *Neofusicoccum*_sp. | **1.998** | 1.846 | 1.581 | −0.011 |
| **Blomberg's *K*** | 0.00061 | 0.00003 | 0.00025 | 0.00001 |

*Blomberg's K statistic of phylogenetic signal and its P-value are reported below. Significance values are reported as: *P < 0.05, **P < 0.01, **Bold** no asterisk = <0.10.*

respiration at any time point in the experiment. Furthermore, although nitrogen fertilization increased respiration rates, this response was independent of phylogenetic community composition: there was no correlation between PD and community resilience. Last, we did not find a phylogenetic signal amongst isolate contributions to community respiration. While previous studies have found significant differences among decomposition rates of fungal isolates (Allison et al., 2009), and some degree of phylogenetic patterning among their substrate utilization (McGuire et al., 2010), we found very few species in our study that correlated with increased or decreased rates of $CO_2$ production.

We can think of at least three reasons for the discrepancy between these past results and the present study. First, the scale of PD considered might matter for the diversity-function relationship. Many of the microbial traits examined are phylogenetically conserved, but at a fine genetic scale (e.g., Martiny et al., 2013). Thus, the scale of PD considered here, spanning three phyla, may not be informative. Constraining communities to phylogenetically narrower membership more consistent with detected levels of trait conservatism may be more conducive to detecting a PD–function relationship.

Second, traits of single isolates may be more likely to show a phylogenetic signal than when they are measured within a community context. Together with other taxa, the isolates do not necessarily perform at their functional potential, but are constrained by interactions with the rest of the community. For fungi, competitive interactions between non-self mycelium, including chemical and physical antagonism, can impact resource allocations and decrease decomposition rates (Boddy, 2000). Similarly, synergistic biotic interactions such as complementary abilities to degrade complex or recalcitrant biomolecules such as lignin among Basidiomycetes (Blanchette, 1991), or specialized enzyme production to decompose cellulose and chitin molecules (Lindahl and Finlay, 2006), may accelerate rates of decomposition.

A third potential reason for a lack of correlation between initial PD and functioning is that the realized PD of the microcosms may have differed from the initial PD. Fungal composition may have changed, perhaps rapidly, due to biotic interactions, nutrient availability, and stochastic processes favoring growth of one species over another (Cleveland et al., 2013; Matulich and Martiny, 2014). A previous study with some of the same fungal isolates did observe changes in community structure over the course of a similar, 60 day microcosm experiment (Matulich and Martiny, 2014). Those community changes were largely driven by changes in relative abundance rather than extinctions, and all measured isolates were able to survive under experimental conditions. In fact a vast, and contradictory, literature predicts both the competitive exclusion of and niche selection for closely related organisms (Maherali and Klironomos, 2007; Mayfield and Levine, 2010; Venail and Vives, 2013; Godoy et al., 2014), making it difficult to predict the outcome of biotic interactions based on relatedness alone.

Although initial PD did not alter respiration rates, nitrogen fertilization significantly increased rates, regardless of phylogenetic relatedness or taxonomic composition of microcosms.

**Table 3 | Relative abundance of isolates and isolate families in a field survey of leaf litter fungi.**

| Isolate name | Family (unless otherwise noted) | Phylum | Taxon relative abundance in survey | Family relative abundance in survey |
|---|---|---|---|---|
| *Tetracladium* sp. | Ascomycota_incertae_sedis (phylum) | Ascomycetes | – | N.D. |
| *Neofusicoccum* sp. | Botryosphaeriaceae | Ascomycetes | – | 0.191 |
| *Davidiella* sp. | Davidiellaceae | Ascomycetes | – | 39.027 |
| *Dothidea* sp. | Dothideaceae | Ascomycetes | – | 0.634 |
| Dothideomycetes *sp.* | Dothideomycetes (class) | Ascomycetes | – | – |
| *Aureobasidium* sp. | Dothioraceae | Ascomycetes | – | 0.863 |
| Helotiales sp. | Helotiales (rank) | Ascomycetes | – | – |
| Helotiales sp. | Helotiales (rank) | Ascomycetes | – | – |
| *Myrothecium roridum* | Helotiales_incertae_sedis (rank) | Ascomycetes | – | N.D. |
| *Capronia brabeji* | Herpotrichiellaceae | Ascomycetes | – | 0.026 |
| *Exophiala* sp. | Herpotrichiellaceae | Ascomycetes | 0.008 | 0.026 |
| *Phaeomoniella* sp. | Herpotrichiellaceae | Ascomycetes | 0.000 | 0.026 |
| *Hypocrea koningii* | Hypocreaceae | Ascomycetes | – | 0.000 |
| Hypocreales sp. | Hypocreaceae | Ascomycetes | 0.000 | 0.000 |
| *Acremonium* sp. | Hypocreales_incertae_sedis (rank) | Ascomycetes | – | N.D. |
| *Cercophora* sp. | Lasiosphaeriaceae | Ascomycetes | – | 0.451 |
| *Gibberella* sp. | Nectriaceae | Ascomycetes | 0.029 | 0.102 |
| *Phaeosphaeria* sp. | Phaeosphaeriaceae | Ascomycetes | – | 6.770 |
| *Alternaria* sp. 1 | Pleosporaceae | Ascomycetes | – | 37.611 |
| *Alternaria* sp. 2 | Pleosporaceae | Ascomycetes | – | 37.611 |
| *Epicoccum* sp. | Pleosporaceae | Ascomycetes | – | 37.611 |
| *Lewia* sp. | Pleosporaceae | Ascomycetes | – | 37.611 |
| Pleosporales sp. 1 | Pleosporales (rank) | Ascomycetes | – | – |
| Pleosporales sp. 2 | Pleosporales (rank) | Ascomycetes | – | – |
| *Pleurophoma* sp. | Pleosporales_incertae_sedis (rank) | Ascomycetes | 0.003 | N.D. |
| Sordariomycetes sp. 1 | Sordariomycetes (class) | Ascomycetes | 0.190 | – |
| Sordariomycetes sp. 2 | Sordariomycetes (class) | Ascomycetes | Singleton | – |
| Sordariomycetes sp. 3 | Sordariomycetes (class) | Ascomycetes | – | – |
| *Penicillium glabrum* | Trichocomaceae | Ascomycetes | Singleton | 0.027 |
| *Rhodotorula minuta* | Sporidiales incertae sedis (rank) | Basidiomycetes | – | N.D. |
| *Rhodotorula* sp.1 | Erythrobasidiaceae | Basidiomycetes | – | N.D. |
| *Rhodotorula* sp. 2 | Erythrobasidiaceae | Basidiomycetes | <0.001 | N.D. |
| *Rhodotorula* sp. 3 | Erythrobasidiaceae | Basidiomycetes | – | N.D. |
| *Rhodotorula* sp. 4 | Erythrobasidiaceae | Basidiomycetes | 0.017 | N.D. |
| *Cryptococcus* sp. 1 | Tremellaceae | Basidiomycetes | – | 3.124 |
| *Cryptococcus* sp. 2 | Tremellaceae | Basidiomycetes | – | 3.124 |
| *Cryptococcus* sp. 3 | Tremellaceae | Basidiomycetes | 0.669 | 3.124 |
| *Cryptococcus* sp. 4 | Tremellaceae | Basidiomycetes | 0.000 | 3.124 |
| *Cryptococcus* sp. 5 | Tremellaceae | Basidiomycetes | – | 3.124 |
| *Cryptococcus* sp. 6 | Tremellaceae | Basidiomycetes | – | 3.124 |
| *Mucor flavus* | Mucoraceae | Zygomycota | – | 0.000 |
| *Mucor racemosus* | Mucoraceae | Zygomycota | Singleton | 0.000 |

*Singletons are found once in the dataset.*
*N.D. indicates that family level taxonomy is uncertain for the genus.*

Earlier research has demonstrated mixed effects of nitrogen availability on decomposition rates, with impacts varying across substrate, taxonomy and functional guild of the microbes under study (Knorr et al., 2005; Allison, 2012). Thus, it appears that community response to nitrogen, and therefore its correlation with phylogenetic patterning, is not consistent across environments, but is context dependent. In low nitrogen environments, for example, nitrogen fertilization has been shown to decrease plant tissue C:N ratios (Bragazza et al., 2011), increase decomposition of cellulose and mineral forms of N (Talbot and Treseder, 2012), and facilitate transcription of lignocellulolytic enzyme genes (Edwards et al., 2011).

Although there is likely to be a mismatch between microbial diversity in natural systems and that amenable to cultivation on lab media, our efforts increased the likelihood that isolate functional and taxonomic diversity were broadly representative of field conditions. Use of multiple media, isolation of a size fraction >100 μm, and dilution to extinction protocols facilitated cultivation of slow growing and less competitive taxa. Further, because the taxa were isolated from the same substrate used in the microcosms, there is the strong likelihood that these fungi are associated with leaf litter decay processes. Cultivation-independent sequence analysis of this field site uncovered more than 800 fungal taxa, of which our isolates comprised approximately 1% of the sequence abundance: a reasonable representation given the typically long-tailed community rank abundance curve. Further, family level taxonomic identities of our isolates represented >88% of the sequence abundance in our field site, indicating that isolate diversity was representative at higher taxonomic ranks.

The complexity of biotic and environmental interactions scales with community and litter complexity and may be difficult to predict. The high species diversity detected in our field site enable an almost unfathomably tangled network of interactions, undoubtedly unique to this site. For this reason, this study highlights the importance of examining traits, particularly those relating to ecosystem function, within the context of the community in which they're found, rather than in isolation. In a recent study of petroleum degrading bacteria, for example, a positive PD ecosystem function relationship was found in both two and four isolate microcosms (Venail and Vives, 2013). Amongst the latter, an increase in positive biotic interactions underpinned this relationship. However, in a natural microbial community, particularly one as species rich as that found within leaf litter, the average distance between community members will decrease as a function of species richness. Therefore, complementarity will be balanced, at some point, by competition amongst species whose niche requirements overlap. Determining this "tipping point" may be a fruitful endeavor for future research into the PD ecosystem function relationship.

## AUTHOR CONTRIBUTIONS

ASA and JBHM designed the experiment, ASA and KLM conducted the experiment, ASA analyzed the data and all authors contributed towards writing and editing the manuscript.

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# Cellulolytic potential under environmental changes in microbial communities from grassland litter

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In many ecosystems, global changes are likely to profoundly affect microorganisms. In Southern California, changes in precipitation and nitrogen deposition may influence the composition and functional potential of microbial communities and their resulting ability to degrade plant material. To test whether such environmental changes impact the distribution of functional groups involved in leaf litter degradation, we determined how the genomic diversity of microbial communities in a semi-arid grassland ecosystem changed under reduced precipitation or increased N deposition. We monitored communities seasonally over a period of 2 years to place environmental change responses into the context of natural variation. Fungal and bacterial communities displayed strong seasonal patterns, Fungi being mostly detected during the dry season whereas Bacteria were common during wet periods. Most putative cellulose degraders were associated with 33 bacterial genera and predicted to constitute 18% of the microbial community. Precipitation reduction reduced bacterial abundance and cellulolytic potential whereas nitrogen addition did not affect the cellulolytic potential of the microbial community. Finally, we detected a strong correlation between the frequencies of genera of putative cellulose degraders and cellulase genes. Thus, microbial taxonomic composition was predictive of cellulolytic potential. This work provides a framework for how environmental changes affect microorganisms responsible for plant litter deconstruction.

**Keywords: cellulase, metagenomics, leaf litter, global change, microbial community composition**

## INTRODUCTION

Establishing the connection between community structure and function has been a longstanding yet elusive goal in microbial ecology. Making such a connection is especially critical for predicting how communities and functions will respond to global environmental change (Allison and Martiny, 2008; Trivedi et al., 2013). Meeting this challenge depends on linking traits that control responses of microbial taxa to the environment ("response" traits) with those that determine ecosystem function ("effect" traits; Lavorel et al., 1997; Gross et al., 2009; Webb et al., 2010). This linkage has been elusive due to the difficulty of isolating microorganisms and characterizing their traits in complex communities. The advent of high-throughput – omics approaches offers a means of linking response and effect traits in microbial communities. When coupled with experimental manipulations of environmental conditions, (meta)genomic approaches can provide a window into both microbial community responses and concurrent changes in functional potential.

Local communities are increasingly being confronted by global-scale environmental changes. For instance, drought and nitrogen deposition are predicted to affect many ecosystems (Cook et al., 2004; Hole and Engardt, 2008; Fenn et al., 2010; Seager and Vecchi, 2010). Nitrogen deposition is known to change plant diversity and to increase primary production (Lamarque et al., 2005; Cleland and Harpole, 2010), whereas drought can lead to reduced primary production and plant diversity (Mueller et al., 2005). Previous studies have shown that microorganisms also respond to changes in water availability (Pesaro et al., 2004; Sheik et al., 2011; Cregger et al., 2012; Barnard et al., 2013; Bouskill et al., 2013). Similarly, N-addition can promote the growth of copiotrophic microbes, whereas other lineages may be negatively affected (Fierer et al., 2012; Philippot et al., 2013). However, we currently have a limited understanding of how environmental changes directly impact the genomic diversity of microbial communities and their associated functional potential.

Plant polymer degradation is a key microbial function that channels plant litter into microbial biomass, where it can be mineralized to $CO_2$ or stabilized as soil carbon (Cebrian, 1999). Cellulose is one of the most abundant polymers in plant litter, and therefore the breakdown of this compound is a key step in the decomposition of plant material. Cellulose-degrading microbes produce cellulase enzymes that catalyze the first step of cellulose hydrolysis and release oligosaccharides that are accessible for many other lineages (Lynd et al., 2002; Goldfarb et al., 2011; Berlemont and Martiny, 2013).

Cellulases belong to glycoside hydrolases (GH) families 5, 6, 7, 8, 9, 12, 44, 45, and 48 (Berlemont and Martiny, 2013). These enzymes are frequently associated with carbohydrate binding modules (CBMs) assumed to increase cellulose hydrolysis. In both isolated microorganisms and complex microbial communities, the redundancy of seemingly similar proteins is assumed to promote synergistic interactions among enzymes with varying regulatory mechanisms and/or biochemical properties (substrate specificity, pH, etc.; Wilson, 2011).

Bacteria that carry cellulase genes are commonly associated with specific genera within the phyla *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* (Haichar et al., 2007; Ulrich et al., 2008; Schellenberger et al., 2010; Goldfarb et al., 2011; Barnard et al., 2013; Berlemont and Martiny, 2013). The initial enzymatic breakdown of cellulose typically results in the release of oligosaccharides like cellobiose. To use oligosaccharides, microorganisms need to express β-glucosidase, which is associated with GH families 1 and 3. A recent genomics analysis suggests that more than 80% of sequenced bacterial lineages carry β-glucosidase (Berlemont and Martiny, 2013) and therefore most lineages may opportunistically benefit from the enzyme production of cellulose degraders.

In arid and semi-arid ecosystems, cellulose degradation and litter decomposition rates may depend on the responses of cellulose-degrading lineages to water and nutrient availability. Previously at our study site in a semi-arid California grassland ecosystem, experimentally induced drought significantly reduced litter decomposition rates and bacterial biomass (Allison et al., 2013). Likewise, decomposition rates and bacterial biomass were markedly lower during the summer dry season. Nitrogen addition had weaker effects on litter decomposition, but there was some evidence for adaptation of microbial communities to nitrogen availability. These results raise the question of whether changes in the abundance of cellulose-degrading lineages contributed to changes in overall litter decomposition rates.

To understand how cellulose degradation might respond to environmental changes, we identified the microbial metagenomic content in leaf litter across seasons and under experimentally manipulated water and nitrogen availability. Specifically, we aimed to characterize the genetic diversity of the leaf litter microbial community, the organisms carrying cellulases, and cellulase genes. We hypothesized that most of the cellulolytic potential (i.e., the collection of detected genes coding for cellulases) would be associated with fungal lineages, consistent with past studies of leaf litter (Schneider et al., 2012). Next, we investigated how cellulolytic potential responded to seasonal and experimental precipitation reduction in this environment. We hypothesized that cellulolytic traits would be correlated with responses to precipitation and nitrogen availability owing to physiological tradeoffs. Enzyme expression requires cellular resources, particularly nitrogen, so cellulolytic traits should be more prevalent in nitrophilic, copiotrophic taxa (Treseder et al., 2011). In contrast, cellulolytic traits should correlate negatively with drought tolerance due to the high resource cost associated with cell walls, osmolytes, and other tolerance traits. Finally, we evaluated if the environmental responses of microbial lineages were correlated with changes in cellulolytic potential. We hypothesized that the frequency of cellulolytic traits in the microbial community is predictable based on the taxa present in the community (e.g., at the genus level). Such a relationship is expected if cellulolytic traits are phylogenetically conserved and would be useful for linking cellulose degradation with other traits conserved among microbial taxa (Allison and Martiny, 2008; Berlemont and Martiny, 2013; Martiny et al., 2013; Zimmerman et al., 2013).

## MATERIALS AND METHODS

### FIELD EXPERIMENT

Analyzed plant litter was collected at the Loma Ridge experimental field in Southern CA, USA (33°44′N, 117°42′E, 365 m elevation; Allison et al., 2013). Plant composition was dominated by exotic annual grasses (e.g., *Avena*, *Bromus*, and *Lolium*) and forbs (e.g., *Erodium*). The climate is semi-arid (mean annual precipitation of 325 mm) with most of the precipitation occurring between October and April (**Figure 1A**). Treatments were ∼50% reduction in precipitation (R), nitrogen addition (N), and control (C). Precipitation and nitrogen manipulations have been established there since February 2007. Precipitation reduction was achieved by covering plots with clear polyethylene during the rain events each winter. Nitrogen was added as 20 kg N/ha ($CaNO_3$) prior to the growing season (October to April) and 40 kg N/ha ($CaNO_3$) ∼3 months after the start of the growing season. Changes in plant community composition, litter chemistry, and microbial biomass across seasons and treatments were previously reported (Allison et al., 2013). For sequencing, 16 samples of plant litter from haphazardly located 0.07-m2 quadrats in each treatment (R, N, and control) were collected, once per season.

### DNA SEQUENCING AND TAXONOMIC ANNOTATION

To balance replication with the cost limitations of sequencing, we pooled eight plots from each treatment (each plot sampled and extracted separately) into two replicate samples for sequencing. This approach allowed us to capture spatial variation across our study site, while still keeping two replicates per treatment. Although two replicates is not ideal, we also sampled those pooled replicates eight times over the course of the experiment (i.e., April 14th, August 20th, and December 17th 2010; February 28th, June 10th, September 21th, and December 14th 2011; and March 12th 2012), which provided us with additional statistical power to capture treatment and temporal effects (see Results).

For each sampling date, eight leaf litter samples (∼20 gr) were collected, grinded in a mixer, subjected to direct DNA isolation as described before (DeAngelis et al., 2010), and normalized to the amount of leaf litter material used for extraction. After fragmentation to 300 bp using Covaris, equal amounts of DNA extracts were pooled to two replicates and prepared for sequencing. In total, 216 litter samples were processed and 54 metagenomic libraries [3 treatments × 8 dates × 2 replicates = 48 samples, two sequencing controls, and four samples from the dual factorial treatment (RN)] were prepared using a Truseq library kit (Illumina, San Diego, CA, USA) and sequenced with an Illumina HiSeq2000 (100 bp-paired ends). Sequences were treated as single reads for downstream analysis. Sequences were uploaded onto the MG-RAST server and made publically accessible (Table S5; Glass and Meyer, 2011). Finally, 107.4 Gbp (passed QC) were obtained.

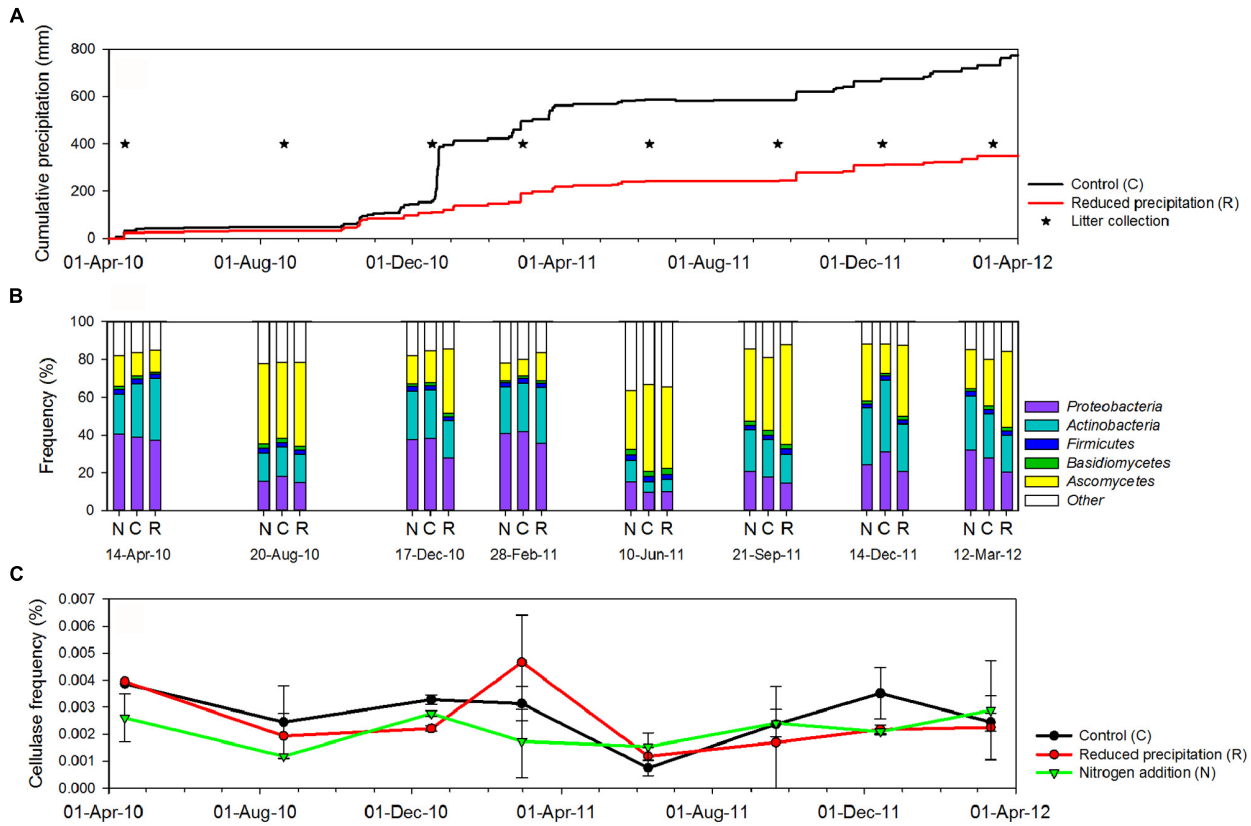


**FIGURE 1 | (A)** Cumulative precipitation at Loma Ridge study site. **(B)** Overall distribution of sequences with annotation in the major microbial phyla detected in the leaf litter metagenomes across treatments [control (C), added nitrogen (N), reduced precipitation (R)] and seasons. **(C)** Fluctuations of the overall frequency of cellulase sequences in the leaf litter metagenomes.

The taxonomic (and functional) diversity of complex plant litter microbial assemblages was investigated using annotations of all the reads (i.e., WGS) in order to evaluate and compare samples without potential assembly biases based on composition or coverage. Taxonomic annotation, using the M5NR database, at the genus level, was considered for sequences with e-value $\leq 10^{-5}$. Using this cut-off, 53% of the sequences were annotated (Figure S1). After rarefaction, the distribution of taxonomically identified reads, was used to estimate and compare the taxonomic composition of samples.

## GLYCOSIDE HYDROLASES/CARBOHYDRATE BINDING MODULE IDENTIFICATION

In order to identify all the sequences associated with GHs and carbohydrate binding modules (CBMs) in the samples, sequences for each GH/CBM family, as defined in the CAZy database (Lombard et al., 2014), were extracted from the Pfam server and mapped against all sequenced genomes using SEED annotations (Overbeek et al., 2005; Berlemont and Martiny, 2013). The SEED functional annotation of these traits was then used as a reference to investigate the SEED-annotated sequences provided by MG-RAST output files (i.e., XXX_650.Superblat.expand.protein) for functional annotations. The resulting hits and their corresponding sequences were then subjected to a Pfam_scan analysis (PfamA db, e-value < 1e-5; Finn et al., 2014) to confirm functional annotations. This approach allowed us to identify short sequences from metagenomes matching GH/CBM from sequenced bacterial genomes.

## STATISTICAL ANALYSIS

Statistical analyses were performed using 'Stat,' 'LME,' and 'Vegan' packages in the R software environment (Pinheiro and Bates, 2000; Oksanen et al., 2012; R Development Core Team, 2012). We focused on describing the effect of date and treatment on groups defined taxonomically (phylum and genus level) and functionally (reads for cellulases). First the sequences were rarefied ($n$ = 1,265,787 reads) and pairs of analytical replicates (from February 2011) were averaged. Next, sequences were binned by phylum/genus based on the taxonomy of the best hit in the M5NR database within the MG-RAST environment (e-value < 1e-5; Wilke et al., 2012). Genera containing cellulase sequences were defined as potential cellulolytic lineages. The genus-specific cellulolytic potential was defined as the total number of cellulase sequences from a genus divided by the total number of sequences from that genus across all samples.

Dependent variables, including taxon, and cellulase frequencies, were analyzed using analysis of variance on linear mixed effects models with repeated measures (Pinheiro and Bates, 2000).

Fixed effects in the model included treatment (control, added N, or reduced precipitation), date, and the treatment by date interaction. The models included the combination of treatment and pooled replicate as a random effect with six levels.

The significance and magnitude of precipitation effects on the frequency of individual taxonomic groups, functional groups (e.g., potential cellulose degraders), and functional traits (e.g., cellulases) were investigated using the Pearson correlation test. The effect of each experimental treatment on the frequency of taxonomic and functional groups was investigated using a paired Welch-*t*-test to compare treatment means with control means.

For each bacterial genus, we determined the response to nitrogen addition and reduced precipitation. The response to treatment was defined as the ratio of the genus frequency in the treatment versus the control. We used the correlation coefficient (Spearman) between the log of the response ratio and the rarefied cellulase abundance for all genera to test for a link between treatment responses and cellulolytic potential.

## RESULTS

### MICROBIAL DIVERSITY

We identified microbial diversity using the taxonomic annotation of all the sequences. Sequences affiliated with Fungi accounted for 9–55% of the annotated reads, depending on the sampling season (**Figures 1A,B**). Sequences from *Ascomycetes* and *Basidiomycetes* dominated Fungi in all samples and accounted for 92.8 and 7.0% of the fungal hits, respectively. Sequences were affiliated with 422 detected families of Fungi. The most abundant Ascomycetes belonged to the families *Pleosporaceae*, *Phaeosphaeriaceae*, *Trichocomaceae*, *Nectriaceae*, and *Sordariiaceae* and accounted for 26.5, 19.8, 14.7, 4.9, and 4.6% of the fungal sequences, respectively. *Basidiomycetes* were primarily composed of the families *Tremellaceae*, *Ustilaginaceae,* and *Tricholomataceae* (2.3, 1.0, and 0.8% of the fungal sequences). 0.5% of the identified sequences were affiliated with *Archaea*, mostly *Euryarchaeota*. Bacteria accounted for 21–88% of the taxonomically identified sequences. *Proteobacteria*, *Actinobacteria*, and *Firmicutes* dominated the bacterial community and represented 43.9, 36.3, and 5.0% of the sequences for bacteria, respectively.

### CELLULOLYTIC TRAIT DIVERSITY

We next identified the distribution of GHs and carbohydrate binding module sequences (CBMs). Using a custom bioinformatics analysis, we detected 442,457, and 17,922 sequences for potential GHs and CBMs, respectively (Figures S1A,B; Tables S1 and S2). Across all samples, GHs accounted for 0.093% of the annotated sequences. Assuming at least few thousands genes per genome, this frequency of GHs indicated that many (if not most) microorganisms contained enzymes from this super-family. Most of the detected GHs appeared to be novel and had low similarity to known enzyme sequences (Figure S1C). Among the identified sequences for putative GHs, 92.7, 0.5, and 6.1% were likely to be derived from *Bacteria*, *Archaea*, and *Eukaryotes*, respectively. For Eukaryotes, 4.3 and 1.5% were likely affiliated to *Ascomycetes* and plants (e.g., *Brassica*), respectively. Sequences for enzymes involved in the processing of oligosaccharides were abundant (e.g., α− and β−glucosidases from GH1-3), whereas enzymes targeting complex structural polymers were less common (Figure S1A). For example, cellulases constituted 0.0025% of the annotated sequences, and thus, only a subset of the microbial organisms appeared to carry this trait. GH6 and 9 were the most abundant cellulase families, followed by GH8, 5, 12, 44, 45, 48, and 7 (Figure S1A; Table S1).

A detailed analysis of the GH sequences involved in cellulose degradation revealed a vast diversity of enzymes likely to be derived from Bacteria and only few enzyme types from Fungi or plants (e.g., Brassica). Putative cellulase sequences were derived from lineages referred to as the potential cellulose degraders. Only 3.9% of the cellulases – mostly GH7 and to a lesser degree GH6 – were affiliated with the genus *Gibberella* (phylum *Ascomycetes*) and 3.7% (all within GH9) were derived from plants. In Bacteria, potential cellulose degraders included lineages within the *Actinobacteria*, *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, and *Chloroflexi* phyla. 33 genera accounted for ∼88% of the reads for potential cellulases detected (**Figure 2A**; Table S3). Together, potential cellulose degraders were estimated to account for 18.2% of the taxonomically annotated sequences.

Some lineages were associated with only one GH family; for example *Clavibacter*, *Caulobacter*, *Xylella,* and *Saccharophagus* carried almost exclusively cellulases from GH5 (**Figure 2A**). Cellulases from *Methylobacterium*, *Rhizobium,* and *Burkholderia* were associated with GH8, and cellulases from *Nocardioides* and *Jonesia* were mostly associated with GH6. *Thermobispora* possessed enzymes associated with GH12 and a few with GH6. In contrast, other genera (e.g., *Streptomyces*, *Mycobacterium*, *Clostridium*, or *Sorangium*) carried cellulolytic enzymes from an array of GH families. Notably, some rare lineages had high numbers of cellulase genes. Across all samples, the frequency of reads from the bacterial potential cellulose degraders was negatively correlated with the corresponding genus-specific cellulase frequency ($P_{\text{Spearman}} < 0.01$, $r_{\text{Spearman}} = -0.83$; **Figure 2B**). Thus, our results showed that most of the reads for potential cellulase were likely to be derived from a limited set of bacterial genera. Some of these potential cellulose degraders were among the most abundant groups of bacteria detected (e.g., *Clavibacter* and *Streptomyces*) but some less abundant genera harbored a higher number of potential cellulases.

### MICROBIAL COMMUNITY FLUCTUATIONS

Monitoring communities seasonally over a period of 2 years allowed us to place environmental change responses into the context of natural climate variation. In order to investigate microbial responses, we ran a linear mixed effects model followed by ANOVA on the frequencies of sequences from taxonomically or functionally identified groups in each dataset. At the phylum level, the relative abundance of microbial lineages in the leaf litter metagenomes was significantly affected ($P < 0.05$) by the sampling date (Table S4). A few phyla (e.g., *Proteobacteria* and *Ascomycota*) were also marginally affected by the treatments ($P < 0.1$). Thus, our results suggested that, at the phylum level, the taxonomic diversity in leaf litter metagenomes was mostly affected by seasonally varying environmental factors and to a lesser extent by the experimental manipulations.

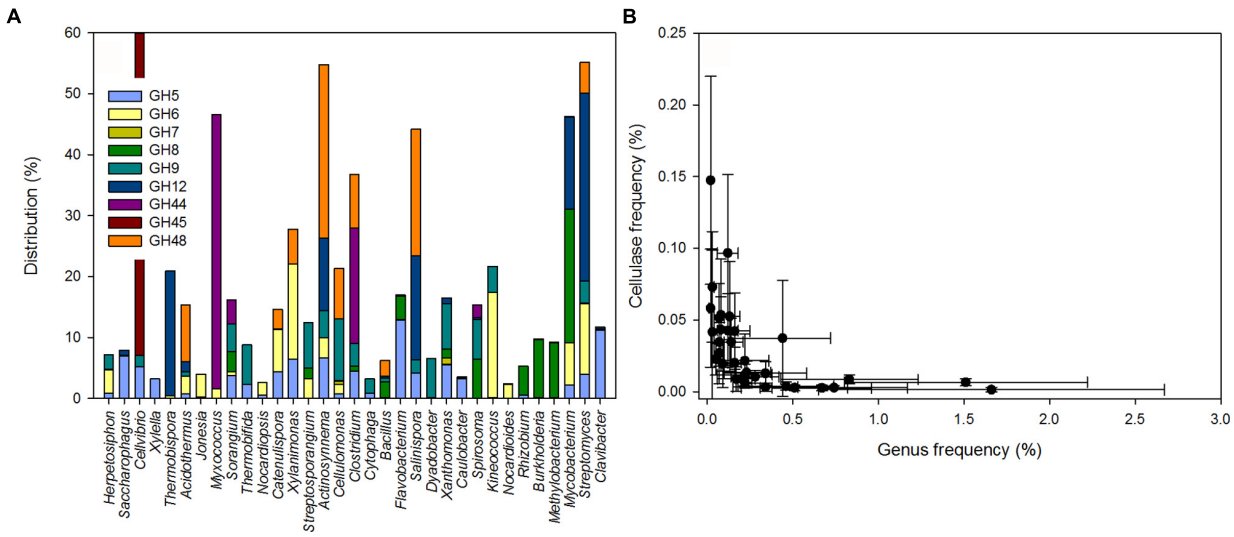


**FIGURE 2 | (A)** Distribution of cellulolytic enzymes in potential bacterial cellulose degraders. **(B)** Relationship between the lineage specific cellulase frequency and the potential cellulose degrading genera in the leaf litter metagenomes ($P_{Spearman} < 0.01$).

## SEASONAL PATTERNS

We investigated the effect of sampling date on overall microbial community composition and observed that the frequencies of sequences from Bacteria and Fungi displayed a seasonal pattern (**Figure 1B**). In the control, sequences from Fungi decreased from 44.2 ± 8.2% during the dry season (i.e., samples 20-August-10, 10-June-11, and 21-September-11) to 16.4 ± 6.4% during the wet season ($P_{Welch-t} < 0.05$). Sequences affiliated with Bacteria, including potential cellulose degraders and non-degraders, displayed the opposite trend, increasing from 36.1 ± 13.6 during the dry season to 70.6 ± 16.0% during the wet season ($P_{Welch-t} < 0.05$). The composition displayed similar temporal trends under reduced precipitation and added nitrogen treatments (**Figure 1B**).

## RESPONSE TO CHANGES IN WATER AND NITROGEN AVAILABILITY

Water availability was affected by seasonal and experimental changes (**Figure 1A**). In the control plots, with few exceptions (e.g., *Clavibacter*, *Kineococcus*, *Bacillus*, and *Clostridium*), the frequencies of the most abundant bacterial lineages were correlated with cumulative precipitation during the 2 weeks prior to litter collection. The frequencies of sequences from *Bacteroidetes* ($P_{Pearson} < 0.05$, $r_{Pearson} = 0.89$), *Proteobacteria* ($P_{Pearson} < 0.05$, $r_{Pearson} = 0.75$), and *Cyanobacteria* ($P_{Pearson} < 0.05$, $r_{Pearson} = 0.76$) were strongly positively correlated with precipitation, whereas *Actinobacteria* were weakly correlated ($P_{Pearson} < 0.05$, $r_{Pearson} = 0.32$). As a whole, potential bacterial cellulose degraders were significantly affected by precipitation ($P_{Pearson} < 0.05$, $r_{Pearson} = 0.57$). Fungal phyla displayed the opposite trends ($P_{Pearson} < 0.05$, $r_{Pearson} = -0.72$), and *Archaea* remained unaffected by the precipitation (**Table 1**; Table S3). This suggested that, among seasonally fluctuating parameters, precipitation strongly affected the microbial community in the leaf litter.

Experimental precipitation reduction reduced the overall bacterial frequencies by ∼10% ($P_{Welch-t} < 0.05$). Nevertheless, the frequency of most bacterial phyla, except *Firmicutes* and *Actinobacteria*, was still significantly correlated with 2-weeks prior precipitation in the reduced precipitation treatment. However, the frequency of bacterial potential cellulose degraders was not correlated with precipitation in this treatment. In contrast, the frequency of sequences for fungi significantly increased under reduced precipitation (**Table 1**).

Cellulase frequencies followed the responses of the bacterial community (**Figure 1C**). GHs ($r_{Pearson} = 0.67$), CBMs ($r_{Pearson} = 0.66$), β-glucosidases ($r_{Pearson} = 0.67$), and cellulases ($r_{Pearson} = 0.49$) were significantly correlated with cumulative precipitation ($P_{Pearson} < 0.05$), under low levels precipitation. Above ∼20 mm precipitation, trait frequency was unaffected by further increase in precipitation (**Figure 3**). We also analyzed how nitrogen deposition affected microbial community structure and the functional potential for litter deconstruction. Under increased nitrogen availability, the frequency of reads from most of the microbial lineages, except *Archaea*, and functional traits remained unchanged (**Table 1**; Table S3).

## LINKING MICROBIAL DIVERSITY TO FUNCTION

The genus-specific cellulase frequency was highly correlated with the abundance of cellulose-degrading genera ($P_{Spearman} < 0.01$; **Figure 4A**). Based on this relationship and the lineage-specific average frequency of cellulases (**Figure 2A**), the total cellulase content in each metagenome sample was highly predictable based on the microbial community composition (at the genus level) of the sample ($P_{Spearman} < 0.01$; **Figure 4B**). As an additional test, we predicted the total cellulase gene content in four samples derived from a combined nitrogen addition and reduced precipitation treatment that were not part of any of the previous analyses (**Figure 4B**). In these four samples, the predicted differed from the observed

**Table 1 | Average frequency (%) of rarefied reads for the most abundant microbial lineages detected in leaf litter metagenomes.**

| | Control | | Reduced precipitation | | Nitrogen addition | |
|---|---|---|---|---|---|---|
| | **Frequency (%)** | **Precipitation $r_P$ (*$P_P$)** | **Frequency (%; *$P_{Welch\text{-}t}$)** | **Precipitation $r_P$ (*$P_P$)** | **Frequency (%; *$P_{Welch\text{-}t}$)** | **Precipitation $r_P$ (*$P_P$)** |
| **Sum of bacteria** | **61.46 ± 22.6** | **0.67*** | **50.38 ± 20.58*** | **0.76*** | **63.63 ± 18.14** | **0.68*** |
| *Proteobacteria* | 27.96 ± 11.61 | 0.75* | 21.81 ± 9.96* | 0.81* | 29.15 ± 10.46 | 0.74* |
| *Actinobacteria* | 22.46 ± 9.67 | | 19.5 ± 9.28 | | 22.85 ± 6.41 | |
| *Bacteroidetes* | 5.29 ± 3.15 | 0.89* | 4 ± 2.31 | 0.72* | 6.15 ± 3.38 | 0.81* |
| *Firmicutes* | 2.5 ± 0.19 | | 2.4 ± 0.48 | | 2.43 ± 0.25 | |
| *Cyanobacteria* | 0.76 ± 0.12 | 0.79* | 0.64 ± 0.07* | 0.69* | 0.72 ± 0.12 | 0.61* |
| *Chloroflexi* | 0.36 ± 0.09 | 0.55* | 0.3 ± 0.06* | 0.78* | 0.33 ± 0.06 | 0.51* |
| *Acidobacteria* | 0.36 ± 0.14 | 0.78* | 0.27 ± 0.1* | 0.70* | 0.33 ± 0.11 | 0.69* |
| *Planctomycetes* | 0.31 ± 0.09 | 0.64* | 0.23 ± 0.05* | 0.74* | 0.28 ± 0.06* | 0.56* |
| *Verrucomicrobia* | 0.3 ± 0.1 | 0.72* | 0.23 ± 0.06* | 0.68* | 0.27 ± 0.08* | 0.60* |
| **Sum of potential cellulase degraders** | **19.36 ± 7.59** | **0.57*** | **15.70 ± 6.76*** | | **19.42 ± 5.37** | **0.60*** |
| **Sum of Archaea** | **0.5 ± 0.05** | | **0.45 ± 0.05*** | | **0.46 ± 0.06*** | |
| *Euryarchaeota* | 0.39 ± 0.03 | | 0.34 ± 0.03 | | 0.35 ± 0.04 | |
| *Crenarchaeota* | 0.1 ± 0.02 | | 0.09 ± 0.02* | | 0.09 ± 0.02$^+$ | |
| *Thaumarchaeota* | 0.01 ± 0 | | 0.01 ± 0 | | 0.01 ± 0$^+$ | |
| **Sum of Fungi** | **27.33 ± 15.12** | **−0.72*** | **38.45 ± 15.52*** | **−0.79*** | **26.45 ± 12.65** | **−0.72*** |
| *Ascomycota* | 25.39 ± 14.56 | −0.72* | 36.39 ± 14.95* | −0.79* | 24.74 ± 12.13 | −0.72* |
| *Basidiomycota* | 1.89 ± 0.61 | −0.56* | 2.01 ± 0.73 | −0.58* | 1.67 ± 0.76 | −0.54* |
| *Unclassified Fungi* | 0.03 ± 0.02 | −0.55* | 0.03 ± 0.02 | −0.70* | 0.02 ± 0.01 | −0.51* |
| *Glomeromycota* | 0.01 ± 0 | −0.68* | 0.01 ± 0 | | 0.01 ± 0 | −0.55* |

*Significant treatment effects relative to controls are denoted with an asterisk (*$P < 0.05$, paired Welch t-test). Effect of cumulative precipitation (Precip., $r_{Pearson}$) on the frequency of lineages in each treatment (*$P_{Pearson} < 0.05$).*

cellulase abundance by 14% (616 cellulases sequences predicted but 530 detected), in total.

In order to test for a relationship between drought or nitrogen responses and potential for cellulose deconstruction, we analyzed the correlation between rarefied reads for potential cellulase and log response ratios for abundance response to experimental precipitation reduction and increased nitrogen availability for the entire microbial community (at the genus level). In contrast to our hypothesis (i.e., correlation between cellulolytic traits and responses to precipitation and nitrogen availability), the frequency of cellulolytic traits were not correlated with response ratios ($P_{Spearman} > 0.05$).

## DISCUSSION

### PATTERNS IN DIVERSITY AND CELLULOLYTIC POTENTIAL

Generally, Fungi are assumed to be the most active microorganisms in litter deconstruction (Boer et al., 2005; Deacon et al., 2006; van der Wal et al., 2013). For example, a recent proteomic analysis suggests that Fungi express many hydrolytic enzymes (Schneider et al., 2012), and another study shows the incorporation of labeled substrates into mainly fungal biomarkers (van der Wal et al., 2013). Here, we observe that the majority of sequences for GHs in grassland leaf litter metagenomes are associated with bacterial lineages. This result is consistent with relatively high bacterial biomass in the leaf litter from Loma Ridge (Alster et al., 2013) and the representation of bacterial GHs in fosmid libraries derived from the same litter and shown to be active on plant polymers (Nyyssönen et al., 2013). As described for forest litter (Stursová et al., 2012), the microbial communities involved in cellulose deconstruction in grassland leaf litter may also be enriched in bacteria due to differences in leaf litter chemistry and climatic conditions. Thus, cellulolytic bacteria may be important for litter decomposition.

We detected sequences in leaf litter communities affiliated with every known cellulolytic GH family. In contrast, cellulases are less diverse in microbial communities involved in plant cell wall deconstruction in the cow rumen (Hess et al., 2011),

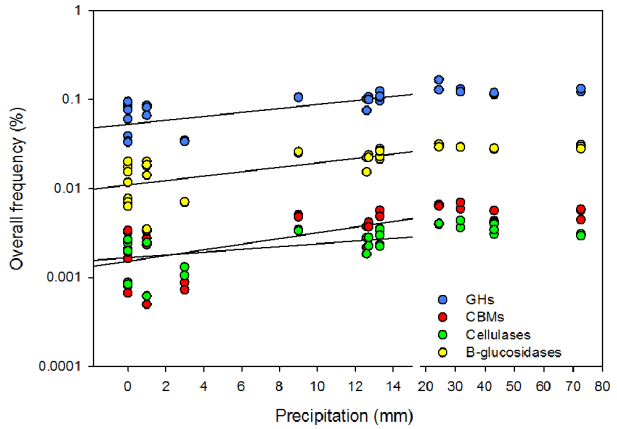


**FIGURE 3 | Effect of the cumulative precipitation during the 2 weeks prior to litter collection on the overall rarefied frequency of microbial glycoside hydrolases (GHs), carbohydrates binding modules (CBMs), β-glucosidases, and cellulases.** Break in the *x*-axis was introduced to discriminate low from high precipitation.

a fungus garden (Suen et al., 2010), or the termite hindgut (Warnecke et al., 2007). Leaf litter potentially contains more complex and heterogeneous substrates and displays more fluctuations in environmental parameters (e.g., substrate chemistry and availability, temperature, and water/oxygen availability). This variation may lead to genetically and functionally diverse communities.

Many of the bacterial potential cellulose degraders we identified are commonly involved in cellulose deconstruction including Streptomyces (Semêdo et al., 2000), *Sorangium* (Hou et al., 2006), *Myxococcus* (Bensmail et al., 1998), *Acidothermus* (Barabote et al., 2009), and *Thermobispora* (Anderson et al., 2012). In most cases, these lineages are associated with cellulolytic activity resulting from the expression of multiple and diverse genes for cellulases (Wilson, 2011). In agreement with the CAZY-genome database (Lombard et al., 2014), some less frequent potential cellulose degraders harbor a higher frequency of reads for putative cellulases including *Herpetosiphon aurantiacus*, *Saccharophagus degradans*, and *Sorangium cellulosum*. This suggests that potential cellulolytic lineages that are less frequent may have an impact on litter deconstruction as shown for some specific fungal taxa (Deacon et al., 2006). On the other hand, the most abundant potential cellulose degraders (e.g., *Streptomyces*) display a lower frequency of cellulase sequences. This result is in good agreement with the hypothesis that reducing the number of cellulase genes in bacteria may reduce the cost of enzyme production and allow a higher growth rate (Allison, 2012). We speculate that such a high variability of the cellulolytic potential, together with other adaptations [i.e., filamentous growth of *Streptomyces* (Boer et al., 2005) and cellulolosome production of *Saccharophagus* and *Clostridium* (Sabathe et al., 2002; Taylor et al., 2006)] increases the decomposition of plant material by providing synergistic biochemical activities that target different fractions of the substrate (Hättenschwiler et al., 2011; van der Wal et al., 2013).

Sequences for putative β-glucosidases in bacterial potential cellulose degraders account for 52% of the detected sequences from GH families 1 and 3. As previously suggested, it is likely that these enzymes are broadly distributed in bacteria including many non-cellulolytic lineages (i.e., opportunists or cheaters; Berlemont and Martiny, 2013). Indeed, many bacterial lineages, including some non-degraders, are stimulated when subjected to labile oligosaccharides (Goldfarb et al., 2011). As a consequence, β-glucosidase activity in environmental samples is likely a poor proxy for the degradation of complex cellulose polymers. However, opportunists may contribute indirectly to plant litter degradation by processing cellulose deconstruction byproducts.

## MICROBIAL RESPONSE TO ENVIRONMENTAL MANIPULATIONS

Our data are consistent with prior findings suggesting that Fungi are less negatively affected by low water availability than bacterial populations and are thus more frequent under seasonally occurring or experimental drought (Allison et al., 2013; Barnard et al., 2013; van der Wal et al., 2013). Indeed, experimental precipitation

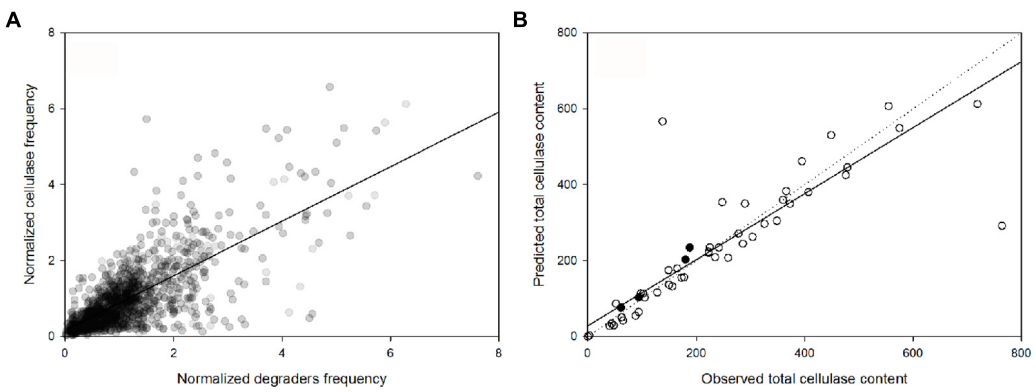


**FIGURE 4 | (A)** Linear dependence of the normalized abundance of potential cellulose degraders and their potential cellulase content, across the samples (PSpearman < 0.01). **(B)** Comparison between the observed and the predicted cellulase content based on microbial community composition [samples used to construct the linear regression model (○) and independent samples from the factorial reduced precipitation and added nitrogen treatment (●)]. The solid and dotted lines are the regression line and the 1:1 line, respectively.

reduction marginally, but significantly, increases fungal relative abundance. At our site, this increased frequency of reads from fungi under dry conditions is likely to result from a reduction in bacterial biomass (Allison et al., 2013). Although most bacterial relative abundances declined under low water availability, there was some variation in response at the genus level within the *Actinobacteria* and *Firmicutes*. In some genera within these phyla, tolerance to desiccation is likely achieved through multiple strategies to survive reduced water potential [e.g., production of a surfactant in *Bacillus* (Straight et al., 2006), exopolysaccharides in *Pseudomonas* (Roberson and Firestone, 1992), osmolytes in *Cyanobacteria* (Rajeev et al., 2013)].

### LINKS BETWEEN TAXONOMY AND FUNCTION

Across seasons and treatments, bacterial responses to reduced precipitation and nitrogen addition were not directly correlated with cellulolytic potential. However, the ratio of Bacteria to Fungi was reduced under seasonal drought and experimental precipitation reduction. Fungi have relatively few cellulases, and litter decomposition experiments show that litter decay rates are lower during seasonal drought periods and in the reduced precipitation treatment (Allison et al., 2013). This pattern could be interpreted as a reduced role of Fungi in comparison to Bacteria in litter decomposition in this ecosystem, but further studies are needed to quantify the contribution from the two groups. Also contrary to our physiological tradeoffs hypothesis, copiotrophic Bacteria that were favored in nitrogen-enriched plots did not show higher genetic cellulolytic potential.

Consistent with our initial hypothesis, the genetic potential for cellulose deconstruction was highly predictable based on the taxonomic composition of the microbial community. This finding aligns with the previously described conservatism at the genus-species level of genes for cellulases in sequenced bacterial genomes (Berlemont and Martiny, 2013). Our current study generalizes this pattern to diverse communities containing poorly sequenced (e.g., *Acidobacteria* and *Chloroflexi*) or hyper-variable taxa (e.g., *Bacillus*; Berlemont and Martiny, 2013), thereby reducing the dependence of trait prediction on sequenced genomes. Furthermore, a phylogeny-function relationship allows for the prediction of the cellulolytic potential in taxonomically resolved communities.

The sequencing of metagenomes involves some important potential limitations. The approach may have a bias due to variations in extraction efficiency among different lineages. Also, poorly characterized bacterial phyla (e.g., *Acidobacteria*) and complex genomes from fungi are unevenly detected in metagenomic studies due the reliance on a small number of previously annotated genes and genomes. Thus, the frequencies and metabolic potentials of phyla with few genome sequences are likely underestimated. In addition, we specifically recognize that some genes from GHs identified as potential cellulases may possibly have other enzymatic functions (Berlemont and Martiny, 2013; Nyyssönen et al., 2013). In addition, some enzymes identified as cellulases may be involved in cellulose biosynthesis or in the interaction between microorganisms and plants (Medie et al., 2012; Berlemont and Martiny, 2013). For example, genera described as plant pathogens [e.g., *Clavibacter* (Jahr et al., 2000)] or plant growth promoting rhizo-bacteria [e.g., *Rhizobium* (Robledo et al., 2008)] may not contribute to cellulose degradation. As is the case for most metagenomic analyses, these biases may influence our results in unknown ways as most traits have not been fully characterized genetically and/or biochemically. Despite these caveats, our metagenomic approach provides a powerful tool for linking microbial community composition and potential function under environmental change. When combined with experimental confirmation of biochemical function (Nyyssönen et al., 2013), highly robust linkages between microbial composition and ecosystem processes may be achieved.

Our data show that fungi are drought resistant, but they are likely not the primary contributors to the cellulolytic potential in this grassland litter community. Rather, changes in cellulolytic potential due to seasonality and experimental precipitation reduction are driven by the dynamics of bacterial taxa that are highly sensitive to drought. Together with previous study (Allison et al., 2013), these results suggest that drought-associated reductions in litter decay and cellulose deconstruction may be related to shifts in microbial community composition and not simply direct moisture limitation. Under nitrogen addition, litter decay is not likely to increase through effects on cellulolytic potential. Describing the effect of precipitation reduction and nitrogen deposition, across seasons, on microbial communities involved in plant material deconstruction is a prerequisite for future investigation of combined effects of these perturbations. More broadly, the phylogenetic conservatism of functional traits and the response of microbial taxa to simulated environmental changes provide a robust conceptual framework to predict how microbial communities will respond to global changes and impact ecosystem functioning.

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## SUPPLEMENTARY MATERIAL

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# Bacterial community structure across environmental gradients in permafrost thaw ponds: methanotroph-rich ecosystems

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Permafrost thawing leads to the formation of thermokarst ponds that potentially emit $CO_2$ and $CH_4$ to the atmosphere. In the Nunavik subarctic region (northern Québec, Canada), these numerous, shallow ponds become well-stratified during summer. This creates a physico-chemical gradient of temperature and oxygen, with an upper oxic layer and a bottom low oxygen or anoxic layer. Our objective was to determine the influence of stratification and related limnological and landscape properties on the community structure of potentially active bacteria in these waters. Samples for RNA analysis were taken from ponds in three contrasting valleys across a gradient of permafrost degradation. A total of 1296 operational taxonomic units were identified by high throughput amplicon sequencing, targeting bacterial 16S rRNA that was reverse transcribed to cDNA. β-proteobacteria were the dominant group in all ponds, with highest representation by the genera *Variovorax* and *Polynucleobacter*. Methanotrophs were also among the most abundant sequences at most sites. They accounted for up to 27% of the total sequences (median of 4.9% for all samples), indicating the importance of methane as a bacterial energy source in these waters. Both oxygenic (cyanobacteria) and anoxygenic (Chlorobi) phototrophs were also well-represented, the latter in the low oxygen bottom waters. Ordination analyses showed that the communities clustered according to valley and depth, with significant effects attributed to dissolved oxygen, pH, dissolved organic carbon, and total suspended solids. These results indicate that the bacterial assemblages of permafrost thaw ponds are filtered by environmental gradients, and are complex consortia of functionally diverse taxa that likely affect the composition as well as magnitude of greenhouse gas emissions from these abundant waters.

**Keywords: bacterial diversity, methanotrophs, permafrost, pyrosequencing, 16S rRNA, thaw ponds**

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## Introduction

One of the impacts of ongoing climate change is the northward migration of the limit of permafrost soils in subarctic landscapes, and this is leading to changes in the distribution and abundance of lakes and ponds caused by permafrost thawing and erosion (Vincent et al., 2013). These so called

thaw ponds (thermokarst lakes and ponds) represent the most widespread aquatic ecosystem type in the circumpolar Arctic and Subarctic (Pienitz et al., 2008; Koch et al., 2014). In some northern regions of the Arctic, thaw lakes are disappearing as a result of evaporation and drainage (Smith et al., 2005), whereas in some southern locations such as subarctic Québec, Canada, permafrost lakes are expanding in size and numbers through increased permafrost thawing and erosion (Payette et al., 2004). Thaw lakes show cycles of expansion, erosion, drainage, and reformation (van Huissteden et al., 2011) that will likely accelerate under warmer climate conditions (Vincent et al., 2013).

Thawing permafrost has global implications for carbon biogeochemical cycling, since carbon that has been sequestered for 1000s of years becomes available for microbial degradation (Tranvik et al., 2009), resulting in the production of greenhouse gasses, especially carbon dioxide and methane. Despite this potential, greenhouse gas emissions from northern lakes and thaw ponds are often ignored in regional and global carbon budgets. These open waters potentially represent a source of around 24 Tg of methane emission per year (Walter et al., 2007). Methane is primarily produced by a few archaeal clades under anoxic conditions (Sowers, 2009), although methanogenesis has also been observed in oxic water columns (Grossart et al., 2011; Bogard et al., 2014). This biologically generated methane is available to aerobic methanotrophic bacteria that occur in the oxic zone or at oxic/anoxic boundaries (Borrel et al., 2011), and this methane oxidation activity may regulate net greenhouse gas emissions (Trotsenko and Khmelenina, 2005; Bodelier et al., 2013); for example, methanotrophy consumed up to 80% of the methane produced in a boreal Finnish lake (Kankaala et al., 2006). Thaw ponds can be either sources of greenhouse gas emissions (Walter et al., 2007; Laurion et al., 2010; Negandhi et al., 2013), or sinks for carbon sedimentation and storage (Walter et al., 2014), but reasons for these differences are poorly understood. Conditions that favor methanotrophy will have the net effect of decreasing methane release to the atmosphere, however, little is known about such processes in the abundant lakes and ponds on degrading permafrost landscapes.

Bacterial communities vary among lakes as a result of differences in physico-chemical and biological properties such as pH (Lindström et al., 2005), productivity and dissolved organic carbon (DOC) availability (Lindström and Leskinen, 2002; Yannarell and Triplett, 2004). For this reason even neighboring lakes in the same region may differ in their bacterial community structure (Casamayor et al., 2000; Van der Gucht et al., 2001). In addition, catchment and underlying soil properties vary, resulting in differences in the quantity and composition of organic matter entering lakes, which in turn may affect their bacterial communities (Judd et al., 2006; Kritzberg et al., 2006). Bacterial community composition in lakes also changes with depth (Shade et al., 2008; Garcia et al., 2013), especially in meromictic lakes where temperature, salinity, and oxygen gradients select for distinct communities down the water column (Hollibaugh et al., 2001; Comeau et al., 2012). Notably, a clone library study by Rossi et al. (2013) found distinct bacterial communities in surface and bottom waters of thaw ponds in northern Québec.

Arctic and sub-arctic ponds occur across a range of conditions that could select for particular bacterial taxa. For example, in northern Québec varied combinations of dissolved organic matter (DOM) and suspended particles result in striking differences in the optical properties of neighboring thaw ponds (Watanabe et al., 2011) and characteristic bacterial communities could be associated with the particular optical properties of individual ponds. The extent and stage of thawing of the surrounding permafrost could also potentially affect bacterial community structure; some thaw ponds occur in highly degraded permafrost while others are surrounded by more than 50% of intact permafrost. These different stages of permafrost thaw influence the landscape characteristics (e.g., vegetation cover, open water extent) and the geomorphology of the ponds (Bouchard et al., 2014), creating a gradient in concentrations of allochthonous DOM. With thawing more pronounced at the southern limits of permafrost, bacterioplankton in these regions would have greater access to this external supply of organic matter. Since microbial communities control biogeochemical processes such as methane balance, understanding factors that influence bacterial community composition is central for predicting net greenhouse gas emissions from the thawing landscape. Clone library analysis of four northern Québec ponds reported the presence of methanotrophs (Rossi et al., 2013), but little is known about how communities might vary over a range of permafrost conditions.

In addition, bacterial α-diversity (number of taxa per lake) could be influenced by local conditions such as depth, light availability, and pond productivity. For example, diversity is expected to be lower where low oxygen and light conditions select for a few specialist taxa (Shade et al., 2008). Lower primary productivity has also been linked with a lower diversity (Cardinale et al., 2006; Ptacnik et al., 2008), and light and nutrient limitation would therefore influence diversity. On a landscape scale, the diversity of animals and plants decreases at higher latitudes (Rahbek, 1995; Falge et al., 2002). However Fierer and Jackson (2006), found that for soil microbes α-diversity is independent of latitude but more related to soil properties, for example pH. If this were the case for thaw ponds then inherent properties of the landscape could also influence the α-diversity.

The goal of the present study was to investigate the diversity and distribution of potentially active bacterial communities in subarctic thaw ponds across a gradient of limnological conditions and permafrost, from discontinuous permafrost in the North to sporadic permafrost in the South. We applied high throughput sequencing, targeting the V6–V8 hypervariable region of 16S rRNA. The use of this hypervariable region enabled us to identify community assemblages in three geographically separate valleys that differed in their underlying stage of permafrost degradation. Specifically we tested the hypotheses that: (1) different bacterial communities develop at the surface and bottom of each thaw pond because of the strong physico-chemical gradient through the water column; (2) bacterial community composition varies across the gradient of permafrost thawing and degradation; and (3) given the known high concentrations of methane in these waters (Laurion et al., 2010), methanotrophic bacteria are well-represented in thaw pond bacterial assemblages.

## Materials and Methods

### Study Sites and Sampling

Samples were collected from 1 to 13 of August 2012 from three different subarctic valleys (**Figure 1**), which were chosen to represent a gradient of thawing permafrost. Ponds from these valleys have been investigated since 2006 and nomenclature follows that of earlier studies (Calmels et al., 2008; Breton et al., 2009; Laurion et al., 2010; Watanabe et al., 2011). The ponds were designated with a three letter prefix and a unique number or number letter combination. The KWK (55°16′N; 77°46′W) and SAS (55°13′N; 77°42′W) ponds are in two separate river valleys close to the village of Whapmagoostui-Kuujjuarapik in the sporadic permafrost zone, while the BGR ponds (56°37′N; 76°13′W) are in the Sheldrake River valley close to the village of Umiujaq, Québec, in the discontinuous permafrost region. The ponds have formed in thawing permafrost mounds that are primarily organic (peat) or mineral; the term palsa refers to organic mounds and lithalsa to mineral mounds (Gurney, 2001; Calmels et al., 2008). KWK and BGR ponds originated from the thawing of lithalsas and SAS ponds from palsas. BGR and KWK ponds are surrounded by shrubs (*Salix planifolia* and *Betula glandulosa*) and sparse trees (*Picea mariana*, *Picea glauca*, *Larix laricina*; Calmels et al., 2008; Breton et al., 2009). The SAS valley vegetation, in contrast, is dominated by *Carex* and *Sphagnum* (Arlen-Pouliot and Bhiry, 2005; Bhiry et al., 2011). Two ponds were selected from the BGR valley (1 and 2), three from the KWK valley (1, 6, and 23) and two from the SAS valley (1B and 2A; **Table 1**). Ponds were sampled from an inflatable boat held near the center of the ponds using ropes tethered to the shore. Temperature, conductivity, dissolved oxygen (DO), and pH profiles were taken using a 600R multiparametric probe (Yellow Spring Instrument). Surface water samples were collected directly into submerged acid and sample rinsed 4-L Cubitainers and near-bottom samples were collected using a horizontally mounted Van Dorn bottle (Wilco) and immediately transferred to 4-L Cubitainers. Care was taken to overfill the Cubitainers when sampling the bottom water and all the Cubitainers were capped to minimize exchange with the atmosphere. The filled Cubitainers were then transported back to the laboratory by helicopter and processed within 2 h.

### Physico-Chemical Analysis

Water samples for DOC, soluble reactive phosphorus (SRP), and major ion analysis were filtered through a MilliQ water pre-rinsed 47-mm diameter 0.22-μm pore size acetate filters (Whatman). DOC concentrations were analyzed using a Shimadzu TOC-5000A carbon analyzer calibrated with potassium biphthalate. Three blank filters of Milli-Q water passed through the filters were analyzed along with the samples and these small background values were subtracted. Water samples for total phosphorus (TP) and total nitrogen (TN) were preserved with $H_2SO_4$ (0.15% final concentration) and analyzed using standard methods (Stainton et al., 1977) at Institut National de la Recherche

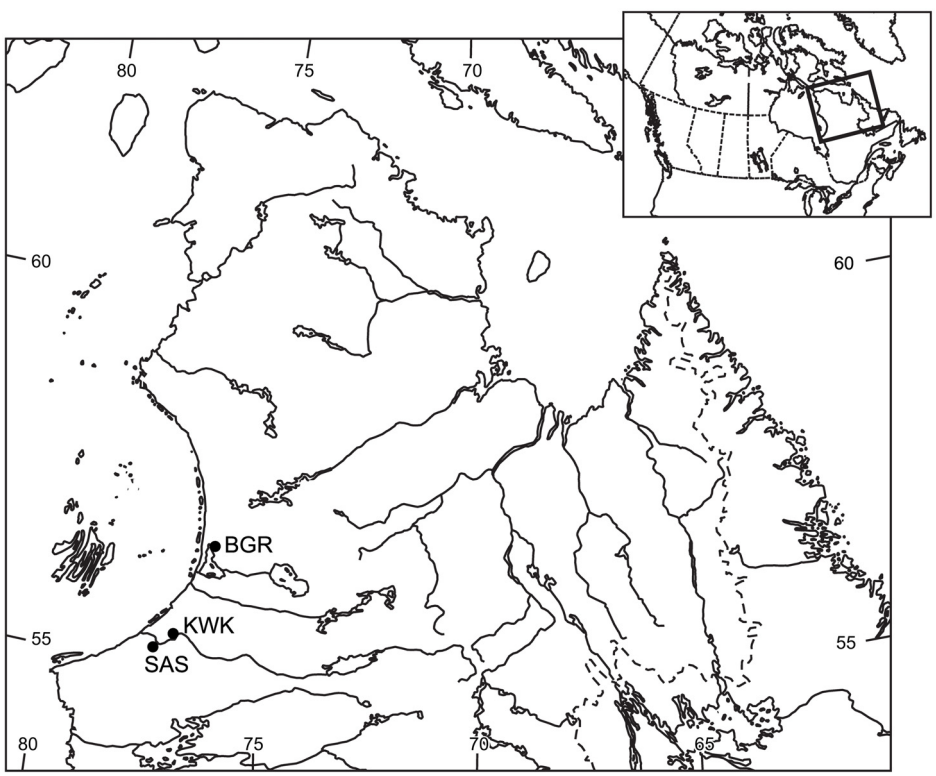


**FIGURE 1 | Location of the three sampling valleys in Nunavik, subarctic Québec, Canada.**

**TABLE 1 | Limnological properties of the sampled thaw ponds: dissolved oxygen (DO), DO % saturation (% sat.), conductivity (Cond), chlorophyll *a* (Chl *a*), dissolved organic carbon (DOC), total suspended solids (TSS), soluble reactive phosphorus (SRP), and total nitrogen (TN).**

| Pond | Depth (m) | DO (mg $L^{-1}$) | DO (% sat.) | pH | Cond ($\mu$S $cm^{-1}$) | Chl *a* ($\mu$g $L^{-1}$) | DOC (mg $L^{-1}$) | TSS (mg $L^{-1}$) | SRP ($\mu$g $L^{-1}$) | TN (mg $L^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|---|
| SAS1B | 0 | 6.37 | 63 | 6.0 | 93 | 4.9 | 15.5 | 27 | 2.7 | 0.9 |
| | 1 | 1.54 | 15 | 5.63 | 212 | 2.9 | 16.2 | 33 | 3 | 1.8 |
| SAS2A | 0 | 5.75 | 64 | 6.2 | 97 | 1.7 | 14.9 | 2.6 | 3.1 | 0.7 |
| | 2.4 | 0.26 | 2 | 5.58 | 300 | 18.1 | 18.9 | 16 | 4.1 | 1.6 |
| KWK1 | 0 | 9.69 | 101 | 6.66 | 63 | 10.9 | 12 | 26 | 3.7 | 0.6 |
| | 1.8 | 0.50 | 4.2 | 6.22 | 150 | 10.3 | 12 | 141 | 12.6 | 1.0 |
| KWK6 | 0 | 9.94 | 97 | 6.36 | 82 | 3.3 | 5.2 | 8.2 | 1.3 | 0.4 |
| | 3 | 1.82 | 17 | 6.35 | 112 | 27.1 | 5.2 | 16 | 1 | 0.7 |
| KWK23 | 0 | 9.8 | 97 | 6.44 | 29 | 1.9 | 7.8 | 8.3 | 5.5 | 0.4 |
| | 3.2 | 0.36 | 2.7 | 6.09 | 259 | 7.2 | 10.9 | 74 | 133.6 | 2.7 |
| BGR1 | 0 | 10.0 | 101 | 7.38 | 168 | 0.9 | 3.5 | 2.4 | 2.4 | 0.2 |
| | 3.2 | 4.06 | 37 | 7.56 | 190 | 1.1 | 3.3 | 3.8 | 2.2 | 0.4 |
| BGR2 | 0 | 9.43 | 94 | 7.31 | 209 | 2.4 | 9.3 | 13 | 3.4 | 0.4 |
| | 1 | 3.47 | 34 | 7.17 | 387 | 3.8 | 8.7 | 57 | 4.5 | 1.2 |

*The surface samples correspond to 0 m and bottom samples to the second depth for each pond.*

Scientifique (Quebec City, QC, Canada). Total suspended solids were collected by filtration onto preweighed 47 mm GF/F filters (Whatman) that had been precombusted at 500°C for 4 h. The GF/F filters were then oven dried for 2 h at 60°C and reweighed. Samples for chlorophyll *a* (Chl *a*) were filtered onto a GF/F 25 mm filters (Whatman) and stored at −80°C. Chl-*a* concentrations were determined using high performance liquid chromatography (ProStar HPLC system, Varian, Palo Alto, CA, USA) following the protocol of Bonilla et al. (2005).

## RNA Collection and Extraction

Water samples were first prefiltered through a 20-μm mesh to remove larger organisms and then filtered sequentially through a 3-μm pore size, 47-mm diameter polycarbonate filter (DHI lab product) and a 0.2 μm Sterivex unit (Millipore) with a peristaltic pump. From 100 to 600 mL of water were filtered and the filtration was stopped after 2 h to minimize RNA degradation. The size fractionation was employed to distinguish between particle-attached bacteria on the 3-μm filter and free-living bacteria on the 0.2-μm filter (Crump et al., 1999; Galand et al., 2008; Mohit et al., 2014). Both filters were preserved in RNAlater (Life Technologies) and the filters were stored at −80°C until processed. The PowerWater Sterivex DNA Isolation Kit was used for extracting the RNA from the Sterivex units for the BGR1, KWK6 surface, KWK23 surface, and SAS1B surface. The large fraction for the same samples was extracted using the PowerWater DNA Isolation kit (MoBio). Protocols were adapted for RNA analysis by adding 1–2% (final concentration) of β-mercaptoethanol as a preliminary step. The extraction column was loaded with 50% ethanol (final concentration) to fix the RNA to the column. RNase-free water was used for the final elution step. The co-extracted DNA was then digested with the RTS DNase Kit (MoBio). Following problems with potential polyphenol contamination in some samples, the remaining samples were extracted with the AllPrep DNA/RNA Mini Kit (Qiagen). This protocol was modified by the addition of cross-linked polyvinylpyrrolidone (PVP, Alfa Aesar) to a final concentration of 10% before loading the samples onto the lysate homogenization column. Prior to use, the PVP was sterilized with UV light and was then added as a reagent to remove potential contaminating organic matter (humic acids and polyphenols). For all samples, the extracted RNA was converted to cDNA immediately with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems-Ambion). The concentrations and quality of cDNA were checked on a 1% agarose gel; cDNA was not detected from the large fractions of KWK23 and the two SAS ponds, and these samples were therefore not further processed. All cDNA was stored at −80°C until analysis.

## High Throughput Multiplex Tag Sequencing

The V6–V8 region of the bacterial 16S rRNA that had been converted to cDNA was amplified using the 454 primers as described in Comeau et al. (2011). PCR was carried out in a total volume of 20 μL; the mixture contained HF buffer 1X (NEB), 0.25 μM of each primer, 200 μM of each dNTPs (Life Technology), 0.4 mg $mL^{-1}$ BSA (NEB), 1 U of Phusion High-Fidelity DNA polymerase (NEB), and 1 μL of template cDNA. Two more reactions with 5X and 10X diluted template were also carried out for each sample, to minimize potential primer bias. Thermal cycling began with an initial denaturation at 98°C for 30 s, followed by 25 cycles of denaturation at 95°C for 10 s, annealing at 50°C for 30 s, extension at 72°C for 30 s and a final extension at 72°C for 420 s. The three dilution reactions were pooled and purified with a magnetic bead kit Agencourt AMPure XP (Beckman Coulter) and then quantified spectrophotometrically with the Nanodrop 1000 (Thermo Fisher Scientific). The amplicons were sequenced on two 1/8 plates of the Roche 454 GS-FLX using the "PLUS" chemistry at the IBIS/Laval University, Plate-forme d'Analyses Génomiques (Québec, QC). Raw 454 sequences have been deposited in the NCBI database under accession number SRP050189.

### Sequence Processing and Statistics

Sequences were analyzed using the UPARSE pipeline (Edgar, 2013). For quality filtering, the sequences were truncated at 340 and 300 bp for the first and second 1/8 plate runs to keep 75% of the reads at the 0.5 expected error rate. Singletons as well as chimeras were then removed and operational taxonomic units (OTUs) were determined at the ≥97% similarity level. Taxonomic assignment of these OTUs was performed using the Mothur classifier (Schloss et al., 2009) with a 0.8 confidence threshold based on the SILVA reference database (Pruesse et al., 2007) modified to include sequences from our in-house, curated northern 16S rRNA gene sequence database. Shannon, Simpson, and Chao1 diversity indexes were estimated for each sample using quantitative insights into microbial ecology (QIIME) pipeline (Caporaso et al., 2010) by creating multiple rarefaction statistics. Species richness and evenness were estimated with Shannon and Simpson indexes while the Chao1 index provides an estimate of true richness (Colwell, 2012). Three-way analysis of variance (three-way ANOVA) was used to assess differences in the diversity indices between valleys, fractions, and depths. As the Simpson index is a proportion, the data were arcsin transformed to achieve normality. An a posteriori Tukey HSD test was run to identify differences between valleys.

β-diversity analysis was used as a means of comparing ponds; for this analysis the dataset was re-sampled to ensure the same number of reads per sample (3071), which corresponded to the sample with the fewest number of sequences. β-diversity was derived using the command multiple_rarefaction_even_depth.py in QIIME. The rarefaction curve for OTUs reached a plateau between 2000 and 4000 reads, indicating that our selection of 3071 reads provided a reasonable representation of the community.

Downstream statistical analyses were performed in R (version 3.0.1; R Core Team, 2013) using the package Vegan (Oksanen et al., 2013). The community data matrix was square root transformed before running the statistical analyses. β-diversity was assessed by the use of Bray–Curtis dissimilarity to estimate compositional difference among sampling sites. Distance-based redundancy analysis (dbRDA) was performed to test and identify the influence of environmental variables on the composition matrix. For the latter analysis, explanatory variables were selected via a forward selection and highly correlated variables were removed from the analysis to ensure that colinearity would not reduce analysis quality. The variance inflation factors (VIFs) were calculated for each variable and none of the coefficients exceeded the value of 5, we note that multicollinearity should be examined when the VIF exceeds 10 (Borcard et al., 2011). Compositional differences among valleys, depths, and fraction were tested with permutation tests (999 permutations) on the Bray–Curtis metric using the function Adonis in the Vegan package (Oksanen et al., 2013).

We defined 'bacterial dominants' as the 10 most abundant OTUs from each valley. Each OTU was submitted to a separate BLASTn search in NCBI GenBank (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to identify the nearest match. In some cases a single OTU corresponded to two separate genera. We constructed a reference tree using longer sequences from clone libraries and GenBank to resolve these uncertainties. The short reads were mapped onto the reference tree using the ParInsert command available in QIIME. In many cases, several different OTUs had matches to a single genus.

## Results

### Limnological Conditions

Temperatures decreased down the water column, with markedly colder bottom waters in all of ponds (**Figure 2A**). The surface waters were well-oxygenated except for the SAS sites, which had only 5–6 mg $L^{-1}$ of DO at the surface (**Table 1** and **Figure 2B**). DO concentrations fell to lower values with depth (**Figure 2B**). Ponds were hypoxic (<2 mg $L^{-1}$) at the near-bottom sampling

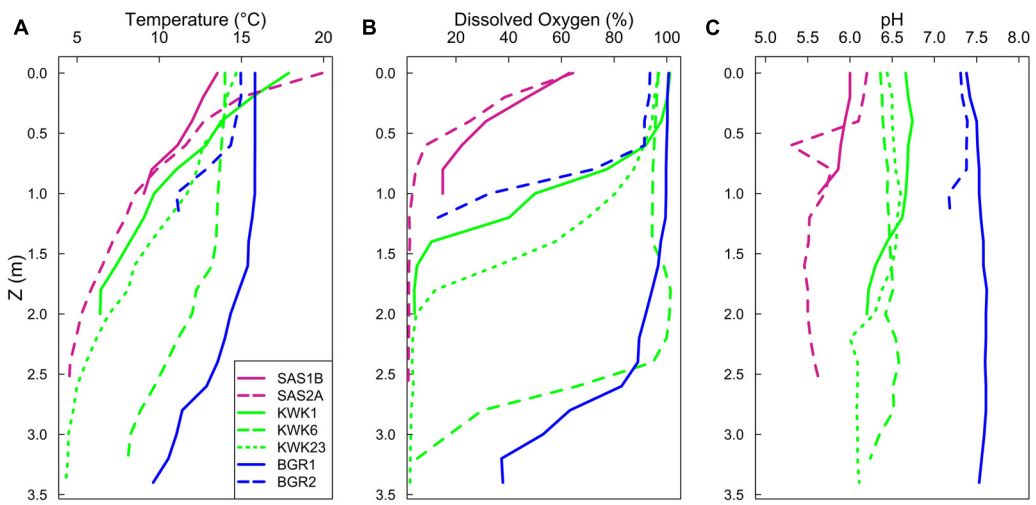


**FIGURE 2 | Profiles of temperature (A), dissolved oxygen (B), and pH (C) as a function of the depth for the seven studied ponds.**

depths, with the exception of the bottom of the BGR ponds where DO levels were around 3–4 mg $L^{-1}$ (**Table 1**). There was a pH gradient across the three valleys: the SAS valley ponds were the most acidic with pH values from 5.3 to 6.2, while those in the KWK valley ranged from 6.0 to 6.7 and in the valley from 7.1 to 7.5. In all ponds, pH decreased slightly with the depth (**Figure 2C**). The other measured limnological variables also showed large differences between valleys, ponds, and depths (**Table 1**). Conductivity was higher in the bottom waters, by up to an order of magnitude in KWK23. DOC concentrations varied from <4 mg $L^{-1}$ in the blue–green colored surface waters of BGR1 to >15 mg $L^{-1}$ in the black SAS ponds. The total suspended solids varied by up to an order of magnitude, even among ponds from the same valley; (for example, SAS1B versus SAS2A and BGR1 versus BGR2), and concentrations increased with depth in all ponds. The indicators of trophic state (Chl *a*, SRP, and TN) were mostly in the oligotrophic to mesotrophic range, with higher values near the bottom of some the ponds, notably KWK23 (SRP and TN), and SAS2A and KWK6 (Chl *a*).

## Bacterial α-Diversity

The sequencing statistics for individual ponds are given in Table S1. For the overall study, 112,479 sequences (reads) were obtained, yielding a total of 1296 OTUs (excluding singletons, defined as OTUs that only occurred once in the entire data set). Three-way ANOVA for the different sample groupings (**Table 2**) showed that there was no significant difference in α-diversity as estimated by either the Shannon or Simpson indices between depths, size fractions, or valleys. However, the Chao1 index showed significant differences between valleys ($p = 0.018$) and fraction ($p = 0.004$), and a significant interaction between valleys and depth ($p = 0.01$). The mean species richness of the KWK ponds was 32% higher than in the BGR ponds, and the Tukey HSD test showed that the significant difference between valleys was only between KWK and BGR ($p = 0.015$). The mean Chao1 index for the large fraction was 22% greater than for the small fraction (**Table 2**).

## Bacterial β-Diversity and Community Composition

All ponds were dominated by Proteobacteria followed by Bacteroidetes, Verrucomicrobia, and Actinobacteria (**Figure 3**).

**TABLE 2 | Sequencing and diversity statistics for samples grouped according to valley, depth, or size fraction.**

| Sample group | OTUs[a] | Shannon[a] | Simpson[a] | Chao1[a] |
|---|---|---|---|---|
| SAS | 183 (11) | 4.35 (13) | 0.88 (4) | 210 (8) |
| KWK | 211 (27) | 5.09 (10) | 0.91 (8) | 224 (23) |
| BGR | 156 (39) | 4.58 (16) | 0.89 (6) | 170 (38) |
| Surface | 176 (26) | 4.98 (14) | 0.92 (5) | 194 (25) |
| Bottom | 196 (34) | 4.56 (13) | 0.87 (7) | 210 (31) |
| Small | 169 (34) | 4.66 (14) | 0.90 (7) | 187 (30) |
| Large | 216 (21) | 4.96 (13) | 0.92 (7) | 229 (22) |

*Values are means (n = 3 to 14) with CV (SD as % mean) in parentheses.* [a]*Calculated with an OTU definition of 97% similarity.*

The maximum Proteobacteria representation (80% of reads) was in the SAS2A surface community. Within the Proteobacteria, β-proteobacteria was the dominant class, except at the bottom of KWK23 where γ-proteobacteria dominated. Chlorobi dominated the bottom of KWK1 and SAS2A. *Gemmatimonas*, Lentisphaerae and *Spirochaetes* were found in low abundance (about 1% of the community) within the KWK valley. There was little difference between the small and large fractions, with the exception of the BGR1 bottom community that had a high proportion (25% of reads) of cyanobacteria in the large fraction only.

## Bacterial Dominants and High Abundance of Methanotrophs

A total of 21 separate taxa were found in the top 10 OTUs from each valley (**Table 3**), which accounted for 38% of the total number of reads. The two most abundant OTUs corresponded to two β-proteobacteria, *Variovorax* and *Polynucleobacter*. These two taxa were in all samples, and accounted for up to 30% of the community reads in each sample. Another OTU in the family Puniceicoccaceae (Verrucomicrobia) was more abundant at the BGR valley and represented up to 40% of the reads in the surface large fraction of BGR2. One OTU of *Pelodictyon* (Chlorobiaceae) represented up to 50% of the bottom community reads for KWK1 and another OTU of *Pelodictyon* was the most abundant taxon in the bottom of SAS2A.

Finer taxonomic analysis revealed the presence of methanotrophic bacterial groups at all sites (**Figure 4**), with 0.1–27% of OTUs matching *Methylobacter* (5% on average per site). Other methanotrophic bacteria belonging to the family Methylococcales (*Methylocaldum*, *Methylomonas, Crenothrix*, or unknown Methylococcales) were found in all ponds, although sometimes in low proportions (<1%). An exception was in the bottom waters of SAS1B where methanotrophs accounted for 23% of the sequences, more than half of which were in the Methylococcales. Sequences corresponding to a newly discovered order of methanotrophic bacteria, Methylacidiphilales (Verrucomicrobia), were found at the KWK and SAS valleys, in smaller proportions compared to other methanotrophs; for example, 1–6% of the total reads in the KWK6 surface samples. Amongst the dominant OTUs, methanotroph sequences were relatively abundant in most samples (**Figures 4** and **5**), with a median representation of 4.9% of total reads per sample, and a maximum of 25% for one single *Methylobacter* OTUs in the KWK23 bottom community reads. Another OTU corresponding also to *Methylobacter* was present at the BGR valley along with the OTU belonging to the order Methylococcaceae.

Some of the most abundant OTUs matching genera *Rhodoferax*, *Rubrivivax*, *Limnohabitans, Curvibacter*, the Actinobacteria ACK-M1, the three *Sediminibacterium* OTUs and the methylotrophic genus *Methylotenera* were distributed evenly across samples. The filamentous cyanobacterium *Dolichospermum* was highly abundant only in the surface of KWK1 and KWK6 where it contributed 20% of the community. Finally, OTUs matching *Synechococcus, Polaromonas* and a taxon in the Chitinophagaceae (Bacteroidetes) were more abundant in samples from the BGR and KWK valleys (**Figure 5B**).

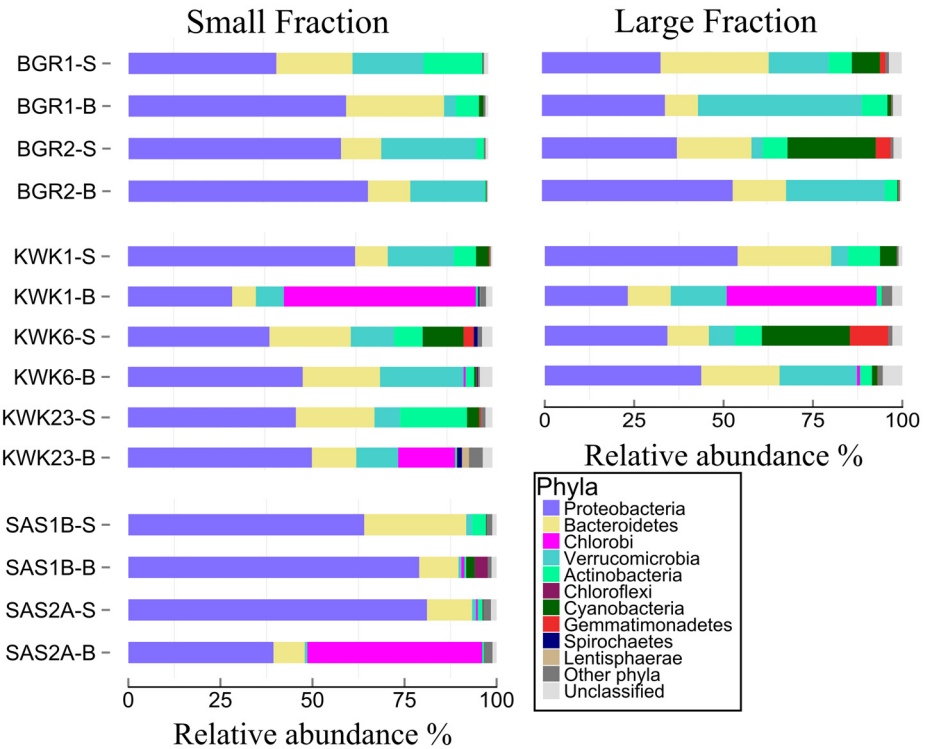


**FIGURE 3 | Relative abundance of the different phyla.** The samples were from the surface (-S) and bottom (-B) of ponds in the three valleys. The small fraction (left) is for samples in the size range 0.2–3 μm, and the large fraction is for 3–20 μm. Phyla that were less than 1% of total abundance are combined under "Other phyla."

**TABLE 3 | Identity of the 10 most abundant OTUs (defined at a level of 97% similarity) in each valley following the SILVA taxonomy.**

| Number of reads | Silva taxonomy | % Identity | Isolation source | Accession number | GenBank taxonomy |
|---|---|---|---|---|---|
| 7461 | Comamonadaceae | 100 | Wheat phyllosphere | KF054966 | *Variovorax soli* |
| 6253 | *Polynucleobacter* | 100 | Lake Grosse Fuchskuhle | KC702668 | *Polynucleobacter* |
| 5757 | Puniceicoccaceae | 100 | Yellowstone Lake | HM856500 | Opitutae |
| 3748 | *Pelodictyon* | 99 | Lake chemocline | AM086645 | *Pelodictyon clathratiforme* |
| 2454 | *Methylobacter* | 99 | Thaw pond hypolimnion | JN656724 | uncultured gamma proteobacterium |
| 1881 | *Rhodoferax* | 100 | Soil | GQ421098 | uncultured *Rhodoferax* |
| 1539 | *Rubrivivax* | 99 | Foodplain | FM886868 | *Rubrivivax gelatinosus* |
| 1317 | Comamonadaceae | 99 | Waterfall | KM035968 | *Curvibacter* |
| 1275 | ACK-M1 | 100 | Irrigation water | JX657295 | uncultured actinobacterium |
| 1260 | Nostocaceae | 99 | Eutrophic pond | FN691914 | *Dolichospermum curvum* |
| 1256 | *Sediminibacterium* | 99 | Yellowstone Lake | HM856392 | uncultured *Sediminibacterium* |
| 1250 | *Sediminibacterium* | 100 | Lake epilimnion | HQ532649 | uncultured Bacteroidetes |
| 1137 | *Pelodictyon* | 97 | Lake chemocline | AM086645 | *Pelodictyon clathratiforme* |
| 1085 | *Limnohabitans* | 99 | Daphnia Digestive tract | HM561466 | uncultured *Limnohabitans* |
| 1003 | *Sediminibacterium* | 99 | Yellowstone Lake | HM856387 | uncultured *Sediminibacterium* |
| 947 | *Methylotenera* | 99 | Biodeteriorated wood | KC172609 | uncultured Methylophilaceae |
| 761 | Chitinophagaceae | 99 | Lake epilimnion | HQ532140 | uncultured Bacteroidetes |
| 739 | Synechococcales | 99 | Meromictic lake | AB610891 | *Synechococcus* |
| 637 | *Methylobacter* | 100 | Thaw pond hypolimnion | JN656784 | uncultured gamma proteobacterium |
| 477 | Methylococcaceae | 96 | Landfill cover soil | HF565143 | *Methylobacter* |
| 450 | *Polaromonas* | 99 | Stems | KF385223 | uncultured *Polaromonas* |

*Following a BLASTn search, nearest matches and the providence of representative reads in GenBank were identified. Several groups appear multiple times because different OTUs match the same group. See* ***Figure 5B*** *for their distribution.*

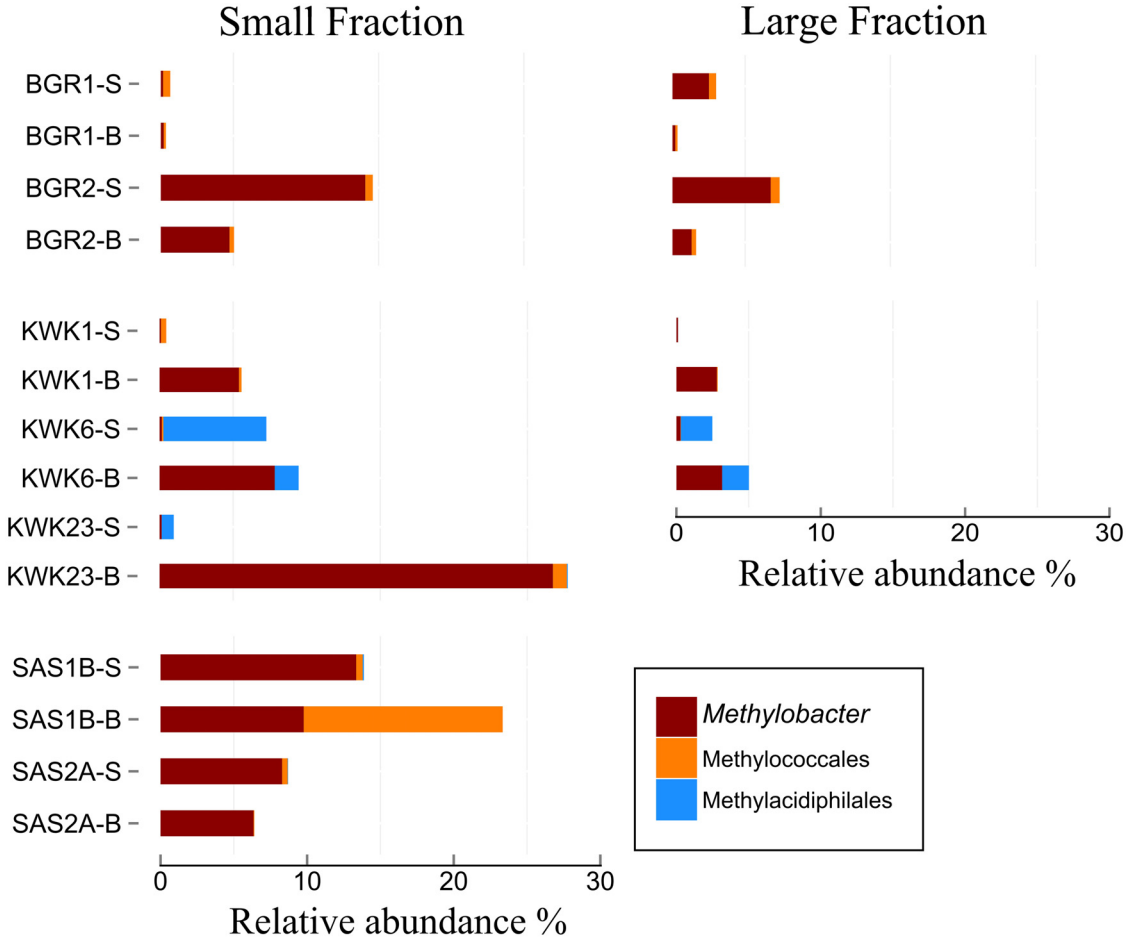


**FIGURE 4 | Relative abundance of methanotrophic bacteria.** The samples were from the surface (-S) and bottom (-B) of ponds in the three valleys. The small fraction (left) is for samples in the size range 0.2–3 μm, and the large fraction is for 3–20 μm. The taxa are labeled according to the highest taxonomical level; genera that represented less than 1% of total abundance were grouped together and labeled by their shared order.

## Bacterial Community Structure as a Function of Environmental Gradients

Community structure mostly followed a regional pattern as ponds from the same valley clustered together in the Bray–Curtis dissimilarity dendrogram (**Figure 5**). There were some exceptions, with the bottom of KWK 1 and 23 clustering closer to the SAS sites and apart from the other KWK samples. Within the BGR cluster, surface and bottom communities clustered separately while the two depths clustered together at the SAS sites. In terms of community composition, with the exception of the more aerobic BGR waters, the bottom communities were strikingly different from those at the surface because of the presence of anoxygenic phototrophs, specifically Chloroflexi and Chlorobi. Small and large fractions from the same sample always clustered together. A permutation test was used to test for differences between valleys, depth and size fraction and indicated significant differences in community composition according to valley ($p = 0.001$) and depth ($p = 0.009$) as well as a significant interaction between valleys and depth ($p = 0.011$), indicating that the difference between surface and bottom communities depended on valley location. Pairwise comparison indicated that amongst the significant differences between sites, communities in the KWK valley were significantly different from those in the BGR and SAS valleys ($p = 0.001$ and $p = 0.015$) but BGR and SAS were not significantly different. Permutation tests were carried out to compare surface versus bottom communities, and all four dominant phyla (Proteobacteria, Bacteroidetes, Verrucomicrobia, and Actinobacteria) showed significant differences between depths ($p < 0.05$). There were no significant differences between fractions ($p = 0.5$).

The dbRDA ordination (**Figure 6**) explained 64% of the variation and confirmed the regional pattern. The first horizontal axis was significantly correlated with pH, DO, TSS, and DOC while the vertical axis was significantly correlated with Chl *a* and conductivity. KWK samples were more dispersed compared to the SAS and BGR samples. The distribution of the BGR sites was mostly explained by the higher pH in this valley. The BGR sites and all of the surface KWK samples were separated from the other samples including bottom layers from KWK ponds and appeared associated with the higher concentrations of DO, while

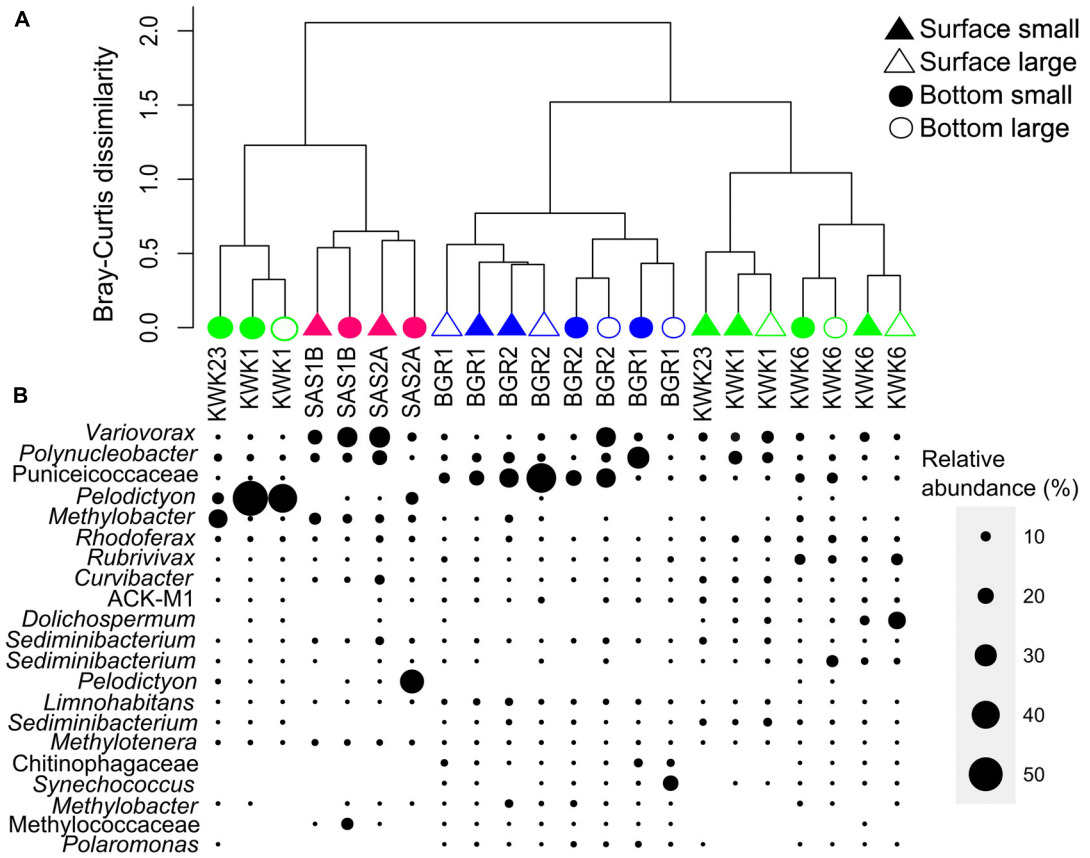


**FIGURE 5 | (A)** Bray–Curtis dissimilarity cluster analysis with the community data matrix (OTUs clustering at >97% of identity) for the study ponds. Surface samples are represented by triangles and bottom samples by circles, either filled (small fraction) or open (large fraction). **(B)** The bacterial dominants in each sample identified by their lowest taxonomical level either found on GenBank or SILVA (see **Table 3**); the size of the filled circle is proportional to their relative abundance.

the bottom of KWK1 and 23, the SAS sites and the bottom of KWK6 were at the low end of the oxygen gradient. The SAS valley was differentiated by its higher DOC, and the bottom samples from KWK1 and KWK23 by their higher concentrations of TSS and SRP. The surface of the KWK sites and KWK6 bottom were characterized by lower conductivity and higher Chl *a*.

## Discussion

### Bacterial α-Diversity

The subarctic thaw ponds at all sites were thermally stratified, consistent with previous observations in summer (Laurion et al., 2010). There were large vertical gradients in chemical properties, with a surface oxic layer (epilimnion) overlying low oxygen bottom waters (hypolimnion). These gradients provided a wide range of potential bacterial habitats within a single pond. The ponds contained diverse bacterial assemblages, with the number of OTUs (99–307 per sample) overlapping that reported for much deeper, stratified water bodies. For example, 280–425 OTUs were reported from a meromictic lake in the High Arctic using the same primers for tag pyrosequencing the 16S rRNA gene (Comeau et al., 2012), and 67–223 OTUs were reported for thermally stratified German lakes (Garcia et al., 2013). The Shannon and Simpson diversity indices in the thaw ponds were greater than those reported from German lakes, which were all less than 4.1 for the Shannon index and 0.36 for the Simpson index (Garcia et al., 2013). However, the taxonomic richness of these thaw pond communities was much lower than that in soils. For example, 4781–6231 OTUs were reported in 10 g samples of German grassland soils (Will et al., 2010) and 1496–1857 OTUs in the same quantity of High Arctic soil crusts (Steven et al., 2013; singletons excluded). Previous studies have documented this large disparity between lakes and soils (Lozupone and Knight, 2007; Tamames et al., 2010), suggesting fundamental difference in microhabitats in the highly structured matrix of soils relative to aqueous planktonic environments. Given the large potential input of soil particles and their associated bacteria from permafrost degradation, higher levels of diversity and species richness might have been expected in these waters. However, the total number of OTUs (1296) for our entire study including all 22 samples was 17% of that observed in the High Arctic soil crust study (7432), and within the range for other aquatic ecosystems.

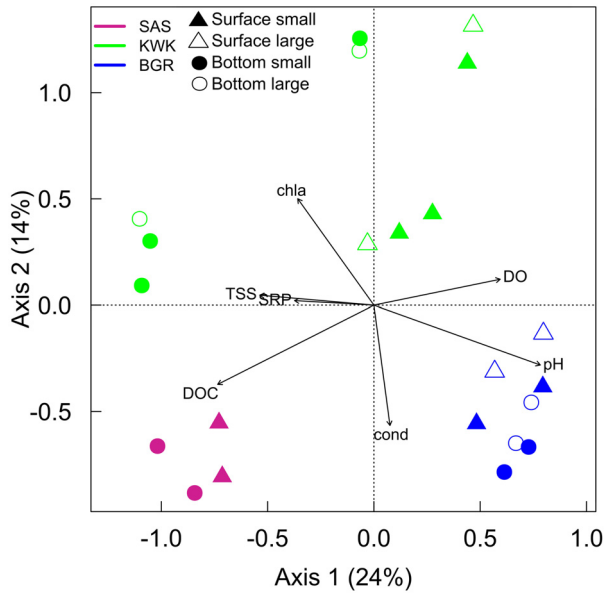


**FIGURE 6 | Distance-based redundancy analysis ordination plot showing selected environmental variables that were significantly correlated with sample distribution.** Abbreviations for the environmental variables are given in **Table 1**.

The Shannon and Simpson diversity indices showed that all samples were equally diverse, with no significant differences among valleys, depths, or size fractions. Steven et al. (2013) similarly observed in their soil crust analyses of six sites in the High Arctic that differences in diversity indices among sites were small relative to comparisons with other systems, including Antarctic soils. It would be of great interest to compare bacterial diversity in these northern thaw ponds with the shallow ponds of Antarctica, but to our knowledge such data are lacking. The significant difference of Chao1 index between KWK and BGR valleys indicated that species richness was higher at the lower latitude site. Species richness values for the surface waters were the same order of magnitude for the three valleys, however, there was a significant interaction of species richness between sites and depth, with species richness of bottom water increasing from BGR to KWK and SAS. This also corresponds to a gradient in DOC, with highest concentrations in the SAS ponds, and may reflect differences in bacterial productivity.

RNA versus DNA templates have been used to distinguish between active and inactive cells (Jones and Lennon, 2010) and to assess potential growth rates (Campbell and Kirchman, 2013). 16S rRNA is thought to provide an estimate of 'potentially active bacteria' (Blazewicz et al., 2013), which would be a subset of the community represented in the environmental DNA. The latter may include dead cells, spores and even free DNA that remains intact in the dark, cold, polar environment (e.g., Charvet et al., 2012). For this reason it is thought that the use of DNA can lead to overestimation of diversity, while rRNA may provide a more conservative and accurate estimation of diversity.

## Bacterial Dominants

For the overall data set, the dominant phylum was Proteobacteria and β-proteobacteria the dominant Class, as in many freshwater ecosystems (Newton et al., 2011). Two genera within that Class were particularly abundant, each on average representing around 10% of the total number of reads: *Polynucleobacter* and *Variovorax*. Both have attributes that make them well-suited to the heterogeneous combination of allochthonous (terrestrial) and autochthonous (aquatic) organic carbon compounds that are likely to occur in these waters. *Polynucleobacter* is a cosmopolitan genus that often dominates planktonic freshwater communities. It produces extremely small cells that are capable of growing aerobically on a wide range of complex media (Hahn, 2003), although genomic analyses of free-living and symbiotic strains of *Polynucleobacter necessarius* indicate a small genome size and the absence of certain functions, including an inability to use sugars as a carbon and energy source (Boscaro et al., 2013).

*Variovorax* includes taxa with diverse nutritional and energy acquisition strategies, including the breakdown and use of a wide variety of plant-derived molecules, as well as denitrification, sulfate reduction, and autotrophic $CO_2$ fixation. Genomic analysis of a plant-associated *Variovorax paradoxus* strain has shown that it has a remarkable combination of features for both heterotrophic and autotrophic lifestyles, and although it lacks the genes for methane monooxygenase, it is known to enhance the activity of methanotrophs in consortia (Han et al., 2011; and references therein). This strain also has three genes encoding aerobic carbon monoxide dehydrogenase, which could be of value given that high levels of carbon monoxide are known to be produced by photochemical reactions in high latitude, DOC-rich waters (Xie et al., 2009).

Subarctic thaw ponds emit methane (Laurion et al., 2010), and the presence and diversity of methanotrophic bacteria is of particular interest. Consist with our hypothesis, methanotrophs were found in all samples, and included three of the most abundant OTUs: two strains of *Methylobacter*, and one OTU with affinities to the family Methyloccocaceae. The methanotrophic bacteria all belonged to Type I methanotrophs which are in the γ-proteobacteria and the Verrucomicrobia. No bacteria belonging to Type II methanotrophs in the α-proteobacteria were identified. Methanotrophic communities are known to be sensitive to temperature, with Type I methanotrophs developing under low temperature conditions (Wartiainen et al., 2003; Börjesson et al., 2004; Wagner et al., 2005; Graef et al., 2011) and Type II under higher temperatures (Mohanty et al., 2007). The absence of Type II methanotrophs from even the warmer surface waters of the thaw ponds in summer implies selection by the low temperature conditions that prevail throughout the water column during most of the year.

Methanotrophs contributed a relatively high percentage of the total number of sequences in the thaw ponds, with a median of 4.9%, and a maximum of 27% in the bottom waters of KWK23. This is particularly high relative to other lakes, for example up to 2% in a meromictic lake in the High Arctic (Comeau et al., 2012) and only up to 3% of sequences in the plankton of eutrophic Lake Pluβsee in Germany (Eller et al.,

2005). This high relative abundance is closer to that observed in tundra soils (Vecherskaya et al., 1993) and anoxic lake sediments (Costello and Lindström, 1999), indicating the biogeochemically distinct nature of thaw pond ecosystems, with continuously high inputs of methane as a bacterial energy source.

Two groups of bacterial phototrophs were conspicuous members of the thaw pond assemblages: cyanobacteria and Chlorobi. The presence and abundance of sequences for these taxa varied among the samples, in part associated with depth and valley specific characteristics (see below). Cyanobacteria are often dominants of high latitude aquatic ecosystems, especially mat-forming Oscillatoriales and picoplanktonic taxa in the genus *Synechococcus* (Vincent, 2000). In the thaw pond data set, Oscillatoriales were little represented (with the exception of the bottom of SAS1B), as might be expected in a planktonic environment, which was in contrast to High Arctic soil crusts where Oscillatoriales are among the dominants. Mat-forming taxa are unlikely to thrive on the sediments of the subarctic thaw ponds given the high turbidity and the poor penetration of photosynthetically active radiation to the bottom (Watanabe et al., 2011). The picocyanobacterial group Synechococcales was represented in many of the ponds, although accounted for only 0.7% of the total bacterial reads. A surprisingly more abundant cyanobacterial group in two of the ponds was the Nostocaceae, with strong affinities (>99%) to the nitrogen-fixing taxon *Dolichospermum curvum* (formerly known as *Anabaena curvum*). Colonial cyanobacteria are largely absent from the plankton in other waters of the north and south polar regions (Vincent, 2000), again underscoring the distinctive properties of permafrost thaw ponds as an ecosystem type.

*Pelodictyon* was the fourth most abundant OTU in the overall data set, accounting for 3.3% of all sequences. This green sulfur bacterium (Chlorobi) is often observed under anoxic conditions in stratified freshwater lakes at depths where there is sufficient light for photosynthesis as well as high concentrations of its electron donor hydrogen sulfide. Chlorobi were earlier reported from 16S rRNA gene clone libraries of the KWK valley (Rossi et al., 2013), and their importance in the deep low- oxygen waters in many of the lakes suggest that anaerobic photosynthesis likely contributes significantly to the production of these subarctic ecosystems.

## Attached and Free-Living Bacteria

In a wide range of marine systems, particle-attached, and free-living bacterial communities are morphologically and phylogenetically distinct (e.g., Crump et al., 1999; Lapoussière et al., 2011; Mohit et al., 2014). Similarly in some freshwaters, for example Lake Erie (Mou et al., 2013) and Lake Bourget (Parveen et al., 2011) differences in attached and free-living bacterial communities have been reported. Here, we found little difference between the communities from our large and small fractions, which should have selected for attached and free-living bacterial communities respectively. The lack of difference in some of the samples may have been due to extremely small particulates in some of these ponds (ca. 1 μm; Watanabe et al., 2011) that would have passed through the 3-μm filter along with their attached bacterial flora, masking any differences. However, Chao1 diversity was significantly greater in the >3 μm size class and this would be more consistent with the >3-μm filters retaining both attached and many free-living bacteria if the filters became blocked. Our inability to filter more than 500 mL of sample suggest this latter explanation is more likely, and it is also consistent with the presence of picocyanobacteria (Synechococcales) in some of the large fraction samples, notably from BGR1 (**Figure 5**). Irrespective of the cause, more detailed size fractionation or a microscopy approach would be needed to accurately resolve the difference between particle-attached and free-living bacteria in these highly turbid ponds.

## Depth Gradients and Bacterial Community Composition

The thaw ponds were stratified with pronounced gradients in temperature and oxygen. Although the ponds are shallow, the high concentrations of CDOM and small wind fetch mean that they stratify early in spring and remain so over the summer (Watanabe et al., 2011). In fact, there is evidence that some ponds may not mix to the bottom at all in some years (Laurion et al., 2010). Within valleys, surface and bottom communities clustered well-apart from each other, with the exception of the SAS site where surface waters were depleted in oxygen compared to the other sites (**Figure 5** and **Table 1**). In terms of community composition, Actinobacteria were often poorly represented at the bottom of all ponds, consistent with their preference for more oxygenated waters (Allgaier and Grossart, 2006; Taipale et al., 2009). Anaerobic sulfate reducers, including the δ-proteobacteria *Geobacter, Anaeromyxobacter* and *Desulfovibri,* were found in small proportions in the bottom of ponds. Other bacterial dominants with matches to the family Chitinophagaceae (Bacteroidetes) including *Sediminibacterium* have the ability to produce $H_2S$ (Qu and Yuan, 2008), consistent with an active sulfur cycle and anaerobic conditions.

Some of the largest depth-dependent differences were for the phototrophic taxa, in keeping with the rapid attenuation of photosynthetically available radiation in these waters (Watanabe et al., 2011), as well as the depth variations in chemical properties of the ponds. The anoxygenic phototroph *Pelodictyon* was one of the most abundant OTUs, but was restricted to low oxygen bottom waters, as expected, and was absent from the more oxygenated BGR sites. This photosynthetic sulfur bacterium is adapted to low light and anoxic, $H_2S$-containing waters (Gich et al., 2001). Cyanobacteria in contrast, tended to be in the upper euphotic zone, with the KWK sites especially having a greater relative representation of cyanobacteria in the surface compared to the bottom of the ponds. The most striking difference was in the surface waters of KWK6 where Nostocaceae were the dominant cyanobacterial group, indicating the likely growth of colonial, potentially nitrogen-fixing species in these surface waters. However, for other ponds the large fraction of BGR1 bottom sample was dominated by Synechococcales, possibly indicating the growth of smaller celled cyanobacteria able to maintain populations in the better illuminated bottom

waters of BGR1, where concentrations of light-attenuating materials (Chl-*a*, DOC, TSS) were less than most of the other ponds. Oscillatoriales, which are filamentous, were found in the bottom of another pond, SAS1B, possibly indicating the sedimentation of aggregates of filaments or sections of mat from shallower depths.

In the KWK and SAS waters, methanotrophs were more abundant in the bottom waters, consistent with the earlier reported profiles of methane in the ponds. For example in KWK23, where the difference between surface and bottom samples was particularly striking, methane concentrations increase sharply at the bottom of the pond by several orders of magnitude (Laurion et al., 2010). Methanotrophs are aerobic and unable to sustain growth under completely anoxic conditions (Chowdhury and Dick, 2013), and our observations imply that the deep pond habitat provides a favorable combination of high methane and adequate oxygen. Interestingly, in the BGR ponds where there was less difference between surface and bottom oxygen, there was a higher proportional abundance of methanotrophs in the surface waters; this might suggest dependence on methane production from decomposition of macrophytes in the littoral zone, or via plant–microbe interactions in this region (Laanbroek, 2010).

### Spatial Variation and Landscape Gradients

Our results showed a clustering of ponds according to the individual valleys (**Figure 5**), implying environmental filtering of community composition based on landscape related properties (**Figure 6**). The dbRDA pointed to DOC concentrations and pH as controlling factors, both of which are known to influence bacterial community structure (e.g., Lindström et al., 2005; Fierer and Jackson, 2006). The higher pH in the BGR ponds separated them from the ponds in the other two valleys, although not all microbial groups followed expected relationships. Verrucomicrobia have been associated with low pH conditions (Lindström et al., 2005), but this group was relatively abundant in the BGR pond with the highest pH, and poorly represented in the SAS pond with the lowest pH. However, the pH range in this study was narrower than in Lindström et al. (2005).

Dissolved organic matter origin and source has been reported to influence bacterial community structure and function (Kritzberg et al., 2006). DOC had the greatest influence on community composition at the SAS site, where the ponds originated from organic palsas and DOC concentrations were higher and likely different in composition from the DOC at the other two valleys, where the ponds were formed by lithalsa thawing. The permafrost thaw gradient was also associated with the availability of DOC. The BGR valley is surrounded by discontinuous permafrost and >50% of the soil carbon would be frozen, and not available for degradation. In contrast, the southern, more degraded KWK and SAS valleys, ponds would be influenced by the eroding permafrost (Bouchard et al., 2014), and the input of allochthonous DOC would be more substantial. Photochemical breakdown of some of the more recalcitrant soil organic materials to lower molecular weight compounds (Laurion and Mladenov, 2013) may additionally enhance substrate availability in these waters.

## Conclusion

Permafrost thaw lakes and ponds are a prominent feature of the northern landscape and are strong emitters of greenhouse gasses. Because of their abundance on the landscape and wide distribution they are also useful for investigating the influence of large scale versus small scale environmental gradients. We found that permafrost gradients influenced the landscape properties, in turn driving bacterial communities composition. Within a pond, the physico-chemical stratification creates oxygen gradients that favor different microbes. In permafrost thaw lakes, the variety of allochthonous substrates derived from terrestrial vegetation and soils, and autochthonous sources including oxygenic photosynthesis by cyanobacteria, microalgae and macrophytes and anoxygenic photosynthesis by green sulfur bacteria, likely provide a heterogeneous range of organic substrates available to diverse heterotrophic taxa. Methanotrophs were among the most abundant sequences at most sites, indicating the potential importance of methane as a bacterial energy source in these waters. Their activities likely reduce the net emission of methane, in the process contributing to the $CO_2$ efflux from these ecosystems. The functionally diverse bacterial taxa in these abundant 'biogeochemical hot spots' across the subarctic landscape likely have a strong effect on the net emission of both greenhouse gasses, as the result of their metabolism of organic carbon from multiple sources. Ancient permafrost soils are now being increasingly thawed, eroded and mobilized as a result of the rapid warming of the North. The diverse bacterial communities identified here will likely assure that at least part of these new transfers from land to water are ultimately converted to $CO_2$ and released to the atmosphere.

## Author Contributions

This study was conceived and designed by SC, WV and CL, field work was conducted by SC, WV and JC, laboratory analysis was by SC, bioinformatics analysis was by SC, JC and CL, and all authors contributed to the writing of the manuscript.

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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.2015.00192/abstract

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# Untangling the fungal niche: the trait-based approach

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Fungi are prominent components of most terrestrial ecosystems, both in terms of biomass and ecosystem functioning, but the hyper-diverse nature of most communities has obscured the search for unifying principles governing community organization. In particular, unlike plants and animals, observational studies provide little evidence for the existence of niche processes in structuring fungal communities at broad spatial scales. This limits our capacity to predict how communities, and their functioning, vary across landscapes. We outline how a shift in focus, from taxonomy toward functional traits, might prove to be valuable in the search for general patterns in fungal ecology. We build on theoretical advances in plant and animal ecology to provide an empirical framework for a trait-based approach in fungal community ecology. Drawing upon specific characteristics of the fungal system, we highlight the significance of drought stress and combat in structuring free-living fungal communities. We propose a conceptual model to formalize how trade-offs between stress-tolerance and combative dominance are likely to organize communities across environmental gradients. Given that the survival of a fungus in a given environment is contingent on its ability to tolerate antagonistic competitors, measuring variation in combat trait expression along environmental gradients provides a means of elucidating realized, from fundamental niche spaces. We conclude that, using a trait-based understanding of how niche processes structure fungal communities across time and space, we can ultimately link communities with ecosystem functioning. Our trait-based framework highlights fundamental uncertainties that require testing in the fungal system, given their potential to uncover general mechanisms in fungal ecology.

**Keywords: fungal niche, functional traits, fungal biogeography, community ecology, dominance-tolerance trade-off**

## INTRODUCTION

Microorganisms are important regulators of global biogeochemical cycling, responsible for mineralising organic nutrients and governing the exchanges of carbon and nutrients between the biosphere and atmosphere. As such, the inclusion of microbial processes into Earth system models (ESM) is integral to our understanding of biogeochemical cycles and their feedbacks to global climate change. Current models incorporate broad climate variables and plant traits as the dominant controls on microbial processes, due to their predictable distributions at global and regional scales (Bonan et al., 2013; Todd-Brown et al., 2013; Wieder et al., 2013; Bradford et al., 2014). A vast body of evidence, however, suggests that microbial-mediated ecosystem functioning can vary substantially within environments, depending on the community composition (Schimel and Schaeffer, 2012; Strickland et al., 2014; van der Wal et al., 2014). This variation in community performance has led researchers to question the validity of broad-scale indicators, highlighting the potential value of including local-scale controls such as direct microbial community attributes to explain and project biogeochemical cycling at global and regional scales (Bradford et al., 2014). Our capacity to incorporate such local community information is, however, restricted by a limited understanding of microbial biogeography or the patterns of microbial traits across broad spatial scales.

Untangling the mechanisms governing patterns in community structure across time and space are foundational goals of community ecology. Despite concerns that the complex nature of natural communities precludes the formulation of unifying principles (Simberloff, 2004), the Hutchinsonian niche concept remains a powerful framework to understand community patterns (Hutchinson, 1957). Like plant and animal communities, the importance of niche processes in shaping bacterial communities across landscapes is increasingly apparent (Barberán et al., 2014). In contrast, environmental characteristics consistently fail to explain the majority of the variation in fungal communities at broad spatial-scales, leading to the widespread belief that neutral processes (e.g., dispersal limitation) are dominant forces governing patterns in fungal taxa (Martiny et al., 2006; Feinstein and Blackwood, 2013; Wu et al., 2013; Cline and Zak, 2014; Talbot et al., 2014). However, the focus on taxonomy, rather than traits, is likely to have restricted the search for predictable patterns in community organization (Aguilar-Trigueros et al., 2014); because niche processes filter communities based on the expression of individual-level traits, it is likely that environmental

characteristics are linked more closely to trait values than to taxonomy at broad spatial-scales.

The niche concept makes competitive advantage or persistence contingent on environmental conditions, given trade-offs in species traits that favor coexistence (Hutchinson, 1957). Multiple biotic and abiotic processes then serve as environmental filters, selecting for traits that allow individuals to survive and compete under those specific conditions. To understand niche processes, then, plant ecologists have invested tremendous efforts in studying communities through the lens of functional traits (Adler et al., 2013; Herben and Goldberg, 2014). By considering trade-offs in ecophysiological, morphological and life-history traits, this approach provides mechanistic linkages between fundamental biology, community dynamics and ecosystem functioning. Despite the prevalence of trait-based approaches in aboveground ecology, fungal ecologists traditionally view communities through a taxonomic lens, often resulting in the loss of ecological generality (Green et al., 2008; Allison, 2012; Powell et al., 2013). In 2013, for example, less than 2% of experimental studies published in the journal *Fungal Ecology* used traits or trade-offs to explain species patterns. In contrast, ∼76% of the experimental studies published in the same year in the corresponding plant journal (*Journal of Ecology*) examined processes through the lens of functional traits. It is important to note that the expression of many traits is linked inexorably to taxonomic identity, so trait filtering often influences taxonomic compositions. However, statements about traits provide generality and predictability, whereas nomenclatural ecology tends toward highly-contingent rules and special cases (only providing information about the study species and closely related taxa; McGill et al., 2006; Adler et al., 2013). Analogous to the example from the plant-based literature (McGill et al., 2006), the statement "faster-growing, cord-forming fungi are more combative than litter-dwelling microfungi" is more useful than "*Resinicium bicolor* out-competes *Mortierella verticillata* in soil" (*sensu* Crowther et al., 2013).

In a classic trait-based framework, Grime (1977) characterized plants, animals and fungi as competitors (C), stress tolerators (S) or colonizers (i.e., ruderals; R), a set of distinctions that encompass the physiological traits governing species distributions and their influences on ecosystem processes. Theory in plant and animal ecology has built on this categorical framework to embrace continuous variation in the expression of various traits across multiple species. Despite calls for a focus on species- and individual-level variation in multiple functional traits in the mycorrhizal literature (Behm and Kiers, 2014; Koide et al., 2014; Parrent et al., 2010) often with a view to understanding host plant dynamics, the trait-based framework is yet to be extended throughout fungal ecology (Aguilar-Trigueros et al., 2014).

We discuss trade-offs in stress-tolerance and combative dominance to exemplify how a trait-based framework might aid in the search for general mechanisms within fungal community ecology. Drawing on examples from across the fungal kingdom, we focus on free-living groups because their physiology is directly linked to the external environment rather than the characteristics of host species. The manuscript is divided into three main sections. First, we discuss the need for a trait-based framework in fungal ecology. The second section proposes this framework, acknowledging that there is a paucity of continuous trait-based datasets for free-living fungi that precludes the empirical testing of specific trade-offs. Instead, we develop a conceptual framework, incorporating specific characteristics of the fungal system, which outlines how trade-offs between competitive ability and drought tolerance can improve our understanding of the processes structuring fungal communities. We believe this framework demonstrates the need to explore fungal traits within the context of environmental gradients and biotic interactions to understand the ecology of all fungal communities (Saunders et al., 2010; Aguilar-Trigueros et al., 2014). In the third section, we highlight the implications of our framework for understanding patterns in fungal communities across time and space. We conclude that trait-based approaches can provide a tractable set of tools for understanding the links between fungal community ecology and ecosystem ecology. These tools can facilitate the conversion of fungal ecology from a phenomenological discipline into a more mechanistic one.

## THE BASIS FOR A TRAIT-BASED APPROACH

### THE TAXONOMIC APPROACH IN FUNGAL ECOLOGY

The fungal kingdom encompasses a huge diversity of species, with micro- and macroscopic taxa that inhabit almost every habitat on Earth. Despite their global distribution, most fungi are generally inconspicuous due to their small size and cryptic lifestyles, living on organic matter in soil and water, or in association with other organisms. Given their inconspicuousness and the fact that many species are indistinguishable from one another without the use of molecular tools, it is not surprising that only 2–5% of the estimated 1.5–6 million fungal species have been formally classified (Blackwell, 2011), and there are few, if any, fungi for which the full geographic distribution is documented.

The practical limitations in identifying fungi and "binning" them into taxonomic groups restricts our capacity to extrapolate to other (even closely-related) species, and the challenges in determining species distributions across landscapes precludes the scaling-up of processes observed in individuals (Green et al., 2008). Furthermore, most fungi are highly plastic, with individuals displaying substantial spatial and temporal variation in morphology and physiology that can obscure differences in life-history strategy observed between taxa (Aguilar-Trigueros et al., 2014). Indeed, individual fungi can display competitive, ruderal or stress-tolerant morphotypes at different stages of development (Pugh and Boddy, 1988). A focus on species identities can, therefore, obscure the detection of niche patterns, depending on the timing of experimental observation. Given that trait expression relates to the strategies of individuals at any one point in time, and under a given set of circumstances, greater emphasis on trait-based approaches in fungal ecology is pragmatic both theoretically and empirically.

### FUNGAL FUNCTIONAL TRAITS

Proponents of the functional approach in fungal ecology have traditionally divided taxa into functional groups. These groups are based on trophic status (mycorrhizal, saprotrophic, pathogenic), and within these, based on size (microfungi and macrofungi),

pigmentation (dark-pigmented and non-pigmented), morphology (e.g., phalanx and guerrilla), physiology (e.g., brown rot and white rot) and life-history strategies (e.g., endo-, ecto- and ericoid-mycorrhiza; Aguilar-Trigueros et al., 2014). Such categorizations provide useful guides to navigate the complex fungal kingdom; they allow for the exploration of within-group generalizations, between-group comparisons and they confirm the positive effect of functional diversity on ecosystem processes (Hättenschwiler et al., 2005; Crowther et al., 2013). Categorical groupings are, however, often limited in their capacity to isolate mechanisms because species within the same groups can display a wide range of trait values (Naeem and Wright, 2003). Many plant endophytes can, for example, be considered early decomposers, as they initiate fungal succession in dead wood (Boddy, 2001), and there is a continuum of life-history strategies between purely pathogenic, mycorrhizal and saprotrophic fungi (Aguilar-Trigueros et al., 2014).

Following aboveground community ecology (e.g., McGill et al., 2006), we refer to "functional traits" as measurable properties of organisms that are used comparatively across individuals and influence an organism's performance or fitness. Measurement of traits on a continuous scale permits regression designs that maximize the predictive power for species responses or effects. Such scales of trait expression can inform community ecologists about the relative distributions of species along environmental filters (Webb et al., 2010; Lennon et al., 2012), and ecosystem ecologists about the specific components of a community that drive ecosystem functioning (Dias et al., 2013).

In this paper, we refer to two categories of continuous traits: "trait complexes" and "true traits." Trait complexes correspond to the performance of an individual relative to co-occurring taxa. These can relate to biotic (e.g., competitive ability) or abiotic factors (e.g., drought tolerance), and are a product of the expression of multiple true traits (e.g., growth rate or osmolyte production; **Figure 1**). Both categories can refer to response traits (that govern how organisms respond to different conditions) and effect traits (that determine how organisms affect their environment).

## ISOLATING AND MEASURING FUNGAL TRAITS

Various techniques allow the measurement of fungal functional traits directly (by measuring expression) or indirectly (using transcriptomics to measure functional genes) across a range of habitats and hosts. In most cases, these traits can be measured at the community-level (Sinsabaugh et al., 2008) to provide a functional fingerprint of the community as a whole. This approach has benefitted from accelerating advances in sequencing-based (e.g., metatranscriptomic) techniques, which allow the mapping of functional genes or genome characteristics across ecosystems

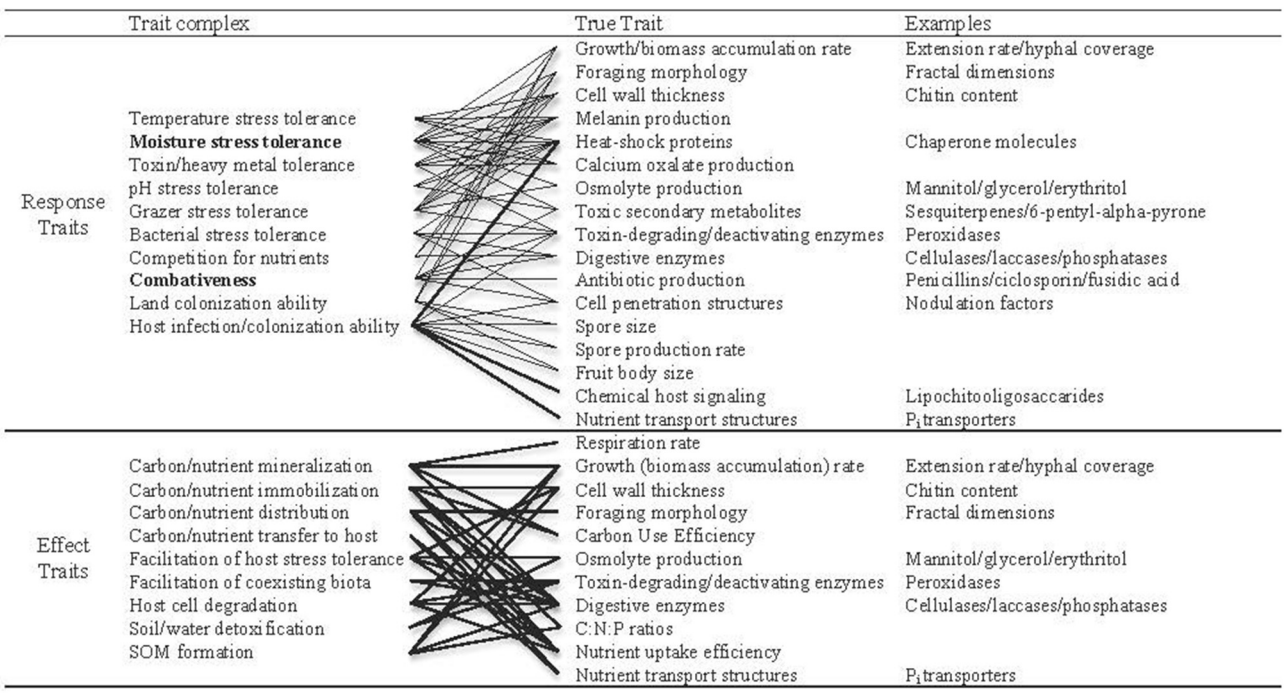


**FIGURE 1 | Examples of trait complexes and corresponding true traits (lines connect trait complexes with the true traits that influence them), each of which can be measured in terms of constitutive or potential expression, and which exist on a continuum across saprotrophic, mycorrhizal and pathogenic fungi.** Even the true traits might be considered trait complexes as they are encoded for by complex sets of genes. Nevertheless, these are all measurable properties individuals that potentially relate to survival under a given set of environmental conditions. These traits can be measured directly or indirectly (by measuring functional gene expression), but are most useful when expression can be related to individuals. Response traits are those that dictate how organisms respond to biotic and abiotic conditions, and effect traits relate to an individual's effect on the environment. Several true traits are predicted to influence both response- and effect-trait complexes (Koide et al., 2014), and each trait complex could be comprised of a suite of interacting true traits. Highlighted in bold are the trait complexes that we propose are dominant structuring forces in free-living fungal communities.

(Green et al., 2008; Barberán et al., 2014). A mechanistic understanding of functional trait organization within communities, however, requires the linking of trait expression with individuals (McGill et al., 2006). For example, having observed high levels of stress-tolerance traits in a given environment it is impossible to determine whether those traits are expressed by one or multiple individuals, and whether trait expression is a prerequisite for survival in that environment or not. The combined use of community-weighted trait measurements (direct or indirect) and metagenomic sequencing data is a powerful and widespread means of linking fungal trait expression with communities (Rajala et al., 2011; Crowther et al., 2013, 2014; Cline and Zak, 2014), but observed relationships between species and trait expression are purely correlative and provide no inferences about the organization of traits within the community. As with plant and animal ecology, a trait-based approach to understanding the structuring of fungal communities requires that trait, or functional gene, expression be attributed directly to individuals within the community. Currently, this generally requires the isolation of fungi.

The plastic nature of most fungal traits reflects that recorded in plants. Development and trait expression can vary drastically when grown under different conditions and on different substrates. As with plant ecology, fungal traits should therefore be estimated under optimal conditions, unless specific goals suggest otherwise (Peìrez-Harguindeguy et al., 2013). This allows the universal comparison of individual-level attributes, and the identification of fundamental physiological trade-offs. These patterns can then be used to understand community dynamics under different conditions (e.g., under different levels of stress or on substrates), where communities may be comprised of different taxa, but still governed by the same fundamental selection pressures.

## A FRAMEWORK FOR A TRAIT-BASED APPROACH

McGill et al. (2006) posit that a trait-based approach within a community context requires consideration of environmental gradients (habitat filters) and the interaction milieu (biotic interactions) to allow the selection of appropriate performance currencies (traits that reflect the performance of an individual in a given environment). In this section, we focus on a dominant environmental gradient (i.e., moisture availability) and biotic interaction (i.e., competition) to guide the selection of performance currencies for the fungal system. From this consideration, we build a framework for application of a trait-based approach in fungal ecology that relies on (i) identifying the dominant trade-offs (between trait complexes) that govern species distributions and abundances (Kneitel and Chase, 2004); and (ii) linking trait complex values with true traits to elucidate the mechanistic basis of these trade-offs and extrapolation beyond the study taxa (Lennon et al., 2012).

### ENVIRONMENTAL GRADIENTS

Identifying how survivorship varies along environmental gradients is the initial step in defining a species' fundamental niche, and this alone can provide a powerful tool to predict the structuring of communities at the broadest spatial scales (Green et al., 2008). As with plants, the fitness of individual fungi, and consequently community composition, varies along gradients of temperature, moisture and a suite of other environmental factors. Notably, however, when grown in isolation, many fungal taxa share overlapping optima toward the center of most environmental gradients (Barcenas-Moreno et al., 2009; Crowther and Bradford, 2013), and are forced to tolerate stressful conditions toward gradient edges (**Figure 2**). The relative importance of different abiotic factors can vary substantially between communities,

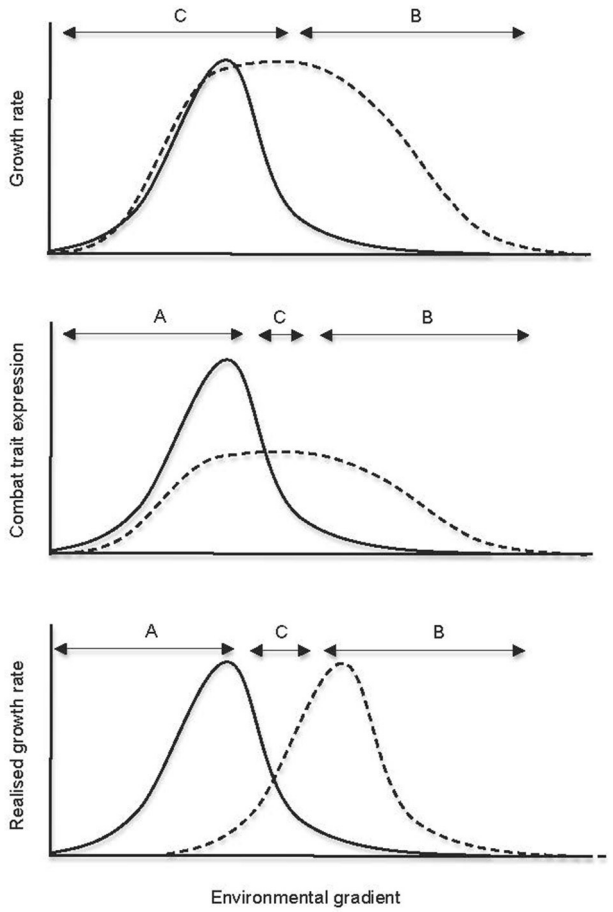


**FIGURE 2 | Plotting the realized niche by estimating variation in a performance currency that represents the interaction milieu along an environmental gradient.** Traditional approaches to exploring individual niche spaces involve estimating survival (e.g., growth or respiration) across environmental gradients (fundamental niche), and then subsequently exploring biotic interactions within this space to approximate the realized niche. Our conceptual diagram shows how the measurement of traits associated with a dominant biotic process along the same environmental gradient can incorporate both biotic and abiotic filters and provide a meaningful estimation of an organism's realized niche. Niche differentiation between two species with identical growth rates can be visualized using this approach. The letters "A" and "B" refer to the dominance of those respective individuals at the corresponding point along the gradient, and "C" indicates coexistence. We stress that, even if maximum growth rates of the two fungi differed substantially from one another in plate "a," the denoted niche spaces would remain unchanged, because growth in the absence of competitors does not imply growth during combat.

but observational studies and multi-factor climate change experiments provide some consistent trends. Most fungi can tolerate a relatively large degree of variation in pH and temperature (Kardol et al., 2010), but are highly responsive to shifts in nutrient status and, in particular, water availability (e.g., Schimel et al., 2007; Kardol et al., 2010; Manzoni et al., 2014). Indeed, moisture stress (caused by disturbance, toxins, fire, drought or freezing) emerges as the dominant abiotic factor regulating the outcome of competitive fungal interactions (Magan and Lacey, 1984; Boddy, 2000). Because fungal cells are semi-permeable and in constant contact with their environment, the physiological challenge of maintaining water potential is essential to sustain turgor pressure, nutrient acquisition via substrate diffusion across membranes and to avoid desiccation (Schimel et al., 2007).

In the vast majority of terrestrial ecosystems, fungi experience drought stress to some extent, during either chronic or pulse events (Schimel et al., 2007). The accumulation of osmolytes reduces internal water potential and limits osmotic losses. Polyols including glycerol, erythritol, and mannitol are commonly regarded as the primary fungal osmolytes (Dijksterhuis and de Vries, 2006), and can constitute between 10 and 60% of total cytoplasmic constituents during stressful conditions (Schimel et al., 2007). Although the specific functions of different polyols vary among fungi (Solomon et al., 2007), their increased production under dry conditions suggests that they constitute a form of induced resistance to drought stress (Son et al., 2012). Heat-shock proteins are also induced to provide structural support, helping to maintain enzyme activity under stress. Chaperone molecules, for example, maintain enzyme-binding sites and facilitate conformational changes under dry conditions (Cowen, 2009). Water loss can also be physically restricted with the use of hydrophobic cell wall proteins, melanin and calcium oxalate crystals on mycelial surfaces (Unestam and Sun, 1995; Fernandez and Koide, 2013). An inability to express these physiological traits under stressful conditions generally forces fungi into dormancy, or ultimately cell death. Although most of these traits are associated with general mechanisms to tolerate a variety of other environmental stressors (e.g., heat, acidity; **Figure 1**), the relative trait expression (constant or potential) will provide a strong indication of a species' ability to persist at a given point along soil moisture gradients (Lennon et al., 2012).

Depending on whether stress-tolerance traits are constitutive or induced, cell maintenance can be energetically expensive, occurring at the cost of other metabolic processes. The cost of constitutive melanin production is, for example, relatively low, with growth rates of melanised individuals being almost equivalent to their non-melanised conspecifics (Fernandez and Koide, 2013). In contrast the induced synthesis of osmolytes and anti-shock proteins consistently leads to sub-optimal cell growth and sporulation (Dijksterhuis and de Vries, 2006). This trade-off between induced stress-tolerance and cell functioning is apparent at the genetic level. The environmental stress response (ESR), a common gene expression response to various biotic and abiotic stressors that has been conserved across the fungal kingdom, generally involves the expression of over 300 genes and the concurrent repression of ∼600 others associated with cell maintenance and growth (Gasch, 2007).

Although the costs of induced stress-tolerance are apparent in most fungi, the degree to which cell growth is limited under sub-optimal conditions varies continuously among species; fungi adapted to specific stressors have the capacity to minimize the number of stress genes activated and repressed (Gasch, 2007). At least in some fungi, an epigenetic process governs this "adaptation." In short, a genetic cascade initiates the expression of stress response genes, and then epigenetic regulation relies on changes in chromatin state and/or nuclear compartmentalization that silences all but a few necessary alleles (Verstrepen and Fink, 2009). This environmental selection and adaptation is likely to explain the niche differences observed between wet-adapted specialists and dry-adapted generalists (Lennon et al., 2012). The environmental gradient therefore acts as a trait filter, allowing inferences about species fundamental niches based on the relative expression of specific functional traits.

## INTERACTION MILIEU

Contracting fundamental into realized niche spaces requires consideration of biotic interactions, the nature of which are contingent upon the environmental conditions. Drought-tolerant fungi are not dry specialists, but "generalists," with the potential to exist along a broad moisture-gradient (Lennon et al., 2012). Drought-intolerant fungi, in contrast, can be considered "specialists," capable of competing under a narrow range of mesic conditions. Drought-tolerant and -intolerant fungi are therefore conceptually analogous to gymnosperm and angiosperm trees, respectively. For example, many coniferous trees have fundamental niche optima that fall in mesic temperate climate but are forced to tolerate colder and drier climates by competitively dominant angiosperms (Bond, 1989). We suggest that competitive ability similarly interacts with climate tolerances to shape the structure of fungal communities.

Under the CSR framework, ruderal and competitive species invest heavily in dispersal and competitive ability, respectively (Grime, 1977). Although the trade-off between these biotic processes undoubtedly exists in the fungal system (Kennedy et al., 2011), the high dispersal rates of most fungi, relative to those of plants and animals, is likely to promote the relative importance of competition, at least at local scales. Dispersal potential can favor early colonizers, with priority effects that determine community development (Fukami et al., 2010), but theoretical models predict that in communities experiencing high dispersal rates, survival in a given environment is primarily contingent upon the ability to compete (Mouquet and Loreau, 2003; Kneitel and Chase, 2004).

In most ecological systems, competition is largely determined by differing abilities to access limiting resources. This is apparent for certain plant symbionts (Chagnon et al., 2013), but most free-living fungal communities are governed by combat (Boddy, 1993, 2000; Saunders et al., 2010; Crowther et al., 2012a). That is, competitive interactions are determined by a species' ability to attack, or withstand attack, from antagonistic competitors. The importance of fungal combat as a structuring force in free-living fungal communities is apparent in that antagonistic ability comes at the cost of multiple other trait complexes. For example, despite the highly combative nature of *Resinicium bicolor* under optimal environmental conditions, its inability to remain combative during

invertebrate grazing or temperature stress prevented the exclusion of fungal competitors in soil microcosms (Crowther et al., 2012b). Thus, the ability of a fungus to persist under a given set of abiotic conditions is immaterial if it cannot effectively overcome co-occurring combatants.

Various strategies allow fungi to attack opponents (e.g., gross mycelial contact, mycoparasitism, chemical interference; Boddy, 1993) and defend against attack (e.g., detoxification, structural alteration of toxins, exudation of absorbed toxins; Saunders et al., 2010). These interactions can be explored using pair-wise combinations of cultured fungi, either in a factorial design or by identifying the relative suppression of a common indicator species (Gaudet and Keddy, 1988). Pair-wise interactions are, however, often non-hierarchical and diffuse, so competitive ability based solely on pair-wise combinations can be misleading (Boddy, 2000). An alternative approach to rank competitors is to explore the growth of individuals within multi-species complexes, relative to growth in the absence of competitors (Fukami et al., 2010). Both of these approaches can generate combative trait complex hierarchies, providing a continuous scale with which to test for the dominant trade-offs that govern coexistence (Kneitel and Chase, 2004).

The tendency of drought stress to alter these combative hierarchies (Boddy, 2000) provides strong evidence for a trade-off between drought stress and combative ability. Indeed, Magan and Lacey (1984) estimated an index of dominance ($I_D$: a numerical score for combative ability) for multiple competing fungi in pairwise combinations in agar cultures, showing that both drought and temperature stress can alter the fungal dominance hierarchy. Re-analysis of this initial data reveals that the magnitude of the drought effect correlated strongly with initial combative ability (recorded under optimal conditions); the most antagonistic species experience the greatest competitive losses during drought stress. In contrast, whilst there was some evidence that temperature stress drives a similar relationship, the trade-off is substantially weaker, and the negative effects of heat stress are relatively consistent across all species (**Figure 3**; Magan and Lacey, 1984). Based on the strength and consistency of the relationship between drought-tolerance and combative ability (Magan and Lacey, 1984; Lennon et al., 2012), we hypothesize that this dominance-tolerance trade-off is likely to be a predominant mechanism structuring free-living fungal communities in predictable ways across time and space.

A mechanistic understanding of these dominance-tolerance trade-off in fungi requires the consideration of true traits associated with combat. The value of linking trait complex values to true traits is well recognized in other systems where, for example, plant height can provide a good approximation of the light competition milieu (McGill et al., 2006). However, linking true traits with fungal combative ability has proven challenging due, in part, to the variety of complex mechanisms of fungal antagonism (Hynes et al., 2007). Growing evidence suggests that fungal combat may be analogous to that observed in clonal animals (e.g., corals; Maguire and Porter, 1988), being governed by traits including growth rate (Boddy, 2000), antibiotic (Aguilar-Trigueros et al., 2014) and toxic secondary metabolite production (e.g., sesquiterpenes; Hynes et al., 2007; Saunders et al., 2010).

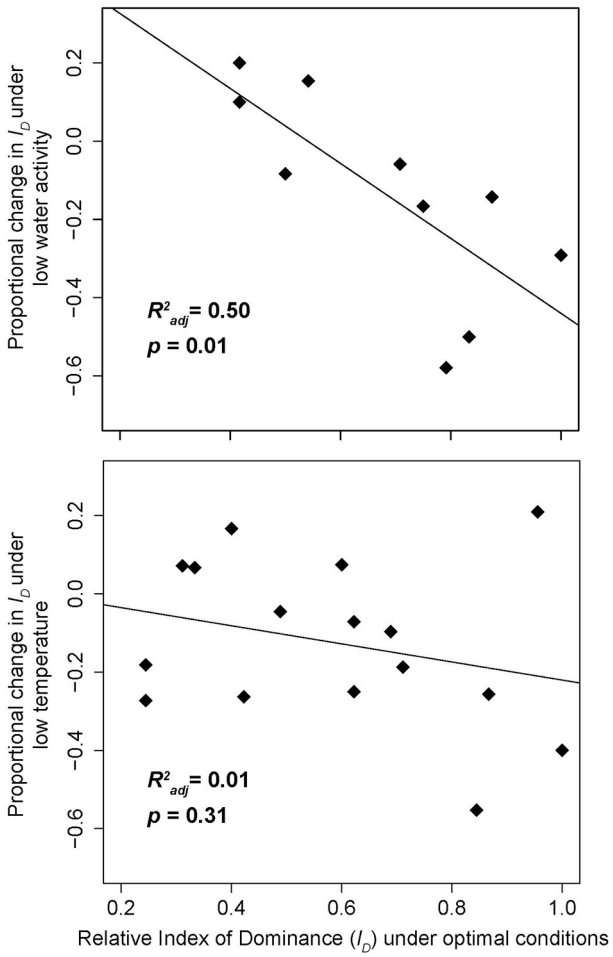


**FIGURE 3 | The effect of reduced water activity (the partial vapor pressure of a substance) and decreased temperature on fungal combative ability.** Data are adapted from Tables 3,6 in Magan and Lacey (1984). Fungi that are the most combative under optimal conditions (25°C and water activity [$A_w$] of 0.98) have a greater reduction in competitive ability under reduced water activity ($A_w = 0.90$) than under reduced temperature conditions. Index of Dominance ($I_D$) was assessed for each fungus by quantifying the outcomes of all pairwise competitions, with higher numbers indicating more competitive fungi (see Magan and Lacey, 1984 for details). (a) "Low water activity" refers to $A_w = 0.90$, holding temperature constant at 25°C (6 of the 16 fungi had missing $I_D$ values at this water activity level); (b) "Low temperature" refers to 15°C, holding $A_w$ constant at 0.98. Relative $I_D$ values were calculated by dividing each fungus' $I_D$ by the maximum $I_D$ across all species. $R^{2adj}$ and corresponding *p*-values were obtained by simple linear regression of relative $I_D$ vs. proportional change in $I_D$.

We are unaware of studies that explore the relative importance of different combat or defense traits across a broad selection of fungi. Identifying a trait or a suite of traits that explains the fungal competition milieu, even within subsets of closely-related, or functionally similar fungi, will be an invaluable step in the formalization of trait-based approaches for understanding realized fungal niche spaces.

As with stress tolerance, fungal combative traits can be either induced or constitutive, but the energetic and genetic

**Box 1 | The dominance-tolerance trade-off model.**

We provide a mathematical framework to illustrate how trade-offs in fungal traits are likely to influence the community organization of species with overlapping environmental optima. Survival in a given environment is contingent on a fungus' ability to compete. This generally requires the diversion of energy from growth and maintenance toward the production of combative allelochemicals and altered growth strategies (Boddy, 2000). Fungal growth can therefore be modeled via a modified "competition for energy" model, as originally given in Schoener (1973). In Schoener's formulation, there is an indirect energetic cost of interaction between two species. We build on this concept by modeling the competition between fungi as having a direct energetic cost. The growth rates of two interacting species can thus be modeled by:

$$\frac{dB_1}{dt} = r_{1g}(g) \cdot \left[E_1(g) - M_1(g) - C_1(g)\right] \cdot B_1 - h_{1,2}\left(B_1, B_2, C_1, C_2, D_{1,2}\right)$$

$$\frac{dB_2}{dt} = r_2(g) \cdot \left[E_2(g) - M_2(g) - C_2(g)\right] \cdot B_2 - h_{2,1}\left(B_1, B_2, C_1, C_2, D_{2,1}\right)$$

Where $B_i$ is the biomass of fungus *i; g* is a given environmental condition along a gradient; $E_i(g)$ is net energy intake (after enzyme production and nutrient uptake) per unit biomass per unit time; $M_i(g)$ is the maintenance energy cost per unit biomass per unit time; $C_i(g)$ is energy invested in competition and combat per unit biomass per unit time; $r_i(g)$ is the energetic cost of building new cells, in terms of unit biomass per unit energy (i.e., growth rate per unit energy); and $h_{i,j}(\cdot)$ is a function representing the death rate of fungus *i* as a function of the biomass of fungi *i* and *j*, the energy invested in competition by both *i* and *j*, and the "degree of interaction" ($D_{i,j}$) between *i* and *j*. The functions $E_i$, $M_i$, $C_i$, and $r_i$ all depend on the environmental condition, *g*.

Regardless of the specific forms of $E_i$, $M_i$, $C_i$, $r_i$ and $h_{i,j}$, it follows that, at a minimum, each fungus must ensure that $E(g) - M(g) \geq C(g)$ and $r(g) \geq 0$ to survive in a given environment *g;* otherwise growth would be negative. These inequalities give rise to a set of intuitive trade-offs between stress tolerance and competitive ability:

(i) *Energetic limitation under stress*, where $E(g) - M(g)$ decreases substantially along the gradient due to increased maintenance demands and/or decreased functional ability (either reduced enzyme production or affinity). Even if the fungus is able to invest most of the net remaining energy in combat, this restricted net energy intake places an upper bound on the amount of energy that it has to invest, thus limiting its competitive ability, growth or maintenance under stressful conditions (**Figure 4**, Species 1). If $r(g)$ remains very large under stress, this species may persist at the landscape-level as a ruderal species, but its existence would be highly contingent on priority effects and on the biomass of its surrounding competitors.

(ii) *Genetic limitation under stress*, where the fungus activates specific stress-response genes to ensure non-negative growth under stressful conditions, (i.e., to ensure that $r(g)[E(g) - M(g)] \geq 0$). In doing so, the fungus necessarily deactivates genes that correspond to competitive ability. Thus, while $E(g) - M(g)$ or $r(g)$ need not substantially differ under high- and low-stress environments, the fungus is fundamentally unable to invest the leftover energy in competition under high-stress environments (**Figure 4**, Species 2).

(iii) *Specialist stress tolerance,* where a fungus maintains competitive ability under high stress by avoiding substantial energetic, growth, and genetic limitations relative to stress-intolerant fungi. This could be achieved through an efficient epigenetic down-regulation of stress response genes or by the presence of constitutive stress-tolerance traits. For such fungi, it must be that both $E(g) - M(g)$ and $r(g)$ remain relatively large even under stressful conditions, and that the mechanisms by which it ensures positive net energy and growth do not substantially inhibit its ability to invest the net energy in antagonism (**Figure 4**, Species 3). The lack of a "Hutchinsonian demon" (a species capable of dominating in all environments) implies that the ability to avoid energetic, growth, and genetic limitations in stressful conditions must correspond to reduced relative competitive ability under optimal conditions, but the genetic basis for this trade-off remains unclear.

These three fungi represent examples selected from a continuous scale of life-history strategies, each of which may be employed to achieve the maximum possible combative ability under certain environmental condition. From this model it also follows that indicators of fungal survival (e.g., growth or respiration) in the absence of competition is not a meaningful surrogate for competitive ability, and is thus not useful for identifying the realized niche of a fungal species under the interaction milieu. Indeed, fungi with energetic and genetic limitations may exhibit growth and activity under stress when measured in isolation, but be out-competed by stress-tolerant species in the interaction milieu.

costs of combative trait expression require further exploration (Saunders et al., 2010). Initial evidence suggests that, in highly combative species, the genes associated with antagonism are clustered together on chromosomes (Mousa and Raizada, 2013), making these fungi highly susceptible to stress-induced gene silencing (Gasch, 2007). It is likely that dormancy represents an important mechanism for these fungi to avoid short-term periods of drought (Manzoni et al., 2014), but leaves them vulnerable to attack from stress-tolerant species in the longer term. In contrast, defensive traits of the less combative stress-tolerants are expected to be more constitutive, requiring minimal gene activation or deactivation during interactions (Iakovlev et al., 2004). This potential trade-off between induced attack and constitutive defense requires further exploration across a broad range of species, but potentially might explain shifts in fungal communities observed along moisture gradients. The highly "combative" species that we describe, therefore, reflect the "competitive" fungi described in Grime's CSR theory (Grime, 1977), although the fungi adapted to xeric conditions are simply those species capable of maintaining intermediate

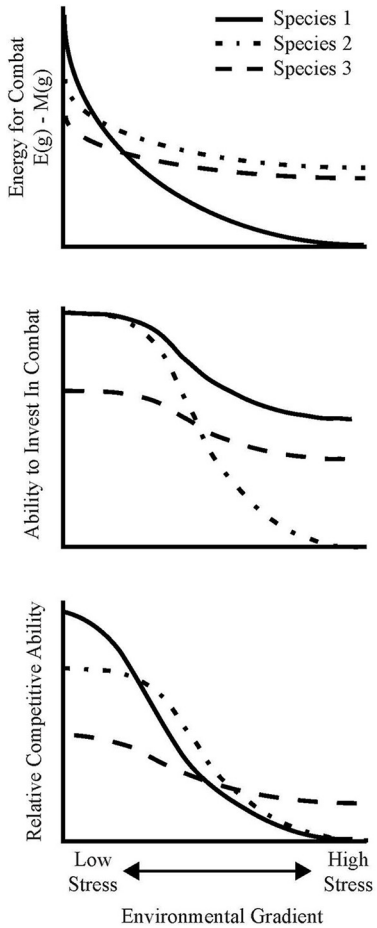


**FIGURE 4 | Conceptual diagram to illustrate how differential investment of energy in stress-tolerance and combative ability can influence the community organization of species with overlapping fundamental niche spaces along environmental gradients.** The different lines refer to do individuals with different life-history strategies: species 1 invests heavily in combat but experiences substantial energetic limitation under stress, whilst species 2 experiences a genetic trade-off whereby combative ability is limited by the activation of environmental stress response genes, and species 3 is a specialist stress-tolerant species, capable of activating stress-response genes with minimal deactivation of combative genes (see **Box 1**).

expression of combative traits under stressful conditions (see **Box 1**).

### PERFORMANCE CURRENCY

A performance currency is a measurable characteristic that relates to the performance (or competitiveness) of individuals under a given environment. The traditional approaches for exploring fungal niche spaces involve measuring changes in biomass, growth or respiration under varying abiotic conditions (e.g., Barcenas-Moreno et al., 2009; Lennon et al., 2012; Crowther and Bradford, 2013). These approaches allow us to visualize fundamental niche spaces (the conditions under which an individual can exist), but are unable to address biotic interactions, or identify the conditions under which fungi can compete and survive. An alternative approach, incorporating biotic and abiotic processes, is to record the outcomes of combative interactions (measured as likelihood of winning or relative growth) under different abiotic conditions (Boddy, 2000; Crowther et al., 2012b). This approach has the potential to approximate the environmental conditions under which dominance can switch, but practical limitations in pairing multiple species in a fully factorial design across a full environmental gradient restrict our capacity to predict realized niches or extrapolate to other fungi. Instead, attempts to relate competitive ability to environmental conditions have generally involved only a handful of fungi and lead to the perhaps trivial conclusion that competitive interactions are context-dependent (e.g., Crowther et al., 2011).

To overcome these limitations and identify how fungal survivorship varies continuously across environments in the context of biotic interactions requires the identification of performance currencies that reflect the interaction milieu. "Combative ability" or associated traits (e.g., toxin production) could provide such a performance currency in many free-living fungal communities. Estimating how relative combative ability shifts along environmental gradients can help to predict, not only the conditions under which a fungus can survive, but under which it can compete for survival (**Figure 2**). Despite the paucity of studies testing trade-offs in free-living fungi, the prevalence of drought stress and combat within the fungal system, and the energetic trade-offs associated with both, provide a testable framework through which we can visualize the realized fungal niche (**Box 1**).

Dominance-tolerance trade-offs are apparent across the fungal kingdom (Boddy, 2000; Gasch, 2007), but the importance of these opposing processes will be context-dependent. Combat is, for example, less important than nutrient acquisition for arbuscular mycorrhizal fungi (Chagnon et al., 2013), and temperature stress is likely to exert a greater selection pressure than drought stress for the internal pathogens of mammals (Kraus and Heitman, 2003). Multiple trade-offs are likely to influence different components of the same community simultaneously. For example, a colonization-competition trade-off has been highlighted as a potential mechanism driving successional dynamics in ectomycorrhizal communities in woodland soil (Kennedy et al., 2011), whereas co-existing saprotrophic species are structured, in part, by trade-offs between combative ability and heat-tolerance (Crowther et al., 2012b). The limited number of individuals represented in these mechanistic studies, however, precludes robust examination and extrapolation of these trade-offs, and the restricted number of traits investigated limits our understanding of their relative importance within communities. Comparing the strength of different trade-offs, between and within communities, is essential to identify the dominant structuring forces within fungal communities and, therefore, the appropriate performance currencies necessary to untangle niche spaces.

## LINKING TRAITS TO THE ENVIRONMENT

Once the dominant traits structuring communities are known they can be linked to the environmental conditions under which those individuals exist, to establish a mechanistic understanding of patterns of fungal biogeography and successional dynamics (Engelbrecht et al., 2007). The traits of individual, free-living

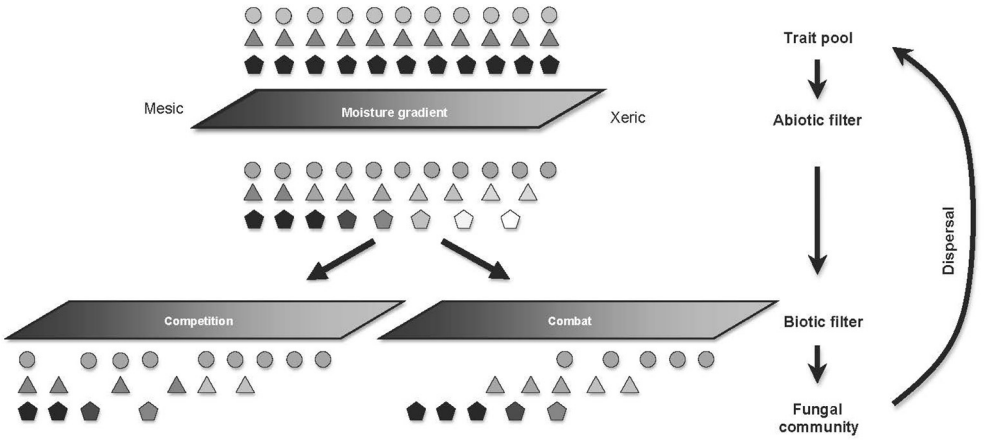


**FIGURE 5 | Biotic and abiotic filters differentially influence trait organization along environmental gradients.** Similar shapes refer to individuals with similar stress-tolerance trait values, and the level of shading refers to relative combative ability of these individuals, with lighter individuals being less combative. On passage through the abiotic filter, stressful conditions reduce the survival and combative abilities of the most combative species, with negligible effects on the stress-tolerant species. Energetic costs of cell maintenance lead to reduced expression of combative traits, ultimately limiting the capacity of stress-intolerant species to compete under xeric conditions. However, the nature of the biotic filtering varies depending on the dominant interactive mechanism. Where combat (the direct killing of opponents) is the dominant interaction mechanism, the stress-intolerant individuals are capable of displacing the stress-tolerant individuals under mesic conditions, leading to under-dispersion of combative traits under mesic conditions. Conversely, competition for nutrients or refuges drives niche differentiation under optimal conditions, ultimately, resulting in trait over-dispersion in these optimal environments.

fungi have yet to be linked explicitly to the environment, but the observed clustering of species within local habitats provides compelling evidence for the importance of habitat filtering at small spatial scales (e.g., Fukasawa et al., 2008; Feinstein and Blackwood, 2013; Crowther et al., 2014). This is especially apparent in the extremophile fungi (Kogej et al., 2007), whose efficient stress responses allow them to compete under temperature and moisture conditions far outside the ranges of most other species. Similar trends exist over time in patterns of fungal succession on decaying wood. For example, fungal diversity is greatest at mid-stages of wood decomposition (Rajala et al., 2011), where combat under optimal conditions is likely to be intense (Boddy, 2001). As decomposition progresses, changes in the chemical composition and physical structure of wood lead to extreme moisture conditions (either too moist or too dry), and/or decreased nutrient availability. These changing environmental conditions eventually select for fungi that can tolerate stressful conditions (Boddy, 2001) or for mycorrhizal fungi (Rajala et al., 2011), whose alternate nutrient-acquisition strategies (e.g., carbon provision by the host plant) provide a competitive advantage under energy-limitation.

Although the role of niche processes in structuring fungal communities is consistently supported at fine spatial scales, recent studies provide limited evidence for habitat filtering at regional and continent-scales (Martiny et al., 2006; Wu et al., 2013; Cline and Zak, 2014; Talbot et al., 2014). By focusing on taxonomic distributions, these studies highlight the importance of dispersal limitation and neutral processes in fungal taxonomic organization. However, we argue that, because environmental conditions have consistently been shown to select for individuals displaying similar characteristics in small-scale studies, the exploration of trait variation at broad scales is also likely to provide reveal more predictable patterns in fungal organization (Talbot et al., 2014). That fungal taxonomic distributions do not appear to reflect environmental conditions provides evidence for a discontinuity between taxonomic identity and trait values. Nevertheless, the observation of trait distributions requires consideration of the different coexistence mechanisms, the relative importance of which remain poorly understood in fungal communities.

## COEXISTENCE MECHANISMS AND TRAIT DISPERSION

Apparent in the majority of observational studies, habitat filtering generally leads to the clustering (under-dispersion) of similar species by selecting for traits that allow individuals to survive in a particular environment. In contrast, "biotic sorting" (competition) can discourage the coexistence of species with similar trait values (Adler et al., 2013; Smith et al., 2013). By promoting niche differentiation, competition for resources generally leads to the over-dispersion of traits values compared to expectations from null models. Despite considerable and growing appreciation for these contrasting coexistence mechanisms in aboveground ecology (e.g., Adler et al., 2013; Smith et al., 2013; Herben and Goldberg, 2014), only one study to date has explored the relative importance of habitat filtering and niche partitioning in the fungal kingdom. Maherali and Klironomos (2012) showed that competition is the dominant structuring force in arbuscular mycorrhizal communities, preventing functionally similar traits from co-existing at fine spatial scales. Drawing inferences from plant communities, it is likely that the relative importance of these processes is scale-dependent, with the effects of habitat filtering being more apparent at the broadest of spatial scales (Smith et al., 2013). This scale effect is also likely to vary between components of the fungal community, with the effect of habitat filtering on endophytes and free-living saprotrophs being apparent at the scale of

individual plants and ecosystems, respectively (Saunders et al., 2010).

Along with spatial scale, the specific mechanisms through which organisms interact also require consideration when exploring how niche processes structure communities. Competition for resources, for example, generally promotes niche differentiation, but intense combat can select for the clustering of similar antagonistic or defensive traits (Mayfield and Levine, 2010; Herben and Goldberg, 2014). These opposing selection pressures can act simultaneously, leading to the over-dispersion of some traits and the under-dispersion of others within the same community (**Figure 5**; Herben and Goldberg, 2014). These dynamics could potentially contribute to the opposing patterns of community assembly observed between free-living, and mycorrhizal fungal communities in Australian sclerophyll forest (Beck et al., in preperation). Exploring trait dispersion across environments therefore requires consideration of the functional roles of those traits and the specific mechanisms through which they promote coexistence.

The use of fungal response traits is, therefore, not only essential to identify the dominant trade-offs that govern a species' potential distribution across environments, but also for understanding how coexistence mechanisms operate within communities. Exploring the equalizing and stabilizing mechanisms governing trait dispersion in the fungal kingdom is essential if we are to understand how niche processes affect patterns of diversity and community responses to environmental change (Adler et al., 2013). Until we have a grasp on these mechanisms, we will be limited in our capacity to explain or predict accurately community patterns observed in broad-scale observational studies. Being small, culturable and rapidly manipulated within laboratory environments, fungi are ideal model organisms to test these mechanisms and explore the predictions of current trait-based models of coexistence (Maherali and Klironomos, 2012).

## CONCLUSIONS

Their capacity for macromolecule degradation allows fungi to fill essential roles in the cycling of nutrients, detoxification of soil and regulation of both autotrophic and heterotrophic productivity (Hättenschwiler et al., 2005). Despite substantial variation between species, our understanding of fungal-mediated biogeochemical processes generally originates from species- or plot-level studies, limiting our capacity to extrapolate effects to regional and global scales. By linking physiological, morphological and biochemical properties of fungi with their environment, the trait-based approach that we outline can provide mechanistic insights into how fungal characteristics vary over time and space. The coupling of these response traits with effect traits can then facilitate the scaling of processes recorded in single sites/species to broader spatial-scales (Lavorel and Garnier, 2002; Pakeman, 2011; Koide et al., 2014). Where habitat filtering is a dominant coexistence mechanism governing trait distribution, relationships between environmental conditions and fungal effect traits are likely to be strong. Indeed, the trade-offs between abiotic stress tolerance and enzyme production are explicitly considered in recent decomposition models (Sinsabaugh et al., 2008; Allison, 2012); re-allocation of energy from enzyme synthesis to osmolyte production under increasing drought stress is a dominant mechanism governing differences in mineralization rates across landscapes (Manzoni et al., 2014). The relationships between response and effect traits are, however, rarely so direct (Pakeman, 2011), due, in part, to the multiple interacting coexistence mechanisms that can lead to complex trait mixtures. It is possible, for example, that the costs of combat under optimal moisture conditions (approximately −0.3 MPa; Lennon et al., 2012) are energetically equivalent to those involved with stress-tolerance under harsh conditions, with similar consequences for soil nutrient mineralization (Snajdr et al., 2011) and organic matter decomposition (Crowther et al., 2011) rates. Identifying the relative physiological costs of tolerating abiotic stress and biotic interactions (see **Box 1**) may be essential to improve the robustness of current decomposition models, and predict how the functioning of fungal communities varies between environments.

Despite the challenges involved in understanding communities governed by multiple coexistence mechanisms, community-level patterns of trait diversity can provide some insights into the functioning of fungal communities. The importance of fungal diversity for nutrient cycling (e.g., Fukami et al., 2010), host (including human) fitness (e.g., Ley et al., 2006) and ecosystem health (e.g., Hättenschwiler et al., 2005), is well appreciated, although the nature of fungal diversity-functioning relationships can vary substantially between communities (Nielsen et al., 2011). Biological diversity can influence ecosystem functioning, either by changing community-weighted mean trait values (related to the mass ratio hypothesis) or functional trait dissimilarity (related to non-additive community effects; Dias et al., 2013). The relative importance of these mechanisms remains unexplored in the fungal system, but may prove to be pivotal in predicting how changes in fungal diversity might affect ecosystem functioning across landscapes. Ultimately, we propose that the use of trait-based approaches is essential to explore whether the processes structuring fungal biogeography and functioning are unique, or equivalent to those in other taxonomic groups. The trait-based framework we provide highlights a number of key uncertainties that require testing in the fungal system, as they have the potential to advance the search for general principles in fungal ecology.

## AUTHOR CONTRIBUTIONS

TWC conceived the study. DSM designed the trade-off model and all authors contributed to manuscrpit writing and preperation.

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# Long-term forest soil warming alters microbial communities in temperate forest soils

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Soil microbes are major drivers of soil carbon cycling, yet we lack an understanding of how climate warming will affect microbial communities. Three ongoing field studies at the Harvard Forest Long-term Ecological Research (LTER) site (Petersham, MA) have warmed soils 5°C above ambient temperatures for 5, 8, and 20 years. We used this chronosequence to test the hypothesis that soil microbial communities have changed in response to chronic warming. Bacterial community composition was studied using Illumina sequencing of the 16S ribosomal RNA gene, and bacterial and fungal abundance were assessed using quantitative PCR. Only the 20-year warmed site exhibited significant change in bacterial community structure in the organic soil horizon, with no significant changes in the mineral soil. The dominant taxa, abundant at 0.1% or greater, represented 0.3% of the richness but nearly 50% of the observations (sequences). Individual members of the Actinobacteria, Alphaproteobacteria and Acidobacteria showed strong warming responses, with one Actinomycete decreasing from 4.5 to 1% relative abundance with warming. Ribosomal RNA copy number can obfuscate community profiles, but is also correlated with maximum growth rate or trophic strategy among bacteria. Ribosomal RNA copy number correction did not affect community profiles, but rRNA copy number was significantly decreased in warming plots compared to controls. Increased bacterial evenness, shifting beta diversity, decreased fungal abundance and increased abundance of bacteria with low rRNA operon copy number, including Alphaproteobacteria and Acidobacteria, together suggest that more or alternative niche space is being created over the course of long-term warming.

**Keywords: climate change, microbial ecology, ribosomal RNA, rrn operon copy number, trophic strategy**

## INTRODUCTION

Earth's climate is warming, and this is exacerbated by both biophysical (e.g., albedo) and biogeochemical [e.g., carbon (C) cycle] feedbacks (IPCC, 2013). Microbes are key players in every biogeochemical cycle, regulating greenhouse gas fluxes between soils and the atmosphere (Falkowski et al., 2008). Despite their pivotal role, we know little about how microbes respond to environmental change, and microbial dynamics are only beginning to be represented in ecosystem models (Reid, 2011; Treseder et al., 2012). Based on the improved predictive capacity of soil carbon cycling models when microbial physiology is considered, it is clear that microbes are important for understanding and predicting ecosystem processes (Allison et al., 2010; Li et al., 2014). New genomic approaches hint at what the most abundant organisms are, though their ecological roles are still unclear. A better understanding of microbial dynamics is critical for projecting the rate and magnitude of climate change (Melillo et al., 1990, 2002; Heimann and Reichstein, 2008).

At the Harvard Forest Long-Term Ecological Research (LTER) site, warming-induced disruptions to ecosystem C cycles have been observed as part of three ongoing warming studies. In the longest-running site (20 years, **Table 1**), soil respiration rates in the warming treatment were initially higher than controls, then slowed to become equal to controls after 12 years (Melillo et al., 2002). Most warming studies, including our own, have observed a short-term increase in $CO_2$ emissions with warming (Rustad et al., 2001; Contosta et al., 2011; Wu et al., 2011; Davidson et al., 2012) as well as slowed $CO_2$ emissions following this initial increase. Slowed $CO_2$-C loss is concomitant with a depletion of labile C (Bradford et al., 2008; Frey et al., 2008; Melillo et al., 2011), as well as a decline in microbial biomass (Frey et al., 2008), thermal adaptation of soil microbes (Bradford et al., 2008) and a shift in microbial carbon use efficiency (Frey et al., 2013). More recently in our longest running (20-year) warming experiment, soil respiration has begun to increase again in warmed soils compared to controls. Partitioning the two components of respiration—microbial and root respiration—showed that on an

**Table 1 | Experimental sites that make up the warming chronosequence at Harvard Forest Long-Term Ecological Research (LTER) site.**

| | Soil Warming x N addition study (SWaN) | Barre Woods | Prospect Hill |
|---|---|---|---|
| Start year | 2006 | 2003 | 1991 |
| Duration[a] (years) | 5 | 8 | 20 |
| Number of replicate plots | 6 | 1 megaplot with 25 subplots | 6 |
| Size of plots | 3 × 3 m | 30 × 30 m | 6 × 6 m |
| Soil pH, O-horizon[b] | 3.72 | 4.29 | 3.82 |
| Soil pH, 0–10 cm mineral[b] | 4.38 | 4.42 | 4.41 |
| Total C (g C $m^{-2}$), O-horizon[b] | 3314 (404) | 1772 (621) | 2565 (247) |
| Total C (g C $m^{-2}$), 0–10 cm mineral[b] | 3478 (121) | 1810 (92) | 2859 (444) |

[a] *Duration of the warming study is listed for the time of sample collection, in fall of 2011.*

[b] *Values listed are for control plot soils only.*

annual basis, the majority of $CO_2$ (70–80%) from forest soil plots was microbial regardless of heat treatment (Melillo et al., 2002, 2011). Climate warming and other cascading effects (such as drying) have been suggested to destabilize stored soil C in a manner that is dependent on organic matter age, protection, and other conditions that may alter microbial access and decay (Fierer et al., 2009; Schimel and Schaeffer, 2012). The non-linear response of soil respiration to warming, combined with the prior observations of microbial adaptation and that most soil respiration is microbial has lead us to the hypothesis that long-term warming has caused changes in the soil microbial community, which may be exacerbating C cycle feedbacks to the climate system.

Studies that have examined both microbial community characteristics and activity as affected by warming, whether as lab experiments (Zogg et al., 1997; Frey et al., 2008) or field studies (Bardgett et al., 1999; Belay-Tedla et al., 2009; Wu et al., 2011; Zhou et al., 2012; Pold and DeAngelis, 2013) reveal that although the physiological response (e.g., $CO_2$ flux) across experiments and biomes tends to be consistent in that warming increases C mineralization, the microbial community response is not, with microbial communities in different ecosystems responding differently to warming. Over the short term the net effect of warming on soil microbes tends to be increased microbial activity, including increased soil respiration (Rustad et al., 2001; Melillo et al., 2011; Wu et al., 2011). This response to warming is likely partly attributable to changes in the active fraction of the biomass, rather than changing community's constituents (Zogg et al., 1997; Andrews et al., 2000; Pietikåinen et al., 2005). A metaanalysis of 75 manipulative climate change experiments showed that not all soil microbial communities respond similarly to warming (Blankinship et al., 2011). Long-term experiments at the Kessler Farm Field Laboratory (KFFL) in the plains of central Oklahoma found increased diversity under warming and drought, suggesting that warming may have somehow "primed" the community to be more resilient and resistant to further disturbance (Zhang et al., 2005). Furthermore, Zhang and colleagues found that warming was associated with decreased net N mineralization and a significant shift in the substrate utilization profiles, indicating a change in the substrate availability for the community. Metagenomic sequencing on grassland soils from experimental plots that had been warmed continuously (+2°C) for 8 years showed that gene signatures for degradation of more labile C compounds—starch, chitin, hemicellulose, and cellulose—were activated, whereas genes involved in recalcitrant C degradation, such as lignin, were not stimulated by warming (Zhou et al., 2012; Luo et al., 2014). They concluded that warming in grasslands could result in a weakened positive feedback between the terrestrial carbon cycle and climate. While results from the early years of the Harvard Forest warming experiment are consistent with the grasslands study, the long-term effects point to a very different conclusion about climate feedbacks.

The Harvard Forest soil warming experiments offer a unique opportunity to understand how climate change affects soil microbial community composition over the course of long-term warming. This is important because bacterial activities are possibly involved in a positive feedback to climate (Melillo et al., 2002; Frey et al., 2008; Brzostek et al., 2012). Three forest plots experienced 5°C above ambient soil temperatures for 5 (SWaN Plots), 8 (Barre Woods) and 20 (Prospect Hill) years at the time of sampling, forming an experimental warming "chronosequence" (**Table 1**). Fatty acid methyl ester (FAME) analysis of the Prospect Hill warming experiment after 12 years of heating 5°C above ambient showed a significant decrease in fungal abundance and an increase in Gram-positive bacteria and Actinobacteria in warmed soils compared to controls (Frey et al., 2008). Previous results indicate observed changes in C source utilization (Frey et al., 2008), plant-microbial interactions (Butler et al., 2012), mass-specific microbial respiration rates (Bradford et al., 2008), and soil chemistry (Melillo et al., 2011, 2002). Together, these results suggest a substantial change in microbial substrate availability which would be consistent with changing bacterial community profiles and potential altered feedbacks to climate.

In this study, we used high-throughput sequencing of the V4 variable region of the 16S ribosomal RNA gene to test the hypothesis that bacterial communities have changed over two decades of simulated climate change in organic and mineral soil horizons. However, utilization of the ribosomal RNA gene for phylogenetic analysis carries the caveat that the rRNA operon often exists at multiple copies per genome, which both confounds estimates of genomic abundance by over-estimating taxa with multiple rRNA operon copies (Crosby and Criddle, 2003; Větrovský and Baldrian, 2013) but also suggests life strategy, where taxa with multiple rRNA operon copies have faster growth rates, while taxa with single copies tend to be associated with oligotrophic environments (Klappenbach et al., 2000; Stevenson and Schmidt, 2004). We estimated copy number by phylogenetic inference using the available complete genome sequences in GenBank (Kembel et al., 2012), and used this to examine the effect of copy number correction on community profiles. We also used these estimates to test the additional hypothesis that average bacterial copy number was decreased by long-term warming, which would be consistent

with observations of microbial adaptation (Bradford et al., 2008) and decreased soil carbon with long-term warming (Melillo et al., 2011). Finally, we performed quantitative PCR (Q-PCR) of bacterial and fungal communities, as well as the phyla Actinobacteria, Acidobacteria, and class Alphaproteobacteria, to measure changes in absolute abundance of these dominant and dynamic groups.

## MATERIALS AND METHODS

### FIELD EXPERIMENT

All three experimental sites at the Harvard Forest Long-Term Ecological Research (LTER) site (Petersham, MA) were established in mixed hardwood forest stands with dominant tree species being paper and black birch (*Betula papyrifera* and *lenta*), red maple (*Acer rubrum*), black and red oak (*Quercus velutina* and *rubra*), and American beech (*Fagus grandifolia*). The soils are coarse-loamy inceptisols. The warmed plots at all three sites have been heated continuously by the use of resistance heating cables buried at 10 cm depth in the mineral horizon (Melillo et al., 2002). Controls in the longest running experiment are disturbance controls (which had cables buried but never activated); effects of disturbance on microbial community composition (Frey et al., 2008), soil inorganic nitrogen, and carbon dioxide flux (Peterjohn et al., 1994) have been minimal. Heating was to 5°C above ambient, a temperature increase which falls within the range of worst-case-scenario model projections for increased global air temperature by the year 2100 as projected by the Intergovernmental Panel on Climate Change (IPCC, 2013). Mean monthly temperatures at Harvard Forest range from 19°C in July to −5°C in January and average annual precipitation is 112 cm, distributed relatively evenly throughout the year.

### SAMPLE COLLECTION

Soil samples were collected October 25–27, 2011 from each of the three sites in the chronosequence with four replicates from each treatment at Barre Woods, Prospect Hill and SWaN (**Table 1**). Soil was collected from the organic horizon (which varies in depth from 1–5 cm) and the upper 10 cm of mineral soil in the heated and control plots of all three studies, with four replicates totaling 48 samples (3 sites × 2 warming treatments × 2 soil horizons × 4 replicates). The organic horizon was collected as intact 20 × 20 cm blocks to the depth of the mineral soil, and the underlying mineral soil was sampled to 10 cm depth in the same locations using a custom-made stainless steel auger (9 cm diameter). Subsamples of both horizons were taken and immediately flash frozen in the field and stored at -80°C until extraction.

### NUCLEIC ACID EXTRACTION

Soils were extracted twice based on previously published methods (DeAngelis et al., 2010) with a few modifications. Frozen soils were extracted for DNA and RNA simultaneously (Griffiths et al., 2000) using modified CTAB extraction buffer (0.25 M phosphate buffer (pH 8), 5% hexadecyltrimethylammonium bromide (CTAB) in 1M NaCl) and 50 μl of 0.1 M ammonium aluminum sulfate (Braid et al., 2003). Three replicate extractions were performed for each sample, then pooled and put through the Qiagen All DNA/ RNA Mini kit (Qiagen, Valencia, CA).

### AMPLIFICATION AND SEQUENCING

Library generation and sequencing of the V4 region of the 16S ribosomal RNA gene were performed as per a recently published protocol (Caporaso et al., 2012). The V4 region of the 16S ribosomal RNA gene was amplified on an Eppendorf ProS thermal cycler using the primers 515F and 806R, where the forward primers contained a subset of 48 of the 8 bp barcoded primers. Reactions were performed in a final volume of 25 μl using Takara ExTaq with 200 pM of each primer, 25 μg of BSA and 2 units of DNA polymerase (Takara Mirus Bio, Madison, WI) with 10 ng template per reaction. PCR amplifications were performed at 50°C annealing temperatures (Tm), with an initial denaturation (5 min) followed by 30 cycles of 95°C (30 s), Tm (25 s) and 72°C (120 s), and a final extension of 72°C (10 min). Triplicate amplification reactions were verified by agarose gel electrophoresis, pooled, then cleaned using Qiagen MinElute kit (Qiagen Sciences, Valencia, CA). Cleaned amplicon pools were quantified using picogreen and quality assessed by nanodrop. Sequencing was performed by the Molecular Biology Core Facility at the Dana-Farber Cancer Institute using standard operating procedures. Due to the low diversity of the library 50% by DNA mass PhiX spike was added to the pooled, barcoded sample just before running. Samples were sequenced using the MiSeq platform to generate 2 × 150 bp paired-end reads.

### SEQUENCING DATA ANALYSIS

Paired-end sequences were assessed for quality using FastQC (Andrews, 2010), and initial quality filtering and assembly was performed using FLASH using default parameters (Magoč and Salzberg, 2011). Sequences were then binned into operational taxonomic units (OTUs) and taxonomies assigned using the subsampled open-referenced OTU picking method in QIIME (Caporaso et al., 2010) at 99% sequence identity based on the October 2012 greengenes taxonomy (DeSantis et al., 2006), where the fasta reference file was truncated to include just the V4 region (Werner et al., 2012). Chimeric sequences were detected using UCHIME (Edgar et al., 2011; Wright et al., 2012). We removed sequences observed only once or twice (singletons or doubletons), as well as erroneous sequences that were probable chimeras (DeSantis et al., 2006). A second community matrix of dominant taxa was generated with only taxa present in the data set at 0.1% relative abundance or greater. Community matrices were rarefied to the number of taxa in the sample with the lowest number of observations for most analyses excluding diversity analyses, since there tends to be a positive correlation between richness and depth of sequence sampling. Sequences are available in GenBank under accession numbers SRP040706, BioProject ID PRJNA242868.

### QUANTITATIVE PCR

Total bacterial and fungal abundances were measured for all 48 samples by quantitative PCR (Q-PCR) using 341F (5′-CCT ACG GGA GGC AGC AG-3′) and 534R (5′-ATT ACC GCG GCT GCT GGC-3′) (Muyzer et al., 1993) primers for total bacteria and ITS1f (5′-TCC GTA GGT GAA CCT GCG G-3′) and 5.8 s (5′-CGC TGC GTT CTT CAT CG-3′) (Fierer et al., 2005) for total fungi. Standard curves were based on linear PCR product

from *Klebsiella* sp. str. BRL6-2 for bacteria and *Saccharomyces cerevisiea* for fungi, after purification, quantification and dilution. All samples were run as technical duplicates on an Eppendorf Realplex2, using the QuantiFast SYBR Green PCR Kit (Qiagen) with 10ng of DNA per 25ul reaction and primers at a final concentration of 1uM. PCR program was 5 min 95°C initially, followed by 40 × (10 s 95°C, 10 s 58°C, 20 s 60°C), and melt curve (60°C–95°C). Efficiencies were 101.6% for fungal, with an $R^2$ of 0.9923, and 107.8% for bacteria, with an $R^2$ of 0.9972. Total Acidobacteria, Actinobacteria and Alphaproteobacteria abundances were measured for all eight Prospect Hill organic horizon samples by quantitative PCR (Q-PCR). Primer sets for Acidobacteria were Acid31 (5′-GAT CCT GGC TCA GAA TC-3′) and Eub518 (5′-ATT ACC GCG GCT GCT GG-3′) (Fierer et al., 2005); for Actinobacteria were Act920F3 (5′-TAC GGC CGC AAG GCT A-3′) and Act1200R (5′-TCR TCC CCA CCT TCC TCC G-3′); and for Alphaproteobacteria were α682F (5′-CIA GTG TAG AGG TGA AAT T-3′) and 908αR (5′-CCC CGT CAA TTC CTT TGA GTT-3′) (Bacchetti De Gregoris et al., 2011). To generate standard curves, we used *Micrococcus luteus* (ATCC 381), *Caulobacter crescentus* str. CB15N (Peter Chien, pers. comm.), and *Acidobacterium capsulatum* (DSMZ 11244) for Actinobacteria, Alphaproteobacteria, and Acidobacteria, respectively. These standard curves were based on PCR product except for Acidobacteria, which was based on plasmid. The identity of all organisms and specificity of amplified products were confirmed by Sanger sequencing. Actinobacteria had a two-step amplification cycle with 10 s at 95°C followed by 30 s at 61.5°C. Acidobacteria and Alphaproteobacteria had a three-step cycle with 10 s at 95°C, 20 s at 55°C, and 10 s at 60°C. Soil dry weights were obtained by drying 10 g fresh soil in a 105°C oven for 3 days, and abundances are reported as counts per dry weight of soil. Counts represent genome equivalents not considering the confounding factor of multiple small subunit ribosomal RNA operon copies per genome.

### STATISTICAL ANALYSIS

The experimental design was a fully replicated design with three warming experiments: Prospect Hill (20 years warming), Barre Woods (8 years warming), and SWaN (5 years warming); one treatment of warming or no-heated control; two soil horizons (organic and mineral); and four field replicates, resulting in 48 samples total. For the copy number correction, we used a recently published method (Kembel et al., 2012), where the copy number corrected OTU table was generated using copy numbers estimated in R based on ancestral state reconstruction (Matsen et al., 2010). Weighted average copy number is based on multiplying relative abundance of OTUs by their estimated copy number, then calculating average copy number per sample. The UniFrac distance matrices were generated using FastUnifrac (Hamady et al., 2009). Beta diversity was estimated based on weighted Unifrac distance matrices used to generate principal coordinates plots, and Procrustes analysis was performed to compare principal component plots of copy number corrected environmental file and uncorrected environmental file. Evenness was measured by Pielou's J, and richness measured as total number of taxa. We used seven methods to identify indicator OTUs of warming in the Prospect Hill organic horizons: (1) indicator value analysis (Dufrêne and Legendre, 1997); (2) volcano plots using two as the minimal fold change, 0.05 as the p value threshold in a *t*-test adjusted for multiple comparisons completed in METAGENassist after normalizing the data using Pareto Scaling (Arndt et al., 2012); (3) Nearest Shrunken Centroid classification (NSC) (Tibshirani et al., 2003); (4) partial least squares discriminatory analysis; (5) Bayesian groupings; (6) rank mobility based on mean abundance and reporting the top 5% of OTUs which showed the greatest change in rank; (7) and paired Student's *T*-test using the Benjamini-Hochberg correction (Benjamini and Hochberg, 1995). To compare whether Q-PCR copies differed between warmed and control plots, we used Bayesian inference (Kruschke, 2013), because this method yields richer inference considering the relatively small sample size. All statistical analyses were performed in R using the RStudio interface (RStudio, 2012; R Core Team, 2014), including packages reshape (Wickham, 2012), vegan (Oksanen et al., 2011), ggplot2 (Wickham, 2009), limma (Smyth, 2004), pplacer (Matsen et al., 2010), indicspecies (De Cáceres and Legendre, 2009), pamr (Tibshirani et al., 2003), caret (Kuhn, 2008), phyloseq (McMurdie and Holmes, 2013), pls (Mevik and Wehrens, 2007), and BEST (Kruschke, 2013).

## RESULTS

Sequencing produced 3,487,689 high-quality sequenced observations of the V4 16S ribosomal RNA gene region (Table S1, Figure S1). Sequencing depths before rarefaction ranged from 33,545 to 112,502 sequences per sample, with a mean of 63,293 reads and median of 62,838 reads. For most analyses, communities were rarefied to 33,545 sequences. Sequence reads were binned into operational taxonomic units (OTUs) based on 99% sequence identity (Werner et al., 2012), both because bacteria with nearly-identical 16S rDNA sequences may represent variable genotypes and different species (Suau et al., 1999; Hold et al., 2002; Konstantinidis and Tiedje, 2005) and because many functional traits are phylogenetically conserved up to 0.01% ribosomal RNA gene sequence dissimilarity (Martiny et al., 2013). After removal of singletons, doubletons and chimeras 2,938,751 observations remained (Table S1) that were binned into 45,875 OTUs, a scale of diversity on par with current estimates of soil bacterial diversity (Torsvik et al., 2002; Gans et al., 2005; Roesch et al., 2007).

### MICROBIAL COMMUNITY RESPONDS TO SOIL WARMING AFTER 20 YEARS

Soil warming had a statistically significant impact on bacterial community structure, but only after 20 years of warming. After 20 years, organic warmed soils were significantly different from those of control plots (**Figure 1A**). The sites warmed for 5 or 8 years showed no significant treatment effect on beta diversity, but bacterial communities in organic horizons warmed for 20 years began to resemble mineral soils. The strongest effect on bacterial community structure in our analysis was soil horizon (organic versus mineral), followed by site and then warming treatment (Table S2), with no significant interaction between soil horizon and treatment.

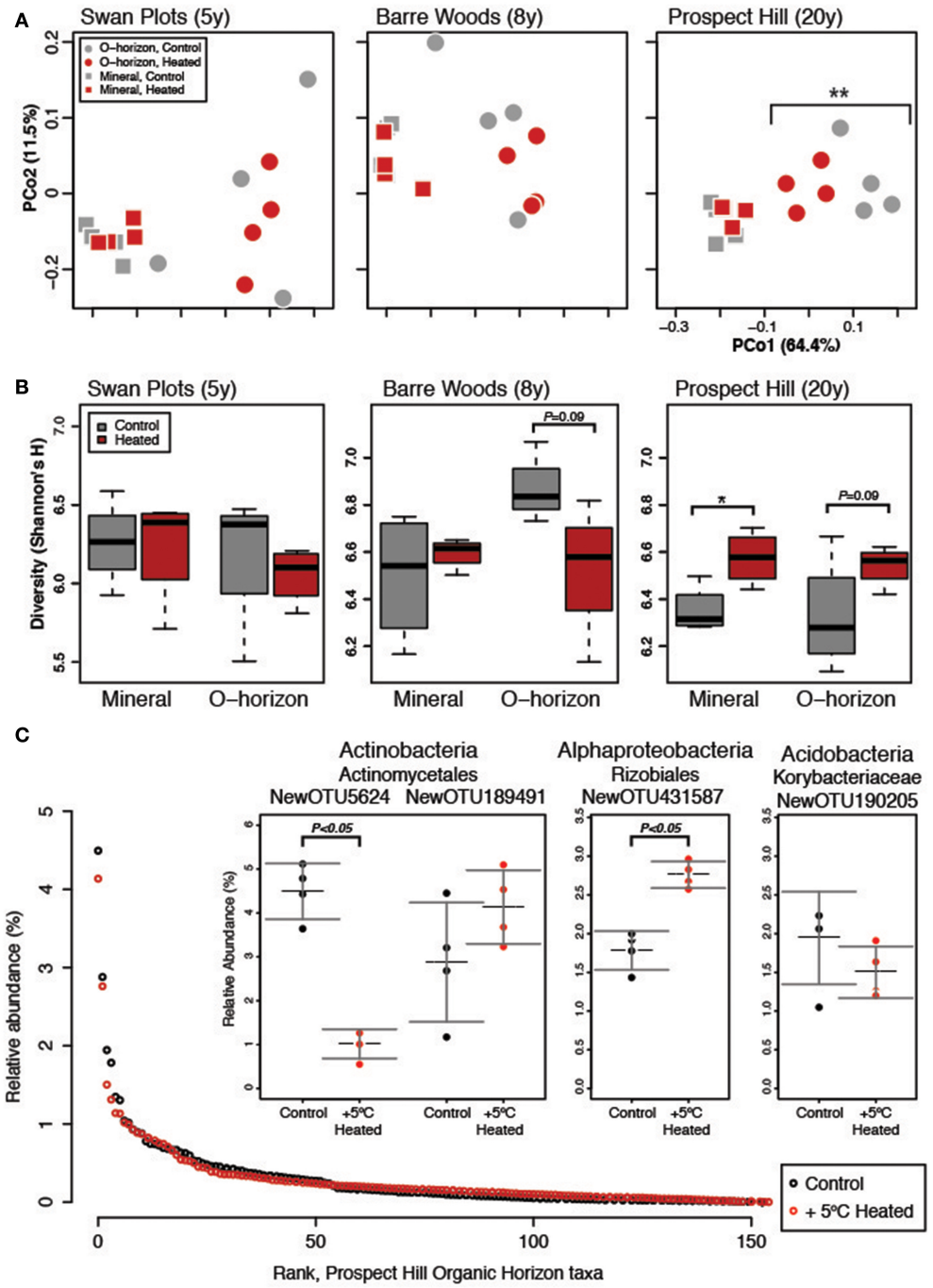


**FIGURE 1 | Measures of diversity for the warming chronosequence, based on the dominant subset community ($N = 155$). (A)** One PCoA ordination was performed on all sites, both treatments and both soil types, but the three sites are shown separately for clarity: SWaN Plots, Barre Woods, and Prospect Hill. **(B)** Diversity as measured by Shannon's H index is shown for the three sites as box (first and third quartiles) and whisker (95% CI) plots where the solid bar is median. **(C)** Rank abundance curve of the 155 dominant species in the community, with inset showing the three most abundant taxa and their relative abundance in heated versus control treatments averaged for the three sites. Statistical significance is indicated as $^{*}P < 0.05$, $^{**}P < 0.01$.

While soil warming affected community structure only in the organic horizon, diversity as measured by Shannon's H index was higher at the 20-year warmed Prospect Hill site ($P < 0.01$, **Figure 1B**, Table S3A), and was on average lower at the SWaN site compared to the other two sites ($P < 0.001$, Table S3B). In the Prospect Hill site, changes in diversity were driven more by increasing evenness ($T$-test $P < 0.01$; Bayesian effect size 1.60, 95% range −3.1 to −0.16) than changes in richness ($T$-test $P =$

0.06; Bayesian effect size −0.983, 95% range −2.05–0.20). The observed increase in bacterial diversity was driven strongly by decreased abundance of a few dominant taxa.

### DOMINANT SUBSET REFLECTS TOTAL COMMUNITY RESPONSE TO WARMING

The bacterial community was observed to be highly uneven: only 155 taxa (0.3% of total) present at 0.1% relative abundance or higher accounted for over half of the 3 million observations (**Figure 1C**). Comparison of the full community to the dominant subset community showed that the two were highly correlated (Procrustes $R = 0.987$, $P < 0.001$; Mantel test $R = 0.980$, $P < 0.001$). The same trends emerged for the dominant community as were observed for the full community, where soil horizon, then site, were major drivers of community structure, and that warming only affected the organic horizon of the 20-year long-term warmed soils (Table S2). This is perhaps not surprising considering that these dominant taxa, though comprising less than 1% of the richness, represented over half of all observations. The treatment effect on organic soil communities was stronger when looking at the dominant compared to the full community (Table S2), suggesting that dominant taxa were more strongly affected by warming.

The composition of the dominant subset community was similar to that of the total community, though the richness and phylogenetic range were much depleted. Compared to the 43,909 OTUs found in 33 phyla and 133 families in the total rarefied community, the dominant subset community was comprised of 155 OTUs found in 6 phyla and only 19 families. These included the phyla (and subphyla) Acidobacteria (57 OTUs), Alphaproteobacteria (40), Actinobacteria (25), Verrucomicrobia (11), Gammaproteobacteria (11), Planctomycetes (6), Firmicutes (2), Betaproteobacteria (2), and Deltaproteobacteria (1).

Most bacterial taxa in the dominant subset responded positively to long-term warming (**Figure 2**), though we observed no change in absolute abundance of total bacteria with warming treatment by Q-PCR (**Figure 3A,** Table S4). In the organic soil horizon, bacterial abundance was unaffected by warming, while fungal abundance was marginally decreased with warming (Bayesian effect size 0.744; $t$-test $P = 0.07$). Power analysis showed that 6.5 biological (field) replicates per group would distinguish the treatment effect on fungi in the organic horizon (for power = 0.90, significance level 0.95). Fungal ITS Q-PCR counts in the mineral horizon were unaffected by warming. The Q-PCR data showed that there were more fungal rRNA operon copies in the organic horizon compared to the mineral soil ($P < 0.05$), with the same amount of bacterial rRNA operon copies in the two soils. Bacteria 16S ribosomal RNA gene counts outnumbered fungal ITS counts by an order of magnitude in the organic layer and mineral layers ($P < 0.001$ for both soils).

### SELECT TAXA WERE ESPECIALLY SENSITIVE TO 20 YEARS OF WARMING

To identify the taxa responsible for the observed changes in the organic horizon bacterial community after 20 years of warming, indicator species analysis was performed using seven different methods (Table S5). Taxa that are more likely indicators of warmed or control conditions for the 20-year warmed site were defined as significant by multiple indicator species analyses (**Figure 2**). These analyses revealed that 19 of the 155 dominant taxa were differentially abundant in the organic soil horizon of the heated compared to control plots, with 15 increased in the warmed plots and four decreased. All six Acidobacteria detected as indicator species were indicators of heating treatment, with increased abundance in warmed compared to control soils. The four Actinobacteria were divided evenly between being indicators for heated or control treatments, and all belonged to the class *Actinobacteria* and order *Actinomycetales*. The last nine species were all Proteobacteria, and the two Proteobacteria that were indicators for control treatments were both Alphaproteobacteria in the order Rhodospirilales. The seven Proteobacteria that were indicators for warming treatments consisted of five Rhizobiales (Alphaproteobacteria), Syntrophobacterales (Delta proteobacteria), and Xanthomonadales (Gammaproteobacteria). Measures of absolute abundance (based on Q-PCR) of Actinobacteria, Alphaproteobacteria and Acidobacteria were performed because taxa in these groups were dominant in our MiSeq observations: 15% Actinobacteria (25 of 155 OTUs), 26% Alphaproteobacteria (40 of 155), and 37% Acidobacteria (57 of 155). Absolute copies of Actinobacteria did not differ between warmed and control plots, though heated plots had more Alphaproteobacteria ($P < 0.05$) and trended toward having increased abundances of Acidobacteria ($P = 0.08$) compared to control plots in the Prospect Hill organic horizon (**Figure 3B**, Table S4).

### MEAN RIBOSOMAL RNA COPY NUMBER IS SIGNIFICANTLY DEPLETED BY WARMING

The organic horizon in the 20-year warmed site (Prospect Hill) had lower average ribosomal RNA copy number of the bacteria present in heated compared to control soils (2.40 Control, 2.28 Heated, $P < 0.05$; **Figure 4**). Community structure did not change substantially when copy number was corrected based on Procrustes analysis of community profiles (Monte Carlo $P < 0.05$), with a high degree of similarity ($R = 0.9511$) between the copy number corrected and uncorrected PCoA community models (Figure S2). The most abundant taxa tended to have fewer than four copies of the 16S ribosomal RNA operon, while lower abundance taxa had as many as 14 estimated copies (Figure S3). Of the top 15 most dominant taxa, 13 had only one or two copies of the 16S ribosomal RNA operon. When copy number correction was applied, only 28 taxa changed rank such that they were no longer in the dominant subset community (comprised of 155 taxa).

## DISCUSSION

Mounting evidence suggests that soil microbes play a role in elevated $CO_2$ emissions and soil organic matter loss that is symptomatic of long-term warming in temperate forest ecosystems (Bardgett et al., 1999; Schimel and Gulledge, 2004; Frey et al., 2008, 2013). We set out to test the primary hypothesis that the soil bacterial community is altered by warming, and statistically significant differences were only apparent after 20 years. There are many factors in the environment that correlate with warming effects on microbial feedbacks to the climate system, including

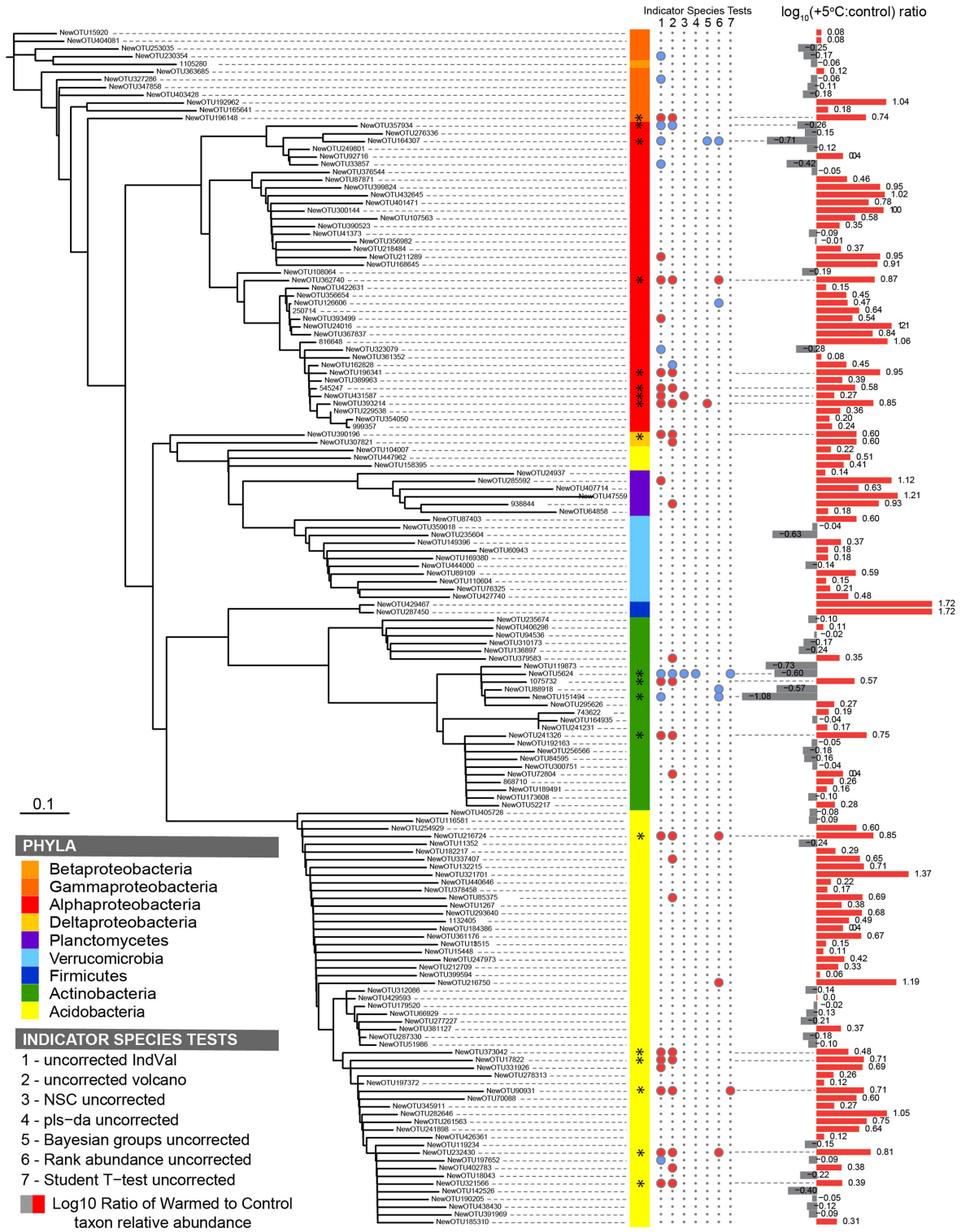


**FIGURE 2 | Phylogenetic tree of the dominant subset community ($n = 155$) in the Prospect Hill 20-year warmed sites, where (from left to right) the color strips denote phylum-level classification; the circles denote a significant indicator species test ($P < 0.05$) with blue circles indicative of control treatments and red circles indicative of heated treatments; bars show fold change in OTU abundance with warming treatment.** The seven indicator species analyses are (1) Dufrêne and Legendre's IndVal, (2) fold change (volcano-plots), (3) nearest shrunken centroid, (4) PLS-DA loadings test, (5) Bayesian group comparison, (6) Rank abundance test, (7) Student's *t*-test. Taxa with two or more significant indicator species tests are marked (*).

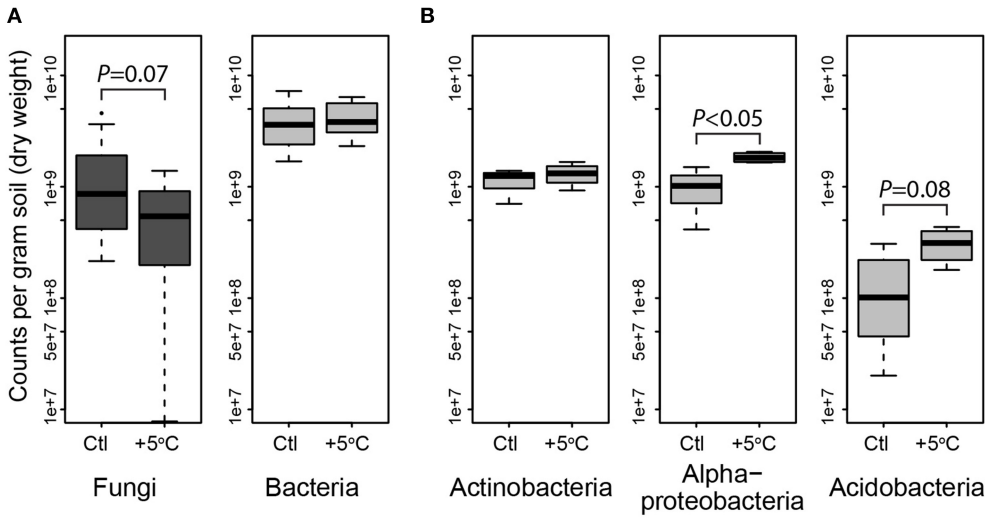


**FIGURE 3 | Quantitative PCR of bacteria and fungi, and the phyla Actinobacteria, Acidobacteria and Alphaproteobacteria showing abundance of dominant microbial phylogenetic groups for both treatments in organic horizon (averaged by site, which was not a significant factor).** Fungi were less abundant, and bacteria unchanged in heated (+5°C) compared to control (Ctl) organic horizon soils across the three sites **(A)**. In the organic horizons of the Prospect Hill site, Actinobacteria were unchanged, while Alpha-proteobacteria and Acidobacteria were enriched by long-term warming **(B)**. Means are shown as as box (first and third quartiles) and whisker (95% CI) plots where the solid bar is median.

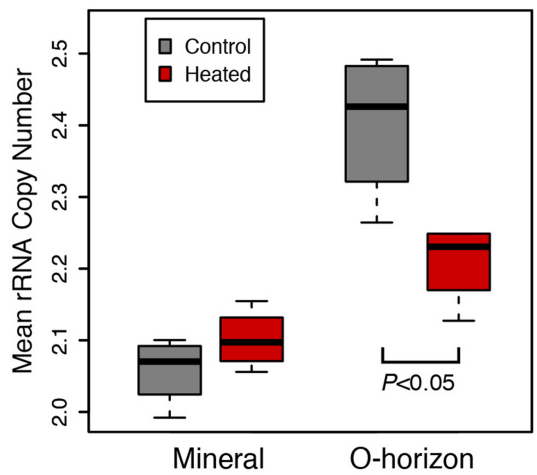


**FIGURE 4 | Mean ribosomal RNA copy number was calculated for Prospect Hill (20 years warmed) soil communities, and these calculations were based on phylogenetic inference (see methods for details).** Means are shown as as box (first and third quartiles) and whisker (95% CI) plots where the solid bar is median; statistical significance was determined based on ANOVA ($P < 0.05$).

associated soil moisture and drought, changes in plant communities, and N deposition. For example, Blankinship and colleagues' meta-analysis of 75 manipulative climate change experiments found that warming was more likely to have a negative effect on microbial abundance (density) in cool, dry locations (Blankinship et al., 2011). Observed changes in beta diversity may be due to the loss of labile C (Frey et al., 2008) that represents a significant change in substrate available to resident microbes. Changes in substrate availability in soil are well known to affect changes in microbial community structure (Cleveland et al., 2007; Fierer et al., 2007; España et al., 2011). The extent to which changes in microbial substrate utilization will result in net changes in carbon cycle feedbacks to the atmosphere remain to be examined, and will necessitate an understanding of why communities are changing, as well as how populations' carbon use efficiencies are changing and the extent to which new soil carbon pools (essentially, new niche space) are being degraded by the changing microbial populations.

Our secondary hypothesis, that average bacterial copy number was decreased by long-term warming, was also supported by the ribosomal RNA copy number estimation evidence, showing that that long-term warming favors bacteria with an oligotrophic lifestyle (Klappenbach et al., 2000; Stevenson and Schmidt, 2004). Within a bacterial genome, the number of rRNA gene operon copies tends to correlate with maximum growth rate (Stevenson and Schmidt, 2004), the ability to change growth rates quickly (Klappenbach et al., 2000), and other traits including limited mobility and fewer types of more high-affinity transporters (Lauro et al., 2009), though there are exceptions to these generalizations (Blazewicz et al., 2013). Organisms with many copies of the rRNA gene operon are broadly considered to be copiotrophs, adapted for exploitation of varying and high-quality substrates, while those with single or few copies are considered to be oligotrophs, adapted to extract maximum resources out of a limited supply (Klappenbach et al., 2000; Stevenson and Schmidt, 2004). However, oligotrophy can also occur under conditions where privatization of resources is possible, e.g., conditions of high spatial structure or high heterogeneity (Pfeiffer et al., 2001; Stevenson and Schmidt, 2004; Lennon et al., 2012; Bachmann et al., 2013). It is possible that long-term warming has caused a change in soil

structure that has increased the spatial heterogeneity, porosity, or other physical structure of the soils including physical or chemical protection of soil carbon that may contribute to the observed decrease in ribosomal operon copy number, though further study would be required to test this hypothesis.

We assume that the dominant subset of the community likely includes taxa that have important functions in the soil due to their success, though this is based on measures of relative abundance. Our measures of relative abundance based on QPCR suggest that of all bacteria, Actinobacteria comprise 8.8% in control and 9.4% in warmed, that Alphaproteobacteria comprise 25% in control and 44% in warmed soils, and that Acidobacteria comprise 3.4% in control and 7.4% in warmed soils (Table S4). However, the fraction of active populations at any one time may be as low as 10% for soil (Lennon and Jones, 2011), which confounds hypothesized links between function and observed community profiles by soil DNA evidence. Our original hypotheses were that long-term warming would induce a shift in the soil microbial communities, and that observed decreased soil C represented a decrease in microbial substrate availability that would increase the incidence of oligotrophy. These hypotheses are supported by our data, which include increased evenness with long-term warming, decreased ribosomal RNA copy number, increased community evenness and increased relative and absolute abundance of known or suspected oligotrophic taxa.

Ultimately, we are interested in changes in community structure insofar as they can reveal indicators of microbial feedbacks to climate, and because of this, we turned to indicator species and QPCR of key dynamic groups: Alphaproteobacteria, Acidobacteria, and Actinobacteria. Of the Alphaproteobacteria that changed with warming, the Rhizobiales mostly increased with warming while the Rhodospirillales mostly decreased with warming. The Rhizobiales include the most dominant Alphaproteobacteria, an unknown *Hyphomicrobium* spp. (**Figure 1C**), as well as members that are purple sulfur and non-sulfur bacteria, a group known for being able to grow under a wide range of conditions (Larimer et al., 2004). These taxa contain well-known plant root-associated microbes, though these experimental plots are too small to take into account changes in plant physiology or community, which also affect the observed microbial community in a warmer world (Bardgett et al., 1999). Acidobacteria can grow on complex polymers, including plant hemicellulose or cellulose and fungal chitin (Eichorst et al., 2011). The phyla Alphaproteobacteria, Acidobacteria and Actinobacteria contain many representative taxa known to degrade recalcitrant C and/or that have plant-specific associations (Barret et al., 2011). Further studies may elucidate the genetic or functional differences between the groups thus far represented only by the V4 region sequence and observed changes in relative abundance in a warmer world, though observation of genetic evolution in response to long-term warming or evolved functional changes, such as extracellular enzyme temperature optima, increased tolerance to low water potential conditions, or increased capacity for uptake and degradation of lower quality and quantity carbon will all require measures of physiology of isolated organisms in the lab.

Though absolute copies of Actinobacteria did not differ between warmed and control plots (**Figure 3B**), increased absolute abundance of Actinobacteria was observed at the Prospect Hill sites by FAME analysis after 12 years of warming (Frey et al., 2008). The most dominant taxon in the control plots, an Actinomycetales (class Actinobacteria, NewOTU5624), decreased 78% in response to warming, declining from 4.5 to 1.02% relative abundance ($P < 0.05$); the second most abundant taxon was also an Actinomycetales (NewOTU189491) and was unaffected by by warming (**Figure 1C**). In a separate study at Harvard Forest, Actinomycetes were shown to increase in relative abundance with the addition of labile, but not recalcitrant C source (Goldfarb et al., 2011), though these isolates were only incubated with lignin as recalcitrant C for 48 h. There are other studies that suggest Actinobacteria may be a rich reservoir of extracellular peroxidases including lignin peroxidases (Godden et al., 1992; Kirby, 2006), though further studies will determine how far lignin activity among the phylum Actinobacteria extends beyond the streptomycetes (Le Roes-Hill et al., 2011). We hypothesize that the first and second most dominant taxa may be representative of copiotrophic and oligotrophic groups, respectively: their respective estimated copy numbers support this (4.07 for NewOTU5624, and 2.75 for NewOTU189491). This would also explain the net zero change in Actinomycetes by Q-PCR. The reduced amount of labile C in the warmed soils (Bradford et al., 2008) would drive opposing responses of two phylogenetically similar but functionally divergent groups to warming–decreased relative abundance of the potential copiotroph and increase of the potential oligotroph.

Observed changes in fungal biomass were observed by FAME analysis after 12 years of warming at the 20-year warmed site, which showed that warming caused decreased fungal abundance in both the mineral and organic soil horizons (Frey et al., 2008). Reduced fungal biomass has also been observed with warming in other sites (Waldrop et al., 2004; Rinnan et al., 2007; Frey et al., 2008) and though this current study focused mainly on bacteria, the importance of fungi in this systems is being separately studied. Fungi generally dominate primary decomposition in the organic horizon of temperate soils (Berg et al., 1998; Thevenot et al., 2010), and fungal laccase, phenol oxidase and peroxidase activities and genes encoding these enzymes have been found in greater abundance in upper layer, high organic-matter content soils relative to deeper, mineral soils (Luis et al., 2005; Sinsabaugh, 2010). The significant loss in organic horizon soil carbon with long-term warming is likely a combination of increased activity of primary decomposers (generally fungi) in the organic horizon, and increased demand (either through activity or abundance) of secondary decomposers (generally bacteria) in the mineral horizon. Like fungi, Actinomycetes are usually filamentous, and their dominance could suggest changing niches in soil and different contributions to soil C cycling due to long-term warming (Six et al., 2006; De Boer et al., 2008, 2005). Functional analyses of organisms from the organic and mineral horizons separately will suggest mechanisms for the observed substantially depleted soil organic layer mass with warming.

While a community shift was only observed after 20 years, functional changes and thermal acclimation were observed much earlier (Melillo et al., 2002; Bradford et al., 2008; Frey et al., 2013). Shorter term studies have also noticed no change in microbial

community by rRNA gene profiles with warming despite increases in respiration, such as temperate mountain forest soil warmed 4°C for 4 years (Kuffner et al., 2012) and mature spruce forest soils warmed 4°C during snow-free seasons for 4 years (Schindlbacher et al., 2011), which is consistent with our observations of the 5-year warmed SWaN plots. Genomic studies such as this permit us to determine whether a change in the community accompanies such changes in function, where no change in community would suggest a high degree of functional redundancy and a capacity for soil community resilience despite the warming treatment (Shade et al., 2012). For example, the KFFL field grassland soils are also dominated by Actinobacteria, Alphaproteobacteria, and Acidobacteria, as are the old-field soils studied by Castro and colleagues, and in both cases, warming alone had a smaller effect than when it was studied in conjunction with elevated $CO_2$ (Castro et al., 2010) or with drought (Sheik et al., 2011). This study is valuable in that it permits examination of the long-term effects of warming without confounding environmental and climate factors. The change in community structure that we observed after 20 years suggests that some other aspect of the soil niche space caused pressure on the community, resulting in the change (Schimel et al., 2007). While in some instances a strong positive correlation has been observed between taxonomic and functional richness of bacteria (Konstantinidis and Tiedje, 2007; Richter and Rosselló-Móra, 2009), we expect that expanded functional diversity that has been observed in these soils (Frey et al., 2013) more likely has its origins in changing active populations or genetic adaptation.

The long-term effects of warming, including altered community structure, decreased fungal biomass, increased evenness and decreased ribosomal copy number as an indicator of oligotrophy, all suggest positive warming-induced climate feedbacks. Indications of increased oligotrophy are perhaps the most alarming, because these may suggest that long-term warming is causing decreased physical protection of older or more recalcitrant soil C pools, assuming that physically protected C pools are more recalcitrant and that accessing these pools requires strategies (different enzymes, exploratory growth) consistent with oligotrophy (Schmidt et al., 2011; Bödeker et al., 2014). Testing hypotheses of mechanisms of how warming changes microbial communities, by distinguishing increased enzyme activity from altered enzyme production (Conant et al., 2011), as well as through decreased soil moisture (Toberman et al., 2008; Peñuelas et al., 2012), would benefit from accompanying direct observations on microbial activity, including comparative microbial physiology and genomics of isolated dominant strains. Genomic studies will remain valuable for understanding genomic context for changes in function, and should ultimately enable incorporation of microbial parameters into modeling efforts for prediction of microbial feedbacks to changing climate in a warmer world.

## CONCLUSIONS

Our data support the main hypothesis that long-term warming induces changes in microbial community composition, changes that are not seen in intermediate lengths of time. The strong community unevenness and similarity between dominant and whole community beta diversity suggests that a few keystone species may be responsible for a large proportion of the soil C cycling activity. The decreased abundance of dominant bacterial taxa as well as of total fungi (Frey et al., 2008) supports our second hypothesis that there is shifting niche space that may be evidence of changing C availability. The reduced ability of fungi and some Actinobacteria to survive may be due to dwindling resources, shifts in C quality, or a reduction in fine root biomass due to long-term warming, and may have created an opportunity for other oligotrophic bacteria (Butler et al., 2012; Koranda et al., 2013). Understanding the specific contributions of the dominant taxa to soil C cycling would be a powerful tool for modeling the relationship between microbial diversity and changes in climate.

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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.2015.00104/abstract

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# Microbial responses to changes in flow status in temporary headwater streams: a cross-system comparison

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Microbial communities are responsible for the bulk of biogeochemical processing in temporary headwater streams, yet there is still relatively little known about how community structure and function respond to periodic drying. Moreover, the ability to sample temporary habitats can be a logistical challenge due to the limited capability to measure and predict the timing, intensity and frequency of wet-dry events. Unsurprisingly, published datasets on microbial community structure and function are limited in scope and temporal resolution and vary widely in the molecular methods applied. We compared environmental and microbial community datasets for permanent and temporary tributaries of two different North American headwater stream systems: Speed River (Ontario, Canada) and Parkers Creek (Maryland, USA). We explored whether taxonomic diversity and community composition were altered as a result of flow permanence and compared community composition amongst streams using different 16S microbial community methods (i.e., T-RFLP and Illumina MiSeq). Contrary to our hypotheses, and irrespective of method, community composition did not respond strongly to drying. In both systems, community composition was related to site rather than drying condition. Additional network analysis on the Parkers Creek dataset indicated a shift in the central microbial relationships between temporary and permanent streams. In the permanent stream at Parkers Creek, associations of methanotrophic taxa were most dominant, whereas associations with taxa from the order Nitrospirales were more dominant in the temporary stream, particularly during dry conditions. We compared these results with existing published studies from around the world and found a wide range in community responses to drying. We conclude by proposing three hypotheses that may address contradictory results and, when tested across systems, may expand understanding of the responses of microbial communities in temporary streams to natural and human-induced fluctuations in flow-status and permanence.

**Keywords: bacterial diversity, microbial ecology, temporary streams, operational taxonomic unit (OTU)**

## Introduction

Temporary streams are fluvial systems that cease to flow over some amount of time or space (Acuña et al., 2014). In many environments, the greatest proportion of temporary streams are located in headwater systems (Dodds et al., 2004), and there is a growing appreciation that temporary headwater streams exert a strong influence on the structure and function of downstream waterbodies (Acuña et al., 2014). For example, temporary systems provide critical habitat, foster unique biota and transfer energy and nutrients (Stehr and Branson, 1938; Williams, 2005; Meyer et al., 2007). Much like other headwater systems, temporary headwater streams link terrestrial landscapes to river networks across space, but also represent a temporal ecotone due to the highly dynamic nature of environmental conditions in these systems (Steward et al., 2012).

Alternating wet-dry states in temporary streams are known to drive environmental gradients and community structure. Moreover, in the transitions between states (i.e., before a temporary stream becomes completely dry or as a stream rewets) environmental conditions are in constant transition. For example, as flow is reduced in a stream and water settles into isolated surface pools, surface water temperature increases, dissolved oxygen decreases, and increased evaporation rates concentrate solutes causing increased conductivity (Smith and Pearson, 1987). This leads to changes in sediments as well, such as decreases in dissolved oxygen and sharper redox gradients with depth and over time. Subsurface sediments (i.e., the hyporheic zone) can maintain elevated moisture content long after surface drying (Schwinning et al., 2011), potentially resulting in hot spots and hot moments of peak biogeochemical activity in wet compared to dry sediments (McClain et al., 2003). Residual moisture left in the hyporheic zone has been known to help sustain habitat refugia for macroinvertebrates taxa, which rely on moist conditions (Stubbington, 2012; Williams and Hynes, 1974).

The ecological impact of drought as a disturbance in temporary streams has been previously explored (Lake, 2003) as have the responses of fish (Labbe and Fausch, 2000; Dodds et al., 2004; Wigington et al., 2006; Colvin et al., 2009) and macroinvertebrate communities (Boulton and Lake, 1992; Stanley et al., 1994; Fritz and Dodds, 2004; Collins et al., 2007). The variable and often unpredictable hydrologic regime in temporary systems may be a driving force behind many ecosystem processes mediated solely by microbial communities. In such a transitional environment, microbes represent a continuum from truly terrestrial communities in soils to aquatically adapted taxa in streambed sediments. However, unlike other biotic components of temporary systems, generalizable relationships between microbial community structure and environmental gradients have not been firmly established. The lack of relationships are likely complicated because microbial community shifts have also been associated with other environmental factors such as organic matter quality (i.e., leaf litter composition; Artigas et al., 2011; Bruder et al., 2011), conductivity (Zeglin et al., 2011) and sediment composition in addition to the degree of desiccation (Marxsen et al., 2010). Thus, local environmental conditions can interact with temporary stream drying resulting in varying responses across ecosystems.

Shifts in stream conditions—such as a drought and rewetting—serve as filters on community structure. Microbial communities can exhibit resilient, resistant or functionally redundant responses (*sensu* Allison and Martiny, 2008) and thereby affect ecosystem processes. For microbes in temporary streams, resistance to and resilience from drought differ in that the dispersal mechanism is passive and facilitated by water flow. Although it has been well-established that the majority of microbial cells among streambed sediments are destroyed by drying events (Van Gestel et al., 1992), drying can take time and the effect of drying as a filter on microbial community structure is less clear. Long periods of desiccation may induce significant responses by microbial communities than brief events. For resistant communities, disturbance from drought causes little or no change to microbial community composition, whereas resilient communities are impacted by disturbance but are quickly restored after disturbance ends (i.e., surface water is restored). A rapid restoration of microbial processing after substantial portions of the community are lost during a drying event implies that resilience is an important trait in these highly dynamic temporary stream environments.

Equally, rewetting of a temporary stream environment can serve as an environmental filter on microbial community structure. Following rewetting of sediment and soils, microbial processing rates are higher than equivalent sediments not subjected to drying (Soulides and Allison, 1961; Van Gestel et al., 1992). This rapid processing may be driven by microbial communities accessing resources from cells that were destroyed during drying (Van Gestel et al., 1992). For heterotrophs, community composition can be driven by resources that become released or altered upon rewetting, such as the nature of organic matter released (Judd et al., 2006). Taxa that are adapted to rapidly access any available resources may be favored and may maintain a competitive advantage even after stream flow is fully restored.

Research to date characterizing microbial communities on either side of the wet-dry transition has yielded conflicting results. Some studies suggest that microbial community structure showed little difference before and after drying (Amalfitano et al., 2008; Zoppini et al., 2010). Other studies observed substantial depletion of microbial diversity (Timoner et al., 2014b) and substantially altered community composition (Rees et al., 2006; Timoner et al., 2014b) after drying. Similarly, several studies found microbial communities of temporary streams to be resilient, quickly regaining functional activity upon re-saturation of sediments (McIntyre et al., 2009; Timoner et al., 2014a,c). By contrast, other research observed depleted microbial activity for extended periods following flow restoration (Rees et al., 2006). These studies have been typically conducted in a single system or systems within the same region. Moreover, the existing studies suggest that flow cessation of a temporary stream does not necessarily result in a discrete state change to microbial communities but perhaps a more continuous shift in community structure. The lack of a definitive microbial response to wet-dry dynamics in temporary streams suggests that other

environmental factors play an important role in these systems, and a regional to global comparison of systems may be warranted.

In practice, the ability to rigorously test hypotheses in temporary streams is a logistical challenge (Lake, 2003). Research efforts are generally hampered by the ability to measure and predict the timing, intensity and frequency of wet-dry events. Unsurprisingly, published datasets are limited in scope and temporal resolution. Moreover human-induced impacts on temporary headwater streams are increasing; true temporary headwater channels are disappearing due to burial (Elmore and Kaushal, 2008) or conversion to perennial status due to urbanization (Roy et al., 2009). At the same time, permanent channels are increasingly becoming temporary and are subjected to more extreme flooding and drying events due to global climate change. This increased variability directly interrupts biotic linkages across the sediment-water interface (Lake et al., 2000). Comparing temporary systems in different regions, using datasets collected from similar environmental conditions, may help address some of these discrepancies and existing knowledge gaps.

We tested the relationships between microbial community structure and environmental conditions, particularly drying and wetting events, in two North American temporary headwater stream systems. Microbial communities were compared for Parkers Creek (Maryland, USA) and Speed River (Ontario, Canada), two different watersheds yet within the temperate zone of eastern North America. Data were collected during different years (Parkers Creek was sampled in 2012; Speed River was sampled in 2007 and 2008). For both systems, we compared sediment and water community composition in streambed sediments before and after seasonal drying events. We further compared stream sediment community composition to that of the stream water column and catchment soils, two sources of colonizing microbes in streams, in order to assess the relative importance of colonization vs. local environmental conditions in sediments.

We predicted that microbial taxonomic diversity in temporary streams would be limited to a subset of groups during periods of stream drying, presumably taxa that are more resistant to desiccation. Thus, we anticipated a substantial shift in community composition and a decrease in taxonomic richness in temporary stream sediments following drying. We also hypothesized that microbial community composition in stream sediments during stream flow would be more similar to water column communities than soil communities. During drying, we expected that the microbial community in stream sediments would change to more closely resemble that of soils. We anticipated that patterns of microbial response to drying would be comparable in both the Speed River and Parkers Creek systems regardless of differing ecosystems and molecular methods. The impacts of temporary stream drying were predicted to persist following rewetting of a stream, a process likely driven by dominance of resistant and resilient taxa that are adapted to dynamic environments.

To place our findings in a broader context, we reviewed published studies and synthesized evidence on the structural and functional response of microbes to drying but found little corroboration across studies due to differences in methodology and analytical resolution among datasets. Therefore, based on the results of this study and our review of the existing literature, three hypotheses are proposed that, when rigorously tested across systems, may strengthen tenuous knowledge of the linkages between environment, community structure, and ecosystem function in temporary headwater streams.

# Methods

## Study Sites

### Speed River Site (Ontario, Canada)

The Speed river watershed is a tributary of Lake Ontario. Samples were collected from one permanent (second order) and one temporary (zero order) stream monthly in 2007 and 2008. The stream sites are both tributaries of the Speed River (Permanent site: 43°43′N, 80°16′W; Temporary site: 43°42′N, 80°17′W; **Figure 1**, Supplemental Table 1). At the permanent site, we focused on a pool-riffle sequence in the stream, measuring approximately 10 m in length and 6 m in width. At the temporary site, we sampled a 15-m section immediately downstream from the springhead that served as the source of the tributary. The temporary stream site was no more than 2 m in width when flooded. Streambed sediments remained saturated throughout the period of study at both sites, however the streambed surface was dry from July to October 2007 and again in July and September 2008.

### Parkers Creek Site (Maryland, USA)

The Parkers Creek watershed is located in the Coastal Plain in Maryland, USA and drains directly into the Chesapeake Bay (**Figure 1**, Supplemental Table 1). Samples were collected from three headwater streams sites, two first order stream reaches

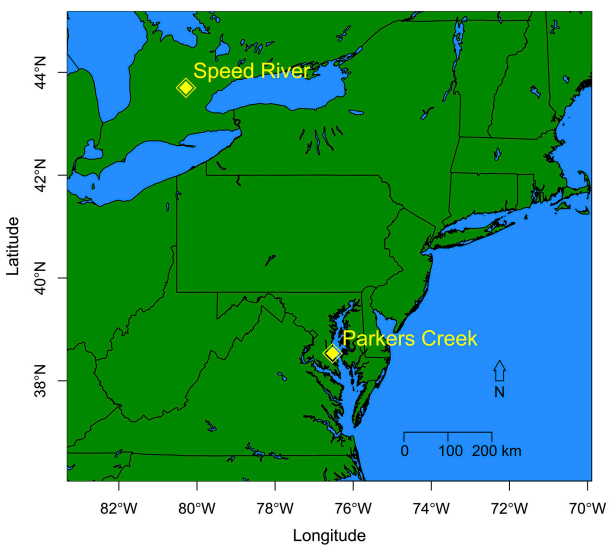


**FIGURE 1 | Location map of the two study systems: Speed River (Ontario, Canada) and Parkers Creek (Maryland, USA).** See Hosen et al. (2014) and Febria et al. (2010, 2012) for detailed maps of Parkers Creek and Speed River sites, respectively.

and one second order stream reach, and are described elsewhere (Hosen et al., 2014). One of the two first order sites was temporary (Site T1: 38°32′51″ N, 76°32′29″ W), the second was permanent (Site P1: 38°33′01″ N, 76°32′39″ W) as was the second order site (Site P2: 38°32′57″ N, 76°32′35″ W).

## Sample Collection

At the Speed sites, surface water fluctuated regularly at the temporary site but logging data were not collected due to limited logistical access. Sediment samples were collected monthly in 2007 and 2008 at the permanent site using a standard PVC sampling core (diameter 2.67 cm). Watershed soil samples were grab samples collected on a single occasion (October 2007) from forested and agricultural soils within 100 m of the stream sites (total $n = 53$). At the Parker's sites, both water column and sediment samples were obtained from all sites. Sites P1 and P2 flowed for the duration of the study while surface flow ceased and site T1 became dry from late July through early October 2012 (Supplemental Table 1).

### Water Samples

Stream water temperature, specific conductivity, dissolved oxygen, and pH were determined in the field using a Hydrolab all-in-one Quanta Probe (Hach Inc., Loveland, Colorado, USA) at the Speed site and a YSI Professional Plus multimeter (YSI Inc., Yellow Springs, Ohio, USA) at the Parkers creek site. Water samples from the Speed sites were collected in previously acid washed Nalgene HDPE bottles; water samples from the Parkers Creek sites for chemical analysis were collected in amber borosilicate bottles that had been acid washed and subsequently combusted at 450°C for 4 h and were sealed with Teflon-coated lids. All samples were placed on ice for transport to the laboratory. Samples for genetic analysis were subsequently stored at −80°C prior to further processing. Samples for chemical analysis were stored at 4°C until sample analysis. Dissolved organic carbon and total dissolved nitrogen were determined on stream water samples using a Shimadzu TOC-Vcph total organic carbon analyzer with attached TNM-1 total nitrogen analyzer (Shimadzu Corporation, Tokyo, Japan). The Parkers Creek samples were also analyzed for dissolved organic carbon quality using the fluorescence index (FI), which indicates whether DOM in a water sample is primarily allochthonous or autochthonous (McKnight et al., 2001). The fluorescence index was determined as the ratio of fluorescence emission intensities at 450 and 500 nm when a water sample was excited at 370 nm (McKnight et al., 2001) on a Horiba Scientific Fluoromax-4 (Horiba Limited, Kyoto, Japan). Physicochemical parameters measured at both sites are summarized in Supplemental Table 1.

### Microbial Community Composition (Speed Site)

Sediment samples were stored at −20°C until DNA analysis in the laboratory. DNA was extracted from approximately 1 g of sediment using PowerSoil DNA extraction kits (MoBio Laboratories, Carlsbad, California, USA). Bacterial communities were characterized using terminal-restriction fragment length polymorphism (T-RFLP) and resultant DNA fragments were digested using *MspI* and *HhaI* as described in Febria et al. (2012). Bacterial communities were identified by the different operational taxonomic units (OTUs) and their relative abundance within a given sample. In total, we included only sample dates for which physicochemistry and bacterial community data were available ($n = 53$).

### Microbial Community Composition (Parkers Site)

Water column samples were collected following Crump et al. (2003). Briefly, in the field, 300–600 mL of stream water were passed through a Millipore Sterivex-GP 0.22 μm filter. Residual water was expelled from the filter and approximately 2 mL of DNA extraction buffer were added after which both ports of the filter were sealed. Sediment samples were collected from streambeds to a depth of 3 cm using 2.67 cm diameter sterile plastic coring devices. Twenty cores were taken from random points along a 20 m reach at each site on each sampling date. All cores taken at a site were combined in a single sterile Nasco Whirlpak bag.

Water column microbial DNA was extracted from Sterivex-GP filters using phenol-chloroform based on established protocols (Crump et al., 2003). Filters were defrosted and 20 μL of 1% proteinase-K and 20 μL of 10% lysozyme. Samples were frozen at −80°C for 15 min and then thawed at 37°C for 5 min a total of three times. Samples were then incubated in a water bath for 37°C for 30 min. Fifty μL of 20% filter-sterilized SDS were added to each sample before a 2 h incubation in a 65°C water bath. Samples were washed twice with buffered phenol-chloroform-isoamyl alcohol and then precipitated at room temperature overnight by adding isopropyl alcohol at 60% of sample volume. Microbial sediment DNA was extracted using PowerSoil DNA Isolation Kits (Mo Bio Laboratories, Inc., Carlsbad, CA). To account for the high water content of stream sediment samples, 0.5 grams of sediment was used for each extraction. PCR amplicons were produced using standard methods for high-throughput sequencing (Caporaso et al., 2012). Amplification of 16S rDNA was conducted using forward primer 515f and barcoded reverse primer 806r obtained from the Earth Microbiome Project. For each sample 12 μL of UV-sterilized PCR-grade water, 10 μL 5-prime HotMasterMix, 1 μL 5 mM forward primer, 1 μL of 5 mM reverse primer, and 1 μL of template DNA were combined in a 96-well PCR plate. Conditions for PCR were as follows: Initial denaturation for 3 min at 94°C followed by 30 cycles first at 94°C for 0.75 min, 50°C for 1 min, and 72°C for 1.5 min. At the conclusion of PCR, temperature was held at 72°C for 10 min before temperature was reduced to 10°C. Amplicons were quantified with Pico-Green dsDNA quantification kit (Life Technologies; Carlsbad, CA), combined in equimolar quantities, and cleaned using an UltraClean PCR Clean-Up kit (MO BIO Laboratories, Inc; Carlsbad, CA). Illumina MiSeq 2 × 150 bp sequencing was conducted at Argonne National Laboratory (Lemont, IL).

## Data Analysis

### Both Sites

Beta diversity across space and time was analyzed using principal coordinate analysis (PCoA) of Bray-Curtis distances calculated between individual samples. Analysis of similarity

(ANOSIM) was conducted to compute similarity between groups. Replication was insufficient to apply statistical tests, but ANOSIM can still be applied to determine the similarity between groups with only one or two members (Cornils et al., 2005). For the Parkers Creek dataset, microbial species richness was estimated using CatchAll (Bunge et al., 2012) on rarified OTU tables. All analysis and plotting were conducted in R version 3.1.2. (R Core Team, 2014). PCoA and ANOSIM were conducted in the vegan package (Oksanen et al., 2013).

### Parkers Site and Network Analysis

Data were analyzed using the package Quantitative Insights into Microbial Ecology (QIIME). Paired end reads were matched using FLASh (Magoc and Salzberg, 2011). USEARCH 6.1 (Edgar, 2010) was used to identify OTUs at 97% similarity from the Silva 111 database (Quast et al., 2013; Yilmaz et al., 2014) and to identify chimeric sequences. Taxonomy was assigned using the RDP Classifier (Wang et al., 2007) at a threshold of 80%. Sequences were subsequently aligned using PyNAST (Caporaso et al., 2010). Sequences identified as belonging to chloroplasts, mitochondria, and the order Thermales were removed from the dataset as well as any OTU that was not identified taxonomically to at least the class level. Each sample was then rarified to 8960 sequences.

Co-occurrence network analysis of microbial OTU data was applied following existing methods (Barberán et al., 2011; Lupatini et al., 2014; Widder et al., 2014; Williams et al., 2014). To avoid spurious correlations and to aid in the interpretation of results, low abundance taxa that represented less than 0.1% of total sequences were filtered prior to analysis. Pairwise correlations were calculated for each pair of OTUs using Spearman's rank correlation. For a co-occurrence event to be included in the final network a threshold of $\rho > 0.75$ and $p < 0.05$ was adopted. To confirm that the network generated is not the product of random correlations, a comparison was made with randomly generated networks following (Lupatini et al., 2014). One thousand random networks with size (i.e., number of nodes and vertices) equal to the network generated from the microbial dataset were produced using the Erdös-Rényi model (Erdős and Rényi, 1960). Mean clustering coefficient, mean path length, and network modularity were calculated for each randomly generated network and were compared to the values generated from the experimentally derived network. The *p*-value of rejecting the null hypothesis that the experimental network was obtained at random was calculated as the proportion of values derived from the randomly generated models that were greater than the values obtained from the experiment for each of the three test statistics. For all three statistics the *p*-value was less than 0.001, indicating the experimental network was not obtained at random.

For each node network, centrality metrics including degree, closeness centrality, and betweenness centrality were calculated. Degree is defined as the number of vertices connected to a node. Betweenness centrality is defined as the number of geodesics that pass through a node when all possible geodesics are considered. Closeness centrality is calculated as the inverse of the average length of all the geodesics connecting one node to each other node in the network (Freeman, 1978). These metrics have the potential to identify keystone species within community networks (Williams et al., 2014). Microbial co-occurrence network analysis on simulated communities with known relationships indicate that both node degree and closeness centrality are positively linked to keystone taxa (Berry and Widder, 2014). Network analysis was conducted using R 3.1.2 with the *vegan* and *igraph* (Csardi and Nepusz, 2006) packages.

## Results

### Speed Site (Ontario, Canada)

Monthly samples over a two-year period confirmed differences in several environmental parameters between the permanent and temporary stream tributaries of the Speed River. Several parameters were found to be higher at the temporary than the permanent stream: conductivity (temporary stream = 542 μS/cm, permanent stream 431 μS/cm), total dissolved nitrogen concentrations (temporary stream = 4.63 mg $L^{-1}$, permanent stream = 1.86 mg $L^{-1}$), and dissolved organic carbon concentrations (temporary stream = 8.6 mg $L^{-1}$, permanent stream = 4.6 mg $L^{-1}$; Supplementary Figure 1).

In the Speed River system, temporary stream sediment microbial communities were highly similar despite seasonal and hydrologic variation over a 2-year period. The most noticeable difference was that despite the environmental changes, sediment microbial communities were most related to specific sites and sample types (i.e., permanent, temporary, or soil; **Figure 2**) and not necessarily season or hydrological status. PCoA revealed that sediment and soil microbial community composition were in large part distinct among sites. Further, wetting and drying did not appear to impact community composition of temporary stream sediments. Samples from both wet and dry sediments had similar PCoA scores.

We further examined the distribution of taxa shared across stream types and soils (**Figure 3**). The number of unique OTUs was roughly comparable in the permanent and temporary stream (134 and 133, respectively) and greater than in watershed soil (72 OTUs). Thirty-one OTUs were shared among the three habitat types with a large proportion of the total number of OTUs found in samples from at least two sites. The permanent stream communities shared a similar number of OTUs with both the temporary and soil habitats (47 and 48, respectively) while fewer OTUs were shared between the soil and temporary stream. Despite being adjacent to riparian areas including watershed soils, the temporary steam sediments had microbial communities that were more similar to the permanent downstream waterway than riparian soils.

### Parkers Site (Maryland, USA)

Stream water temperatures, DOC and TDN concentrations, and carbon quality (as measured by fluorescence index) were similar across all three sites. At temporary stream site T1, mean conductivity was 316 μS/cm, which was substantially higher than sites P1 (137 μS/cm) and P2 (139

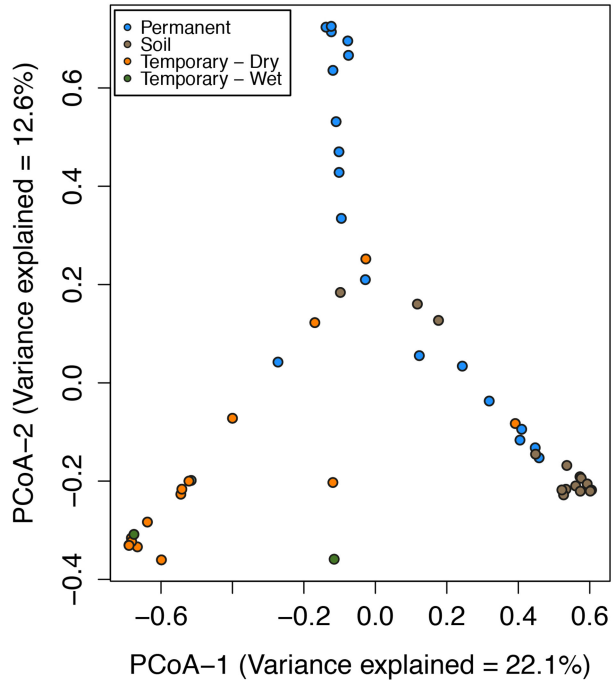


**FIGURE 2 | Ordination of the first two axes of the principal coordinate analysis (PCoA) of microbial community composition Bray-Curtis distances for the Speed River sites.** Indicated are communities from multiple dates at the permanent stream, temporary stream, and watershed soils. Points closer together in the plots indicate similar community composition than that those farther apart. Variance explained in PCoA Axis 1 = 22.1% and PCoA Axis 2 = 12.6%.

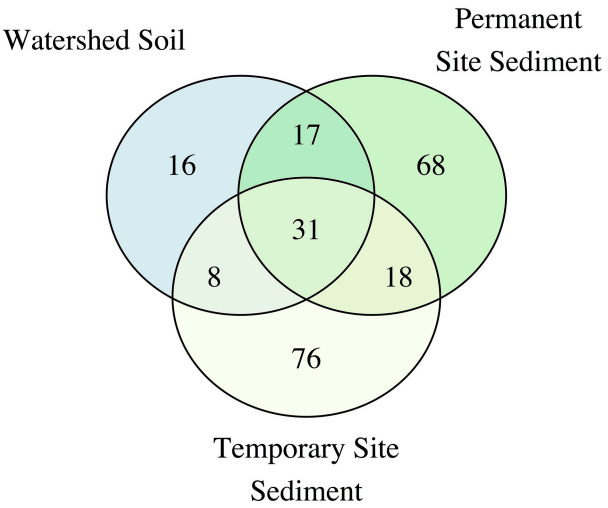


**FIGURE 3 | Venn diagram describing overlap in OTUs in the complete dataset.**

μS/cm). Dissolved oxygen levels were consistently lower at site T1 (mean: 6.0 mg/L) compared to sites P1 (8.4 mg/L) and P2 (7.5 mg/L).

As in the Speed River study, microbial community composition in temporary stream sediments at site T1 did not differ substantially between wet and dry conditions when comparing the percent of sequences belonging to the most common taxonomic classes (**Figure 4**). To provide a more detailed examination of microbial beta-diversity, PCoA was conducted on the 16S rDNA dataset using Bray-Curtis distances between OTUs for each sample collected and the first two principal coordinates were plotted (**Figure 5**). The first axis accounted for the 34.4% of variance and separated sediment samples on the left from water column samples on the right. Interestingly, site location appeared to be a stronger factor than stream drying; community composition at site T1 was more similar between the wet (Nov.) and dry (Aug.) seasons than between this site and other sites (**Figure 5**). This pattern was confirmed by comparing global and pairwise R statistics obtained by ANOSIM. The global R statistic, comparing all four combinations of temporary vs. permanent and sediment vs. water column, was 0.75. Lower R statistic values were identified for sediment vs. water column communities (0.67) and permanent vs. temporary (0.39) comparison.

As in the Speed River system, levels of taxa shared were similar among temporary and permanent sites in the Parkers Creek watershed. Sediment microbial community richness was lower at site T1 during drying conditions in August 2012 (4664; s.e.: 184) than in November 2012 (9523; s.e.: 1693), however this pattern was also true for site P1 which had an estimated 4791 (s.e.: 276) OTUs in August and 6679 (s.e.: 1472) in November. The number of OTUs exclusive to temporary stream site T1 was greater than either permanent stream site (**Figure 6**). The proportion of shared OTUs in sediments across sampling dates was similar in both first order streams; 30.7% of OTUs at site T1 and 34.8% of OTUs at site P1 were shared between August and November. The microbial community at site P2 appeared to be more stable; 56.7% of OTUs were found in both sediment samples taken at site P2.

## Network Analysis Identifies Distinct Assemblages in Permanent and Temporary Streams

Network analysis of microbial co-occurrence patterns incorporated a total of 167 nodes, each representing a distinct OTU, and 1085 edges connecting these nodes. The resulting network clusters OTUs into two groups (**Figure 7**). Each OTU was labeled according to the site type—permanent, temporary (flowing), and temporary (dry)—and assigned the maximum sequence abundance observed among samples (**Figure 7B**). The resultant clusters generated represented two distinct communities: one associated with permanent sites, and, a second associated with temporary sites.

The network statistics and taxonomic classification of the OTUs with greatest degree of centrality are identified in **Table 1**. High-centrality OTUs from the network cluster associated with permanent stream sites were almost entirely members of the Proteobacteria phylum with only one of the top ten OTUs from this group belonging to Bacteroidetes. Seven of the ten permanent stream OTUs were from the order Methylococcales, which is comprised of methantrophs (Bowman, 2005). Another

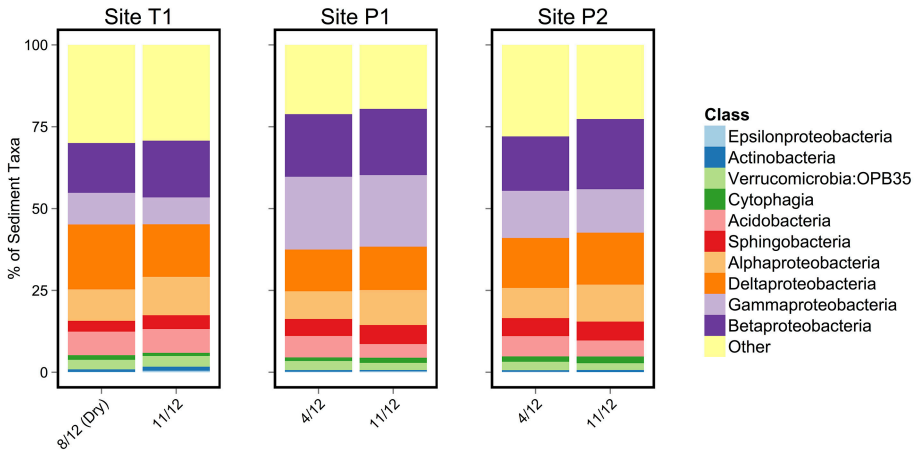


**FIGURE 4 | Class level microbial taxonomic composition of sediment samples.** The 10 most abundant classes are identified in the legend.

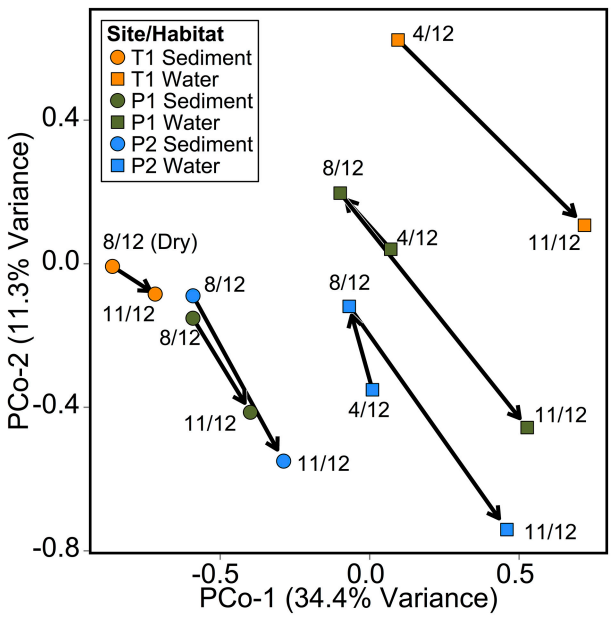


**FIGURE 5 | Principal coordinate analysis (PCoA) of microbial community composition Bray-Curtis distances between samples based on OTU tables.** Points individual microbial sediment or water column samples. Arrows indicate temporal relationship between samples (April 2012, August 2012, and November 2012).

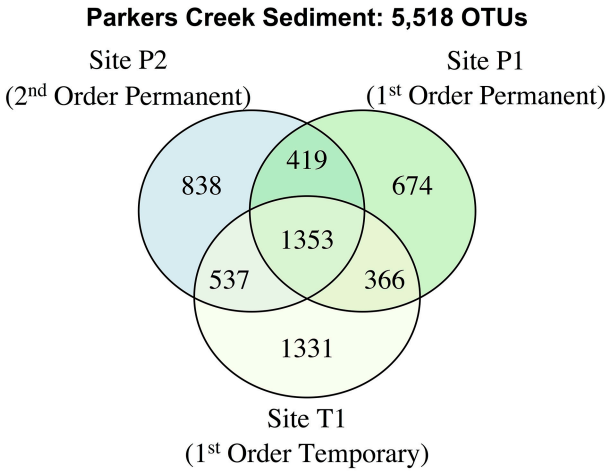


**FIGURE 6 | A venn diagram of sediment OTUs collected from sites T1, P1, and P2 on August and November 2012.**

permanent stream OTU that had high network centrality was from the order Methylophilales, a group of methylotrophic bacteria (Qiu et al., 2009). The OTU with the highest closeness centrality was from the family Nitrosomonadaceae; a family that exclusively includes ammonia oxidizing bacteria (Arp et al., 2007).

In contrast to the network cluster associated with permanent stream sites, the temporary stream cluster included a much lower proportion of Proteobacteria OTUs. Six out of the 10 most central OTUs were from the Phylum Nitrospirae, which is dominated by nitrite-oxidizing bacteria often found in wastewater treatment systems (Daims et al., 2001). A heatmap of the OTUs presented in **Table 2** (**Figure 8**) shows that following re-wetting at site T1 (11/2012), OTUs associated with the dry temporary stream decreased in sequence abundance. Despite this shift there were no concomitant increases in sequence abundances of OTUs associated with permanent stream samples.

## Discussion

### Weak Relationship between Community Composition and Drying

The Speed River and Parkers Creek have different climates and exist in landscapes with distinct geologies, yet microbial communities responded similarly to temporary stream drying in both systems. Contrary to our hypothesis, results suggest that drying events did not lead to large changes in microbial communities in streambed sediments. Instead, for both systems,

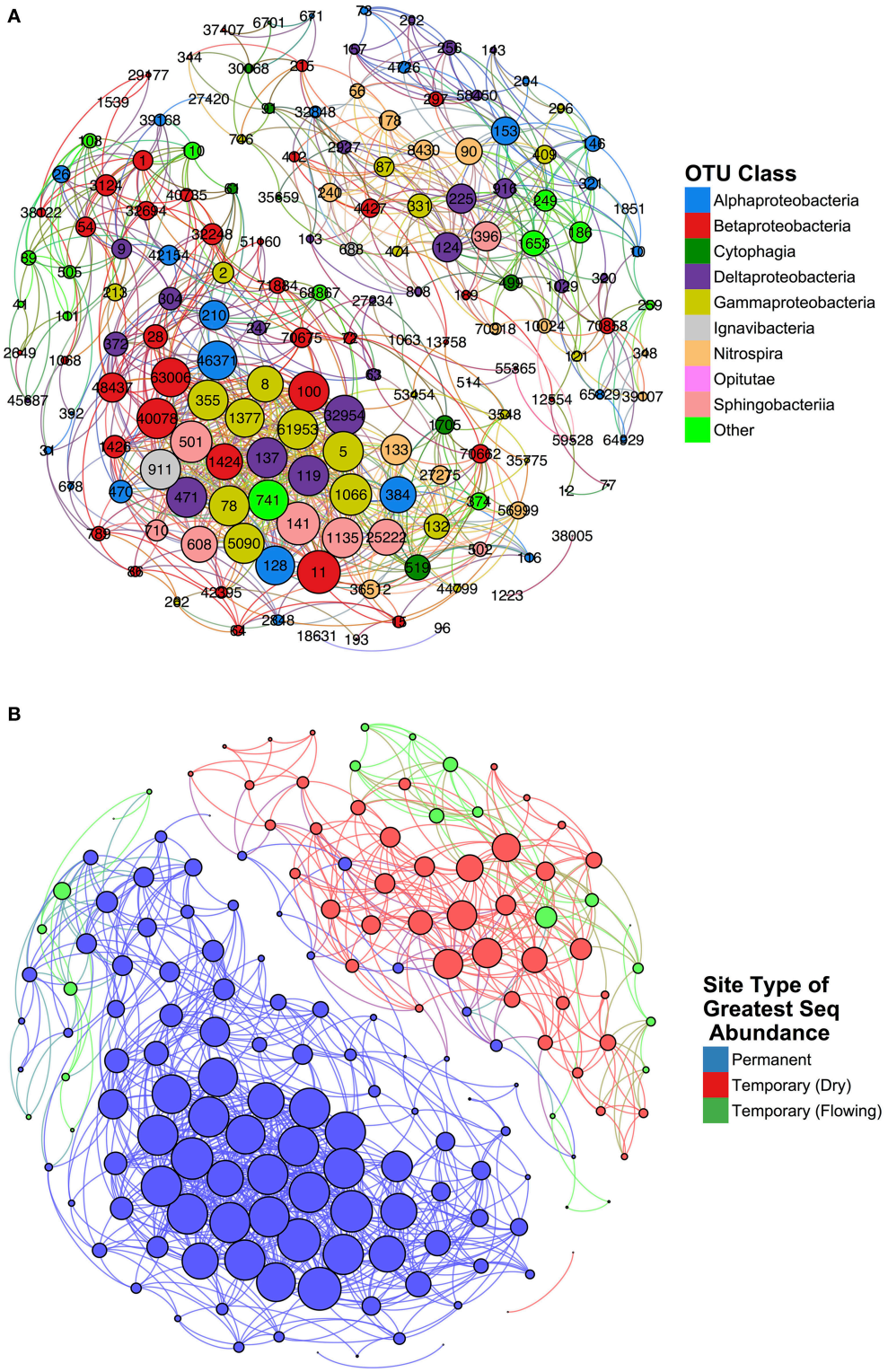


**FIGURE 7 | Results of network analysis conducted on temporary and permanent stream samples.** Nodes represent individual OTUs and edges represent significant spearman correlations ($\rho > 0.75$ and $p < 0.05$). Node size is determined by weight of that node (i.e., the number of edges connected). Nodes are color-coded according to **(A)** class and **(B)** the site type for which that OTU is most abundant.

**TABLE 1 | Parkers Creek PCoA loadings of principal coordinate axis 1 and principal coordinate axis 2 for the taxa with greatest loadings from PCoA displayed in Figure 4.**

| Taxonomy | PCo-1 | PCo-2 | Description of Taxa |
|---|---|---|---|
| Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Comamonadaceae | 2.2383 | −1.4288 | Family *Comamonadaceae* have been identified as primary denitrifiers in activated sludge (Khan et al., 2002) and associated with younger soils following deglaciation (Nemergut et al., 2007). Multiple taxa in this family have been identified as motile rods (Willems et al., 1991; Khan et al., 2002; Spring et al., 2005). Also includes iron reducing bacteria (Ramana and Sasikala, 2009). |
| Bacteria;Proteobacteria; Betaproteobacteria; Nitrosomonadales; Gallionellaceae | 1.1895 | 0.7054 | Iron-oxidizing bacteria commonly found in streams (Hedrich et al., 2011; Reis et al., 2014). |
| Bacteria; Proteobacteria; Betaproteobacteria; Burkholderiales; Burkholderiaceae | 0.8038 | −0.6179 | Found with increasing depth in soils (Sait et al., 2002). Includes pathogenic taxa (Ulrich et al., 2006). |
| Bacteria; Actinobacteria; Actinobacteria; Frankiales; Sporichthyaceae | 0.7306 | −0.4518 | Slow growing taxa associated with compost (Normand, 2006). Grows on a medium containing soil humic acid as the sole source of carbon (Suzuki et al., 1999). |
| Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales; Sinobacteraceae | −0.7111 | −0.0170 | Includes non-motile gram-negative taxa obtained from polluted soils (Zhou et al., 2008). |
| Bacteria; Proteobacteria; Epsilonproteobacteria; Campylobacterales; Helicobacteraceae | 0.4602 | 0.5021 | Includes potential anaerobic, nitrate-reducing taxa found in the Baltic Sea (Labrenz et al., 2007) and wetlands in rural Spain (Ansola et al., 2014). |
| Bacteria; Proteobacteria; Gammaproteobacteria; Methylococcales; CABC2E06 | 0.6389 | 0.1747 | Includes type 1 methanotroph taxa that have been found in association with iron-oxidizing bacterial communities in riparian wetlands (Wang et al., 2012) and anoxic reservoir water (Quaiser et al., 2014). |

microbial community structure was strongly tied to a location with changes across time apparently related more to seasonal changes than flow status. Thus, the hypothesis that temporary stream sediment communities would resemble soil communities during drying was also not supported as temporary stream communities did not change substantially during drying (**Figure 2**). In contrast to sediment communities, Febria et al. (2012) found that surface and pore water communities varied significantly within days to weeks of a re-wetting event, whereas sediment microbial communities remained more stable over the same time period and across seasons. Instead community composition was more related to individual sites and site type (i.e., soils, permanent stream sediments, temporary stream sediments).

The weak relationship between community composition and drying was demonstrated in both systems by comparing communities associated with different flow regimes or sample types (e.g., sediments from permanent vs. temporary streams, water vs. sediments). We reported results for comparisons between the four categories (temporary sediment, temporary water, permanent sediment, and permanent water) and confirmed that habitat (sediment and water) or stream type alone (i.e., temporary and permanent) was less compelling. Despite differences between temporary and permanent stream microbial communities at the Parkers site, samples from permanent sites P1 and P2 each shared as many OTUs with temporary site T1 as with each other.

Again at the Parkers site, differences across season were related to seasonal impacts exerted on all samples, rather than a specific effect from stream drying (**Figure 4**). For example, observed community shifts from August to November was driven by an increase in sequences from the families Comamonadaceae, Burkholderiaceae, Sporichthyaceae, and to a lesser extent Gallionellaceae, and uncultured clone CABC2E06 from the order Methylococcales. There was also a decrease in the number of sequences from the family Sinobacteraceae. These taxonomic shifts from August to November across the study sites suggest establishment of community assemblages associated with iron oxidizing bacteria. Members of the family Gallionellaceae, iron-oxidizers found in stream environments (Hedrich et al., 2011; Reis et al., 2014), increased from summer to fall. The same was true of the family Comamonadaceae, which includes iron-reducing (Ramana and Sasikala, 2009) and denitrifying (Khan et al., 2002) taxa. Another family that increased in sequence abundance from August to November was Helicobacteraceae, which includes potential anaerobic nitrate-reducing taxa that have been found in high abundance in wetland sediments in northern Spain (Labrenz et al., 2007; Ansola et al., 2014). The sequence abundance of Methylococcales clone CABC2E06, a methanotroph that has previously been associated with iron-oxidizing communities also increased from August to November (Wang et al., 2012; Quaiser et al., 2014). Similar assemblages of bacteria have been found in in association with iron-oxidizing bacterial communities in both riparian zones (Wang et al., 2012) and anoxic reservoir water (Quaiser et al., 2014). While more data are needed, this suggests that anoxic conditions near the sediment water interface impact microbial communities in both permanent and temporary headwater streams.

**TABLE 2 | The network OTUs identified with greatest centrality as defined by closeness, centrality, and node degree.**

| OTU ID | Closeness Centrality | Betweenness Centrality | Degree | Site type of greatest OTU sequence abundance | Taxonomy |
|---|---|---|---|---|---|
| OTU_48437 | 0.12 | 937 | 22 | Perennial | Proteobacteria\|Betaproteobacteria\|Burkholderiales\|Comamonadaceae |
| OTU_00078 | 0.127 | 67.4 | 30 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Crenotrichaceae\|Crenothrix |
| OTU_00100 | 0.132 | 811.8 | 30 | Perennial | Proteobacteria\|Betaproteobacteria\|Nitrosomonadales\|Nitrosomonadaceae\|Unclassified |
| OTU_53454 | 0.129 | 2385.5 | 5 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Crenotrichaceae\|Crenothrix |
| OTU_01066 | 0.132 | 820 | 31 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Methylococcaceae\|Methylosoma |
| OTU_61953 | 0.132 | 811.8 | 30 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Crenotrichaceae\|Crenothrix |
| OTU_00141 | 0.127 | 106.9 | 32 | Perennial | Bacteroidetes\|Sphingobacteriia\|Sphingobacteriales\|Chitinophagaceae\|Terrimonas |
| OTU_35775 | 0.129 | 1029.8 | 4 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Crenotrichaceae\|Crenothrix |
| OTU_00011 | 0.127 | 221.2 | 32 | Perennial | Proteobacteria\|Betaproteobacteria\|Methylophilales\|Methylophilaceae |
| OTU_00005 | 0.128 | 175.5 | 30 | Perennial | Proteobacteria\|Gammaproteobacteria\|Methylococcales\|Crenotrichaceae\|Crenothrix |
| OTU_12554 | 0.103 | 320 | 4 | Temporary (flowing) | Proteobacteria\|Betaproteobacteria\|Rhodocyclales\|Rhodocyclaceae |
| OTU_00297 | 0.11 | 131.4 | 11 | Temporary (flowing) | Proteobacteria\|Betaproteobacteria\|Nitrosomonadales\|Gallionellaceae |
| OTU_00249 | 0.108 | 281 | 16 | Temporary (flowing) | Spirochaetes\|Spirochaetes\|Spirochaetales\|Spirochaetaceae\|Spirochaeta |
| OTU_01068 | 0.104 | 34.1 | 6 | Temporary (flowing) | Proteobacteria\|Betaproteobacteria\|Burkholderiales\|Comamonadaceae |
| OTU_00010 | 0.105 | 237.2 | 8 | Temporary (flowing) | Proteobacteria\|Alphaproteobacteria\|Rhizobiales\|Bradyrhizobiaceae |
| OTU_00153 | 0.113 | 581.3 | 21 | Temporary (dry) | Proteobacteria\|Alphaproteobacteria\|Rhizobiales\|Xanthobacteraceae\|Unclassified |
| OTU_08430 | 0.12 | 494.2 | 15 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|4-29\|Unclassified |
| OTU_00396 | 0.117 | 450.7 | 22 | Temporary (dry) | Bacteroidetes\|Sphingobacteriia\|Sphingobacteriales\|Saprospiraceae\|Unclassified |
| OTU_00090 | 0.113 | 256.6 | 20 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|Nitrospiraceae\|Unclassified |
| OTU_00178 | 0.12 | 494.2 | 15 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|Nitrospiraceae\|Unclassified |
| OTU_10024 | 0.111 | 469.7 | 11 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|4-29\|Unclassified |
| OTU_00124 | 0.117 | 450.7 | 22 | Temporary (dry) | Proteobacteria\|Deltaproteobacteria\|Syntrophobacterales\|Syntrophaceae\|Syntrophus |
| OTU_00056 | 0.118 | 822.4 | 11 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|4-29\|Unclassified |
| OTU_00240 | 0.119 | 296.6 | 14 | Temporary (dry) | Nitrospirae\|Nitrospira\|Nitrospirales\|Nitrospiraceae\|Nitrospira |
| OTU_00087 | 0.12 | 494.2 | 15 | Temporary (dry) | Proteobacteria\|Gammaproteobacteria\|Xanthomonadales\|Sinobacteraceae\|Unclassified |

*The site type at which the sequences abundance of a given OTU is greatest is also identified.*

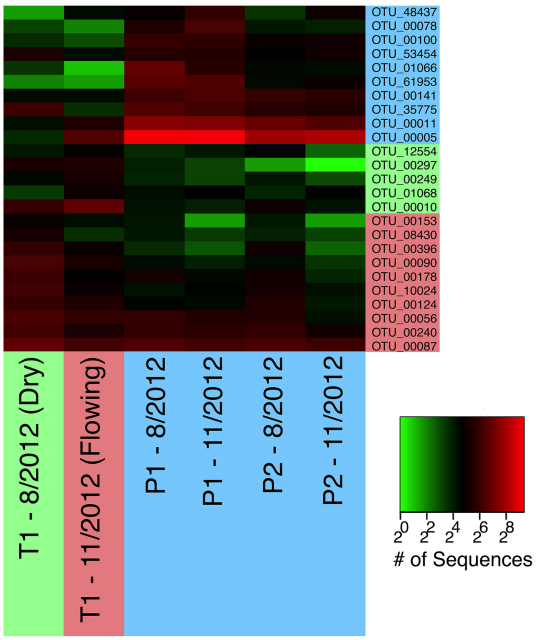


**FIGURE 8 | A heatmap of OTUs presented in Table 2.** OTUs are color-coded as in **Figure 7B** to indicate whether they are most common in permanent (blue), wet temporary (green), or dry temporary (red) samples. Samples are also color coded using the same scheme.

Muted impacts of streambed drying on microbial community structure highlight an important question for future research: What factors control microbial community structure in these streams? In both systems, water column and sediment communities were distinct from sediment microbial communities, suggesting that dispersal via the water column did not have a strong impact on streambed sediment microbial dynamics. While stream drying did not have a strong impact on microbial community composition, environmental gradients associated with drying may play a role. Temporary streams in both the Speed and Parkers Creek systems demonstrated higher stream water conductivity and lower stream water dissolved oxygen compared to permanent reaches. These differences are seen typically in association with drying as evaporation increases solute concentrations and low flows decrease oxygen mixing (Boulton and Lake, 1990; Boulton, 2003). Both conductivity and dissolved oxygen levels have been related to stream microbial community structure in studies of other ecosystems (Lawrence et al., 2004; Zeglin et al., 2011). This suggests that drying may indirectly impact microbial community structure via changes to environmental conditions.

Determining whether functional impact is tied to environmental conditions or the microbial community composition of a temporary headwater stream is an important avenue of future research and beyond the scope of this study and many studies to date. Biogeochemical function may be related more to which fraction of the microbial community is most active, rather than which fraction is the most numerically abundant (Fulthorpe et al., 2008; Shi et al., 2012; Manis et al., 2014). Such a pattern has been observed in studies attempting to link denitrification rates to the abundance of denitrifying taxa. Studies have failed to find a relationship between microbial structure and denitrification rates (Iribar et al., 2008; Song et al., 2012), including one conducted in an ephemeral stream system (Manis et al., 2014). Comparisons between microbial community composition and functional measurements using RNA expression are needed to determine what proportion of temporary stream communities are active and to uncover the sources of functional diversity in temporary streams.

## Central Microbial Associations Differ between Temporary and Permanent Streams

While the overall microbial community structure in Parkers Creek temporary stream sediments was largely similar to the communities in permanent stream sediments, network analysis revealed that different taxonomic associations were dominant in the two types of samples. In permanent stream samples, OTUs from the order Methylococcales and a single OTU from the family Methylophilales were highly central to the microbial network. Taxa from the order Methylophilales do not oxidize methane, but experimental results have indicated that taxa from this group utilizes byproducts of methane oxidation from methanotrophic taxa like Methylococcales (Qiu et al., 2009; Beck et al., 2013; Liu et al., 2014). Similar evidence of cooperative metabolism between the Methylococcaceae and Methylophilaceae families has also been identified (Beck et al., 2013; Liu et al., 2014). Thus, it appears that the network cluster identified in this study represented a community of methanotrophs and other microbes adapted to oxidize methane and associated compounds produced in saturated, anoxic sediments. By contrast, the dominant microbial associations in temporary stream sediments are among OTUs from the order Nitrospirales, indicating that nitrite-oxidizing bacteria play an important role in temporary stream sediments.

Multivariate analysis using Bray-Curtis distances and PCoA demonstrated that bacterial community composition responded weakly or not at all to temporary stream drying. Network analysis presents a useful complement to this approach, revealing that different microbial associations were favored in sediments of temporary vs. permanent streams. Although analysis of overall community composition showed little difference between temporary and permanent stream sediments, network analysis indicated that different assemblages were dominant in the respective stream types and are indicative of functional differences between temporary and permanent stream sediments. Whether this change is representative of functional shifts must be explored with more direct analysis. Network analysis also revealed the presence of microbial associations between taxa that have previously been identified experimentally. This result highlights the utility of this approach as a screening technique to identify previously unrecognized microbial associations.

## Published Studies of Temporary Stream Microbial Communities Yield Conflicting Results

The results reported here indicated that temporary stream drying and rewetting is not strongly related to shifts in sediment

microbial communities of Speed and Parkers sites. To place these findings in context, we compared the results presented here with the limited but growing dataset from temporary headwater streams across the globe (**Table 3**). This review found that temporary stream studies, including those reported here, report a range of responses to changes in temporary stream flow status.

In a study of two European temporary streams, researchers found that the microbial community structure in sediments was resistant to desiccation and rapidly regained function following re-wetting, whereas sediment communities in a stream another system did not (Marxsen et al., 2010). The authors of that study hypothesized that microbial communities in the first stream were protected by higher sediment moisture content (Marxsen et al., 2010). Sediment composition, size distribution, and organic matter content are just a few of the factors that impact sediment drying rate (Gupta and Larson, 1979). Thus, temporary stream pore water, which has been identified as harboring substantial microbial diversity (Febria et al., 2012), may be an important refuge during brief periods of drying. Given that drying periods are predicted to lengthen with climate change (Brooks, 2009; Palmer et al., 2009), we anticipate that the moisture retention capacity of temporary streambed sediments will determine how individual temporary streams respond to climate change. Antecedent conditions may also explain seemingly contradictory results. Fierer et al. (2003) showed that microbes from soils that had been previously exposed to alternating wet/dry conditions were less impacted by experimental drying and re-wetting.

In the studies reviewed, the methods employed to measure microbial community structure or function varied; community composition was largely measured using techniques based on the amplification of the 16S rDNA gene including clone library analysis (Zeglin et al., 2011), degenerating gradient gel electrophoresis (DGGE; Frossard et al., 2013), terminal restriction fragment length polymorphism (T-RFLP; Fierer et al., 2003; Rees et al., 2006; Febria et al., 2012; Manis et al., 2014; this study, Speed River), automated ribosomal intergenic spacer analysis (ARISA; Amalfitano et al., 2008; Frossard et al., 2013), phospholipid-derived fatty acid (PLFA) analysis (McIntyre et al., 2009), fluorescence in-situ hybridization (FISH; Amalfitano et al., 2008; Zoppini et al., 2010), pyrosequencing (Timoner et al., 2014b), and Illumina MiSeq (this study, Parkers Creek). A wide variety of PCR primers were used, the only primer pair that was repeated across studies was 27F/1492R (Rees et al., 2006; Febria et al., 2012, this study, Speed River; **Table 3**), which limited our ability for direct comparison across systems. While inconsistent methods may be one factor contributing to conflicting results, it is important to note that the results obtained from these two systems were largely similar despite different community analysis techniques. Thus, at least some of the disparate results found across studies is likely due to true differences in environment.

## Conclusions and Future Research

In this study, community composition was weakly linked to flow status, with variability in community structure in temporary streams related to other factors. This adds some support to the idea that changes in the function of temporary stream microbes over time is a factor more of changing environmental conditions than shifting microbial community composition. By contrast, network analysis did show that the dominant microbial interactions shifted with stream wetting and drying. This suggests that a subset of the overall microbial community is more responsive to stream flow status than the overall microbial population. Future research should be conducted to determine the functional impacts of these changing associations. Our review of data from the Speed and Parkers systems and others from around the globe suggests that research on sediment microbial communities in temporary headwater streams is a rich but not yet unified pursuit. Identification of key controls on microbial community structure in temporary headwater streams hinders efforts to develop predictive models that elucidate links between microbial structure and function to ecosystem-scale processes and the impacts of human actions on these processes.

Thus, future research should directly address these knowledge gaps by identifying the factors leading to the inconsistent findings highlighted here. We identified the following hypotheses that may explain our results, and when tested across other systems, may fill in critical knowledge gaps and address broader questions about controls on microbial community structure and function in temporary headwater streams and related water management needs:

(1) Degree of sediment water retention, not flow status, determines whether a temporary microbial community is resistant to drying. Our analysis suggests that flow status itself had marginal impact on community structure in some temporary headwater streams but substantial impact in other systems. Rather, the degree of sediment drying, which is controlled by a number of factors including sediment composition, may be a more important factor. Prior exposure to highly variable conditions may also play a role. Experimental studies show that soil microbial communities previously exposed to drying change less in response to experimental drying (Fierer et al., 2003). Studies that directly examine these factors are needed to address this issue.

(2) Contradictory results can be resolved by standardized field and molecular methods. The collection of physicochemical data including the timing and frequency of wet-dry events is especially challenging. Our limited ability to generalize findings across studies are due to the intermittent nature of surface flow in these headwater systems and the resultant lack of temporal and spatial resolution in the available datasets. For example, in both the Speed and Parkers systems, *in situ* data collection was either logistically infeasible (due to their remote location or unpredictable surface flow conditions), or instrumentation were either damaged or stolen during critical periods. Moreover, the tools with which to characterize microbial communities vary widely, making cross-site comparisons difficult. New technology and lowering costs promise to make high-throughput sequencing a standard practice and allow for more comparable datasets.

(3) Functional rates in temporary streams are more related to environmental conditions than to community composition.

**TABLE 3 | A summary of microbial community compositional and functional studies on temporary streams and general relationships established.**

| Citation | Study system | Microbial community metric(s) | Microbial functional metric(s) | Primers or probes used | Summary of results |
|---|---|---|---|---|---|
| Amalfitano et al., 2008 | Mulargia River (Sardinia, Italy), River Krathis (Peloponnesus, Greece), River Pariela (Portugal), River Tagliamento (Italy). | Bacterial abundance and biomass: DAPI; Community composition: Fluorescence in-situ hybridization (FISH), Automated ribosomal intergenic spacer analysis (ARISA). | Bacterial production ([3-H]leucine incorporation). | Probes: ARCH915, EUB338, EUB338-II, ALF1b, BET42a, GASM42a, PLA46a, CF319a, HGC69a, LGC354abc. | Following experimental desiccation, bacterial carbon production and biomass decreased strongly. Limited change in community structure with increase in Alphaproteobacteria and Betaproteobacteria with drying. |
| Frossard et al., 2012 | Terrestrial soils, ephemeral and permanent stream channel sites in the Chicken Creek watershed (Germany). | Community composition: Denaturing gradient gel electrophoresis (DGGE). | Extracellular enzyme activity: Phosphatase, $\beta$-glucosidase, $\beta$-xylosidase, cellobiohydrolase, chitinase, leucine-aminopeptidase, aspartate-aminopeptidase, glutamate-aminopeptidase, phenol oxidase, phenol perioxidase. | Primers: Eub338f/Eub518r | Bacterial community structure did not show differences between permanent and ephemeral stream sediments. Enzyme activity was seasonally variable but was not related to microbial community composition. |
| Febria et al., 2012 | In-stream colonization corers at 1 permanent and 1 temporary headwater stream (Ontario, Canada). | Community composition: 16S T-RFLP. | | Primers: 27F/1492R. | Strong temporal differences in hyporheic porewater community structure both before and after a drying event |
| Fierer et al., 2003 | Soils from Sedgwick Ranch Natural Reserve (Santa Ynez, CA, USA). | Community composition: 16S terminal restriction fragment length polymorphism (T-RFLP). | | Primers: 8 F hex/1389R. | During experimental drying/wetting cycles community composition was varied by environment. Soils without less history of moisture stress, but not in soils with a history of moisture stress. |
| Frossard et al., 2013 | Succession of microbial community in flumes filled with dry stream bed sediments from the Chicken Creek watershed (Germany). | Community composition: ARISA | Extracellular enzyme activity: Phosphatase, $\beta$-glucosidase, $\beta$-xylosidase, cellobiohydrolase, chitinase, leucine-aminopeptidase, aspartate-aminopeptidase, glutamate-aminopeptidase, phenol oxidase, phenol perioxidase. | Primers: 1406F-FAM/23Sr. | Strong temporal differences in community structure during succession experiments. Enzyme activity changes were linked to shifts in microbial community structure. |
| Manis et al., 2014 | Survey of known temporary streams in agricultural landscapes (USA). | Community composition: 16S and nosZ T-RFLP and 16S and nosZ quantitative polymerase chain reaction (qPCR). Community abundance: DAPI fluorescence microscopy. | Dentrification enzyme assays. | 16S T-RFLP Primers: Eub338F-0-III, Eub338F-I-II/1392R. 16S qPCR Primers: Eub339, Eub339 II/ Eub518. nosZ T-RFLP Primers: nosZ-F-1181/nosZR. nosZ qPCR: nosZ1F/nosZ2R. | Greater denitrification rates were observed in ephemeral vs. perennial channels, but potential denitrification was not correlated to denitrifier abundance. |
| McIntyre et al., 2009 | Barnett Creek (Pilbara region, Western Australia). Ephemeral stream located in lowland floodplain. | Microbial biomass: phospholipid fatty acid (PLFA) analysis. | Carbon mineralization assay; carbon dioxide flux assay; carbon and nitrogen stable isotopes. | | Landscape position (e.g., riparian soils, floodplain soils, and channel sediments) was less important to microbial activity than soil saturation once water content was greater than 40%. Mineralization of carbon and nitrogen occurs more slowly following complete saturation of sediments compared to brief events that rapidly stimulate microbial activity. |

*(Continued)*

**TABLE 3 | Continued**

| Citation | Study system | Microbial community metric(s) | Microbial functional metric(s) | Primers or probes used | Summary of results |
|---|---|---|---|---|---|
| Rees et al., 2006 | Semi-permanent stream near Binalong, New South Wales, Australia | Community composition:16S T-RFLP. | | Primers: 27F/1492R. | Community composition varied by hydrological condition and within riffles. Communities were changed after drying and did not recover to pre-drought conditions one month after flow was restored. |
| Timoner et al., 2014a | Dam Creek (South-East Queensland, Australia). First order intermittent headwater stream. | Bacterial abundance: fluorescence microscopy. | Microbial carbon degradation: BiologEcoPlates. | | Before re-wetting biofilms differed based on time since drying. Rewetting rapidly increased biofilm functional diversity and functional patterns became more similar across sites. Low counts of bacteria were found in both wet and dry isolated pools in an intermittent channel. |
| Timoner et al., 2014b | Fuirosos temporary stream (Spain). | Community composition: 16S pyrosequencing. | | Primers: 28F/519R. | Differences between biofilm, shallow streambed hyporheic bacterial communities related to flow, drying stress/desiccation and sediment type. |
| Timoner et al., 2014c | Fuirosos (Iberian Peninsula, Spain). Third order temporary stream. | Community structure and abundance: Chlorophyll-a concentration, pigment composition. | | | Chlorophyll-a concentrations went down in response to drying but quickly returned following re-wetting. Tendency toward production of protective carotenoids and desiccation resistance structures (e.g., increased membrane thickness and spore production) during drying. |
| Zeglin et al., 2011 | Onyx River (McMurdo Dry Valleys, Antarctica); Rio Salado (New Mexico, USA). Both ephemeral desert streams. | Community composition: DGGE, Clone library analysis. | | Primers: 8F/1391R or 1492R. 519R, 515F, 1100R, and 1492R. | Bacterial diversity at both sites was not correlated with sediment water content but was instead most strongly related to conductivity. Community composition was strongly related to water content. |
| Zoppini et al., 2010 | Mulargia River (Sardinia, Italy). Second-order temporary river. | Bacterial abundance/community composition: FISH, DAPI fluorescence microscopy. | Bacterial production ([3-H]leucine incorporation); extracellular enzyme activity. | Probes: EUB338, EUB338-II, EUB338-III, ALF968, BET42a, GAM42a, CF319a, PLA46a, and LGC354abc. | Metrics including bacterial cell counts, bacterial productivity, and enzyme activity were largely comparable during wet and dry conditions. Community composition was not substantially different between wet and dry conditions. Enzyme activities increased during flooding event. |
| Febria et al. (This study) | Speed River system, Ontario, Canada. | Community composition: 16S T-RFLP. | | Primers: 27F/1492R. | Similar community composition in sediment between sites, highly varied surface water communities. |
| Febria et al. (This study) | Parkers Creek system, Maryland, USA. | Community composition: 16S Illumina MiSeq. | | Primers: 515F/806R. | Community composition similar by site. Seasonal changes in microbial community composition were not linked to flow status. |

Evidence reviewed here suggests that microbial community structure is often similar between wet and dry conditions even as the processing rates of some functions, such as denitrification, change between wet and dry conditions. This suggests that environmental changes may alter functional processing rates of stream microbial communities, a pattern that must be tested for other microbial functions.

Despite increasing human-induced impacts on headwater streams, appreciation for temporary streams and the contributions of these systems to ecosystem processes are building. Understanding the critical drivers of microbial community diversity and function in these systems will inform restoration efforts focused on enhancing or supporting nutrient cycling and food web interactions across space and time.

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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.2015.00522/abstract

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# Stoichiometric flexibility in diverse aquatic heterotrophic bacteria is coupled to differences in cellular phosphorus quotas

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It is frequently presumed that heterotrophic bacteria from aquatic environments have low carbon (C) content, high phosphorus (P) content, and maintain homeostasis at low C:P in their biomass. Dissolved and particulate organic matter from primary producers in terrestrial and aquatic environments typically has high C:P ratios, suggesting that heterotrophic bacteria consuming this resource experience stoichiometric imbalance in C and P. The strength of elemental homeostasis is important for understanding how heterotrophic bacteria couple C and P cycles in response to environmental change, yet these generalizations are based upon data from only a few species that might not represent the physiology of bacteria in freshwaters. However, recent research has indicated that some strains of bacteria isolated from freshwaters have flexible C:P stoichiometry and can acclimate to changes in resource C:P. Although it is apparent that strains differ in their biomass C:P and flexibility, the basis for these characteristics has not been explained. We evaluated biomass C:P homeostasis in 24 strains of bacteria isolated from temperate lakes using a uniform relative growth rate in chemostats. Overall, the strains exhibited a range of homeostatic regulation from strong homeostasis to highly flexible biomass stoichiometry, but strains that were isolated using P-rich media formulations were more homeostatic than strains isolated using P-poor media. Strains exhibiting homeostatic biomass C:P had high cellular C and P content and showed little morphological change between C and P limitation. In contrast, stoichiometrically flexible strains had low P quotas and increased their C quotas and cell size under P limitation. Because stoichiometric flexibility is closely coupled to absolute P content in bacteria, anthropogenic inputs of P could lead to prevalence of more homeostatic bacteria, reducing the ability of natural assemblages to buffer changes in the availability of P and organic C.

**Keywords: aquatic heterotrophic bacteria, phosphorus content, ecological stoichiometry, chemostats, lakes, element quotas, cell morphometry**

## INTRODUCTION

Heterotrophic bacteria couple multiple biogeochemical cycles within terrestrial and aquatic ecosystems (Azam, 1998; Cole, 1999; Schlesinger et al., 2011) and experience imbalance between the chemical composition of their biomass and the chemical composition of their resources. Elemental imbalances and nutrient limitation are not absolute, but rather, they occur relative to the availability of other resources and the physiological requirements of the organism. Adaptations for dealing with resource limitation include reducing growth rate and/or metabolic activity, increasing consumption or uptake rates, minimizing resource loss rates, and minimizing the quantity of the resource required within biomass. Although all organisms exhibit at least one of these mechanisms, flexible biomass composition is particularly important because realized growth rate and resource acquisition rates are coupled indirectly by internal nutrient concentrations (Droop, 1973; van den Berg, 2001). In their role as "gatekeepers" of nutrients within aquatic ecosystems (Kirchman, 1994), the nutrient content and, stoichiometry of bacterial biomass affects the rates at which bacterial communities can remineralize or sequester carbon (C), nitrogen (N), and phosphorus (P) when the supply of these elements is unbalanced relative to their demands (Goldman et al., 1987).

Stoichiometric homeostasis describes how organisms maintain or alter their biomass element composition in response to resource imbalance and environmental change. The strength of stoichiometric homeostasis for C, N, and P differs among major phylogenetic groups (Persson et al., 2010), but there is substantial variation within each group in both the strength of regulation and the range of biomass stoichiometry. Generalizations about the strength of elemental homeostasis within groups of taxa are common in the field of ecological stoichiometry and allow reduction of complex physiological mechanisms to a more tractable mass balance problem. Although such simplifications enable modeling of resource limitation, elemental imbalance, and nutrient regeneration within an assemblage, the biomass stoichiometry and

strength of homeostatic regulation for entire trophic levels has been characterized using data from only a few species or strains and might not represent the physiologies present within natural assemblages.

Stoichiometric regulation in bacteria was initially examined using *Escherichia coli*, which exhibited strong homeostasis in biomass C:N:P across two orders of magnitude in P supply (Makino et al., 2003). In a subsequent study, an assemblage of bacteria cultured from a temperate freshwater lake was non-homeostatic, suggesting that assemblages could exhibit non-homeostasis as the result of shifts in relative abundance of strains driven by nutrient availability (Makino and Cotner, 2004). Indeed, assemblages of bacteria from multiple lakes exhibit non-homeostasis and this is partly attributable to selection for different stoichiometric strategies (Godwin and Cotner, 2014). Recent work with bacterial isolates (Scott et al., 2012) has demonstrated that strains encompass a wider range of homeostatic regulation than was presumed, but none of the strains exhibited the strong homeostasis or high growth rates characteristic of *E. coli*. Also, assemblages of bacteria subject to ecological selection at high P availability (low C:P in resources) can exhibit strong homeostasis (Godwin and Cotner, 2014), indicating that homeostatic strains are likely present within natural assemblages. Despite these recent advances, the strength of stoichiometric homeostasis present within assemblages remains poorly characterized.

Just as the ratio of C:N:P within biomass has implications for competition and coexistence within local assemblages (Andersen et al., 2004; Moe et al., 2005), variability in the strength of stoichiometric regulation among related taxa is key to understanding species-level interactions in environments where resource stoichiometry varies across space or time (Jeyasingh et al., 2009; Hood and Sterner, 2010). Due to experimental constraints, measurements of stoichiometric homeostasis are seldom performed for more than a few taxa in a single study. Combining data on stoichiometric regulation from multiple studies introduces unrecognized effects of experimental design and culture conditions, potentially masking important patterns. However, determining the ranges of elemental content and stoichiometric homeostasis within a functional or phylogenetic group is essential to understanding how assemblages regulate their stoichiometry in natural ecosystems.

One problem in studying stoichiometric homeostasis of bacteria from natural environments is that few strains are readily culturable (Colwell and Grimes, 2000) and there may be bias if the medium used for isolation selects for specific physiologies. Specifically, studies of stoichiometric homeostasis in bacteria have been restricted to a small number of strains that were isolated using nutrient-rich media. Bacteria that readily colonize nutrient-rich media often exhibit high growth rates (Staley and Konopka, 1985) and rapidly growing organisms have high P content and P requirements due to the abundance of P-rich ribosomes required at high growth rates (Elser et al., 2000, 2003). Therefore, strains isolated using nutrient-rich media could have different P physiology and stoichiometry than other strains in an assemblage. We hypothesized that P-rich media formulations select for strains with higher P content and more homeostatic physiology than bacteria isolated using dilute P-poor media. We also propose that this bias due to isolation medium has contributed to an under-representation the range of stoichiometric strategies present in natural assemblages.

We sought to answer two questions: (1) Does the medium used for isolation select for different stoichiometric strategies? and (2) Do quotas of C and P differ in systematic ways among homeostatic strains and those with flexible stoichiometry? To address these questions, we characterized growth rates, stoichiometric regulation of C:N:$P_{biomass}$, cell quotas, and morphometry in 24 strains of bacteria isolated from lakes using multiple isolation methods and culture media. Both within and among assemblages, the isolates exhibited a range of element content and degree of stoichiometric regulation, from non-homeostatic strains with low P quotas to strongly homeostatic strains with high P content.

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND PROCESSING

We isolated bacteria from Lake Itasca and Long Lake, both located in or adjacent to the Itasca State Park, Clearwater County, Minnesota, USA. Lake Itasca is moderately productive (7.0 $\mu$g $L^{-1}$ chlorophyll-a and 1.07 $\mu$moles $L^{-1}$ total dissolved phosphorus), has a maximum depth of 12 m, and a surface area of 431 hectares. Long Lake is less productive (3.1 $\mu$g $L^{-1}$ chlorophyll-a and 0.25 $\mu$moles $L^{-1}$ total dissolved phosphorus), has a maximum depth of 24 m, and a surface area of 64 hectares. Water samples were collected from the upper mixed layer of each lake during the spring using acid-soaked and sterilized polyethylene bottles. Samples were processed within 1 h of collection. The bacteria-sized fraction was separated by filtration through a sterile Whatman GF/B filter (Hall et al., 2009). Cell-free lake water was prepared by filtering the lake water twice using a 0.22 $\mu$m pore-size sterile filter (Fisher SteriTop).

### BACTERIAL ISOLATION AND CULTURE METHODS

#### *Dilution isolation and MPN method*

To quantify the number of culturable cells obtained using each medium treatment, we performed dilution to extinction isolation (Schut et al., 1993; Page et al., 2004) and most probable number (MPN) assays (Klee, 1993) using the bacterial-sized fraction from each lake. To detect growth of cells, resazurin was added to a final concentration of 20 $\mu$moles $L^{-1}$ in both the inoculum water and the sterile media. Resazurin becomes highly fluorescent when it is reduced by bacterial respiration (Nix and Daykin, 1992; Haines et al., 1996). We diluted the inocula into four media treatments: cell-free lake water, a complex medium (Difco Nutrient Broth, 8 g $L^{-1}$), a defined medium with high phosphorus, and a defined medium with low phosphorus. The composition of the nutrient broth was 1.39 mmoles P $L^{-1}$ (total phosphorus), 1.38 mmoles P $L^{-1}$ soluble reactive phosphorus, and 274 mmoles C $L^{-1}$, resulting in a molar C:N:P of 198:67:1. The defined medium was Basal Microbiological Medium (BMM), prepared following Tanner (2002) using deionized water, with glucose (23.9 mmoles C $L^{-1}$) as the sole source of carbon. Additional minerals, vitamins, and trace metals were supplied at concentrations described in Tanner (2002) and the medium was buffered between pH 7.2 and 7.4 using 11 mmoles $L^{-1}$ 3-(N-Morpholino)propanesulfonic acid (MOPS). Phosphorus was added as potassium phosphate

at two levels to create molar C:P of 100:1 (239 μmoles-P $L^{-1}$) and 100,000:1 (0.239 μmoles-P $L^{-1}$). The nutrient broth and BMM medium with C:P of 100:1 are categorized as P-rich media and the cell-free lake water and BMM with C:P of 100,000:1 are categorized as P-poor media.

Dilution cultures were performed in black 96-well microtiter plates (Nunc) at 11 dilutions between 1 and $2.39 \times 10^{-7}$ (total culture volumes of 170 μL) relative to lake water (**Figure 1**). Sixty replicate dilution series were performed for each lake in the BMM 100:1 and BMM 100,000:1 treatments. One hundred and eighty replicate dilution series were performed for each lake in the cell-free lake water and nutrient broth treatments. Each microtiter plate contained eight control wells of medium without any inoculum. The plates were sealed with sterile transparent film (Excel Scientific, ThinSeal) and incubated at 20°C in the dark. Fluorescence of the dilution plates was monitored using a using a Fluoromax 3 spectrofluorometer with a MicroMax 384 plate reader (Horbia Jobin Yvon). Fluorescence was measured using

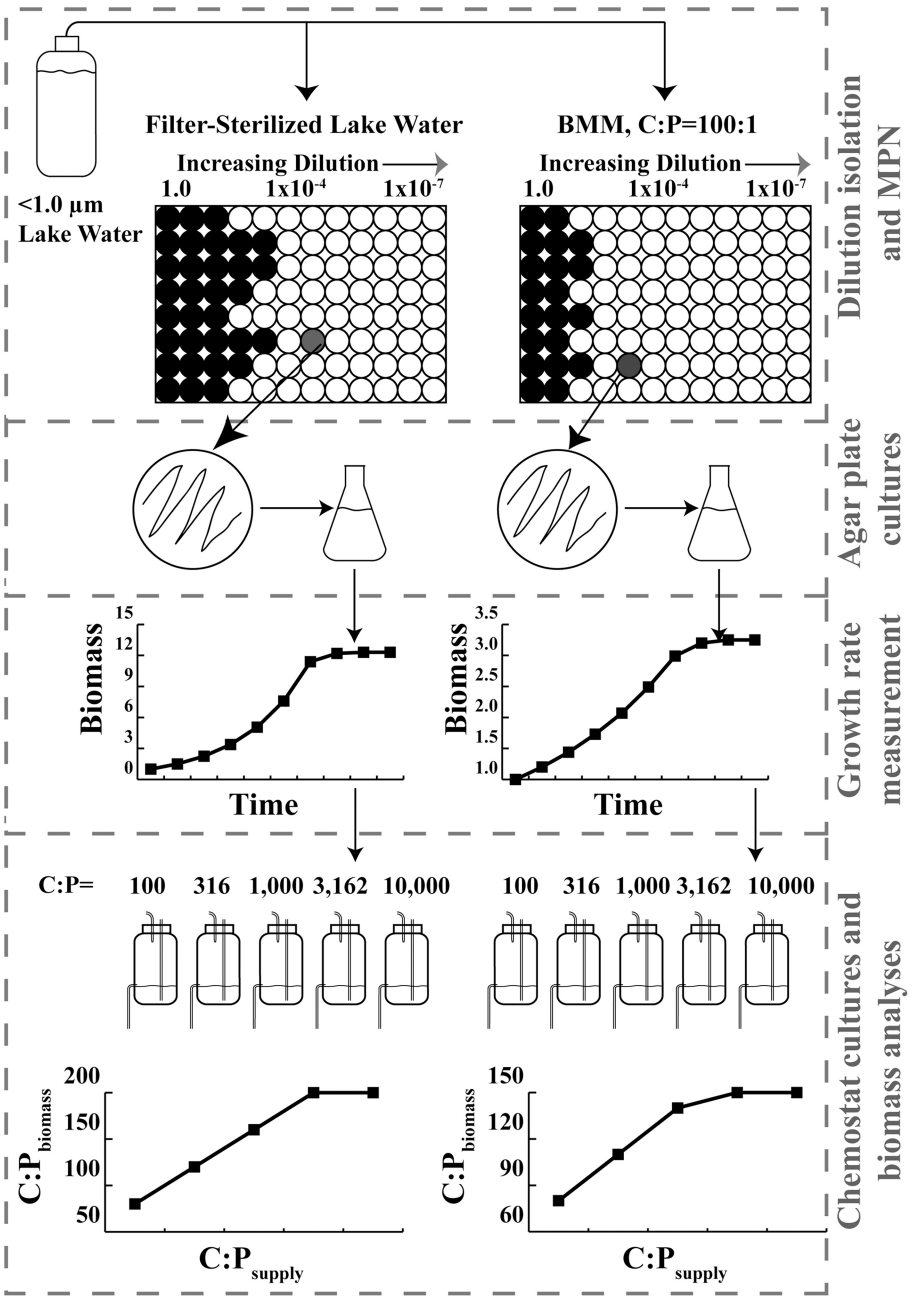


**FIGURE 1 | Schematic diagram of isolation, growth rate measurement, chemostat culture, and results for two different isolates.** Dashed boxes denote Materials and Methods subsections.

excitation at 560 nm and emission at 585 nm, both with 5 nm band pass slit widths. Fluorescence in each well was averaged for 1 s and the plates were returned to the dark incubator between readings. The fluorescence of the plates was measured seven times between 3 and 38 days after inoculation.

After 18 and 38 days, we identified the wells with positive growth as those where the slope of fluorescence vs. time and the absolute fluorescence were both greater than the 90% upper confidence interval for the slope of the control wells for each plate. We computed the MPN estimates and confidence intervals for each lake and medium type following Jarvis et al. (2010). To obtain cultures with a high probability of being axenic, we harvested wells at the highest dilution where the next lowest dilution did not show detectable growth. The contents of these wells were diluted again into the same medium type with resazurin and the fluorescence was monitored for 19 days. Following the second dilution of the potential isolates, we harvested the highest dilution with detectable growth and plated the cultures onto agar made with the same medium. Distinct colony morphologies were preserved as described below.

#### *Agar plate cultures*

We diluted the bacterial-sized fraction from each lake into cell-free lake water (dilution 1 – 1000×) and plated 100 μL onto agar plates. The plates were prepared using 15 g agar $L^{-1}$ (Fluka number 05038) and each of the following medium formulations: nutrient broth (prepared as above), BMM at C:P of 100:1, and BMM at C:P of 100,000:1. We analyzed the phosphorus content of the agar and it contributed less than 0.263 μmoles P $L^{-1}$ as soluble reactive phosphorus and less than 0.97 μmoles P $L^{-1}$ to the finished media. Five replicate plates were used for each dilution of nutrient broth and 10 replicate plates were used for the BMM formulations. The plates were incubated at 22–24°C and visible colonies were characterized, enumerated, and harvested after 6 and 12 days. For each set of replicate plates, single colonies of each distinct morphology were harvested and streaked onto agar plates with the same media. Permanent cultures were established by adding glycerol to liquid cultures of each isolate (final concentration 15% v/v) and freezing the cultures at −70°C (Morrison, 1977).

#### *Isolates selection, identity, and growth rates*

Isolates for use in the experiments were randomly selected from the list of potential isolates in each combination of medium treatment and lake. Strains that did not exhibit sufficient growth in liquid batch culture were excluded and other candidates were evaluated until each combination was represented by at least one strain. The strains were assigned to taxonomic affiliation using partial 16S rRNA sequences derived using the primers 8F and 1492R, and subsequent alignment against sequence libraries using the Basic Local Alignment Search Tool (BLAST, National Institutes of Health). To ensure that the strains for this study were not biased toward the isolation methods described above or the biogeography of Long Lake and Lake Itasca, we included seven additional strains provided by Stuart Jones (University of Notre Dame). The strains were isolated from lakes in Indiana and Michigan (Livermore et al., 2014). A strain of *Polynucleobacter necessarius* (Pnec), was obtained from the DSMZ collection (Leibniz Institute, Germany). The source, isolation conditions, and taxonomic affiliation of all of the study strains are given in **Table 1**.

Because the absolute and relative growth rates of microorganisms affect their stoichiometry and flexibility (Makino and Cotner, 2004; Hillebrand et al., 2013), we normalized the dilution rate for each strain to its apparent maximum growth rate. The apparent maximum growth rate of each isolate ($\mu_{max}$) was measured in BMM medium with high P availability (C:P = 100:1) using batch cultures with a volume of one milliliter. Since these culture conditions are not specific to each isolate, the apparent $\mu_{max}$ likely underestimates the actual maximum growth rate. Cultures of each isolate were inoculated from permanent cultures and incubated at 22–24°C on an orbital shaker. At each time point, two replicates of each isolate were rapidly frozen using liquid nitrogen and were stored at −70°C until analysis. The population growth rate of bacteria in each culture was determined from the change in the concentration of double-stranded DNA using the PicoGreen reagent (Invitrogen Quant-It PicoGreen Kit) and fluorescence measurement (Tranvik, 1997; Cotner et al., 2001). Poor sensitivity was observed when the PicoGreen reagent was added directly to cells growing in medium. Sensitivity was improved substantially by extracting the DNA prior to quantification. After thawing the cultures, 125 μL of extraction buffer (29.1 mmoles $L^{-1}$ sodium lauryl sarcosine, 54 mmoles $L^{-1}$ tris(hydroxymethyl) aminomethane, and 5.4 mmoles $L^{-1}$ ethylene(diamine)tetraacetic acid at pH 8.0) was added to the samples (Gorokhova and Kyle, 2002) and the samples were incubated at 22–24°C on a rotary shaker for 1 h. DNA standards (Invitrogen) were prepared by dilution into TE buffer (10 mmoles $L^{-1}$ tris(hydroxymethyl) aminomethane and 1 mmoles $L^{-1}$ ethylene(diamine)tetraacetic acid at pH 8.0). The PicoGreen reagent was diluted 1:470 in extraction buffer and 150 μL was added to each sample, followed by mixing. The samples were incubated in the dark for at least 10 min and transferred to 1 cm polymethylmethacrylate cuvettes (VWR Scientific). The fluorescence was measured using excitation of 500 nm and emission at 523 nm (5 nm band pass slit widths) using a Fluoromax 3 fluorometer (Horbia Jobin Yvon). Fluorescence values were averaged over 1 s. The working range of the assay was 50 pg to 200 ng DNA $mL^{-1}$. Growth rates were estimated from cultures where the DNA concentration increased exponentially (log-linear $R^2 > 0.9$) for at least three successive time points and the DNA concentration was less than 40 ng $mL^{-1}$. Based upon a range of 3–20 fg DNA $cell^{-1}$ in cultured cells (Cotner et al., 2001; Makino and Cotner, 2004), the approximate cell densities used for growth rates were between $3.3 \times 10^4$ and $1 \times 10^7$ cells $mL^{-1}$. The growth rate estimates from replicate cultures ($\mu_{max}$) were within 10%.

### CHEMOSTAT CULTURES AND BIOMASS ANALYSES

#### *Chemostat cultures*

Each isolate was cultured in 100 mL polypropylene chemostats diluted at 33% of $\mu_{max}$(**Figure 1**). The P content of the BMM formulation was manipulated to achieve molar C:P ratios of 100, 316, 1000, 3162, and 10,000:1 (2.4–239 μmoles P $L^{-1}$). Batch cultures for each treatment were inoculated with aliquots of

**Table 1 | Source and taxonomic affiliation of the strains used for this study.**

| Strain ID | Taxonomic affiliation | Source | Isolation method |
|---|---|---|---|
| D111 | *Betaproteobacteria, Hylemonella* | Lake Itasca, MN | BMM 100:1 |
| D201 | *Betaproteobacteria, Hylemonella* | Long Lake, MN | BMM 100:1 |
| D206 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | BMM 100:1 |
| D301 | *Alphaproteobacteria, Rhizobium* | Long Lake, MN | BMM 100,000:1 |
| D304 | *Alphaproteobacteria, Rhizobium* | Long Lake, MN | BMM 100,000:1 |
| D611 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | Sterile lake water |
| D703 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | Sterile lake water |
| D712 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | Sterile lake water |
| D801 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | Sterile lake water |
| D905 | *Alphaproteobacteria, Brevundimonas* | Long Lake, MN | Sterile lake water |
| D909 | *Alphaproteobacteria, Brevundimonas* | Lake Itasca, MN | Nutrient broth |
| D1207 | *Betaproteobacteria, Achromobacter* | Long Lake, MN | Nutrient broth |
| D1303 | *Betaproteobacteria, Achromobacter* | Long Lake, MN | Nutrient broth |
| P026 | Unique partial sequence | Long Lake, MN | BMM 100,000:1 |
| P045 | Alphaproteobacteria, Agrobacterium | Long Lake, MN | Nutrient broth |
| P078 | Actinobacteria, Microbacterium | Lake Itasca, MN | Nutrient broth |
| P089 | Not sequenced | Lake Itasca, MN | Nutrient broth |
| UND-FW12 | *Betaproteobacteria, betII (Pnec)* | Pleasant Lake, IN | WC minimal medium |
| UND-L13 | *Betaproteobacteria* | Little Long Lake, MI | WC minimal medium |
| UND-L18 | *Gammaproteobacteria, gamIV gamIV-A Pseudo A1* | Little Long Lake, MI | WC minimal medium |
| UND-L41A | *Alphaproteobacteria, alfII Brev* | Little Long Lake, MI | WC minimal medium |
| UND-Pnec | *Polynucleobacter necessarius* | DSMZ culture collection | – |
| UND-WG21 | *Bacteroidetes, Flavobacteria* | Wintergreen Lake, MI | WC minimal medium |
| UND-WG36 | *Gammaproteobacteria, gamII gamII-A* | Wintergreen Lake, MI | WC minimal medium |

the permanent cultures. After the batch cultures reached optical density at 600 nm greater than 0.05 $cm^{-1}$, 5 mL of the batch cultures was used to inoculate duplicate chemostats at each level of C:$P_{supply}$. Chemostats were maintained at 20°C in darkness, aerated, and mixed with 0.2 μm-filtered air, and harvested after 9 complete turnover times.

### *Dry mass and elemental content*

Samples of biomass were collected from each chemostat using Whatman GF/F filters that were combusted at 450°C. Three replicate filters were stored in a desiccator until weighing to the nearest 0.1 μg for determination of dry mass. The filters were rinsed with 10% hydrochloric acid and then with deionized water prior to harvesting the cells under low vacuum (<100 mm Hg). The filter samples were rinsed with deionized water to remove excess media, and stored at −20°C until analysis. Filters for dry mass were dried at 60°C until constant mass (7 days) and weighed again, and the blank-corrected difference was used as dry biomass. Blank filters prepared with deionized water and filters from sterile chemostats showed no significant difference in biomass accumulation (Godwin and Cotner in review). One filter from each chemostat was randomly selected for direct measurement of C and N content using a CHN analyzer (Perkin-Elmer 2400CHN) with acetanilide (Elemental Microanalysis Ltd.) as a primary standard and a zooplankton-derived recovery standard. Three filter samples from each chemostat were analyzed for bacterial P content. Following digestion in 25 g $L^{-1}$ potassium persulfate at 121°C for 30 min (APHA, 1995), the phosphorus content was determined using the ascorbic acid molybdenum method. Spinach leaf reference material (NIST) was used as a recovery standard for all phosphorus analyses (mean recovery 94.4%). The apparent yields of C and P were computed as the proportion of C and P available in supply that was recovered as biomass from the chemostats.

### *Cell abundance and morphometry*

Aliquots of the chemostat cultures were preserved with 0.2 μm-filtered formaldehyde (3.7% by volume) and stored at 4°C. One sample from each chemostat was prepared for microscopic enumeration by dilution in sodium pyrophosphate and sonication (Velji and Albright, 1993). Each sample was stained with acridine orange, filtered onto black polycarbonate membrane filters (Nucleopore, 0.2 μm pore size), washed with cell-free deionized water, and mounted to slides for microscopy (Hobbie et al., 1977). Cell counts and morphometry measurements were performed at 1000× magnification using an Olympus BX40 microscope. For cell counts, at least 10 fields and 300 cells on each filter were enumerated manually. Photomicrographs were obtained using a digital camera (Spot 2, Diagnostic Instruments) at 1000× magnification. Cell dimensions (length, width, planar area, and planar perimeter) were measured for at least 100 cells from each chemostat using image analysis software (Image Pro Plus, Media Cybernetics). Cell shape was measured as cylinders capped with two hemispheres (Hillebrand et al., 1999). Due to the high proportion of curved cells, cell dimensions were calculated using the planar area and perimeter, rather than the box length and

width. The equations used for estimating cell length, width, surface area, and volume from planar area and perimeter are given in the Supplementary Materials.

### STATISTICAL ANALYSES

The mean blank-corrected measurements for each chemostat were used to calculate molar ratios of elements ($C:P_{biomass}$, $N:P_{biomass}$, and $C:N_{biomass}$). The elemental quotas of the cells (P $cell^{-1}$ and P relative to dry mass) were computed as the mean particulate P divided by the cell density. In several strains, quotas of each C, N, and P increased under P limitation ($C:P_{supply}$ of 10,000:1) relative to P sufficiency ($C:P_{supply}$ of 100:1 ). For these strains, the C:N:P of the added biomass was calculated as the molar ratio of the increase for each element. For each strain, the strength of stoichiometric regulation was assessed using segmented linear regressions (Kim et al., 2004) of $\log_{10}$ $C:P_{biomass}$ against $\log_{10}$ $C:P_{supply}$ (Sterner and Elser, 2002). The break point was selected by iteratively bisecting the data series at each level of $C:P_{supply}$ from 316:1 to 10,000:1 and performing standard linear regression on the lower (flexible) and upper (homeostatic) ranges. The breakpoint was chosen as the level of $C:P_{supply}$ that minimized the total sum of squares for both segments. Strains were separated into three even categories (stoichiometric categories) based upon the degree of flexibility in $C:P_{biomass}$ observed in the chemostat cultures.

Morphometric data were $\log_{10}$-transformed prior to analysis to meet assumptions of approximate normality and homogeneity of variances (Sokal and Rohlf, 1995). Biomass elemental content, morphometric data, and yields were analyzed by analysis of covariance (ANCOVA) tests, using stoichiometric category as a fixed effect and $C:P_{supply}$ as a quantitative treatment. When a significant interaction was observed, separate One-Way analysis of variance (ANOVA) tests were performed for each level of $C:P_{supply}$. Pairwise differences in the One-Way ANOVA analyses were evaluated using Tukey's Honest Significant Difference tests, with a significance cutoff of $p < 0.05$. To determine the effect of isolation medium on homeostatic classification of the isolates, the strains from Lake Itasca and Long Lake were grouped into those isolated using P-rich media (nutrient broth and BMM with C:P of 100:1) and P-poor media (sterile lake water and BMM with C:P of 100,000:1). The proportions of isolates belonging to each stoichiometric category were compared for P-rich and P-poor media using a chi-squared test with the null hypothesis that the categories are evenly distributed among medium types (Sokal and Rohlf, 1995).

## RESULTS

### CULTIVATION AND MEDIUM FORMULATIONS

The bacterial assemblages diluted into a defined medium (BMM) with C:P of 100:1 showed significantly higher MPN estimates than the samples diluted into cell-free lake water or nutrient broth (**Figure 2**). The MPN estimates for Long Lake were higher than those for Lake Itasca in all medium treatments except for the nutrient broth. Samples plated onto solid media showed a similar pattern of reduced counts for nutrient broth compared to both BMM treatments (**Figure 2**). The ANCOVA for CFU $mL^{-1}$ revealed significant effects of medium treatment ($p < 0.003$), and

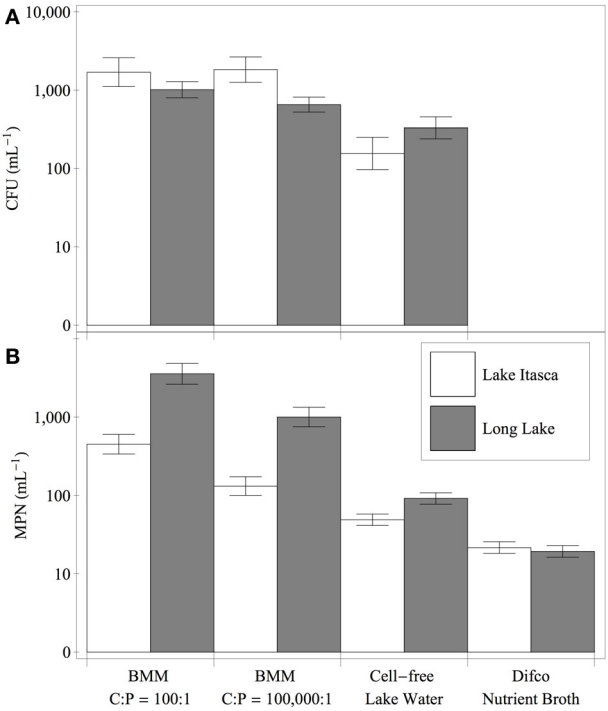


**FIGURE 2 | Cultivation yields of bacteria from Long Lake and Lake Itasca on P-rich and P-poor media formulations.** Panels show colony-forming units **(A)** and most probable number estimates **(B)** for water samples inoculated into different medium treatments. Error bars denote the 95% confidence intervals for the estimates.

lake ($p < 0.03$), but no interaction ($p > 0.10$). *Post-hoc* tests indicated that the nutrient broth had significantly lower CFU $mL^{-1}$ than the BMM medium formulations (Tukey HSD, $p < 0.05$). The growth rates of the isolates ranged from 0.07 to 0.43 $h^{-1}$ (**Table 2**). For the isolates from Lake Itasca and Long Lake (MN), there was no effect of isolation medium richness on growth rate (Wilcoxon two-sample test, $p > 0.05$).

### BIOMASS STOICHIOMETRY

The strains exhibited variable stoichiometry in chemostat cultures, with $C:P_{biomass}$ ranging from 47:1 to 994:1 and $N:P_{biomass}$ ranging from 8.2:1 to 132:1 (**Table 2**). $C:N_{biomass}$ was less variable, ranging from 2.3:1 to 11:1. ANCOVA tests on $C:P_{biomass}$, $N:P_{biomass}$, and $C:N_{biomass}$ indicated significant effects of strain, $C:P_{supply}$, and an interaction (all $p < 0.0001$). Separate One-Way ANOVA tests for each strain indicated significant effects of $C:P_{supply}$ for a subset of the strains (**Table 2**). The regression slopes of log $C:P_{biomass}$ vs. log $C:P_{supply}$ (Sterner and Elser, 2002) below the breakpoint ranged from −0.09 to 0.93 (**Table 2**, **Figure 3**). The strains were assigned into three arbitrary categories using the lower, middle, and upper third of the range in $C:P_{biomass}$. Homeostoichs exhibited ranges of $C:P_{biomass}$ less than 83, mesostoichs had ranges of $C:P_{biomass}$ from 83 to 210, and heterostoichs had ranges of $C:P_{biomass}$ greater than 210 (**Figure 3**). For the isolates from Lake Itasca and Long Lake (MN), medium types produced different

**Table 2 | Growth rate, biomass stoichiometry, and strength of homeostasis for isolates.**

| Isolate | $\mu_{max}$ ($h^{-1}$) | C:$P_{biomass}$ | N:$P_{biomass}$ | Slope |
|---|---|---|---|---|
| D111 | 0.116 | 88–132* | 13–19 | 0.037 |
| D201 | 0.153 | *68–78* | *25–33* | 0.036 |
| D206 | 0.089 | – | – | – |
| D301 | 0.142 | 64–280* | 15–38* | 0.926 |
| D304 | 0.089 | 75–399** | 16–43* | 0.730 |
| D611 | 0.091 | 103–374*** | 19–53*** | 0.497 |
| D703 | 0.091 | 104–869*** | 19–124*** | 0.570 |
| D712 | 0.124 | 87–297*** | 16–44** | 0.384 |
| D801 | 0.120 | 80–421*** | 15–67*** | 0.548 |
| D905 | 0.112 | 90–287*** | 17–43** | 0.480 |
| D909 | 0.091 | 91–566** | 17–78** | 0.528 |
| D1207 | 0.086 | 86–160* | 19–28 | 0.079 |
| D1303 | 0.138 | 53–116** | 12–22* | 0.400 |
| P026 | 0.112 | *62–160* | *9–22* | 0.295 |
| P045 | 0.219 | 67–146** | 15–28* | 0.464 |
| P078 | 0.074 | *66–85* | *15* | 0 |
| P089 | 0.222 | *67–102* | *15–27* | −0.011 |
| UND-FW12 | 0.078 | 80–179*** | 18–36*** | 0.146 |
| UND-L13 | 0.342 | 50–241* | 9–40* | −0.117 |
| UND-L18 | 0.249 | 52–222* | 11–25*** | 0.140 |
| UND-L41A | 0.144 | 100–372* | 15–40*** | 0.158 |
| UND-Pnec | 0.204 | *71–192* | *17–44* | −0.040 |
| UND-WG21 | 0.097 | 79–162*** | 16–34*** | 0.350 |
| UND-WG36 | 0.432 | 72–190** | 13–32*** | 0.579 |

*Stoichiometry data are the ranges of mean values for each level of C:$P_{supply}$. The slope is the linear regression below the breakpoint in C:$P_{supply}$. The p-value associated with the One-Way ANOVA of each parameter vs. C:$P_{supply}$ is denoted by *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Italics denote strains where fewer than 10 chemostats were within detection limits for the parameter.*

proportions of homeostoich, mesostoich, and heterostoich strains (chi-squared test, $p < 0.018$). P-rich media formulations produced 6 homeostoichs, 1 mesostoich, and 1 heterostoich and P-poor media yielded 1 homeostoich, 1 mesostoich, and 5 heterostoichs.

At C:$P_{supply}$ of 100:1, there were no significant differences in biomass stoichiometry among the stoichiometric categories (all $p > 0.05$), with C:N:$P_{biomass}$ ranging from 52:11:1 to 104:19:1 and a median ratio of 81:16:1. Under P limitation, mean C:N:$P_{biomass}$ for each isolate ranged from 116:21:1 to 869:124:1. Using the stoichiometric categories as groups (**Figure 4**), C:$P_{biomass}$ and N:$P_{biomass}$ each showed significant effects of C:$P_{supply}$, category, and an interaction (all $p < 0.0001$). All three categories of strains exhibited increased N:$P_{biomass}$ under P limitation ($p < 0.05$). Mean C:$N_{biomass}$ was also affected by C:$P_{supply}$ ($p < 0.0001$), category ($p < 0.0001$), and an interaction ($p < 0.05$). For heterostoichs and mesostoichs, C:$N_{biomass}$ increased under P limitation ($p < 0.05$), but homeostoich C:$N_{biomass}$ did not change (**Figure 4**). For the isolates from Lake Itasca and Long Lake (MN), P-rich media produced isolates with lower ranges in C:$P_{biomass}$, N:$P_{biomass}$, and C:$N_{biomass}$ (Wilcoxon test, all $p < 0.01$). Minimum C:$P_{biomass}$ showed a negative correlation with $\mu_{max}$ ($r^2 = 0.24$, $p < 0.02$).

Abundance of the cells in the cultures decreased with increasing C:$P_{supply}$ and the strength of the decrease was proportionally different among the categories (ANCOVA, $p < 0.0001$). At all levels of C:$P_{supply}$, heterostoichs had higher cell abundance than mesostoichs or homeostoichs. As a percentage of available C, biomass C yield was higher in the heterostoich strains compared to the mesostoichs and homeostoichs at all levels of C:$P_{supply}$ above 100:1 (**Figure 5**, $p < 0.05$). Between C:$P_{supply}$ of 316:1 and 1000:1, heterostoichs also exhibited higher P yield (mean 75%) than the homeostoichs and mesostoichs (35–45%, $p < 0.05$). Although the residual P was not measured in the chemostats, the recovered biomass P was lowest at C:$P_{supply}$ of 10,000:1. Inorganic P was supplied at 2.39 μmoles $L^{-1}$ in the 10,000:1 medium treatment and the mean biomass P was 1.19 μmoles $L^{-1}$ for heterostoichs, 1.44 μmoles $L^{-1}$ for mesostoichs, and 1.103 μmoles for homeostoichs (ANOVA by category $p < 0.002$).

## CELLULAR C AND P CONTENT

Phosphorus quotas of the isolates ranged from 0.013 to 1.57 fmoles $cell^{-1}$ and C quotas ranged from 1.04 to 143 fmoles $cell^{-1}$ (**Table 3**, **Figure 6**). The ANCOVA tests on cell quotas indicated a significant effect of C:$P_{supply}$ and an interaction between C:$P_{supply}$ and strain (all $p < 0.0001$). One-Way ANOVA tests on C and P quotas indicated significant effects of C:$P_{supply}$ in only a subset of the strains (**Table 3**). Relative to cell volume, P content ranged from 0.009 to 1.53 fmoles $\mu m^{-3}$ and C content ranged from 2.9 to 126 fmoles $\mu m^{-3}$. Under P limitation, P quotas relative to cell volume increased significantly only in one strain, but decreased significantly in eight strains (**Table 3**).

For the isolates from Lake Itasca and Long Lake (MN), P-poor media produced isolates with lower minimum P quotas (by volume, Wilcoxon test, $p < 0.03$) and lower maximum P quotas (per cell, $p < 0.02$). Isolation medium type did not affect minimum C quotas, but P-poor media produced isolates with significantly lower maximum C quota (by volume, $p < 0.004$). At C:$P_{supply}$ of 100:1, $\mu_{max}$ was positively correlated with P quotas ($r^2 = 0.37$, $p < 0.003$) and carbon quotas ($r^2 = 0.30$, $p < 0.008$) among the isolates. Relative to dry mass, P content of the isolates ranged from 0.032 to 2.08% and showed significant effects of stoichiometric category and C:$P_{supply}$ (ANCOVA, all $p < 0.001$). Under P limitation, all of the strains exhibited decreased P content relative to dry mass, although this decrease was statistically significant in only 16 of the strains (**Table 3**).

Under P-replete conditions, C:$P_{biomass}$ of the isolates was not strongly related to biomass C and P content (**Figure 7**). Under P limitation, there was a significant negative relationship between C:$P_{biomass}$ and P quota, but there was no relationship with C quota. Of the 11 strains that increased both their absolute C quota and P quota under P limitation, the C:P of the added biomass between C:$P_{supply}$ of 100:1 and 10,000:1 ranged from 205:1 to 5866:1. For the seven heterostoich strains exhibiting increased quotas of C and P under P limitation, the mean C:P of the added biomass was 1964:1. In 13 of the strains, both C and N quotas increased under P limitation and the C:N of added biomass ranged from 5:1 (P045) to 63:1 (UND-WG36). In the 10 strains

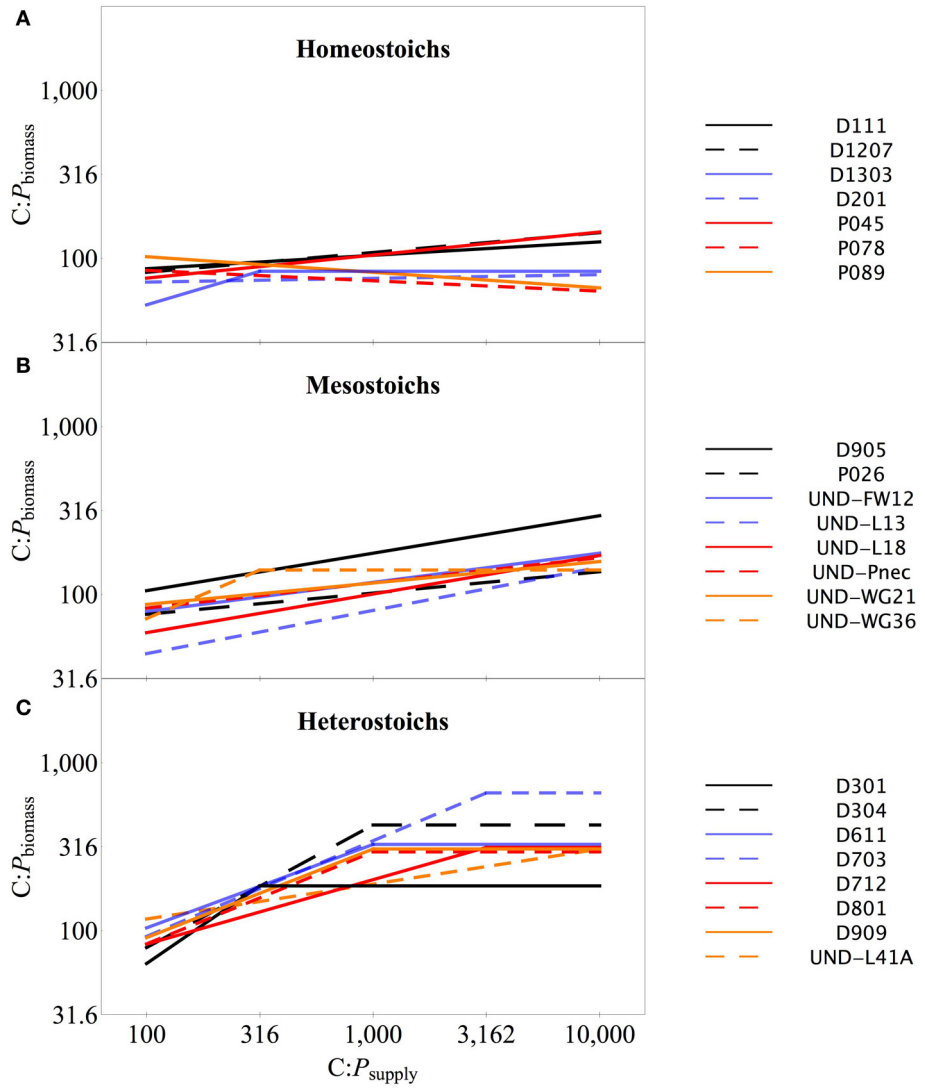


**FIGURE 3 | Biomass C:P stoichiometry across C:$P_{supply}$ for isolates in each category.** Biomass C:P stoichiometry for the isolates in each category: homeostoichs **(A)**, mesostoichs **(B)**, and heterostoichs **(C)**. Lines denote the segmented linear regression as described in the text.

where both N and P increased under P limitation, N:P of the added biomass ranged from 17:1 to 372:1.

### CELL MORPHOMETRY

The isolates exhibited a range of morphological responses to P limitation (**Figure 8**) and these responses were related to stoichiometric category. At high C:$P_{supply}$, homeostoich strains increased less in length, volume, surface area, and L:W than the mesostoich and heterostoich strains (Supplement Figures S1–S3). Mean cell length was significantly affected by stoichiometric category, C:$P_{supply}$, and an interaction (all $p < 0.0001$). Cell L:W was affected by category ($p < 0.0001$) and C:$P_{supply}$ ($p < 0.0001$) without a significant interaction ($p > 0.05$). Eighteen strains exhibited significantly increased length:width (L:W) under P limitation (Supplement Figures S1–S3). Overall, cell SA:V did not show significant effects of category or C:$P_{supply}$. Eight homeostoich and mesostoich strains exhibited significantly increased surface area:volume (SA:V) in response to P limitation (2–25% change). In contrast, all but one of the heterostoich strains showed a significant decrease in SA:V under P limitation. Cell volume showed significant effects of stoichiometric category, C:$P_{supply}$, and an interaction (all $p < 0.05$), again with heterostoichs increasing most in volume under P limitation. Cell surface area showed significant effects of category, C:$P_{supply}$, and an interaction (all $p < 0.001$).

## DISCUSSION

The strains examined in this study showed more variability in their elemental content and stoichiometric regulation than was previously known for heterotrophic bacteria. Data from these experiments can be used to provide insight into three areas. First, these cultures illuminate a gradient of stoichiometric strategies

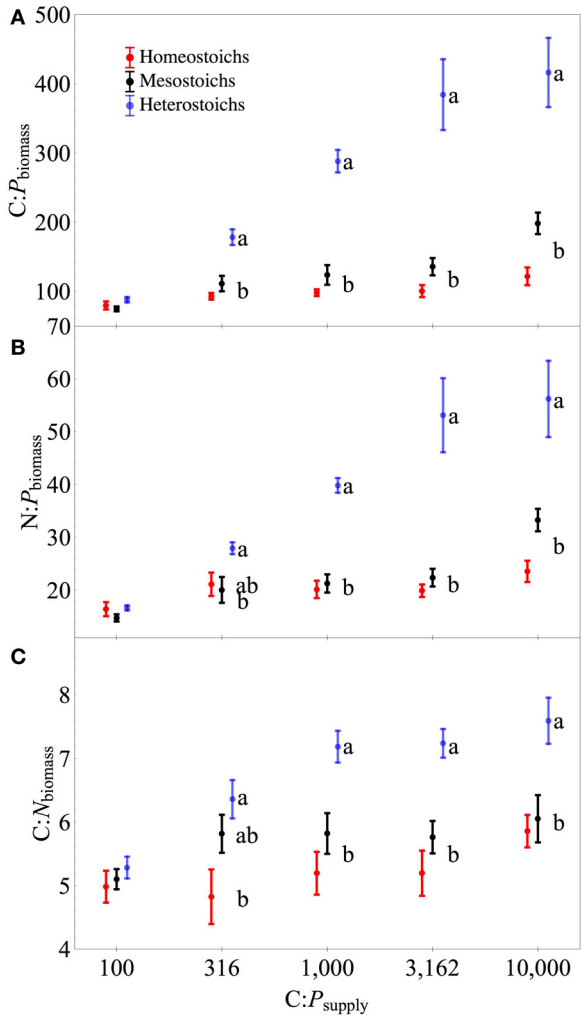


**FIGURE 4 | Biomass stoichiometry of isolates by stoichiometric category.** Separate panels for C:P **(A)**, N:P **(B)**, and C:N **(C)** stoichiometry across C:$P_{supply}$. Error bars denote one standard error of the mean. Lower case letters denote significantly different subsets of the stoichiometric categories at each level of C:$P_{supply}$ (Tukey HSD, $p < 0.05$). Symbols for each category are staggered horizontally at each level of C:$P_{supply}$ to improve clarity.

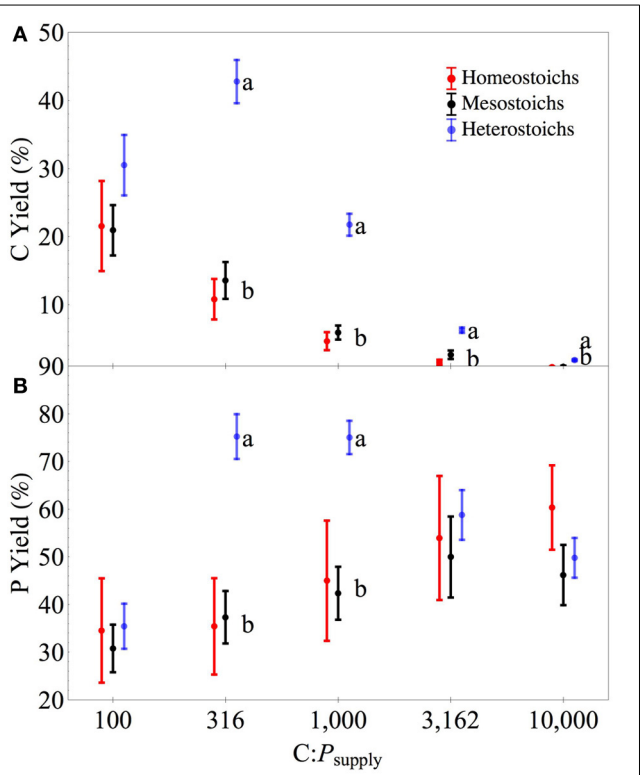


**FIGURE 5 | Carbon and phosphorus quotas of isolates by stoichiometric category.** Separate panels for cellular carbon **(A)** and phosphorus **(B)** quotas across C:$P_{supply}$. Error bars denote one standard error of the mean.

and show that isolates from a single assemblage may exhibit strong homeostasis or flexible stoichiometry depending upon the isolation conditions used. Second, heterostoich strains achieved flexibility in their stoichiometry through low P quotas at all P supply levels and dynamic C content, whereas homeostatic strains had high quotas of both C and P. Last, cell morphology and stoichiometric flexibility are related in most strains, but existing hypotheses receive only partial support from the data presented here.

## ISOLATION CONDITIONS SELECT FOR STOICHIOMETRIC REGULATION

The medium formulations employed in this study differed in their effectiveness for culturing strains from the bacterial assemblages in Lake Itasca and Long Lake (MN). Notably, more strains were viable in the BMM formulations than in the nutrient broth medium. Previous studies have demonstrated that only a small portion of the bacterial community can be easily cultured (Staley and Konopka, 1985; Eilers et al., 2000) and that nutrient-rich media are often poorly suited to isolate bacteria from aquatic environments (Barer and Harwood, 1999). For the defined medium dilution cultures, there was a decrease in apparent cultivability when the P availability was decreased by three orders of magnitude. Thus, there were distinct effects of C source (large effect) and P availability (smaller effect) on the number of culturable cells. Fewer bacteria were capable of utilizing the animal-derived carbon substrates in the nutrient broth compared to the defined medium, which could be attributed to inhibition of growth in some strains by high concentrations of substrates (Morita, 1997).

The medium formulations used to isolate strains from Long Lake and Lake Itasca effectively selected for strains with different types of stoichiometric regulation. Both the P-rich and P-poor media formulations yielded isolates exhibiting a range of stoichiometric regulation, but the P-rich media produced disproportionately more homeostatic strains compared to P-poor media. Previous studies on the stoichiometry of bacterial isolates have examined only strains that were isolated on nutrient-rich media (Nakano, 1994; Løvdal et al., 2008; Scott et al., 2012). Although some isolates obtained using nutrient-rich media have exhibited non-homeostasis and high C:$P_{biomass}$ (Scott et al., 2012), these studies have described a comparatively small portion

**Table 3 | Elemental content of the isolates.**

| Isolate | P/dry mass (%) | P cell$^{-1}$ (fmoles) | C cell$^{-1}$ (fmoles) | P volume$^{-1}$ (fmoles $\mu m^{-3}$) | C volume$^{-1}$ (fmoles $\mu m^{-3}$) |
|---|---|---|---|---|---|
| D111 | 0.57–1.09 | 0.191–0.245 | 17.7–31.1 | 0.726–0.960 | 67.7–121.61 |
| D201 | *0.17–0.38* | *0.015–0.074* | *1.03–5.79* | *0.054–0.341* | *3.68–26.7* |
| D206 | – | *0.042–0.051* | – | *0.030–0.033* | – |
| D301 | 0.38–1.80** | 0.062–0.075 | 4.4–17.5* | 0.052–0.110* | 7–14.5 |
| D304 | 0.33–1.74 | 0.028–0.06* | 2.1–22.0** | 0.028–0.039 | 2.9–12.7** |
| D611 | 0.36–1.05*** | 0.019–0.031** | 2.0–11.5*** | 0.042–0.062 | 6.3–18.8*** |
| D703 | 0.03–0.76*** | 0.013–0.028 | 1.0–11.6*** | 0.009–0.059 | 3.6–24.8* |
| D712 | 0.06–1.25** | 0.023–0.042 | 2.1–8.4*** | 0.041–0.09 | 6.8–14.8** |
| D801 | 0.11–1.20*** | 0.021–0.029 | 1.7–12.1*** | 0.037–0.096** | 7.7–17.6** |
| D905 | 0.28–1.18*** | 0.026–0.038 | 2.9–9.6*** | 0.044–0.127* | 9.2–15 |
| D909 | 0.11–0.85*** | 0.033–0.084 | 3.1–37.5* | 0.048–0.16 | 13.1–83.5 |
| D1207 | 0.68–1.42* | 0.032–0.13** | 2.6–17.5*** | 0.106–0.493** | 8.3–54.7*** |
| D1303 | 1.01–2.08** | 0.076–0.12 | 4.0–12.5*** | 0.155–0.259 | 8.2–25** |
| P026 | 0.32–1.35 | *0.143–0.302* | *18.0–21.9* | *0.568–1.264* | *71.7–91.2* |
| P045 | 0.29–1.52*** | 0.11–0.23 | 11.7–27.8* | 0.11–0.284 | 12.3–33.7 |
| P078 | *0.21–0.31* | *0.071–0.146* | *6.07–9.25* | *0.335–0.603* | *28.5–38.3* |
| P089 | *0.27–1.03* | *0.440–0.768* | *41.19–50.96* | *0.756–0.888* | *58.9–71.8* |
| UND-FW12 | 0.24–0.84** | 0.037–0.088* | 5.4–9.2 | 0.124–0.38* | 17.8–32.5 |
| UND-L13 | 0.21–1.92* | *0.92–1.57* | *73.7–80.8* | *0.892–1.526* | *72.1–85.4* |
| UND-L18 | *0.28–1.85*** | 0.28–1.18 | 27.6–142.6 | 0.29–1.031* | 29.2–125.2 |
| UND-L41A | 0.28–1.13** | 0.024–0.45** | 4.7–46.5 | 0.06–1.238** | 11.5–126.4* |
| UND-Pnec | *0.05–0.36* | *0.118–0.306* | *22.4–24.0* | *0.484–1.32* | *92.3–103.6* |
| UND-WG21 | 0.20–1.18** | *0.039–0.15** | *4.7–12.0* | *0.106–0.335** | *12.7–26.6* |
| UND-WG36 | 0.25–1.40*** | 0.10–0.17* | 12.1–21.9 | 0.173–0.362* | 25.9–36.5 |

*Data are the ranges of mean values for two chemostats at each level of C:$P_{supply}$. The p-value associated with the one-way ANOVA of each parameter vs. C:$P_{supply}$ is denoted by $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Underlining denotes samples with insufficient replication for ANOVA, italics denote strains where fewer than 10 chemostats were within detection limits for the parameter.*

of the stoichiometric strategies culturable from natural assemblages. Although our isolation methods produced a broader range of physiologies than previous work, the defined medium used for the chemostat cultures restricts the number of strains that could be cultured. Further work using other carbon substrates and strains would help to determine if the gradient of stoichiometric flexibility is representative for freshwater isolates.

#### GRADIENT OF STOICHIOMETRIC REGULATION IN ISOLATES

The isolates described here showed a substantial range of stoichiometric regulation, from strong homeostasis to highly flexible stoichiometry. This finding unequivocally demonstrates that bacteria differ in their strength of stoichiometric regulation and that assemblages contain multiple stoichiometric strategies. The first systematic examination of stoichiometric homeostasis in heterotrophic bacteria was performed with *E. coli*, which was strongly homeostatic (Makino et al., 2003). Other studies did not find such strong homeostasis as in *E. coli*, with most strains exhibiting only weak or moderate homeostasis (Chrzanowski and Kyle, 1996; Løvdal et al., 2008; Scott et al., 2012). From these experiments, it appears that *E. coli* could represent an aberrant observation due to its high absolute growth rate relative to other bacteria (dilution rates of 0.5–1.5 $h^{-1}$) or due the culture conditions used. However, the homeostoich strains characterized here exhibited modest growth rates that did not differ from the mesostoich or heterostoich strains, suggesting that strong homeostasis is not simply a signature of a high growth rate, either absolute growth rate ($\mu_{max}$) or realized growth rate. This is contrary to our prediction, but the BMM formulations used in this study do not reflect the maximum growth rate of the strains *in situ*. Instead, the existence of strongly homeostatic strains could represent physiological adaptation to environments with high nutrient availability and low imbalance. Furthermore, homeostatic strains of bacteria can be dominant at low C:$P_{supply}$ (Godwin and Cotner, 2014), but heterostoich physiology is dominant at high C:$P_{supply}$, suggesting that homeostatic strains are poorly adapted to dealing with resource imbalance.

The range of stoichiometric regulation present within these isolates (slopes from 0 to 0.93) is equivalent to the range of stoichiometric regulation measured in all previously published studies of bacterial isolates and assemblages. Furthermore, this range is comparable to the extent of stoichiometric flexibility associated with species of phytoplankton (Persson et al., 2010 and references therein). Although it is often assumed that all heterotrophic bacteria are strongly homeostatic (Tambi et al., 2009; Tanaka et al., 2009; Fanin et al., 2013), this study and other recent studies with environmental isolates demonstrate that non-homeostasis is common among culturable bacteria. The range of C:N:$P_{biomass}$ exhibited by the cultures was comparable to the range observed in assemblages of bacteria cultured from lakes (Godwin and Cotner,

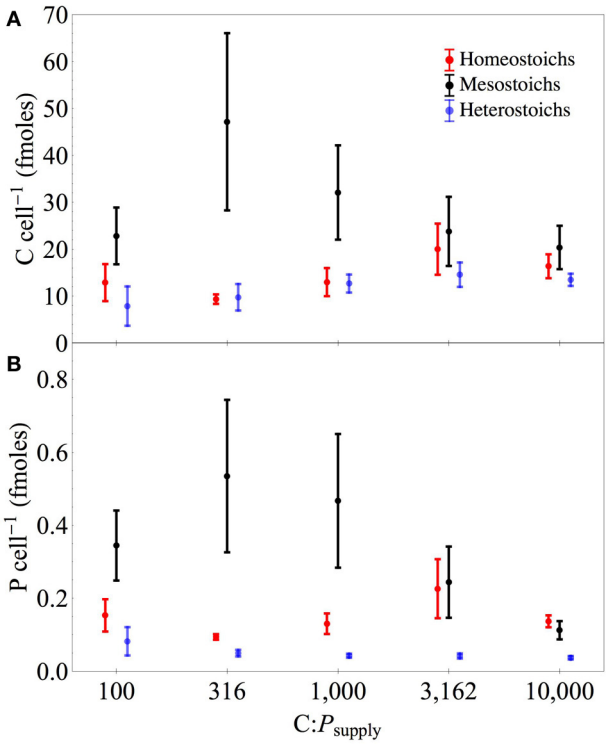


**FIGURE 6 | Carbon and phosphorus yields of isolates by stoichiometric category.** Separate panels for carbon **(A)** and phosphorus **(B)** yields across C:$P_{supply}$. Error bars denote one standard error of the mean.

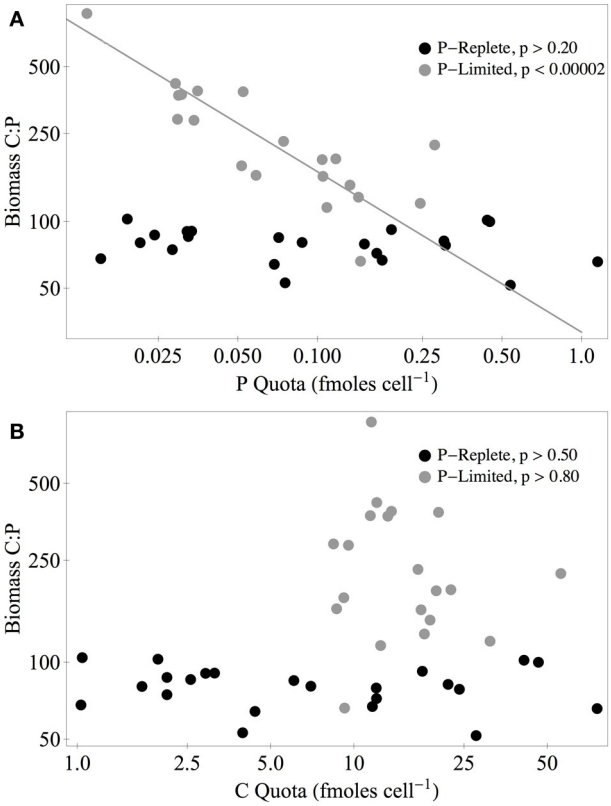


**FIGURE 7 | Biomass C:P vs. P (A) and C quotas (B) of all isolates under P-replete (C:P = 100:1, black circles) and P-limited conditions (C:P = 10,000:1, gray circles).** The solid gray line represents the standardized major axis regression (Warton et al., 2006) of log-transformed data. *P*-values are from ANOVA tests of the regression slope ($H_0$: slope = 0).

2014). Taken together, these results indicate that assemblages of bacteria likely contain strains with a range of stoichiometric regulation and suggest a flexible and diverse role in carbon and nutrient cycling. The wide range of responses by mesostoich strains (**Figures 6**, **8**) could be attributable to the arbitrary cutoff values used to assign stoichiometric categories. Further work with a large number of strains from different environments would help to determine whether distinct subgroups exist with assemblages or there is a continuous gradient of stoichiometric regulation.

## STOICHIOMETRY AND CELL QUOTAS

Although the strains differed in the extent of plasticity in their C and P quotas, several key patterns are apparent at the level of the stoichiometric categories. The first pattern is that although the isolates had similar C:N:$P_{biomass}$ under P-replete conditions, heterostoichs as a group had lower P content than the other categories at all levels of C:$P_{supply}$. This pattern of limited variation in C:$P_{biomass}$ under P sufficiency (Scott et al., 2012) suggests that heterotrophic bacteria have an essential C:$P_{biomass}$ under conditions of low imbalance. This is similar to the convergence of phytoplankton species at N:$P_{biomass}$ of approximately 16:1 under P-sufficiency (Hillebrand et al., 2013). Heterostoichs achieved plasticity in C:$P_{biomass}$ by the combination of a uniformly low cellular P content and accumulated C under P-stress. The C and P quotas of the homeostoich strains were higher and changed less compared to the heterostoichs. No single measure is sufficient to definitively diagnose P nutritional status or resource imbalance. In particular, neither C:$P_{biomass}$ nor P quotas could be used to reliably diagnose P limitation in homeostoich strains. Instead, alternative measures such as transcriptional profiling (Boer et al., 2010), phosphatase activity (Cotner and Wetzel, 1991), or growth rate bioassays (Cotner et al., 1997; Sterner et al., 2004) would be more informative.

Since the P content of the heterostoichs was lower than the other categories even at high P availability, it seems likely that a lower overall P quota is required for highly flexible biomass stoichiometry. Reduced P content and flexible C quotas of the heterostoich strains can also explain higher cell abundance and higher apparent yields of C and P. The Growth Rate Hypothesis (GRH) predicts that the P content of an organism is proportional to its growth rate due to the role of P-rich ribosomes in growth (Elser et al., 2000). The strength of stoichiometric homeostasis was not correlated with maximum growth rate, but the P content of the isolates under P sufficient conditions was positively correlated with maximum growth rate ($r^2 = 0.37, p < 0.003$). This can be explained by high variability in P content among the mesostoichs strains. From the available genome sequence data, several of the mesostoich strains are predicted to exhibit fast growth rates and exploit temporally or spatially variable resource conditions

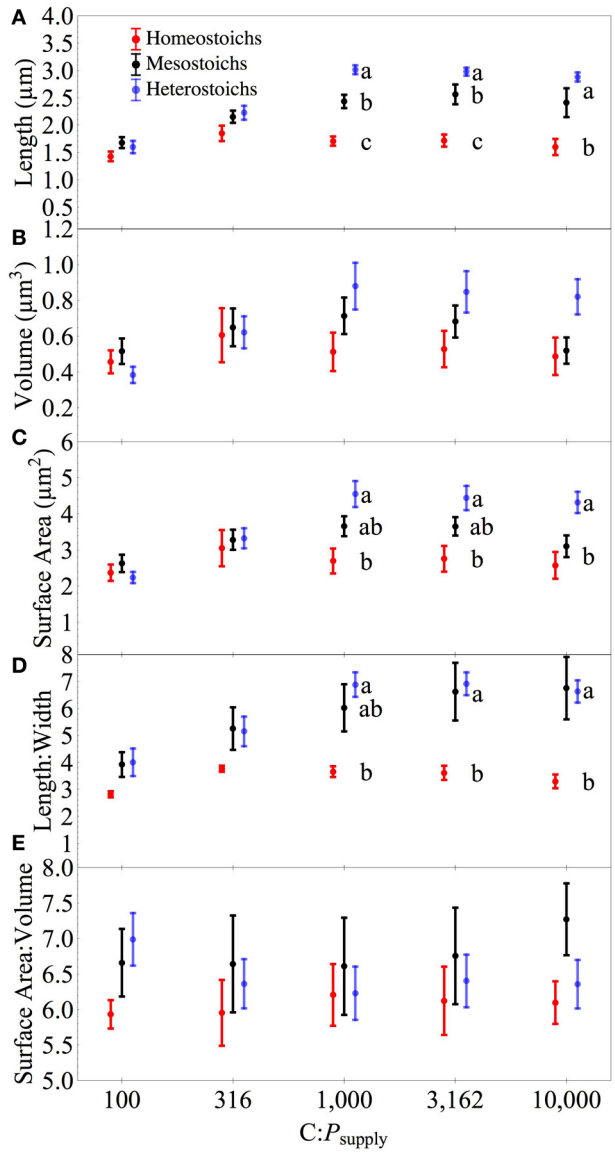


**FIGURE 8 | Morphometry of isolates by stoichiometric category.** Cell morphometry of the isolates across C:$P_{supply}$. Error bars denote one standard error of the mean. Panels for length **(A)**, volume **(B)**, surface area **(C)**, length: width **(D)**, and surface area: volume **(E)**. Lower case letters denote significantly different subsets of the categories at each level of C:$P_{supply}$ (Tukey HSD, $p < 0.05$).

(Livermore et al., 2014). Thus, their stoichiometry under steady state chemostat culture might not represent their response to variable C and P *in situ* within aquatic ecosystems.

The second key result is that several strains increased their C content under P limitation, contributing to the elevated C:$P_{biomass}$. This shows that the heterostoichs achieved stoichiometric flexibility by maintaining low cellular P content and increasing cellular C under P limitation and suggests that heterostoichs could alter their biomass stoichiometry via accumulation of C-rich molecules (e.g., glucose, glycogen, extracellular polymers). However, most of the isolates had relatively constrained C:$N_{biomass}$ compared to C:$P_{biomass}$ or N:$P_{biomass}$, which suggests that any macromolecules used for surplus storage of C also contained a substantial amount of N. One exception to this pattern is strains D301 and D304, which accumulated biomass with high C:N (43–44), characteristic of accumulation of a C-rich material such as poly-B-hydroxybutyrate or glycogen. Part of the measured increase in C:$P_{biomass}$ is attributable to an increase in cell volume (see below).

The third key result is that in addition to lower P content and variable stoichiometry, the heterostoich strains had higher apparent C and P yields than mesostoich and homeostoich strains, regardless of the C:$P_{supply}$. The higher apparent C yields for the heterostoichs could be explained by high carbon use efficiency (Sinsabaugh et al., 2013) or surplus uptake of C when P limited. Assuming that the heterostoichs were C limited at 100:1 and consumed all of the available glucose, the apparent yields represent a carbon use efficiency of approximately 30%. The apparent P yield at C:$P_{supply}$ of 100:1 was low across categories, reflecting P sufficiency and incomplete consumption of P. At intermediate levels of C:$P_{supply}$, the heterostoichs utilized more of the available P due to low P quotas. Apparent P yields for homeostoichs and mesostoichs increased with increasing C:$P_{supply}$, but the heterostoich apparent P yield decreased. As the input of P decreases, the steady state residual P becomes large relative to the assimilated portion of the available P, making the apparent P yield lower. Together with the observation that many of the heterostoichs had regression breakpoints (C:$P_{TER}$, Sterner and Elser, 2002) of 1000:1 or greater (**Figure 3**), this pattern indicates that the heterostoichs became P-limited at higher C:$P_{supply}$ than the mesostoichs or homeostoichs. An increased C:$P_{TER}$ supports the hypothesis that heterostoichs have superior competitive ability at intermediate and high C:$P_{supply}$.

## ROLE OF MORPHOMETRY IN STOICHIOMETRY AND QUOTAS

Thingstad et al. (2005) documented an increase in cell length to width ratios under P limitation and hypothesized that this is an adaptation to increase the surface area for uptake of P across the cell membrane. This hypothesis is partially supported by the present study: the mesostoich and heterostoich strains increased their surface area but there was no change relative to their cellular volume. The allometric scaling of cell size and surface area is dependent upon cell shape and also the absolute dimensions of the cells (Grover et al., 2004; Okie, 2013). At the dimensions of these cells, increasing length without changing width increases the surface area of the cell, but also increases volume, leading to little change in SA:V. In contrast, decreases in cell width would lead to increased L:W and also increased SA:V. Although morphometric elongation was a common response to P limitation among these isolates, there was an important difference between the stoichiometric categories. Most of the homeostoich and mesostoich strains increased both their L:W and SA:V slightly under P limitation and accumulated biomass with a modest C:P ratio. For heterostoichs, the morphological change resulted in tight coupling of surface area and volume. Since the heterostoich strains increased their surface area under P limitation (but decreased SA:V) by adding biomass that was deplete in P, the increase in cellular volume might not represent a significant cost, supporting

the hypothesis that surplus C can be used to increase diffusive uptake of P (Thingstad et al., 2005).

### IMPLICATIONS

This study highlights the importance of physiological constraints in biomass stoichiometry. Bacteria in aquatic ecosystems are commonly assumed to have low C:$P_{biomass}$, high P content, and little flexibility in their elemental composition, but these assumptions are challenged by the physiology of the isolates presented here and other recent work. Compared to an assemblage consisting of only homeostatic high-P bacteria, an assemblage composed of multiple stoichiometric strategies should be more sensitive to changes in the availability of C and P. Bacteria with flexible biomass stoichiometry can buffer changes in ambient C and P by altering their biomass composition. If stoichiometric flexibility is linked to competitive ability for P (through minimum P quotas), anthropogenic inputs of inorganic P may lead to decreased abundance of flexible strains and would serve to decouple the consumption of organic C and uptake of inorganic P. Such assemblage-level dynamics are important to understanding how bacteria link multiple element cycles and underscore the need to describe functional diversity of strains present within ecosystems.

Although the bacterial strains described in the present study were isolated using a range of culture methods, these strains do not necessarily represent the physiology of the dominant taxa in freshwater ecosystems. Also, all of the physiological measurements were obtained using a defined medium with a single carbon substrate. This could cause overestimation of stoichiometric flexibility since the bacteria experienced strong resource imbalance at high C:$P_{supply}$. While all of the isolates genera have been detected in lakes using 16S sequencing (Newton et al., 2011), their global representation within lake assemblages is not known. The isolate Pnec (*Polynucleobacter necessarius*) is a comparatively well-studied representative from lakes (Livermore et al., 2014), often representing a large fraction of bacterial assemblages (Jezberova et al., 2010; Hahn et al., 2012). Because Pnec is ubiquitous in lakes and was moderate in its biomass stoichiometry and growth rate, it could serve as a model strain for bridging culture-based physiological information with sequence-based characterization of *in situ* assemblages. None of the isolates exhibited homeostasis at high C:$P_{biomass}$ or N:$P_{biomass}$, but single-cell measurements of bacterial element content from lakes show significant variation in C:$P_{biomass}$ and N:$P_{biomass}$(Fagerbakke et al., 1996; Cotner et al., 2010). Together, these findings suggest that most cells present *in situ* have flexible stoichiometry and experience elemental imbalance. Assessing the relative abundance of homeostatic and flexible strains within lakes is essential for the development of assemblage-scale models with stoichiometric constraints, particularly where assemblages are subject to strong resource imbalance.

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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.2015.00159/abstract

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# Soil bacterial community composition altered by increased nutrient availability in Arctic tundra soils

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The pool of soil organic carbon (SOC) in the Arctic is disproportionally large compared to those in other biomes. This large quantity of SOC accumulated over millennia due to slow rates of decomposition relative to net primary productivity. Decomposition is constrained by low temperatures and nutrient concentrations, which limit soil microbial activity. We investigated how nutrients limit bacterial and fungal biomass and community composition in organic and mineral soils within moist acidic tussock tundra ecosystems. We sampled two experimental arrays of moist acidic tussock tundra that included fertilized and non-fertilized control plots. One array included plots that had been fertilized annually since 1989 and the other since 2006. Fertilization significantly altered overall bacterial community composition and reduced evenness, to a greater degree in organic than mineral soils, and in the 1989 compared to the 2006 site. The relative abundance of copiotrophic α-Proteobacteria and β-Proteobacteria was higher in fertilized than control soils, and oligotrophic Acidobacteria were less abundant in fertilized than control soils at the 1989 site. Fungal community composition was less sensitive to increased nutrient availability, and fungal responses to fertilization were not consistent between soil horizons and sites. We detected two ectomycorrhizal genera, *Russula* and *Cortinarius* spp., associated with shrubs. Their relative abundance was not affected by fertilization despite increased dominance of their host plants in the fertilized plots. Our results indicate that fertilization, which has been commonly used to simulate warming in Arctic tundra, has limited applicability for investigating fungal dynamics under warming.

**Keywords: soil, bacteria, fungi, Arctic tundra, fertilization, nitrogen, phosphorus, mycorrhizae**

## INTRODUCTION

Arctic tundra soils represent one of the largest terrestrial carbon (C) pools on earth (Loya and Grogan, 2004; Tarnocai et al., 2009), due to low rates of organic matter decomposition relative to net primary productivity over millennia (Marion and Oechel, 1993). Decomposition of soil organic matter (SOM) in the Arctic is constrained by low temperature, anoxic conditions due to poor drainage associated with underlying permafrost, and nutrient limitations to microbial heterotrophic activity.

Arctic tundra may be among the most nitrogen (N) limited ecosystems in the world, and N often limits both plant productivity and microbially driven SOM decomposition. Much of our understanding of the role of N in Arctic ecosystems comes from long-term field N-addition experiments. Long-term experimental N-addition has led to a significant increase in plant biomass in different types of tundra ecosystems including moist acidic tundra (Chapin and Shaver, 1985; Chapin et al., 1995; Shaver et al., 2001), wet sedge (Shaver et al., 1998), and dry heath (Gough et al., 2002). Nutrient addition has also accelerated SOM decomposition (Mack et al., 2004; Nowinski et al., 2008). Specifically, in moist acidic tundra, where above-ground net primary productivity is strongly limited by N (Shaver and Chapin, 1986; Chapin et al., 1995), 18 years of chronic N and phosphorus (P) fertilization accelerated SOM decomposition, resulting in net C loss, despite significantly increased plant biomass, relative to non-fertilized control (Mack et al., 2004). At an adjacent study site with a similar design and treatment regime, Hobbie and Gough (2004) demonstrated that long-term N and P fertilization in moist acidic tundra accelerated litter decomposition. This finding was supported by Koyama et al. (2013) who found that the fertilization stimulated soil microbial production of C-degrading enzymes, which are proximate drivers of soil organic carbon (SOC) decomposition in the same study site for Hobbie and Gough (2004). Thus, N availability plays a key role in determining the balance between net primary productivity and SOM decomposition, and whether tundra ecosystems are a net C sink or source.

Soil microbial communities in Arctic tundra, which are responsible for SOM decomposition, are as diverse as those found in other biomes (Neufeld and Mohn, 2005; Chu et al., 2010), despite the harsh environmental conditions. Soil microbial diversity in Arctic tundra is likely to be driven by many environmental factors, including N availability. In Arctic tundra soils, where N is stored in numerous chemical forms of different recalcitrance (Schulten and Schnitzer, 1997), the diversity may be an important aspect in N cycling as the diversity can increase nutrient recycling efficiency

via greater intensity in organic compound exploitation and/or complementary functional niches (Loreau, 2001).

To what extent does N-availability structure soil microbial communities and influence microbially mediated functions in an Arctic tundra ecosystem? To address this question, we considered how N availability directly and indirectly influenced the microbial community structure (abundance and diversity). Increased levels of readily available N (e.g., $NH_4^+$, $NO_3^-$, urea) via fertilization can favor nitrophilic over nitrophobic taxa, resulting in a less even community composition. For instance, Lilleskov et al. (2001) assessed nitrophobic and nitrophilic ectomycorrhizal taxa using a N deposition gradient in forests dominated by *Picea glauca* (Moench) Voss in Alaska, USA. The study showed that some genera, including *Cortinarius, Russula, Tricholoma, Lactarius,* and *Hebeloma*, were categorized as nitrophobic for being less abundant in forests with high N deposition. Other taxa, including *Lactarius theiogalus, Laccaria, Paxillus involutus,* and *Hygrophorus olivaceoalbus*, were categorized as nitrophilic as their abundance did not decline in response to the N deposition (Lilleskov et al., 2001). Increased N availability can stimulate microbial production of C-degrading extracellular enzymes (Koyama et al., 2013), resulting in increased available C, which in turn can restructure heterotrophic microbial communities.

Nitrogen can also indirectly affect microbial communities via increased net primary productivity of vegetation, especially shrubs (Chapin and Shaver, 1985; Chapin et al., 1995; Shaver et al., 2001). In moist acidic tussock tundra ecosystems, N and P addition significantly increased cover of deciduous shrubs, primarily *Betula nana*, in only 2–3 years (Shaver et al., 2001; Hobbie et al., 2005). Hobbie et al. (2005) reported that mean coverages of deciduous shrubs 2–6 years after the initiation of annual fertilization treatments were 71 and 26% for fertilized and control plots, respectively. This greater coverage of deciduous shrubs with fertilization led to significantly greater above-ground biomass and almost doubled above-ground net primary productivity of vascular plants compared to control (Hobbie et al., 2005; Gough et al., 2012). Shaver et al. (2001) reported that a 15-year fertilization of N and P increased above-ground biomass and net primary productivity of vascular plants by 2.5 times. Increased nutrient availability also affected root structures in moist acidic tussock tundra. Sullivan et al. (2007) reported that long-term fertilization over a decade significantly increased fine root biomass, but decreased production of fine roots at the community level. This result was attributed to the replacement of annual fine root system of tussock, *Eriophorum vaginatum*, with longer-lived fine roots of *B. nana* (Sullivan et al., 2007). Changes in vegetation of this sort can alter the quantity and quality of plant materials affecting the abundance and activities of certain microbial taxa. Increased net primary productivity can result in increased labile C flow to soils via root exudates (Coleman, 1985; Wall and Moore, 1999; Moore et al., 2003). Increased availability of labile C has been shown to favor bacteria and their consumers over fungi and their consumers (Moore et al., 2003), and at a finer resolution, favor copiotrophic over oligotrophic microbes (Fierer et al., 2007). The increased C flow to soils via plant roots has also been shown to stimulate biological activities in deeper mineral soils (Sistla et al., 2013).

Changes in the types of roots and root biomass affect mycorrhizal fungi associations as well (Hobbie, 2006; Hobbie and Hobbie, 2006; Hobbie et al., 2009). In Arctic tundra ecosystems, however, responses of root biomass to fertilization have varied from no change (Mack et al., 2004; Gough et al., 2012) to significant increase (Jonasson et al., 1999; Nadelhoffer et al., 2002) at a community level. Clemmensen et al. (2006) found that a 13-year fertilization of N and P significantly increased fine root biomass of shrubs associated with ectomycorrhizal fungi in moist acidic tundra, but no such increase occurred in heath tundra. Such a range in responses of root biomass to added N raises uncertainty in predicting effects on the mycorrhizal community. The relative abundance of mycorrhizal fungi can increase along with increased root biomass (Clemmensen et al., 2006), but at some point mycorrhizal infection rates decrease with fertilization (Treseder, 2004; Johnson et al., 2006; Hoeksema et al., 2010).

Addition of nutrients, typically N and P, has been used to surrogate a warming effect in Arctic tundra ecosystems (Mack et al., 2004; Aerts, 2010). The rationale is that warming often increases nutrient availability by accelerating decomposition of SOM (Chapin et al., 1995; Hartley et al., 1999; Rustad et al., 2001; Schmidt et al., 2002; Schimel et al., 2004; Aerts et al., 2006). However, Rinnan et al. (2007, 2013) found that long-term warming and fertilization treatments over a decade affected soil microbial communities differently in a Swedish Arctic tundra ecosystem, concluding that nutrient addition might not be a suitable means to mimic warming to assess soil microbial dynamics.

In this study, we investigated how bacterial and fungal biomass and their community composition were altered by long-term fertilization treatments in a moist acidic tussock tundra ecosystem of the Alaskan Arctic. We selected two study sites with different fertilization durations; one site had been fertilized for 23 years as of the sample collection since 1989 (1989 site) and the other for 6 years since 2006 (2006 site). At the two sites, we collected soil samples from two levels of treatments, fertilized (10 $g\ N{\cdot}m^{-2}{\cdot}year^{-1}$ and 5 $g\ P{\cdot}m^{-2}{\cdot}year^{-1}$) and control. We predicted that fertilization had altered bacterial and fungal community composition and decreased diversity through its influence on plant community structure and the quality and quantity of plant by-products (e.g., roots, detritus, rhizodeposition including root exudates), and that these effects were greater in the 1989 than 2006 sites, and in organic compared to mineral soils. We also predicted that the fertilization treatments had significantly increased the relative abundance of copiotrophs (e.g., α-Proteobacteria) and reduced the abundance of oligotrophs (e.g., Acidobacteria) through increased nutrient availability as a direct fertilization effect and/or increased C input via stimulated net primary productivity as an indirect fertilization effect (Ramirez et al., 2010). Finally, we predicted that the fertilization treatments altered fungal community composition by increasing the relative abundance of mycorrhizal fungi associated with shrubs, including *B. nana*, which became dominant over other vegetation forms in response to fertilization (Shaver et al., 2001; Hobbie et al., 2005). Our results contribute to the debate as to whether nutrient addition is a suitable means to mimic a warming effect in Arctic tundra

ecosystems (Mack et al., 2004; Rinnan et al., 2007, 2013; Aerts, 2010).

## MATERIALS AND METHODS

### STUDY SITE AND SAMPLE COLLECTION

Soils were collected from the Arctic Long-Term Ecological Research (LTER) site at Toolik Lake, AK, USA (68°38'N, 149°38'W) in late July 2011. The soils in moist acidic tussock tundra on the hillslopes near Toolik Lake are classified as Typic Aquiturbels (Bockheim, 2007), consisting of an organic horizon of varying thickness overlaying a mineral soil with imbedded permafrost. The average annual temperature and precipitation are –7°C and 400 mm, respectively, with approximately half of the annual precipitation as snow. The growing season is limited to between 50 and 70 days in July and August when a mean temperature is ∼10°C. Moist acidic tussock tundra is the dominant habitat type where vegetation consists of graminoids (*E. vaginatum* and *Carex microchaeta*), deciduous shrubs (*B. nana*), evergreen shrubs (*Ledum palustre* and *Vaccinium vitis-idaea*), and mosses (*Sphagnum spp.*, *Hylocomium splendens*, and *Aulacomnium spp.*; Shaver and Chapin, 1991; Chapin et al., 1995; McKane et al., 1997; Gough et al., 2007). *B. nana* is a shrub species with obligate symbiosis with ectomycorrhizal fungi (Molina et al., 1992) including *Russula* and *Cortinarius* spp. Evergreen shrubs, *Ledum palustre* and *V. vitis-idaea* are ericaceous associated with ericoid mycorrhizal fungi dominated by members of the order *Helotiales* (Walker et al., 2011).

Samples were collected from two experimental sites established in 1989 and 2006 that contained annual fertilization treatments and controls. The two different sites were located on adjacent hillslopes in moist acidic tussock tundra, 175 m apart from each other. The 1989 site is arranged in a randomized complete block design with four blocks, each containing a fertilization treatment (10 *g* $N.m^{-2}.year^{-1}$ as $NH_4NO_3$ and 5 *g* $P.m^{-2}.year^{-1}$ as $P_2O_5$) and control. The 2006 site is also arranged in a randomized complete block design with three blocks, each containing a control and a fertilization treatment applied at the same rate as the 1989 site. Responses of above-ground vegetation to the fertilization treatments at the sites assessed in late July and early August of 2011 were similar to those in the same area reported in previous studies (Chapin and Shaver, 1985; Chapin et al., 1995; Shaver et al., 2001); deciduous shrubs became dominant over other functional types with fertilization relative to non-fertilized controls in the 1989 site, and such vegetation shift was in transition in the 2006 sites (unpublished data). Two sub-samples were collected from each plot. Each soil sample was separated into three horizons: organic, organic/mineral interface and mineral soils based on organic matter content and degree of decomposition. Depths of the organic soils varied from 6 to 12 cm, and the interface from 4 to 15 cm. Mineral soils were collected from the top 5 cm of the horizon beneath the interface soils. Samples were frozen at –20°C at Toolik Field Station up to 2 weeks, transported in a cooler on dry ice to the EcoCore Analytical Laboratory at the Natural Resource Ecology Laboratory, Colorado State University, Fort Collins, CO, USA, and stored at −80°C until DNA was extracted from the soils.

### SOIL PROPERTY MEASUREMENTS

Soil samples were quantified for soil properties, including bulk density, soil water content, SOC, and total N contents. All the soil properties, except bulk density, were previously reported by Koyama et al. (2013) and summarized in **Table 1**. Soil water content was determined by drying soil samples at 105°C for 48 h. To measure SOC and total N contents, samples were first dried out at 60°C, and ground finely using a Brinkmann Retsch mill (Haan, Germany). Total C and N contents of the ground samples were quantified via dry combustion using a LECO TruSpec® (Leco Corporation, St. Joseph, MI, USA). To measure bulk density, we used sub-samples of known dimensions (i.e., width × depth × height in cm) sliced from harvested organic and mineral soils. The sub-samples were dried at 60°C for up to 10 days until their weights did not decline further. Bulk density of each sample was

**Table 1 | Soil properties (mean ± 1 se) assessed for microbial community compositions in this study.**

| Site | 1989 Site | | 2006 Site | |
|---|---|---|---|---|
| **Treatment** | **Control** | **Fertilized** | **Control** | **Fertilized** |
| **Organic** | | | | |
| Bulk density (*g* $cm^{-3}$) | 0.13 ± 0.03 | 0.31 ± 0.22 | 0.07 ± 0.01 | 0.11 ± 0.03 |
| Horizon depth (cm) | 11.88 ± 3.63 | 9.50 ± 1.55 | 4.67 ± 0.44 | 4.50 ± 1.04 |
| SOC (%) | 39.34 ± 1.98 | 39.18 ± 5.09 | 45.74 ± 0.81 | 33.90 ± 6.50 |
| Total *N* (%) | 0.85 ± 0.11 | 1.81 ± 0.24 | 0.89 ± 0.08 | 1.24 ± 0.26 |
| C:N ratio | 48.45 ± 5.70 | 21.69 ± 0.82 | 52.10 ± 4.98 | 27.51 ± 2.37 |
| **Mineral** | | | | |
| Bulk density (*g* $cm^{-3}$) | 1.09 ± 0.22 | 0.97 ± 0.36 | 0.95 ± 0.14 | 1.08 ± 0.26 |
| Horizon depth (cm) | 10.75 ± 3.25 | 15.25 ± 2.95 | 16.67 ± 3.33 | 17.67 ± 2.33 |
| SOC (%) | 8.75 ± 2.42 | 9.05 ± 3.08 | 3.14 ± 0.51 | 3.70 ± 0.79 |
| Total *N* (%) | 0.39 ± 0.11 | 0.44 ± 0.15 | 0.17 ± 0.03 | 0.20 ± 0.03 |
| C:N ratio | 22.52 ± 1.34 | 20.80 ± 0.33 | 18.93 ± 0.62 | 18.35 ± 0.72 |

calculated using its volume ($cm^3$) measured upon harvest, and its dry weight ($g$).

### MICROBIAL BIOMASS

Bacterial and fungal biomass was estimated using a direct count method modified from Bloem (1995) and Frey et al. (1999). A five-gram soil sample was added to 45 mL filtered and sterilized (autoclaved) de-ionized water and blended in a Waring blender for 1 min. A 1 mL aliquot was immediately added to 9 mL of filtered sterile de-ionized water, from which five 10 μL sub-samples were pipetted onto one side of a sterile, 10-well (6 mm) microscope slide and allowed to air dry. Separate slides were used for bacteria and fungi, but the 10 μL sub-samples on each slide were from the same soil solution. Bacterial samples were then stained with DTAF (5-(4,6 dichlorotriazin-2-yl) aminofluorescein) while fungal samples were stained with calcifluor M2R fluorescence brightener (Bloem, 1995), rinsed and allowed to air dry. A drop of immersion oil (type FF) was placed on each well and a cover slip was affixed to each slide. All finished samples were stored in the dark at 4°C until direct counts could be made. Bacterial cell counts and fungal hyphal length estimation were made using a confocal microscope at 1500 and 400× magnification, respectively. Bacterial cell counts were converted to bacterial biomass assuming an average dry weight of $6.65 \times 10^{-13}$ $g$ C per bacterial cell (Ilic et al., 2001). Fungal hyphal lengths were estimated using a grid intercept technique by counting the number of times hyphae crossed an ocular lens grid. Fungal hyphal length was estimated using the equation

$$R = \frac{\pi NA}{2H}$$

where $R$ is the total hyphal length, $N$ is the number of times hyphae crossed the horizontal lines on the grid, $A$ is the area of one slide well, and $H$ is the total length of the horizontal grid lines. Fungal biomass was estimated assuming $2.3 \times 10^{-6}$ $g$ C $m^{-1}$ of hyphae (Frey et al., 1999).

### DNA EXTRACTION, PCR, AND PYROSEQUENCING

DNA was extracted from each 0.25 $g$ sub-sample of soil using MoBio PowerSoil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA) following the instructions provided by the manufacturer. Eluted DNA samples were stored at –80°C before processing. The 16S and 18S rRNA genes were amplified for each sample using primer sets of F515/R806 (Bates et al., 2010) and SSU817R/SSU1196 (Borneman and Hartin, 2000), respectively, which were modified for the 454 pyrosequencing platform (Rousk et al., 2010).

Polymerase chain reactions were performed using 25 μL assays; 12.5 μL of KAPA2G Fast Multiplex Mix (Kapa Biosystems, Woburn, MA, USA), 1.25 μL of BSA (10.0 ng $\mu L^{-1}$), 1.25 μL of each primer (10.0 μM), 8.5 μL of PCR grade water and 1.0 μL of a genomic DNA template (1.0 ng $\mu L^{-1}$). The PCR thermal profile was developed following the protocols provided by the manufacturer (Kapa Biosystems, Woburn, MA, USA), which included an initial denaturation and enzyme activation step of 95°C for 3 min, followed by 30 cycles of 95°C for 10 s, 50°C for 10 s and 72°C for 1 s. PCR products were evaluated for amplification and their lengths by agarose gel electrophoresis, and purified with the UltraClean® PCR Clean-UP Kit (MO BIO Laboratories, Inc., Carlsbad, CA, USA). Purified PCR products were quantified using Quant-iTTM PicoGreen® (Invitrogen, Molecular Probes, Inc., Eugene, OR, USA) and pooled with an equal quantity of each PCR product for 16S and 18S separately following instructions by Selah Genomics (Greenville, SC, USA) where the pooled PCR products were sequenced on a Roche 454 FLX sequencer.

### SEQUENCING DATA PROCESSING

Sequences were processed using the QIIME 1.8.0 toolkit (Caporaso et al., 2010a). Sequences were assigned to operational taxonomic units (OTUs) at the ≥97% similarity level (Stackebrandt and Goebel, 1994). Taxonomy was assigned to each OTU via the Ribosomal Database Project (RDP, Wang et al., 2007; Cole et al., 2009) classifier for bacteria and NCBI BLAST (Johnson et al., 2008) for fungi. After singletons were removed, the remaining sequences were aligned using PyNAST (Caporaso et al., 2010b) and filtered to construct a phylogenetic tree using FastTree (Price et al., 2009). The bacterial and fungal sequences were normalized via random sub-sampling at 509 and 1136 reads per sample, respectively, for downstream analyses. We used UniFrac (Hamady et al., 2010), distant-based redundancy analysis (dbRDA, Legendre and Anderson, 1999), Phylocom (Webb et al., 2008), and four additional indices to assess differences in bacterial and fungal community composition. The four additional indices to assess microbial diversity included Shannon (Ludwig and Reynolds, 1988), observed OTUs, Chao 1 (Chao, 1984), and phylogenetic diversity (PD, Faith and Baker, 2007). UniFrac distances, both weighted (quantitative with relative abundances considered) and unweighted (qualitative with only presence or absence of OTUs considered), were computed among the samples and principal coordinate analyses (PCoA) were conducted using the QIIME. Using Phylocom analysis in the QIIME, we calculated net relatedness index (NRI) and nearest taxon index (NTI), which measure phylogenetic dispersion (Swenson, 2009). Relative bacterial abundance at the phylum and class levels and fungal abundance at the class level were used for dbRDA to assess relative contributions of taxa to the differences among the fertilization treatments, sites and soil horizons. Sequences were deposited to the MG-RAST server (http://metagenomics.anl.gov/) and are available to the public (accession numbers from 4574203.3 to 4574311.3, total 109 data sets).

### STATISTICAL ANALYSES

All computations were carried out using the *vegan* and *lme4* packages in R Development Core Team (2013). The *vegan* package was used for dbRDA. For the other statistical analysis, we used a mixed-effect analysis of variance (ANOVA) via the *lmer* function in the *lme4* package with the sites (i.e., the 1989 and 2006 sites), fertilization levels (i.e., control and high) and horizons (i.e., organic and mineral soils) as fixed effects, and blocks as a random effect. In all the mixed-effect ANOVA, we used average values of sub-samples nested within each plot, which was the experimental and statistical unit. A significance level of $P \leq 0.10$ was employed to assess statistical significance due to high soil heterogeneity and relatively

small sample sizes in this study, and all *P*-values are for two-sided confidence intervals.

## RESULTS

### MICROBIAL BIOMASS

Neither bacterial nor fungal biomass was affected by the fertilization treatments (**Figure 1**). Both bacterial and fungal biomass was greater in organic than mineral soils (**Figure 1**). Organic soils had 2.3 and 1.5 times greater bacterial biomass than mineral soils at the 1989 and 2006 sites, respectively (**Figure 1**). Organic soils had 4.4 and 4.5 times greater fungal biomass than mineral soils at the 1989 and 2006 sites, respectively (**Figure 1**).

### MICROBIAL COMMUNITY COMPOSITION

#### *Bacterial community composition*

Bacterial community composition at the OTU level was altered by fertilization in organic soils, but not in mineral soils (**Figure 2**). This alteration was supported by significant fertilization × horizon interaction effects in PC2 scores in both weighted and unweighted UniFrac ($P = 0.006$ and 0.023, respectively; **Table 2**) in combination with significant main fertilization effects in PC2 scores of weighted and unweighted UniFrac ($P = 0.057$ and 0.017, respectively; **Table 2**). The bacterial community alteration in organic soils was more pronounced in the 1989 site with a longer fertilization history than the 2006 site (**Figure 2**). This was supported by a significant three-way interaction (i.e., fertilization × site × horizon) for PC2 scores of weighted UniFrac ($P = 0.017$; **Table 2**). At the coarser phylogenetic levels of phylum and class, fertilization also altered bacterial community composition in organic soils, but not in mineral soils (**Figure 3**). However, the alteration in organic soils was not directional between the two sites. Fertilization increased Proteobacteria, and reduced Actinobacteria and Acidobacteria at the phylum level, and increased α-, β-, and γ-Proteobacteria and decreased Actinobacteria at the class level in relative abundances at the 1989 site, but those changes in relative abundance were opposite at the 2006 site (**Figures 3** and **4**). Those bi-directional alterations by fertilization in organic soils were supported by significant three-way interactions (i.e., fertilization × site × horizon) in axis 2 scores of dbRDA at the phylum ($P = 0.016$) and class ($P = 0.020$) levels (**Table 3**). Shannon Index was reduced by the fertilization treatments ($P = 0.077$; **Table 4**; **Figure 5**).

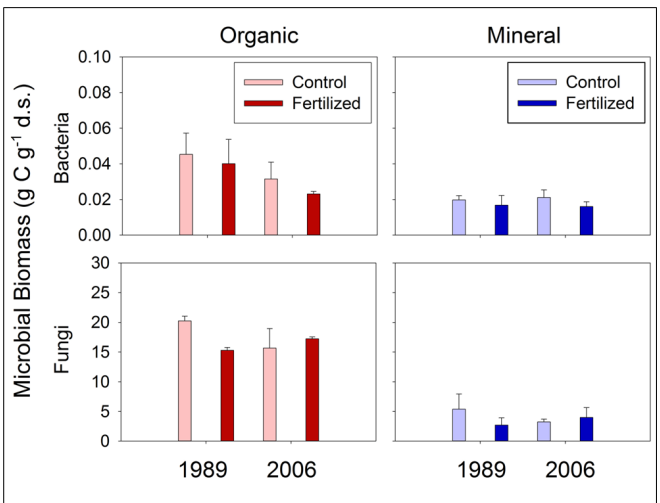


**FIGURE 1 | Bacterial and fungal biomass in organic and mineral soil horizons.** There was no significant effect of treatment or site found by mixed-effect ANOVA.

Most of the variation in bacterial community composition at the OTU level was attributed to soil horizon (**Figure 2**; **Table 2**). There were significant differences due to soil horizon in PC1 scores of both weighted and unweighted UniFrac analyses ($P < 0.001$; **Table 2**), which explained 35.1 and 12.6% of variability, respectively, in the bacterial community composition at the OTU level (**Figure 2**). These differences in community composition between the two soil depths were also evident in relative abundances among taxa at the phylum and class levels assessed via dbRDA; main effects of the soil horizons were significant for axis 1 scores at both phylum and class levels ($P < 0.001$; **Table 3**), which explained 75.7 and 69.3% of variation, respectively (**Figure 3**). At the phylum level, the difference between the two soil horizons was primarily derived by relatively higher abundances of Proteobacteria (35.3 vs. 28.3% in organic and mineral soils, respectively) and Actinobacteria (27.7 vs. 11.1% in organic and mineral soils, respectively) in the organic soils and AD3 (0.9 vs. 13.0% in organic and mineral soils, respectively) and Acidobacteria (17.7 vs. 23.4% in organic and mineral soils, respectively) in the mineral soils (**Figures 3** and **4**). At the class level, the differences between soil horizons resulted from relatively higher abundances of Actinobacteria (15.8 vs. 5.3% in organic and mineral soils, respectively), γ-Proteobacteria (11.5 vs. 3.8% in organic and mineral soils, respectively) and α-Proteobacteria (20.2 vs. 14.2% in organic and mineral soils, respectively) in the organic soils and ABS 6 (0.5 vs. 10.0% in organic and mineral soils, respectively) in the mineral soils (**Figures 3** and **4**). The two soil horizons were also significantly different in diversity evident in observed OTUs ($P < 0.001$), Chao 1 ($P < 0.001$) and PD ($P = 0.001$) and phylogenetic dispersion measured as NTI ($P = 0.017$; **Figure 5**; **Table 4**). Observed OTU's and scores of Chao 1 and PD were significantly lower for the organic than mineral soils, and NTI scores were higher for the organic than mineral soils (**Figure 5**; **Table 4**).

The 1989 and 2006 sites had inherently different bacterial community compositions, indicated by significant main site effects in PC2 scores of weighted and unweighted UniFrac ($P = 0.002$ and 0.046, respectively; **Table 2**) as well as axis 2 scores of dbRDA at both phylum and class levels ($P = 0.002$ and 0.001, respectively; **Table 3**). At the phylum level, the 1989 site had a relatively greater abundance of Proteobacteria and lower abundance of Actinobacteria than the 2006 site (**Figures 3** and **4**). At the class level, the 1989 site had a relatively higher abundance of α- and γ-Proteobacteria, and a lower abundance of Actinobacteria than the 2006 site (**Figures 3** and **5**).

Almost all of the bacterial 16S rRNA amplicons were identified at least to the phylum level (30027/30031; **Figure 4**) and 98.0% to the class level (29444/30031; **Figure 4**). At the

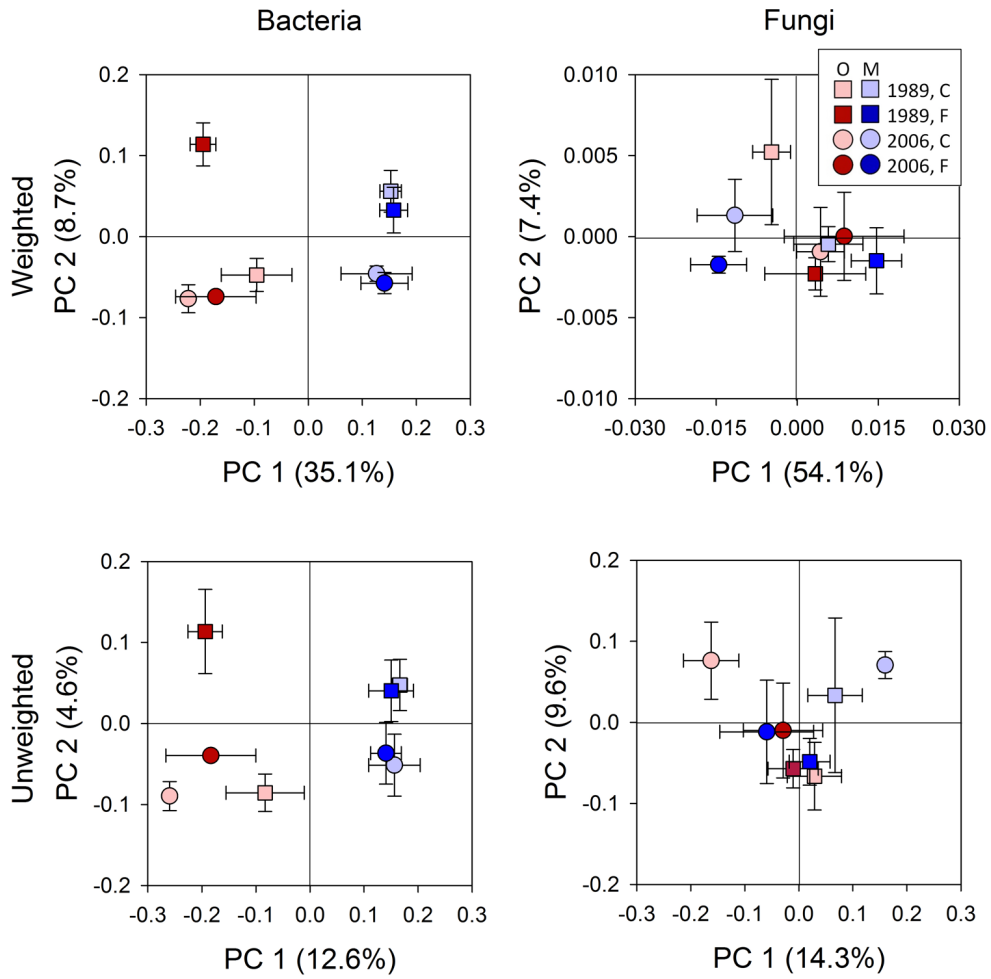


**FIGURE 2 | Results of principle coordinate analysis of bacterial and fungal communities based on weighted and unweighted UniFrac distance metrics.** Statistical results using mixed-effect ANOVA to test effects of fertilization treatments, sites, soil horizons, and their interactions are shown in **Table 2**. Legend: O; organic soils, M; mineral soils, C; control, and F; fertilized.

phylum level, Proteobacteria was the most dominant taxon comprising 33.1%, followed by Acidobacteria (19.3%), Actinobacteria (18.2%), and AD3 (6.9%, **Figure 4**). These four phyla constituted 78.7% of the overall abundance of bacteria found in this study (**Figure 4**). In organic soils, Proteobacteria (35.3%), Acidobacteria (17.7%), and Actinobacteria (27.7%) constituted 80.7% (**Figure 4**). In mineral soils, Proteobacteria (28.3%), Acidobacteria (23.4%), AD3 (13.0%) and Actinobacteria (11.1%) constituted 75.8% (**Figure 4**). At the class level, α-Proteobacteria was most abundant across the soil horizons, constituting 17.2% in the overall abundance (**Figure 4**). In the organic soils, α-Proteobacteria (20.2%), Actinobacteria (15.8%) and γ-Proteobacteria (11.5%) were three most abundant taxa, constituting 47.5% of the overall abundance in the soil horizon (**Figure 4**). In the mineral soils, α-Proteobacteria (14.2%), ABS-6 (10.0%) and β-Proteobacteria (8.4%) are three most abundant taxa, constituting 32.7% of the overall abundance of bacteria found in the mineral soils (**Figure 4**).

### *Fungal community composition*

The fertilization treatments did not influence fungal community composition. None of the primary scores from UniFrac or dbRDA had a significant main effect of fertilization (**Tables 2** and **3**). No significant fertilization effect was found for any of the diversity or phylogenetic dispersal indices, except NTI which was increased by fertilization ($P = 0.048$; **Table 4**; **Figure 6**).

The soil horizons were not a strong driver of fungal community composition structure as the only significant horizon effect was found in PC1 scores of unweighted UniFrac ($P = 0.026$; **Table 2**; **Figure 2**). There was no significant main effect of the soil horizons for the diversity or dispersal indices (**Figure 6**; **Table 4**).

Three diversity indices (Shannon Index, observed OTUs and Chao 1) and the two dispersion indices (NRI and NTI) showed similar trends in fungal community composition (**Figure 6**). Fertilization consistently increased these indices in mineral soils in both 1989 and 2006 sites (**Figure 6**). However, such consistent fertilization effects were not observed in organic soils; fertilization increased these indices in the 1989 site, but decreased them in

**Table 2 | Results of statistical analyses (*P*-values) for two primary PCoA scores from weighted and unweighted UniFrac for bacteria and fungi.**

| Independent variables | Weighted | | Unweighted | |
|---|---|---|---|---|
| | PC1 | PC2 | PC1 | PC2 |
| **Bacteria** | | | | |
| F | 0.935 | **0.057** | 0.789 | **0.017** |
| S | 0.405 | **0.002** | 0.344 | **0.046** |
| H | **<0.001** | 0.278 | **<0.001** | 0.285 |
| F × S | 0.171 | **0.036** | 0.177 | 0.245 |
| F × H | 0.778 | **0.006** | 0.823 | **0.023** |
| S × H | 0.362 | 0.676 | 0.131 | 0.914 |
| F × S × H | 0.112 | **0.017** | **0.071** | 0.119 |
| **Fungi** | | | | |
| F | 0.357 | 0.158 | 0.265 | 0.136 |
| S | 0.115 | 0.815 | 0.346 | 0.105 |
| H | 0.386 | 0.493 | **0.026** | 0.522 |
| F × S | 0.433 | 0.360 | 0.995 | 0.544 |
| F × H | 0.739 | 0.695 | **0.026** | 0.580 |
| S × H | **0.005** | 0.405 | 0.137 | 0.466 |
| F × S × H | 0.679 | 0.121 | **0.031** | 0.548 |

*F, S, and H represent fertilization, site and soil horizon, respectively. F × S, F × H, S × H, and F × S × H represent their corresponding interactions. P-values equal to or less than 0.100 are shown bold.*

the 2006 site (**Figure 6**). These site- and horizon-dependent fertilization effects were supported by significant fertilization × site interaction effects for Shannon Index ($P = 0.071$), observed OTUs ($P = 0.015$), Chao 1($P = 0.021$), NRI ($P = 0.019$) and NTI ($P = 0.038$), significant fertilization × horizon interaction effects for NRI ($P = 0.068$) and NTI ($P = 0.031$), and significant three-way interaction effects (i.e., fertilization × site × horizon) for Shannon Index ($P = 0.049$), observed OTUs ($P = 0.031$), Chao 1 ($P = 0.085$), NRI ($P = 0.031$), and NTI ($P = 0.021$; **Table 4**).

For fungal 18S rRNA amplicons, 93.0% could be identified at least at the phylum level (66587/71568; **Figure 7**) and 63.8% at the class level (45673/71568; **Figure 7**). At the phylum level, Ascomycota and Basidiomycota constituted 50.5 and 40.7% of overall abundances, respectively (**Figure 7**). At the class level, Agaricomycetes was most abundant (38.7%) followed by Leotiomycetes (11.5%) and Dothideomycetes (5.5%; **Figure 7**).

### *Relative abundances of ectomycorrhizal taxa*

The relative abundances of mycorrhizal fungi among all fungal OTU's are shown in **Figure 8**. Three taxa of mycorrhizal fungi were detected among the sequenced fungal taxa; two ectomycorrhizal taxa of *Russula* and *Cortinarius* spp. (Newsham et al., 2009), and members of the order *Helotiales* which include many ericoid mycorrhizal spp. (Walker et al., 2011). There was a significant effect of the soil horizons for the abundances of *Cortinarius* spp. and the total ectomycorrhizal taxa ($P = 0.076$ and 0.061, respectively; **Table 5**). This suggested that relative abundances of *Cortinarius* spp. and the three ectomycorrhizal taxa combined were higher in mineral than organic soils among all fungal OTU's found in this study (**Figure 8**).

## DISCUSSION

Long-term fertilization altered bacterial community composition to a greater degree in the organic than mineral soils, and at the 1989 compared to the 2006 site. The change in bacterial composition was accompanied by a reduction in evenness among OTUs. Long-term fertilization at the 1989 site increased copiotrophic α- and β-Proteobacteria, and decreased oligotrophic Acidobacteria as predicted, most likely as a result of increased labile C input via rhizodeposition caused by stimulated net primary productivity of vascular plants (Ramirez et al., 2010). These bacterial responses to fertilization were consistent with our *a priori* predictions based on the generalized ecology of these taxa with respect to nutrient and C availability (Fierer et al., 2007). In contrast to the bacteria community, fungal community composition was not as sensitive to the fertilization treatments. The insensitivity of the fungal communities included the mycorrhizal taxa associated with shrubs, which became dominant in response to fertilization. This fungal response to fertilization is different from results found in warming experiments using greenhouses in the same ecosystem (e.g., Deslippe et al., 2012). Though fertilization is often used as a means to mimic an effect of warming (i.e., increased nutrient availability in soils, Mack et al., 2004; Aerts, 2010), the fungal dynamics in response to fertilization do not appear to emulate the fungal dynamics under warming in Arctic tundra (Rinnan et al., 2007, 2013).

### BACTERIAL COMMUNITIES

Reduction in the Shannon Index for bacterial communities by the fertilization treatments was significant (**Figure 6**; **Table 4**). Given no significant difference in observed numbers of OTUs between the treatments (**Figure 6**; **Table 4**), the fertilization treatments reduced evenness of bacterial community compositions at the OTU level (Ludwig and Reynolds, 1988). These observations are consistent with long-term fertilization experiments in several ecosystems, including Arctic tundra (24-year fertilization, Campbell et al., 2010), grassland (20-year fertilization, Coolon et al., 2013), coniferous forest (10-year fertilization, Burke et al., 2006), and agricultural fields including pasture (12-year fertilization, Jangid et al., 2008) and corn fields (*Zea mays*; 8-year fertilization, Ramirez et al., 2010). However, reduction of microbial diversity by fertilization is not a universal phenomenon; a fertilization experiment in a hardwood forest at Harvard Forest in Massachusetts, USA, showed increased bacterial diversities (20-year fertilization, Turlapati et al., 2013), and no significant change was found in a grassland ecosystem at Cedar Creek in Minnesota, USA (27-year fertilization, Ramirez et al., 2010; Fierer et al., 2012).

Bacterial community composition at the phylum level was significantly different between the organic and mineral soil horizons (**Figure 3**; **Table 3**), as organic soils had a relatively higher abundance of Bacteroidetes and a lower abundances of Acidobacteria (**Figures 3** and **4**). The differences in the relative abundances

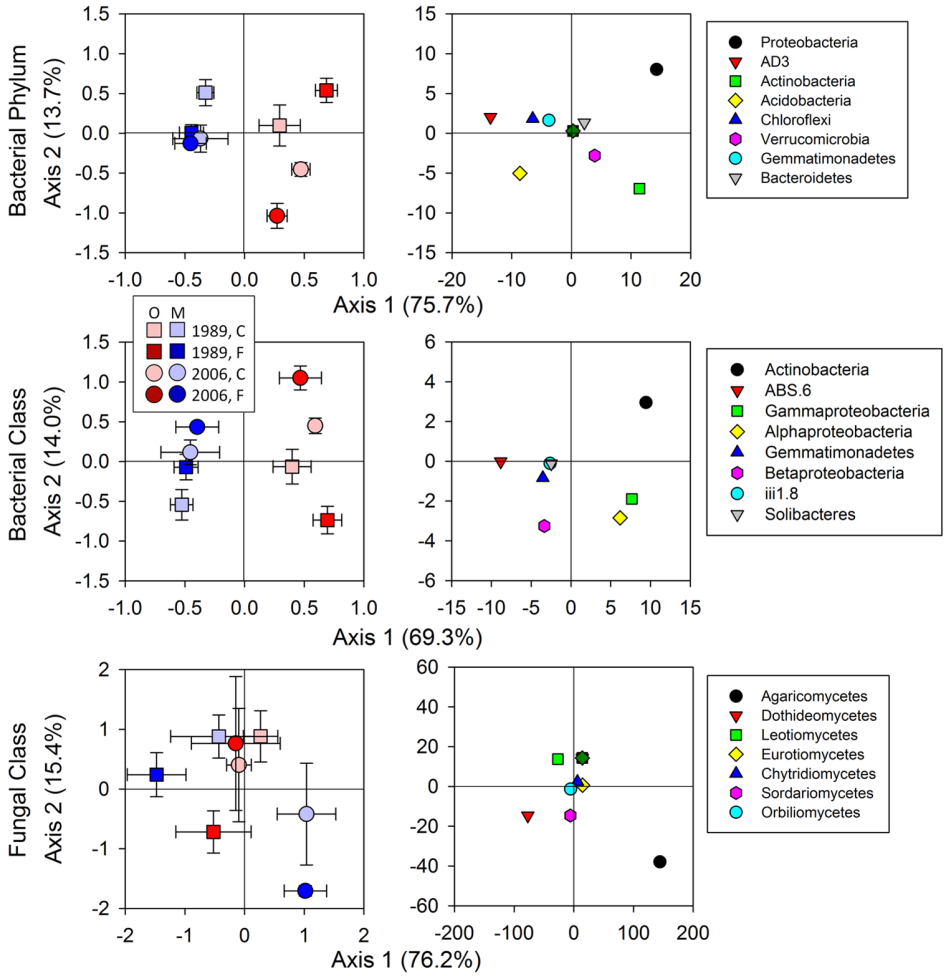


**FIGURE 3 | Results of dbRDA for relative abundances in bacterial and fungal communities.** Bacterial communities were assessed at the Phylum and Class levels and fungal at the Class level only. Statistical results using mixed-effect ANOVA to test effects of fertilization treatments, sites, soil horizons, and their interactions are shown in **Table 3**. Legend: O; organic soils, M; mineral soils, C; control, and F; fertilized.

can be explained by differences in the availability of C substrates within the two soil horizons (Fierer et al., 2007): the organic soils had more organic C than the mineral soils (**Table 1**), thus copiotrophic Bacteroidetes and oligotrophic Acidobacteria were expected to be relatively more abundant in the organic and mineral soils, respectively. This was also the case for copiotrophic α-Proteobacteria, which were relatively more abundant in the organic soils compared to the than mineral soils (**Figures 3** and **4**). However, β-Proteobacteria were more abundant in the mineral than organic soils even though the phylum is considered copiotrophic (**Figures 3** and **4**; Fierer et al., 2007). These differences in the bacterial community composition between the organic and mineral soils in this study are consistent with those reported by Campbell et al. (2010) who investigated bacterial community structures using high-throughput sequencing in the same study area. However, in contrast to the results found at both 1989 and 2006 sites in this study (**Figure 3**) and by Wallenstein et al. (2007), Campbell et al. (2010) found that bacterial community composition was relatively similar between shrub organic and mineral soils at the phylum/class levels at this study area.

The responses of bacterial phylum and class community composition to fertilization were not uniform across sites, fertilization histories or soil horizons, as indicated by the a significant three-way interaction effect of fertilization × site × horizon in axis 2 scores of dbRDA for both bacterial phylum and class (**Table 3**; **Figure 3**). The organic soils from the fertilized plots within the1989 site, which were expected to be most affected by fertilization because of their position and longer treatment history, had a lower relative abundance of oligotrophic Acidobacteria, and higher relative abundances of copiotrophic α-, β-, and γ-Proteobacteria than soils from the control plots within the site (**Figures 3** and **4**). These observations supported our first prediction that fertilization should increase the relative abundance of copiotrophs and reduce the abundance of oligotrophs (Cleveland et al., 2007; Fierer et al., 2007, 2012; Nemergut et al., 2010). We cannot separate a direct effect of increased N and P from an indirect effect via plants (i.e., increased productivity and community

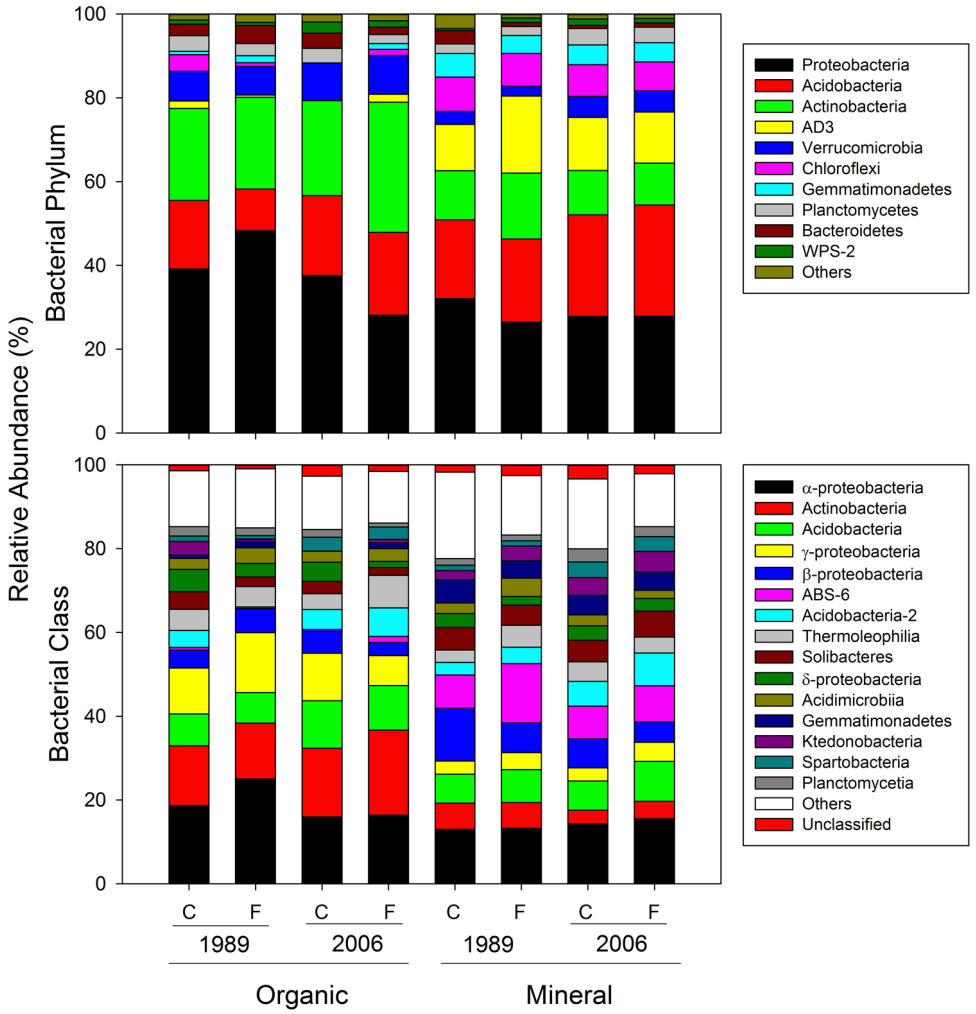


**FIGURE 4 | Mean relative abundances of bacterial taxa at the phylum and class level.**

shift) on the altered bacterial community compositions. However, we can speculate that the consistent shifts in community compositions of the copiotrophs and oligotroph were likely caused by increased net primary productivity of vascular plants (Fierer et al., 2007; Ramirez et al., 2010), especially shrubs (Chapin and Shaver, 1985; Chapin et al., 1995; Shaver et al., 2001). We ruled out a potential mechanism that fertilization stimulated microbial production of C-degrading extracellular enzymes (Koyama et al., 2013), resulting in increased C availability, which in turn altered bacterial community composition. Koyama et al. (2013) found that fertilization consistently increased C-degrading extracellular enzyme activities in organic horizons of both 1989 and 2006 sites, but bacterial community shifts at the phylum and class levels were opposite between the two sites (**Figure 3**). Another mechanism that fertilization increased below-ground litter input was also unlikely as Sullivan et al. (2007) demonstrated that long-term fertilization decreased root production at the community level. Our finding of bacterial community shift with fertilization in the 1989 site was consistent with Campbell et al. (2010) who found increased relative abundances of α- and γ-Proteobacteria and a reduced relative abundance of Acidobacteria by a 24-year fertilization in the same ecosystem.

The results from analyses using finer taxonomic levels, including UniFrac, need to be interpreted with caution, given seasonal variation in communities and the single sample date used in this study. Even though bacterial community compositions are relatively stable across seasons in this ecosystem (e.g., Deslippe et al., 2012) especially at coarse taxonomic levels (e.g., phylum), they can be different in finer taxonomic levels (Wallenstein et al., 2007).

## FUNGAL COMMUNITIES

We found that fertilization did not significantly affect the relative abundance of mycorrhizal fungi detected in this study (**Figure 8**; **Table 5**). These findings contradicted our prediction that the fertilization treatments had increased relative abundances of these ectomycorrhizal fungi associated with shrubs, including *B. nana*, which became dominant in response to fertilization. There are

**Table 3 | Results of statistical analyses (*P*-values) for two primary scores from dbRDA for bacteria and fungi.**

| Independent variables | Bacterial phylum | | Bacterial class | | Fungal class | |
|---|---|---|---|---|---|---|
| | Axis 1 | Axis 2 | Axis 1 | Axis 2 | Axis 1 | Axis 2 |
| F | 0.768 | 0.218 | 0.806 | 0.224 | 0.290 | 0.138 |
| S | 0.759 | **0.002** | 0.681 | **0.001** | **0.057** | 0.267 |
| H | **<0.001** | **0.042** | **<0.001** | 0.172 | 0.696 | 0.166 |
| F × S | 0.135 | 0.301 | 0.275 | **0.083** | 0.325 | 0.497 |
| F × H | 0.364 | 0.421 | 0.814 | 0.127 | 0.893 | 0.669 |
| S × H | 0.800 | **0.018** | 0.875 | **0.053** | **0.034** | **0.022** |
| F × S × H | **0.035** | **0.016** | 0.083 | **0.020** | 0.863 | 0.126 |

*F, S, and H represent fertilization, site and soil horizon, respectively. F × S, F × H, S × H, and F × S × H represent their corresponding interactions. P-values equal to or less than 0.100 are shown bold.*

three possible explanations for this observation; (1) fertilization reduced infection rates of ectomycorrhizal fungi in shrub roots; (2) increased nutrient availability did not increase below-ground shrub biomass; and (3) fertilization increased saprotrophic fungi, which in turn reduced relative abundances of ectomycorrhizal fungi. The first explanation was consistent with a finding by Urcelay et al. (2003) who reported that ectomycorrhizal infection rates of *B. nana* root were reduced almost by half following 3-year fertilization in this study area. Both *Russula* and *Cortinarius* spp. are considered nitrophobic and have been shown to decrease in abundance when N availability is high (Lilleskov et al., 2002). Other studies have shown that mycorrhizal infection rates often decrease following fertilization in many ecosystems. In a meta-analysis, Treseder (2004) found that fertilization of N and P tended to decrease mycorrhizal abundances across biomes, though this meta-analysis did not include Arctic tundra. The second explanation was supported by Gough et al. (2012) who reported that an 11-year fertilization treatment significantly increased above-ground plant biomass, especially *B. nana*, without significant change in below-ground plant biomass in moist acidic tundra adjacent to our study sites. Such lack of change in below-ground biomass was most likely caused by reduced allocation of resources to roots by plants with abundant nutrients (Treseder and Vitousek, 2001). We ruled out the third explanation given that the fungal biomass between the two treatments did not differ significantly (**Figure 1**). Taken together, our finding of no change in relative abundances of ectomycorrhizal fungi was most likely caused by little change in root biomass of shrubs and reduced infection rates in shrub roots under increased nutrient availability.

## COMPARISON WITH WARMING EFFECTS ON ARCTIC TUNDRA ECOSYSTEMS

Fertilization, typically N and P, has been used to obtain insights for ecosystem responses to warming in Arctic tundra ecosystems (Mack et al., 2004; Aerts, 2010); warming stimulates SOM decomposition, resulting in increased nutrient availability for plants and soil microbes (Chapin et al., 1995; Hartley et al., 1999; Rustad et al., 2001; Schmidt et al., 2002; Schimel et al., 2004; Aerts et al.,

**Table 4 | Results of statistical analyses (*P*-values) for the indices to assess bacterial diversity.**

| Independent variables | Shannon | Observed OTUs | Chao 1 | PD | NRI | NTI |
|---|---|---|---|---|---|---|
| **Bacteria** | | | | | | |
| F | **0.077** | 0.136 | 0.267 | 0.164 | 0.204 | 0.195 |
| S | 0.250 | 0.616 | 0.437 | 0.856 | 0.606 | 0.363 |
| H | **0.089** | **0.001** | **<0.001** | **0.001** | 0.611 | **0.017** |
| F × S | 0.869 | 0.947 | 0.428 | 0.799 | 0.367 | 0.353 |
| F × H | 0.188 | 0.292 | 0.740 | 0.125 | 0.765 | 0.991 |
| S × H | 0.172 | **0.073** | 0.226 | **0.018** | **0.003** | 0.556 |
| F × S × H | 0.521 | 0.781 | **0.077** | 0.989 | 0.692 | 0.406 |
| **Fungi** | | | | | | |
| F | 0.983 | 0.209 | 0.103 | 0.564 | 0.110 | **0.048** |
| S | 0.904 | 0.385 | 0.558 | 0.974 | 0.305 | 0.468 |
| H | 0.624 | 0.908 | 0.516 | 0.107 | 0.147 | 0.317 |
| F × S | **0.071** | **0.015** | **0.021** | 0.165 | **0.019** | **0.038** |
| F × H | 0.466 | 0.269 | 0.309 | 0.457 | **0.068** | **0.031** |
| S × H | 0.791 | 0.481 | 0.278 | 0.228 | 0.696 | 0.329 |
| F × S × H | **0.049** | **0.031** | **0.085** | 0.576 | **0.031** | **0.021** |

*F, S, and H represent fertilization, site and soil horizon, respectively. F × S, F × H, S × H, and F × S × H represent their corresponding interactions. P-values equal to or less than 0.100 are shown bold.*

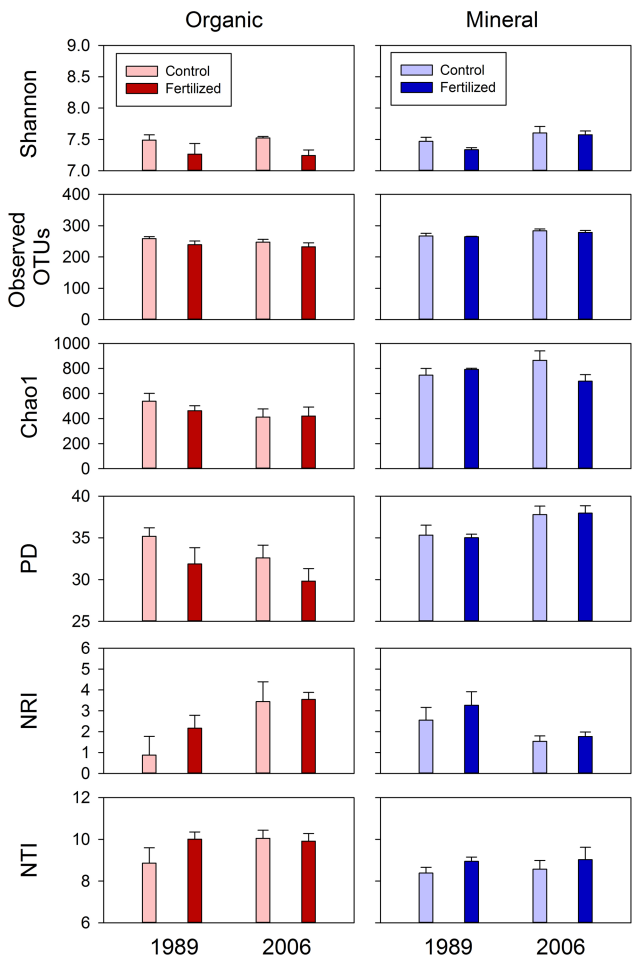


**FIGURE 5 | Diversity (Shannon Index, observed OTUs and Chao 1 and PD) and dispersion (NRI and NTI) indices of bacterial communities for organic and mineral soils from control and fertilized treatments collected from the 1989 and 2006 sites.** Statistical results using mixed-effect ANOVA to test effects of fertilization treatments, sites, soil horizons, and their interactions are shown in **Table 4**.

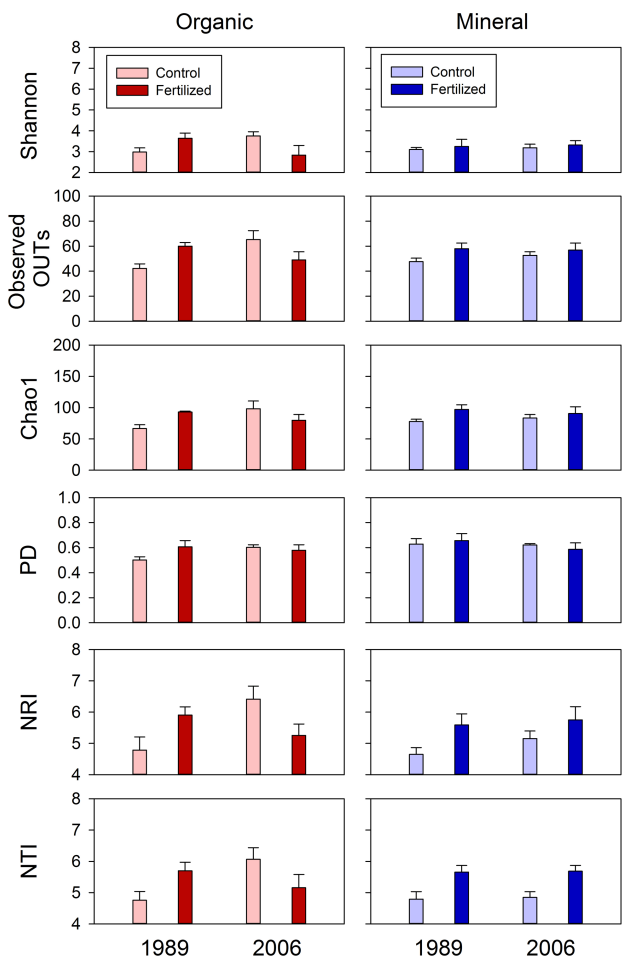


**FIGURE 6 | Diversity (Shannon Index, observed OTUs and Chao 1 and PD) and dispersion (NRI and NTI) indices of fungal communities for organic and mineral soils from control and fertilized treatments collected from the 1989 and 2006 sites.** Statistical results using mixed-effect ANOVA to test effects of fertilization treatments, sites, soil horizons, and their interactions are shown in **Table 4**.

2006). In a few Arctic tundra ecosystems, warming and fertilization showed similar effects on above-ground vegetation dynamics (e.g., Michelsen et al., 1996) and ectomycorrhizal fungal abundance (Clemmensen et al., 2006). However as noted below, these cases appear to be exceptions rather than rules. Warming and fertilization experiments tended to show different effects on plant community composition (Chapin et al., 1995; Press et al., 1998; Campioli et al., 2012), above-ground biomass (Chapin et al., 1995; Sorensen et al., 2008), soil microbial biomass (Rinnan et al., 2013), soil microbial community composition (Deslippe et al., 2011), and carbon storage (Mack et al., 2004; Sistla et al., 2013). Thus, fertilization does not appear to be an appropriate proxy for warming in this ecosystem.

## MICROBIAL DIVERSITY AND STABILITY IN ECOSYSTEM PROCESSES

High plant community diversity can stabilize temporal productivity via species asynchrony (Tilman et al., 2006; Isbell et al., 2009; Hector et al., 2010); as environmental variability reduces productivity of some plant species, other species compensate the reduction. This type of response has been observed in Arctic tussock and wet meadow tundra in the same site as this study was conducted (Chapin and Shaver, 1985). Chapin and Shaver (1985) observed that community level above-ground net primary productivity was relatively stable from year to year, even though production of individual plant species showed great variation along with environmental fluctuations over time. Species asynchrony in these communities was supported by an environmental manipulation experiment that demonstrated no single factor limiting productivity of all the species in the same study site (Chapin and Shaver, 1985). Fertilization experiments that included a network of 41 different grassland sites demonstrated that increased nutrient availability decreased stability in temporal productivity, not because species diversity at the sites decreased, but because fertilization had increased temporal variability in productivity and,

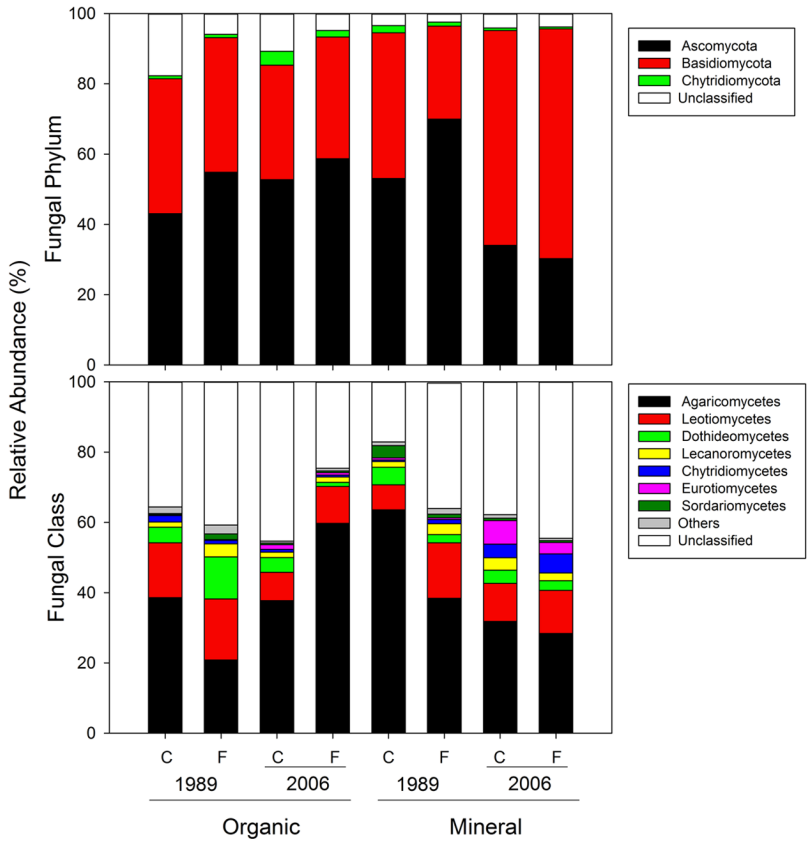


**FIGURE 7 | Mean relative abundances of fungal taxa at the phylum and class levels for organic and mineral soils from control and fertilized treatments collected from the 1989 and 2006 sites.**

at the same time, decreased asynchrony of diverse plant communities, which would otherwise stabilize plant biomass production over time (Hautier et al., 2014).

How the concept of species asynchrony applies to soil microbial diversity and its effects on the stability of ecosystem processes mediated by soil microbes is unclear. In a meta-analysis, Allison and Martiny (2008) showed that microbial communities altered by disturbances often change ecosystem process rates, suggesting a potential relationship between microbial diversity and stability. In this study, fertilization reduced soil bacterial diversity, indicated by a reduced Shannon Index (**Figure 5**; **Table 4**). In particular, the relative abundance of oligotrophic Acidobacteria was reduced, and that of copiotrophic taxa of Proteobacteria was increased in the organic soils in the longer fertilization (**Figures 3** and **4**). These observed changes in microbial diversity could contribute to increased potential activities of C-degrading enzymes and altered stoichiometry between C- and N-degrading enzyme activities in the same soils used to assess microbial community structures in this study (Koyama et al., 2013). However, we do not know how these changes in microbial diversity and processes (e.g., extracellular enzyme production) result in short-term (seasonal within a year) and long-term (year to year) temporal stability of ecosystem processes. Two characteristics of soil microbes make it challenging to predict the relationship; (1) higher diversity of soil microbes than plant species and (2) microbial dormancy. One gram of soil can contain more than 10,000 OTUs of bacteria (Roesch et al., 2007) and their functions can be redundant (Allison and Martiny, 2008). Thus, extreme abundance and diversity, and functional redundancy of soil microbes can buffer change in stability in ecosystem processes caused by reduced microbial diversity. In addition, not all the microbes found by the method employed in this study were active at a given time. McMahon et al. (2011) demonstrated that active bacterial communities were different between summer and winter in shrub soils in the same study site. This microbial dormancy will add another complexity to assess microbial diversity and its effects on stability in ecosystem processes.

## CONCLUSION

Given the dominant role of N in limiting both plant and microbial activity in Arctic tundra, it was not surprising that long-term fertilization strongly affected bacterial diversity and community composition. Long-term fertilization reduced bacterial evenness at the OTU level, increased copiotrophic classes (α-Proteobacteria and β-Proteobacteria) and reduced a dominant oligotrophic phylum (Acidobacteria). This diverse soil bacterial community plays a critical role in cycling nutrients, including N, stored in SOM of varying recalcitrance in Arctic tundra. Thus, changes in the

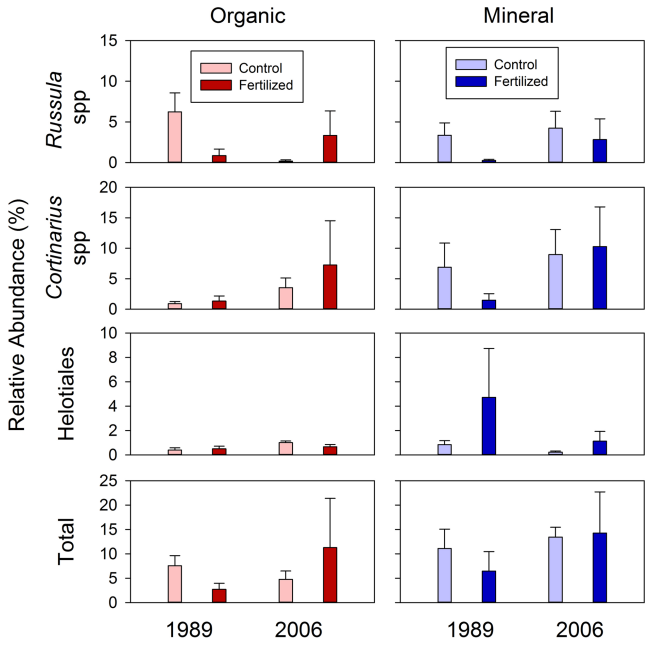


**FIGURE 8 | Relative abundances of ectomycorrhizal taxa, *Russula* and *Cortinarius* spp., and members of the order *Helotiales* among rarefied fungal taxa for organic and mineral soils from control and high fertilized treatments collected from the 1989 and 2006 sites.** Statistical results using mixed-effect ANOVA to test effects of fertilization treatments, sites, soil horizons and their interactions are shown in **Table 5**.

**Table 5 | Results of statistical analyses (*P*-values) for the indices to assess relative abundances of ectomycorrhizal taxa.**

| Independent variables | *Russua* spp. | *Cortinarius* spp. | *Helotiales* | Total |
|---|---|---|---|---|
| F | 0.163 | 0.411 | 0.338 | 0.169 |
| S | 0.966 | 0.357 | 0.706 | 0.527 |
| H | 0.566 | **0.076** | 0.447 | **0.061** |
| F × S | 0.112 | 0.788 | 0.652 | 0.273 |
| F × H | 0.319 | 0.548 | 0.192 | 0.942 |
| S × H | 0.154 | 0.602 | 0.118 | 0.364 |
| F × S × H | 0.212 | 0.366 | 0.878 | 0.634 |

*F, S, and H represent fertilization, site and soil horizon, respectively. F × S, F × H, S × H, and F × S × H represent their corresponding interactions. P-values equal to or less than 0.100 are shown bold.*

composition of this community due to N availability are likely to impact SOM cycling. On the other hand, chronic fertilization did not significantly affect fungal community composition, including ectomycorrhizal fungi, despite increases in the abundance of their host plants (e.g., *B. nana*). This insensitivity of ectomycorrhizal fungi suggests reduced resource allocation to below-ground of their host plants in response to the chronic fertilization. Nitrogen appears to affect bacteria and fungi in different ways, indicating that changes in N availability could restructure below-ground communities and food webs.

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# Microbial lipid and amino sugar responses to long-term simulated global environmental changes in a California annual grassland

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Global environmental change is predicted to have major consequences for carbon cycling and the functioning of soil ecosystems. However, we have limited knowledge about its impacts on the microorganisms, which act as a "valve" between carbon sequestered in soils versus released into the atmosphere. In this study we examined microbial response to continuous 9-years manipulation of three global change factors (elevated $CO_2$, warming, and nitrogen deposition), singly and in combination using two methods: lipid and amino sugar biomarkers at the Jasper Ridge Global Change Experiment (JRGCE). The two methods yielded important distinctions. There were limited microbial lipid differences, but many significant effects for microbial amino sugars. We found that $CO_2$ was not a direct factor influencing soil carbon and major amino sugar pools, but had a positive impact on bacterial-derived muramic acid. Likewise, warming and nitrogen deposition appeared to enrich residues specific to bacteria despite an overall depletion in total amino sugars. The results indicate that elevated $CO_2$, warming, and nitrogen deposition all appeared to increase bacterial-derived residues, but this accumulation effect was far offset by a corresponding decline in fungal residues. The sensitivity of microbial residue biomarker amino sugars to warming and nitrogen deposition may have implications for our predictions of global change impacts on soil stored carbon.

**Keywords: lipid, amino sugar, microbial biomass, microbial residue, warming, nitrogen deposition, elevated $CO_2$, soil carbon stabilization**

## Introduction

Within the context of global change, it is recognized that carbon (C) stabilization in soils is of critical importance, and a better understanding of C biogeochemistry is needed (Lal, 2004; Davidson and Janssens, 2006). Because soil C cycling is ultimately the consequence of microbial growth and activity, the mechanistic basis for understanding C decomposition, transformation, and stabilization in soils lies in a detailed understanding of general microbial physiology and activities, which may act as a "valve" between C sequestered in soils versus released into the atmosphere. It has been well established that the dynamics of the terrestrial C pool are heavily influenced by the catabolic

and anabolic activities of microorganisms (Balser, 2005; Schimel and Schaeffer, 2012), and that these activities are essential for biogeochemical cycling, climate change, and ecosystem sustainability (Schimel et al., 2007; Bardgett et al., 2008; Liang and Balser, 2011). However, the direct incorporation of microbial residues (microbial cellular components from both living and senesced biomass), into stable soil C pools (specifically those whose turnover time can be on the order of centuries) has received less attention (Liang et al., 2011; Miltner et al., 2012; Lee and Schmidt, 2014).

Microorganisms can be considered responsible for both the formation and turnover of stable soil C. They decrease the stable C pool by the process of decomposition, but also can contribute to it by production and turnover of their biomass (Liang et al., 2011; Miltner et al., 2012). Historically, direct microbial contribution to soil C sequestration has been regarded as low, and has been considered negligible or even ignored in many instances, as active microbial biomass makes up <5% of soil organic matter (Wardle, 1992; Dalal, 1998), and has been reported as <4% of soil organic C (Anderson and Joergensen, 1997). However, this may be misleading as living biomass alone does not properly indicate long-term C dynamics (Potthoff et al., 2008). Microorganisms can utilize easily degradable substrates for biomass synthesis, and residual parts of their biomass accumulate in soils when they turn over. In the iterative process of cell generation, growth and death, microorganisms continuously add to soil stable C pool. Therefore, microbial "necromass" (senesced cell components) rather than standing biomass may be a better indicator of microbial contribution to soil C pools.

Microbial residues are now thought to play a far greater role in the sequestration of C into soil stable C pools than traditionally believed (Kindler et al., 2006, 2009; Simpson et al., 2007a; Miltner et al., 2009; Liang et al., 2011). Microbial necromass can exist as relatively recalcitrant polymers, some of which are resistant to decomposition, and have been suggested as important components of the relatively stable C pool in soils (Guggenberger et al., 1999; Glaser et al., 2004; Liang et al., 2008). In fact, because of their rapid growth and constant turnover, microbial residues accumulate greatly over time, and therefore the contribution of microbial-derived C in soils is potentially quite large (Liang et al., 2011). Recent analytical work also confirms this: using nuclear magnetic resonance analysis, it has been found that microbial components and metabolic products are shown to have similar structures to stable humic substances that qualitatively indicates a significant incorporation of microbial-derived C (Simpson et al., 2007a,b). Further, microbial-derived sugars are stabilized in finer soil over time, as indicated by high ratios of hexose to pentose (Guggenberger et al., 1994; Kiem and Kogel-Knabner, 2003). In sum, microbial residues can result in a net contribution of microbial-derived C to the soil stable C pool. As it is well established that the stable soil organic C pool is the most important for long-term C sequestration (Swift, 2001), research on formation, storage and transport of microbial residues in soils is critical for understanding microbial involvement and control over the stabilization of organic C, and further global C cycling.

To date investigations about potential ecosystem C storage in response to climate change have not been focused on the degree to which soil microbial-derived C persists and changes, but rather have been focused more generally on the transformation of plant-derived C. However, given the potential significance of microbial contribution to, and control over, stable soil C, accurate prediction of the impact of climate change drivers on soil organic C will likely require understanding the response of microbial-derived recalcitrant compounds to a range of environmental factors that affect microbial growth and activities (such as soil water, nitrogen deposition, temperature). While there are studies addressing these independently (Millar et al., 2004; van Groenigen et al., 2007; Zhang et al., 2014), few have explicitly investigated their simultaneous impact.

A detailed understanding of C transformation and sequestration driven by microbial communities can not be obtained by analysis of bulk microbial biomass alone, as incorporation of microbial biomass C into soil organic C does not significantly increase the total C to the soil (Potthoff et al., 2008). Also, measurement of total microbial residues is difficult since reliable differentiation between the C bound in microbial residues and soil extant organic C is still unavailable. Alternatively, biomarker molecules can be used to trace the microbial origin of soil organic C (Boschker and Middelburg, 2002; Joergensen and Emmerling, 2006). Microbial residues contain characteristic amino sugars that can be used as time-integrated biomarkers because of their absence in plants (Amelung, 2001), and their stability against degradation (Nannipieri et al., 1979; Chantigny et al., 1997). Microbial amino sugars have been shown to be a relatively stable fraction of the microbial biomass, and persist after cell death, thus the proportion of total amino sugars to total soil C has been used to characterize the relative contribution of the microbial community to soil C turnover and storage (Guggenberger et al., 1999; Amelung, 2001; Glaser et al., 2004; Joergensen and Emmerling, 2006; Niggemann and Schubert, 2006).

In this study, we quantify living microbial biomass using lipid analysis and microbial residues by amino sugar analysis in a California annual grassland ecosystem continuously exposed for 9 years to elevated $CO_2$, water addition, warming, and nitrogen (N) deposition, alone or in combination, at the Jasper Ridge Global Change Experiment (JRGCE) facility. These four factors have widely been shown to impact above ground C dynamics (plant production and turnover; i.e., Dukes et al., 2005), and our intent with the work reported here was to increase our corresponding understanding of their impact on below ground C, using microbial residues as a proxy. The two methods – lipid and amino sugar analysis reflect microbial components with very different turnover times. Lipids (with their rapid turnover following cell death) represent the extant, active community, and amino sugars (which have been shown to persist indefinitely in soil) are reflective of both extant and past soil communities. We propose that by using both methods it is possible to identify nuances of the long-term effects on soil C that would be missed by conventional bulk C analysis (Schmidt et al., 2015). Based on prior work indicating no significant impact of water addition on general microbial community structure (Gutknecht et al.,

2012), we chose to focus on $CO_2$, temperature, and nitrogen. We hypothesized that above-ground elevated $CO_2$ treatment,would have little direct impact on microbial community structure or residues and that temperature and nitrogen treatments would have an effect on microbial residues after 9 years [though the exact direction of change was unclear; both elevated temperature and nitrogen have been shown to increase or decrease microbial stable C (Wixon and Balser, 2009; Wang et al., 2014a,b; Griepentrog et al., 2015)]. More specifically our objectives were to: (1) examine and quantify soil microbial response to 9 years of continuous global environmental changes using methods reflecting two distinct timescales of microbial biomass production and turnover; and (2) explore the mechanism and potential feedbacks of microbial-derived C contribution to soil C storage under global environmental changes.

## Results

### Microbial Lipids

We did not find any statistically significant differences ($P > 0.05$) in fungal, bacterial, or total microbial lipid biomass (nmol/g-soil) among all treatments (**Figure 1A**). General linear model (GLM) analysis showed an insignificant impact of elevated $CO_2$, warming and N deposition on general lipid indices (**Table 1**). With regard to more specific microbial group abundance among simulated global change treatments, the N addition treatment had lower levels of arbuscular mycorrhizal fungi (AMF) and higher saprotrophic fungi (SF) compared with other global change treatments, resulting in a significantly ($P < 0.05$) lower AMF/SF ratio for N deposition alone (**Figure 1B**). Gram-positive ($Gm^+$) bacteria and actinomycetes tended to show similar patterns among global change treatments as those of SF (**Figure 1C**), with an increase under N deposition and decrease with other factors. In treatments with more than one factor, the abundance of the microbial groups (AMF, SF, $Gm^+$, and actinomycete) fell between the values of those in single-factor treatments (**Figure 1**).

### Microbial Amino Sugars

We found that total amino sugars and the amounts of GluN, GalN, and ManN (μg/g-soil) were depleted, while the bacterial-derived MurA was enriched, under all the JRGCE global change treatments (**Figure 2**). The GLM analysis showed that temperature and N impacted the overall size of the amino sugar pool as well as the proportions of the total amino sugars in the total C pool; $CO_2$ had little effect on total amino sugars but did significantly affect the individual amino sugar MurA (**Table 1**). More specifically, temperature and N were both associated with a significant decrease in the total amino sugar abundance by 52.8 and 23.8% respectively (**Figure 2A**), and a significant enrichment in MurA by 18.7 and 37.8% respectively (**Figure 2E**). Elevated $CO_2$, on the other hand, showed a suppressive effect in the amount of MurA when in combination with elevated temperature or N addition (**Figure 2E**). The ratios of GluN/GalN and GluN/MurA, a way to measure the amino sugar pattern shift, showed distinct trends. We found that elevated temperature was significantly related to lower GluN/GalN and GluN/MurA ratios, while elevated $CO_2$ had no effect. Nitrogen deposition tended to be related to an increased GluN/GalN ratio but a decreased GluN/MurA ratio (**Figure 3**).

### Microbial-Derived C Contribution

For our second objective, we used the proportion of amino sugars to total soil C as an indication of the relative contribution of microbial-derived residues to stored soil C. Total soil C significantly ($P < 0.01$) increased under N deposition. We found that the amino sugar proportion of total C was not significantly affected by elevated $CO_2$, but was negatively affected by warming and N deposition. Also, the negative effects of warming and N deposition were not significantly altered by the addition of $CO_2$ as a factor (there was no interactive effect of $CO_2$ with these treatments; **Figure 4**). An interactive effect of warming with elevated N and $CO_2$ does, however, appear to have a suppressive effect compared with N deposition alone or N deposition coupled with elevated $CO_2$ (**Figure 4**).

## Discussion

Our objectives were to investigate response to 9 years of continuous environmental manipulation using two methods of microbial biomarker analysis and to examine contribution of microbial-derived C to soil stable C pools. Changes in microbial lipids reflect an immediate/rapid response to a changed environment, and amino sugars reflect cumulative long-term change. We used lipids and amino sugars simultaneously to study the responses of microorganisms under simulated global environmental changes, and to evaluate the role of microbial ecology in soil C dynamics. Through these simultaneous measurements, we also assess and compare potential long-term sensitivity of soil C to global change.

### Responses to Global Change Treatments

#### Microbial Biomass (Lipids)

We found that total living microbial biomass (lipid abundance) did not differ significantly under any treatment. Changes in microbial biomass under global change scenarios could be important, since microbial biomass has been known as an early indicator of changes in total soil organic C (Powlson et al., 1987). Past studies have shown inconsistent trends for microbial biomass under elevated $CO_2$, warming, or N deposition. Reported responses vary in direction and magnitude, and much uncertainty exists on whether microbial biomass will increase, decrease or remain the same under elevated $CO_2$ (Zak et al., 2000), warming (Zhang et al., 2005; Frey et al., 2008; Bell et al., 2010) or N deposition (Treseder, 2008). In addition to various mechanisms that contribute to observed microbial abundance, the inconsistency among studies could be a result of high variations in the analysis of microbial living biomass, or in quickly changing dynamics of the living microbial biomass. However, despite the lack of biomass response in this study, microbial community structure did differ in some ways after 9 years of continuous treatment. In particular, fungal community composition appeared altered (AMF to SF ratio). This is aligned with results from Rillig et al. (2002) and Treseder (2004), who found

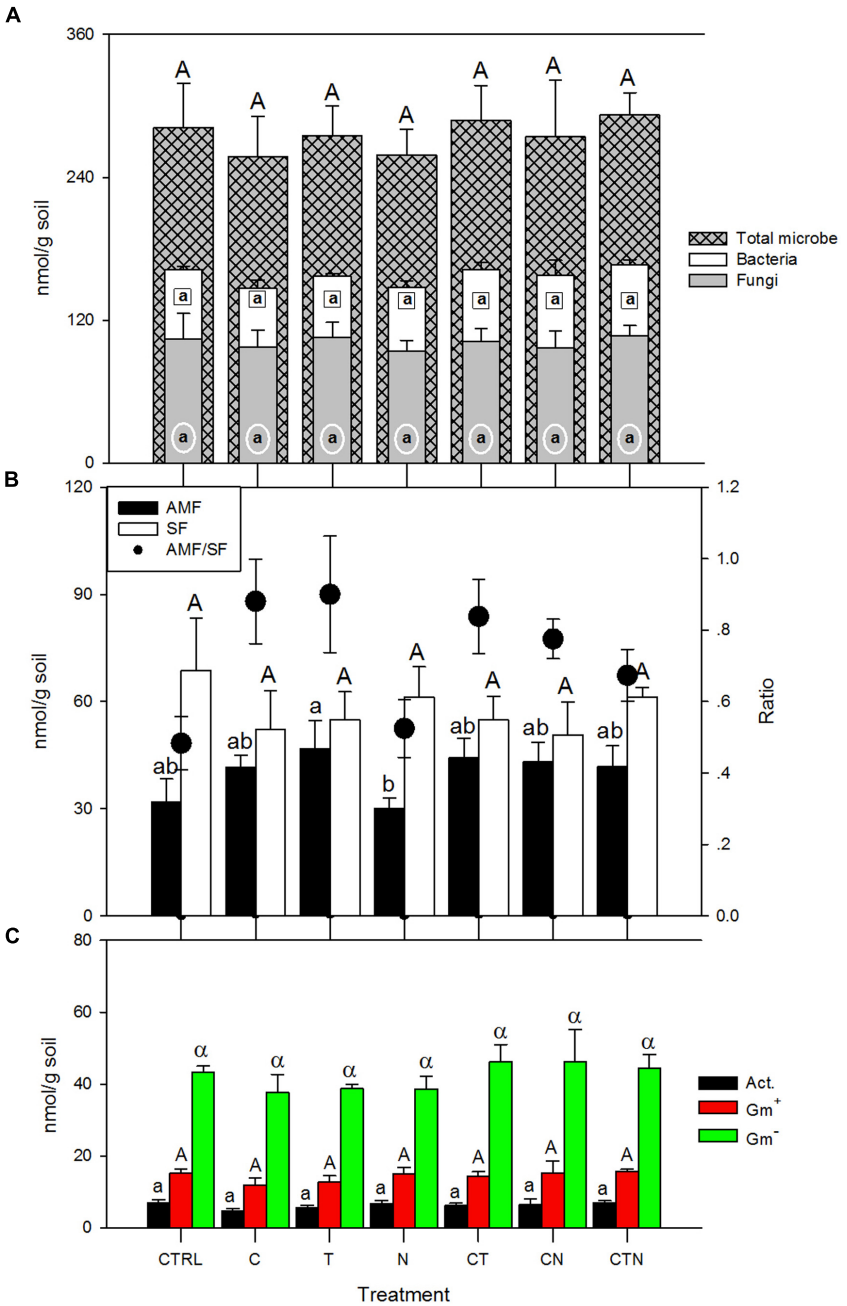


**FIGURE 1 | Lipid guild abundance (nmol/g soil) under single and combined global change treatments.** Bars indicate SE of the mean ($n = 4$ or 5). Bars sharing the same letter are not significantly different ($P = 0.05$, LSD test). CTRL, control; C, elevated $CO_2$; T, elevated temperature; N, nitrogen deposition; AMF, arbuscular mycorrhizal fungi; SF, saprotrophic fungi; $Gm^+$, gram-positive bacteria; $Gm^-$, gram-negative bacteria; Act., actinomycete.

that AMF abundance increased under elevated $CO_2$ and soil warming. In contrast, AMF abundance here did not significantly change under N fertilization compared with the control (**Figure 1B**). Two mechanisms have been proposed regarding the N addition influence on AMF: (1) increased N has a negative effect on AMF abundance as AMF become C-limited when N is more available and plants correspondingly reduce allocation of C to mycorrhizal association (Treseder, 2004; van Diepen et al., 2007); (2) AMF proliferation following N addition has also been reported in some ecosystems, possibly because of extreme N-limitation and an increased plant recruitment of AMF (Anderson and Liberta, 1992; Treseder and Allen, 2002). Here, we did not find a significant difference in AMF abundance compared with the control, possibly indicating that multiple mechanisms were involved, or that after 9 years the fungal communities have acclimated to the new level of N-availability.

**TABLE 1 | Summary of F-ratios from a general linear model, testing for the integrative effects of treatments on amino sugars, lipids, total carbon and nitrogen after 9-years global change manipulation.**

| | Treatment | | | | | |
|---|---|---|---|---|---|---|
| **Variable** | **C** | **T** | **N** | **C·T** | **C·N** | **C·T·N** |
| TC% | 0.10 | 0.00 | 19.60**** | 3.14* | 1.05 | 2.20 |
| TN% | 0.10 | 0.00 | 15.78**** | 2.99* | 0.87 | 0.53 |
| Total lipids (nmol/g) | 0.17 | 0.73 | 0.08 | 0.01 | 0.02 | 0.04 |
| Fungal lipids (nmol/g) | 0.01 | 0.88 | 0.03 | 0.07 | 0.00 | 0.04 |
| AMF lipids (nmol/g) | 1.44 | 2.62 | 0.01 | 2.68 | 0.00 | 0.15 |
| SF lipids (nmol/g) | 1.44 | 2.62 | 0.01 | 2.68 | 0.00 | 0.15 |
| Bacterial lipids (nmol/g) | 0.86 | 0.18 | 0.47 | 0.25 | 0.17 | 0.89 |
| SF to AMF ratio | 5.06 | 0.48 | 0.65 | 4.09* | 0.07 | 0.51 |
| $Gm^+$ to $Gm^-$ ratio | 2.78 | 0.18 | 0.46 | 1.79 | 0.23 | 0.53 |
| Total AS (ug/g) | 0.26 | 37.36**** | 1.61 | 0.78 | 2.27 | 5.39** |
| GluN (ug/g) | 0.63 | 40.11**** | 0.73 | 0.64 | 2.63 | 5.21** |
| GalN (ug/g) | 0.13 | 21.67**** | 4.66** | 0.55 | 1.31 | 5.30** |
| MurA (ug/g) | 16.59**** | 0.92 | 14.2*** | 0.16 | 18.11**** | 0.43 |
| AS/TC (mg/g) | 0.22 | 32.82**** | 12.05*** | 4.05* | 4.68** | 6.57** |
| GluN/TC (mg/g) | 0.47 | 35.58**** | 9.40*** | 3.69* | 4.99** | 6.59** |
| GalN/TC (mg/g) | 0.11 | 21.10*** | 14.81*** | 3.04* | 3.05* | 5.77** |
| MurA/TC (mg/g) | 8.32*** | 0.39 | 0.74 | 0.17 | 6.53** | 1.46 |
| GluN/GalN ratio | 0.77 | 14.41**** | 14.85**** | 0.01 | 1.46 | 0.28 |
| GluN/MurA ratio | 5.78** | 28.2**** | 3.76* | 0.66 | 13.01*** | 3.00* |

*F-ratio statistic is shown with asterisks indicating the P-value level. *$P < 0.1$; **$P < 0.05$; ***$P < 0.01$; ****$P < 0.001$. C, elevated $CO_2$; T, elevated temperature; N, nitrogen deposition; AMF, arbuscular mycorrhizal fungi; SF, saprotrophic fungi; $Gm^+$, gram-positive bacteria; $Gm^-$, gram-negative bacteria; AS, amino sugar; GluN, glucosamine; GalN, galactosamine; MurA, muramic acid.*

## Microbial Residues (Amino Sugars)

We found that total amino sugars, GluN, GalN, and ManN were significantly depleted in warming and N deposition treatments after 9 years, but the MurA was in greater abundance overall. This indicates that 9 years of warming and elevated N significantly reduced the amount of total microbial residues and fungal residues, but augmented bacterial residues. In spite of the differing effects by warming and N deposition on fungal and bacterial residues, they both consistently indicate that N addition has a generally positive effect, and warming has a generally negative effect on microbial residue accumulation. Although the complexity of soil processes makes straightforward interpretations of microbial residue dynamics difficult, we believe that the treatment-induced changes both directly and indirectly influence microbial growth, death and residue decay. The N addition-induced enhancement of plant growth at the JRGCE (Zavaleta et al., 2003; Dukes et al., 2005) could increase C inputs to the soil as litter and root exudation, which may directly benefit microbial growth (Treseder and Allen, 2002). However, plants may also compete with microbes for limited nutrients, which may hamper microbial growth due to nutrient limitation (Treseder, 2004). The treated plots (not N amended plots) at Jasper Ridge are likely a nutrient-limited environment (Menge and Field, 2007; Gutknecht et al., 2010), and plant competition likely limits microbial growth, biomass production, and accumulation. In the case of the warming treatment, changes in microbial physiology may explain the decline in the fraction of assimilated C that is allocated to microbial growth (Allison et al., 2010). In addition, the dominant amino sugars might be decomposed as a type of N nutrient for plant and microbial growth under warming and extreme nutrient-limited conditions. The distinct increase in MurA under treatments could be explained by faster bacterial life cycles associated with faster turnover rate, or a bacterial community shift from $Gm^-$ to $Gm^+$ bacteria which contain thick murein layers (Eudy et al., 1985; Kogel-Knabner, 2002).

Not surprisingly, there was no significant relationship between elevated $CO_2$ and total amino sugars and GluN suggesting that elevated $CO_2$ does not influence total microbial residues and fungal residues in soil (**Table 1**, **Figures 2A,B**). In addition, we only found a slight additive influence of elevated $CO_2$ coupled with other global change factors (**Figures 2A,B**), which is in accord with the previous work on lipid dynamics over time (Gutknecht et al., 2012). In contrast, elevated $CO_2$ was related to moderately higher bacterial-derived MurA compared to the control, suggesting that elevated $CO_2$ may contribute to an increased concentration of bacterial-derived residues. In line with this finding, Drigo et al. (2007) found that bacterial community structure in the rhizosphere was most affected by elevated $CO_2$, whereas fungal community structure was less influenced.

Direct effects of elevated $CO_2$ on soil microbial communities could be expected to be negligible since the $CO_2$ concentration in the soil pore space is much higher than the atmospheric level above the soil. However, elevated $CO_2$ can indirectly affect soil microbial communities via plant metabolism and root secretion (Drigo et al., 2008), decreased evapotranspiration (Ainsworth and Long, 2005). The Jasper Ridge site, as with typical grassland systems, has a high volume of rhizosphere soil, which was dominated by $Gm^-$ bacteria (**Figure 1C**). The increase in

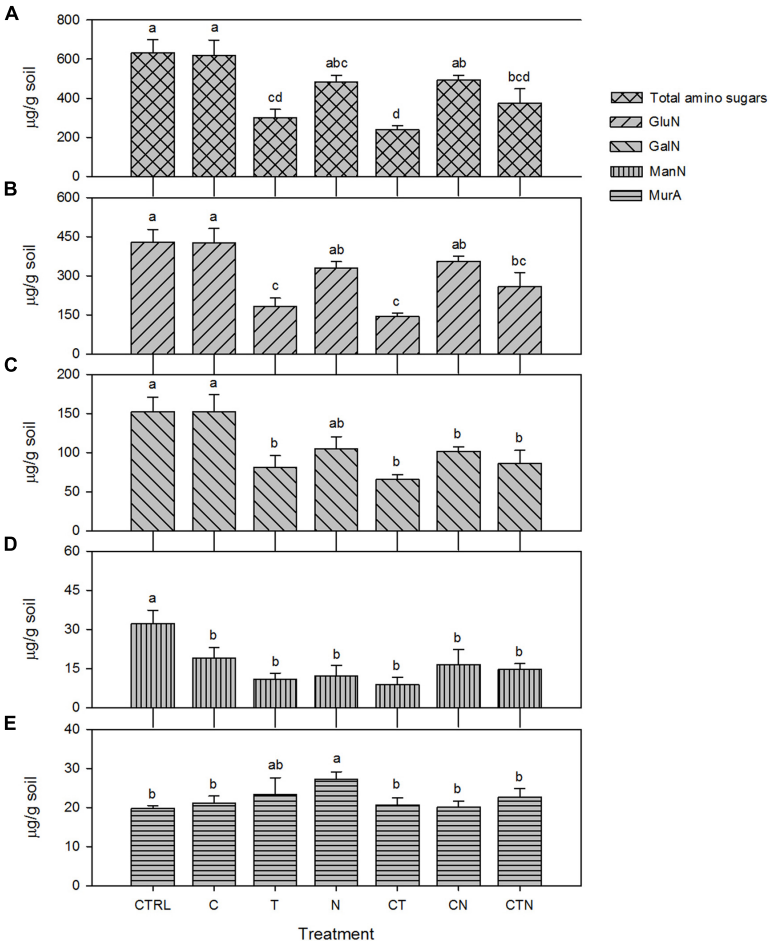


**FIGURE 2 | Absolute abundance in bulk and individual amino sugars (μg/g soil) under single and combined global change treatments.** Bars indicate SE of the mean ($n = 4$ or 5). Bars sharing the same letter are not significantly different ($P = 0.05$, LSD test). CTRL, control; C, elevated $CO_2$; T, elevated temperature; N, nitrogen deposition; GluN, glucosamine; GalN, galactosamine; ManN, mannosamine; MurA, muramic acid.

MurA under elevated $CO_2$ may thus be a result of indirect plant "fertilization," increased levels of labile C and nutrient excreted into the rhizosphere leading to higher $Gm^-$ bacterial growth and turnover.

Variation in amino sugar ratios can be used as a qualitative indicator of time-integrated compositional changes in the soil microbial community (Guggenberger et al., 1999; Glaser et al., 2004). We found that the abundance of GalN was different from MurA among treatments, and thus ratios of GluN/GalN and GluN/MurA showed different tendencies, especially under N deposition where MurA was significantly increased. We suggest using the GluN/GalN ratio to describe overall amino sugar accumulation patterns (long-term microbial turnover), and the GluN/MurA ratio to understand the relative long-term contribution to soil C by fungi versus bacteria.

## Evaluation of Rapid versus Long-Term Responses of Soil Microbes

Another interest of ours was to compare the use of the two microbial methods, each indicating something distinct about soil C. Precise prediction of microbial response to global changes has proven difficult within a specific ecosystem due to the large uncertainty regarding microbial community (Treseder et al., 2012). This could be due both to method limitations and limitations due to the timing and frequency of sampling. A single sampling of microbial biomass may not reveal microbial response to prolonged global changes, as rapid and ephemeral changes in the living microbial community may confound the perceived treatment effects. Another limitation is that the standing biomass of microbes does not necessarily reflect microbial contribution to soil C storage, since microbial-derived residues in nascent humic substances could have increased even while standing living biomass remains constant or decreases (Potthoff et al., 2008). It is reasonable to expect that the different microbial community responses to simulated global changes in our study should sustain microbial residue accumulations to a different degree, and the effects could be further amplified over time; while that measures of standing biomass, for instance lipids, may not reveal these long-term changes in microbial

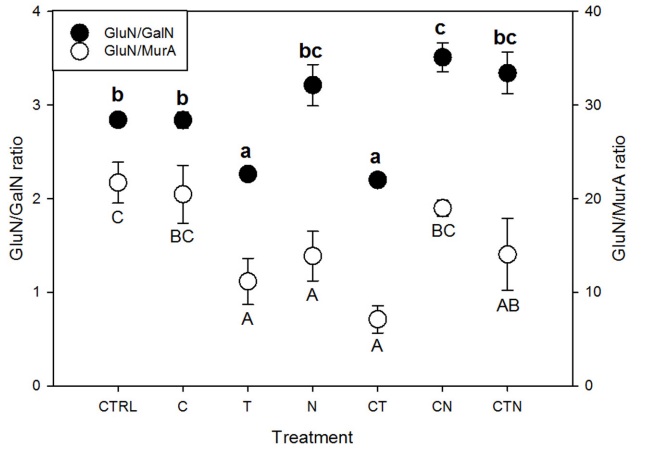


**FIGURE 3 | Amino sugar ratios under single and combined global change treatments.** CTRL, control; C, elevated $CO_2$; T, elevated temperature; N, nitrogen deposition; GluN, glucosamine; GalN, galactosamine; MurA, muramic acid. Bars indicate SE of the mean ($n = 4$ or 5). Dots sharing a letter are not significantly different ($P = 0.05$, LSD test).

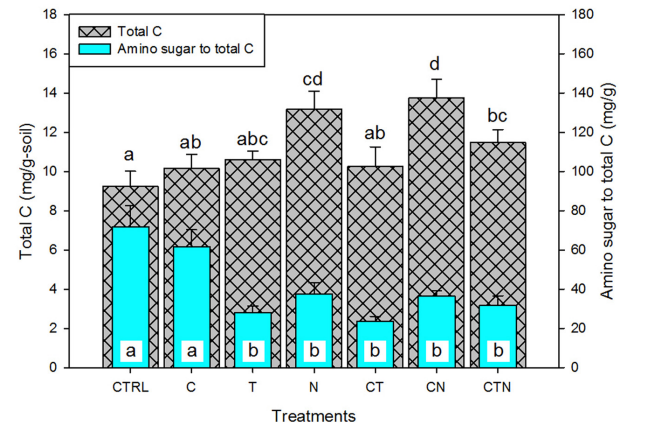


**FIGURE 4 | The effect of single and combined global environmental change treatments on the total carbon pool and the proportion of bulk amino sugars in that pool.** CTRL, control; C, elevated $CO_2$; T, elevated temperature; N, nitrogen deposition. Bars indicate SE of the mean ($n = 4$ or 5). Bars sharing a letter are not significantly different ($P = 0.05$, LSD test).

contributions to soil C (**Table 2**). As microbial cell walls appear more stable and turn over more slowly than living microbial biomass (Guggenberger et al., 1999; Amelung, 2001; Glaser et al., 2004), microbial cell wall amino sugars provide a more useful indicator of chronic, long-term soil microbial responses and microbial necromass contributions to changes in soil C storage.

## Microbial Residue Amino Sugar Contribution to Soil C Storage

Our second goal with this research was ultimately to go beyond describing microbial responses following 9 years of manipulation and assess the potential significance of a given response for below ground C storage. Toward that end, the amino sugar proportion of total soil C may be used to indicate microbial residue accumulation, or microbial-derived C contribution to soil C sequestration over time (Liang and Balser, 2012). In addition to examining absolute amino sugar amounts (indicating extant and past microbial biomass and coarse community structure), it is interesting to consider how the proportion of amino sugars to soil total C was altered by the global change manipulations. In the Jasper Ridge grassland ecosystem, elevated $CO_2$ had no significant effect on total soil C or its proportional amino sugar content. However, under increased nitrogen and warming, total soil C showed two responses: 9-years of N deposition significantly increased soil total C storage, while warming did not statistically alter the total soil C pool (**Figure 4**). In contrast, the amino sugar proportion of total soil C was lowered under both warming and N deposition (**Table 1**; **Figure 4**). Thus microbial residues appeared to decline in contribution to total soil C under warming and N deposition compared with other C-containing compounds. This has potential implication for long-term C stabilization or sequestration under warming or N deposition. The decline in contribution of microbial C may reflect differing underlying impacts of the two environmental changes on microbial dynamics (Liang and Balser, 2012). Temperature is a global modulator affecting respiration, enzyme activity, and membrane fluidity – all living organisms must respond in some way to a change in temperature. Increased temperature has been shown to correlate to a decline in microbial biomass (Gutknecht et al., 2012), and this decline may be responsible for the lowered contribution of amino sugars-C to total soil C. Nitrogen is a resource, not a modulator. Organisms compete for it, and it is often related to increased overall biomass. Thus the lower microbial amino sugars-C relative to total soil C in this case is likely due to an increase in newer plant C when N constraints on plant production are alleviated rather than a decline in microbial contribution.

This highlights the need to look at soil C as more than a bulk measure, as is also being recognized in other research efforts

**TABLE 2 | Summarized dynamics of lipids and amino sugars under 9-years simulated global changes in JRGCE.**

| | Lipid | | | Amino sugar | | | |
|---|---|---|---|---|---|---|---|
| | **Total lipids** | **Fungal lipids** | **Bacterial lipids** | **Total amino sugars** | **Glucosamine** | **Muramic acid** | **Proportions of amino sugars in soil C** |
| Elevated $CO_2$ | No change | No change | No change | No change | No change | Increase | No change |
| Warming | No change | No change | No change | Decrease | Decrease | Increase | Decrease |
| N deposition | No change | No change | No change | Decrease | Decrease | Increase | Decrease |

(Poirier et al., 2005; Schmidt et al., 2015). Assuming soil C is a single homogeneous pool ignores the fact that separate fractions have different origins or turnover time (Trumbore, 1997, 2000). The soil stable C pool is critical in the role of soil as a C sink within the global C cycle, as even a small change in the stable C pool could have large consequences for climate change (Rustad et al., 2000). In this study, microbial residue biomarker analysis allowed us to separate organic C of microbial origin from other fractions. While their percentage of total C is relatively small, microbial amino sugars may act as a canary in the mineshaft for soil C – indicating possible vulnerabilities or changes in long term stability. The sensitivity of microbial residues to warming and N deposition observed in this study may prove important in our predictions of global change impacts on soil stored C.

## Conclusion

The overall purpose of this study was to explore soil microbial communities and potential responses of stable soil C to long-term simulated global environmental changes. We used microbial biomarkers, lipids and amino sugars, to investigate microbial responses and contribution of microbial residues. Amino sugars, as compared to lipids, provide a more useful indicator of long-term soil microbial responses, and reflect microbial residue contributions to soil C storage under chronic simulated global environmental changes. We found that an environmental modulator (temperature) and a nutrient resource (nitrogen) differed in their impact on microbial residue accumulation. Warming and N deposition both resulted in significantly depleted total microbial residues, and enriched bacterial residues (though offset by the corresponding decline in fungal residues).

This study highlights the power of the amino sugar analysis by directly comparing potential microbial responses on short and long time scales (using lipid and amino sugar biomarkers as reflective of these timescales, respectively) in a grassland after continuous 9-years manipulation of global multifaceted factors. We suggest that single time point microbial lipid measurements may not reveal the long-term microbial contribution to soil C in the same way that analysis of microbial amino sugars can. The study also highlights the sensitivity of microbial residues in the soil C stock in the context of warming and N deposition. We suggest the dynamics of microbial residues should be incorporated into current soil C research and that integration may substantially improve our predictions of global change impacts on soil stored C.

## Materials and Methods

### Site Description

The JRGCE contains manipulations of each of the following global change factors: (1) atmospheric $CO_2$ concentration (ambient and 700 ppm), (2) temperature (ambient and +1 degree Celsius at the soil surface), (3) N deposition (ambient and 7 g N $m^{-2}y^{-1}$), 4) water addition (ambient and augmented to twice ambient), with all factors crossed in a complete full factorial design (Shaw et al., 2002; Zavaleta et al., 2003). In this study we did not include samples from the water addition plots. The JRGCE was started in 1998 to test future global change scenarios for this region (Hayhoe et al., 2004). The site is located in the eastern foothills of the Santa Cruz Mountains at the Jasper Ridge Biological Preserve in the San Francisco Bay area. The region experiences a Mediterranean climate, and is generally dominated by annual non-native grasses *Avena barbata* and *Avena fatua* (Zavaleta et al., 2003). The soil at the JRGCE is a loam texture with a pH ranging from 6.5 to 7.0, ∼3% organic matter and a cation exchange capacity of 9.5 meq 100 g $soil^{-1}$. Soils were sampled on 16–17 April, 2007 from the JRGCE, corresponding with annual peak plant biomass, 9 years following the start of the experiment. Four soil cores (4 cm diameter by 15 cm depth) were taken from each experimental plot, 10-g sub-samples from the homogenized cores were frozen immediately, shipped to the University of Wisconsin – Madison, and freeze dried for microbial analysis. Other sub-samples were air dried for total C and N analysis. Total soil C and N data were determined by combustion analysis and provided by the JRGCE facility support staff.

### Microbial Analysis

We assayed the soils for microbial amino sugars and lipids. We determined four amino sugars [glucosamine, galactosamine (GalN), mannosamine (ManN), and muramic acid] by gas chromatograph (GC) after their conversion to aldonitrile acetates according to the protocol of Guerrant and Moss (1984) and Zhang and Amelung (1996), as modified by Liang et al. (2012). Briefly, ∼1 g soil samples were hydrolyzed with 6M HCl at 105°C for 8 h, and then the solution was filtered and purified by neutralization. After drying the supernatant, methanol was used to wash amino sugars from the residues, transferred to 3 mL vials, and then evaporated to dryness on a Labconco 79000 RapidVap (Labconco Co., Kansas City, MO, USA). The residues were re-dissolved in 1 mL distilled deionized water, lyophilized overnight, and then processed for aldononitrile acetate derivatization. To prepare the aldononitrile derivatives, the amino sugars were dissolved in a derivatization reagent consisting of hydroxylamine hydrochloride (32 mg $mL^{-1}$) and 4-(dimethylamino) pyridine (40 mg $mL^{-1}$) in 4:1 pyridine-methanol (v/v), and heated to 75–80°C for 35 min. Then, 1 mL acetic anhydride was added, and the solution was reheated for 25 min for acetylation. After cooling, 1.5 mL dichloromethane and 1 mL 1M HCl were successively added and the mixture was vortexed to transfer the amino sugar to organic phase. The solution was washed thrice with 1 mL deionized $H_2O$ to remove excess anhydride. In the last washing step, the aqueous phase was removed as completely as possible. Finally, the organic phase was dried at 45°C in a Labconco 79000 RapidVap, and re-suspended in 300 μL ethyl acetate-hexane (1:1) for GC analysis. Separation of amino sugar derivatives was carried out on an Agilent 6890 GC (Agilent Technologies, Wilmington, DE, USA) equipped with a J&W Scientific Ultra-2 column (25 m by 0.2 mm by 0.33 μm) and flame ionization detector (FID). Sample extracts (2 μL) were injected onto the column using $H_2$ as the carrier gas at a constant flow rate of 0.4 mL $min^{-1}$. The GC inlet was set to 250°C and operated in split mode with a 30:1 ratio. The individual

amino sugar derivatives were identified by comparing their retention time with those of authentic standards. Quantification was gained relative to the internal standard myo-inositol, which was added to the samples prior to purification, and the recovery standard *N*-methylglucamine was added before derivatization. The recovery standard was used to assess the reliability of derivatization step. Glucosamine (GluN, made by fungi and bacteria) and muramic acid (MurA, made by bacteria) are both particularly useful biomarkers. Fungal GluN is often the most abundant amino sugar found in soils, while MurA is uniquely derived from bacterial peptidoglycan (Amelung, 2001; Joergensen and Emmerling, 2006). The others, GalN and mannosamine, are less useful due to questions about their origins (Coelho et al., 1997; Amelung, 2001; Engelking et al., 2007).

We used a hybrid procedure of phospholipid fatty acid (PLFA) and fatty acid methyl ester (FAME) analysis to assay microbial community structure (Kao-Kniffin and Balser, 2007). We extracted lipids from ~3 g freeze-dried sub-samples using a phosphate buffer, chloroform and methanol (0.9:1:2) extraction solution. Samples were extracted twice, and then the supernatant were combined together. After phase separation overnight, the organic phase isolated and evaporated to dryness using a RapidVap (LabConco, Kansas City, MO, USA). We carried out FAME analysis as described by Microbial ID Inc on the dried organic residue. Lipids were saponified, and then subjected to alkaline methanolysis. Lipids were isolated from the samples in a hexane extraction. We analyzed extracts using a Hewlett-Packard Agilent 6890 GC (Agilent Technologies, Wilmington, DE, USA) equipped with an Agilent Ultra-2 (5% phenyl)-methylpolysiloxane capillary column (25 m by 0.2 mm by 0.33 μm) and FID. Lipid peaks were identified by MIDI peak identification software ("Sherlock microbial identification system," MIDI Inc., Newark, DE, USA). Two internal standards, 9:0 nonanoic methyl ester and 19:0 nonadecanoic methyl ester, were used as internal standards to convert fatty acid peak areas to the absolute abundance at nmol/g-soil. We quantified the abundance of different microbial groups in each treatment using the abundance of lipids in chemically similar 'guilds'. Microbial biomass was represented by the sum of all identifiable fatty acids (detectable at >0.05% and C number <20). Fungal, bacterial and actinomycetal biomass were represented by summing all representative PLFAs for each group. In specific, we used the sum of 14:0iso, 15:0anteiso, 15:0iso, 16:0iso, 17:0anteiso, and 17:0iso to indicate $Gm^+$ bacteria, and the sum of 16:1ω7c, 16:1ω9c, 17:0cyclo, 17:1ω8c, 19:0cyclo and 18:1ω7c to indicate $Gm^-$ bacteria. The sum of 10Me18:0; 10Me17:0; 10Me16:0 represents actinomycete. AMF is indicated by 16:1ω5c and SF by the sum of 18:1ω9c and 18:2ω6c.

### Statistics

In order to test the global change effects, we took single and combined treatments as the different levels of global change, and inferred the effects with one-way ANOVA for the abundance of individual and total lipids and amino sugars, ratios of lipids and amino sugars, and soil total C. A *post hoc* separation of means by LSD was performed in the cases where main effects were significant at $p < 0.05$. We used a GLM to test the integrative effects of elevated $CO_2$, warming, N deposition and their interactions on microbial lipids and amino sugars. Statistical analyses were performed with the SPSS (SYSTAT Software, Inc.) software for Windows, and figure preparations were accomplished using Sigma Plot (SYSTAT Software, Inc.).

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# Transplant experiments uncover Baltic Sea basin-specific responses in bacterioplankton community composition and metabolic activities

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Anthropogenically induced changes in precipitation are projected to generate increased river runoff to semi-enclosed seas, increasing loads of terrestrial dissolved organic matter and decreasing salinity. To determine how bacterial community structure and functioning adjust to such changes, we designed microcosm transplant experiments with Baltic Proper (salinity 7.2) and Bothnian Sea (salinity 3.6) water. Baltic Proper bacteria generally reached higher abundances than Bothnian Sea bacteria in both Baltic Proper and Bothnian Sea water, indicating higher adaptability. Moreover, Baltic Proper bacteria growing in Bothnian Sea water consistently showed highest bacterial production and beta-glucosidase activity. These metabolic responses were accompanied by basin-specific changes in bacterial community structure. For example, Baltic Proper *Pseudomonas* and *Limnobacter* populations increased markedly in relative abundance in Bothnian Sea water, indicating a replacement effect. In contrast, *Roseobacter* and *Rheinheimera* populations were stable or increased in abundance when challenged by either of the waters, indicating an adjustment effect. Transplants to Bothnian Sea water triggered the initial emergence of particular *Burkholderiaceae* populations, and transplants to Baltic Proper water triggered *Alteromonadaceae* populations. Notably, in the subsequent re-transplant experiment, a priming effect resulted in further increases to dominance of these populations. Correlated changes in community composition and metabolic activity were observed only in the transplant experiment and only at relatively high phylogenetic resolution. This suggested an importance of successional progression for interpreting relationships between bacterial community composition and functioning. We infer that priming effects on bacterial community structure by natural episodic events or climate change induced forcing could translate into long-term changes in bacterial ecosystem process rates.

**Keywords: bacterial community functioning, salinity, DOM, terrigenous carbon, climate change, marine bacteria, bacterial diversity**

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## Introduction

A fundamental question in ecology focuses on whether shifts in diversity and community composition due to changes in environmental conditions also result in changes in bacterial community functioning (Loreau, 2000; Gamfeldt and Hillebrand, 2008). Overall, little is known about how bacterial community composition affects bacterial community functioning and how sensitive or resistant bacterial communities and individual taxa are to environmental disturbances (Allison and Martiny, 2008; Comte and Del Giorgio, 2011). It is, therefore, desirable to examine the adaptability (i.e., sensitivity, resistance, and responsiveness) and metabolic plasticity (i.e., the potential to achieve similar ecosystem process rates) of bacterioplankton populations responding to environmental disturbances. Most bacterial populations are sensitive to environmental disturbances, and changes in bacterial community composition can influence the rates of ecosystem processes, suggesting that populations are functionally dissimilar (Bell et al., 2005; Langenheder et al., 2005; Judd et al., 2006; Allison and Martiny, 2008; Comte and Del Giorgio, 2011; Comte et al., 2013). However, little is known about the changes in population dynamics and ecosystem ecology in response to climate change consequences, such as increased temperature, lower pH, or increased river runoff (Degerman et al., 2013; Lindh et al., 2013; Von Scheibner et al., 2014). Potentially, knowledge of the responses of bacterioplankton populations to anthropogenically induced environmental change could extend the understanding of the links between population dynamics and ecosystem ecology and might help to predict and monitor future change in marine environments.

Projections from climate change models highlight increased annual levels of precipitation in Northern Europe, decreasing salinity and increasing loadings of terrigenous (allochthonous) dissolved organic matter (DOM) to coastal waters through river outflows (Meier, 2006). Changes in salinity and increased terrigenous carbon inputs have been shown to influence growth and activity of bacterioplankton (del Giorgio and Bouvier, 2002; Langenheder et al., 2003; Kritzberg et al., 2004; Rochelle-Newall et al., 2004; Laghdass et al., 2010; Fasching et al., 2014). Salinity is an important factor shaping bacterial community composition in that it influences the spatial distribution of bacterial populations (Lozupone and Knight, 2007; Herlemann et al., 2011; Dupont et al., 2014). On the other hand, bacterial community composition is also much dependent on the quantity and quality of DOM (Lindström, 2000; Eiler et al., 2003; Kisand and Wikner, 2003; Kirchman et al., 2004; Rochelle-Newall et al., 2004; Kritzberg et al., 2006; Kisand et al., 2008; Teira et al., 2009; Gómez-Consarnau et al., 2012; Grubisic et al., 2012; Rocker et al., 2012). Yet, empirical data for how bacterial community functioning and the cycling of carbon will be affected in coastal or semi-enclosed waters like the Baltic Sea under conditions simulating potential future climate change influences are scarce. Detailed knowledge on the combined effects of climate change driven changes in salinity and DOM for bacterial community composition and metabolic activity would be desirable.

As a semi-enclosed sea, the Baltic Sea is characterized by seasonally changing inputs in the quality and quantity of allochthonous DOM (Zweifel et al., 1993). In addition, the prominent salinity gradient ranges from truly marine in the southern to freshwater salinities in the northern basins of the Baltic Sea, where large river discharges cause lower salinity. The cause for differences in the distribution of microbial populations due to salinity is likely related to the long residence time in the Baltic Sea (>5 years), allowing niche differentiation and adaptions to optimum salinity levels (Riemann et al., 2008; Herlemann et al., 2011; Dupont et al., 2014). The combined environmental disturbances projected from climate change models imply substantial effects on the structure and function of both macro- and microorganism communities, including bacterioplankton, in the Baltic Sea (Wikner and Andersson, 2012). One of the major consequences of such anthropogenically induced disturbances for marine microbes is expected to be a change in biogeochemical cycling of carbon that may allocate more energy for heterotrophic bacterial production in the Baltic Sea (Sandberg et al., 2004; Wikner and Andersson, 2012).

Transplant experiments have provided insights into key factors that regulate bacterial community structure, diversity, and functioning in different aquatic environments (Gasol et al., 2002; Kirchman et al., 2004; Rochelle-Newall et al., 2004; Langenheder et al., 2005; Bonilla-Findji et al., 2009; Sjöstedt et al., 2012; Comte et al., 2013). The aim of the present study was to investigate how the quality of water originating from geographically distinct basins of the Baltic Sea influences bacterial community composition and metabolism. This was done under the premise that projections of future climate change influence on the Baltic Sea indicate that increased precipitation will lead to environmental conditions in the Baltic Proper similar to those currently found in the northern basins of the Baltic Sea (Bothnian Sea or Bothnian Bay). We designed a transplant and re-transplant microcosm experiment and monitored the effects on bacterial community composition (by using 16S rRNA gene Illumina Miseq tag sequencing) and functioning (i.e., bacterial abundance, production, and enzyme activities). A conceptual model of potential outcomes of this study is presented in **Figure 1**. We hypothesized that: (i) bacterial community composition would change after both transplantation and re-transplantation disturbances relative to controls following the replacement scenario (pathway B in **Figure 1**) *sensu* Allison and Martiny (2008) and Comte and Del Giorgio (2011) and (ii) bacterial community functioning would be affected due to limited functional redundancy.

## Materials and Methods

### Field Sampling

Culture media for the experiments was prepared from seawater collected from the Baltic Sea Proper (BAL; Linnaeus Microbial Observatory station, LMO; N 56°55.851, E 17°03.640) and the Bothnian Sea (BOT; NB1; N 63°31.0000, E 19°48.1166) on the 1 and 2 July 2013, respectively (**Figure 2**). Seawater was taken using a Ruttner sampler at a depth of 2 m. BAL and BOT water were transported in the dark to the laboratory in acid-washed Milli-Q rinsed polycarbonate bottles and at *in situ* temperatures within 1 or 12 h, respectively.

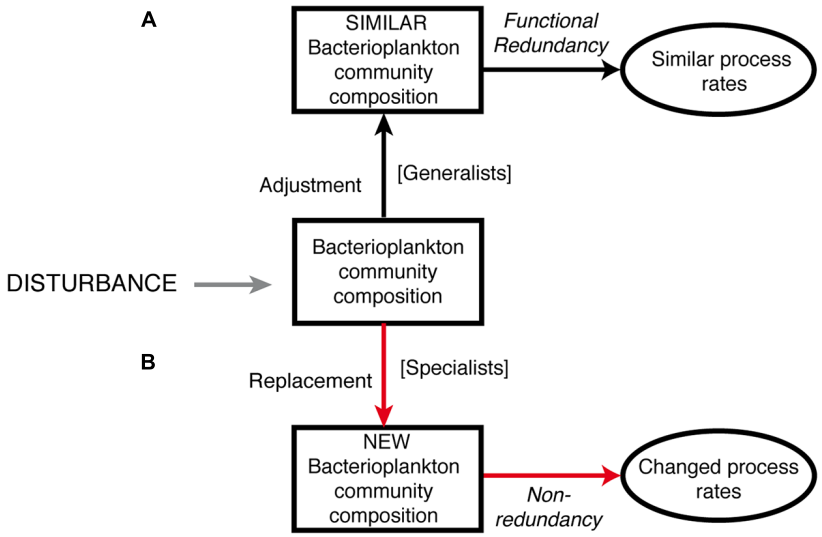


**FIGURE 1 | Conceptual model of the potential outcome of this study.** We hypothesized that bacterioplankton responses in community composition and metabolic activity would follow pathway **B**; red arrows, i.e., replacement of OTUs, leading to changes in community composition and functioning. Our null-hypothesis is therefore pathway **A**; black arrows; i.e., adjustment of OTUs, leading to unchanged community composition and bacterial community functioning.

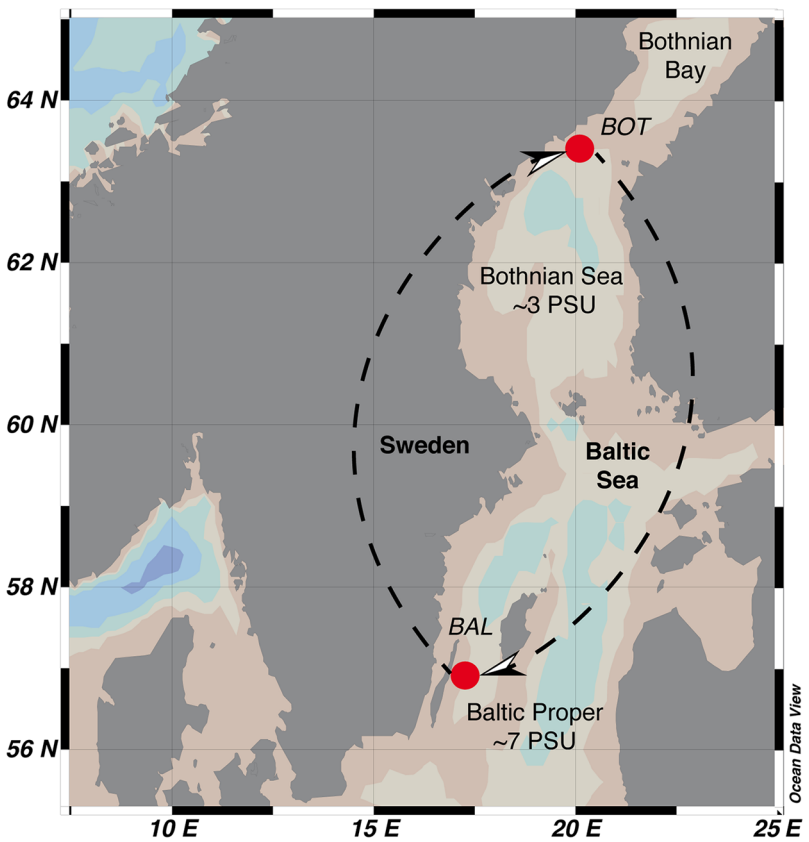


**FIGURE 2 | Geographic location of the Baltic Sea Proper (BAL) and Bothnian Sea (BOT) stations in the Baltic Sea.** Arrows indicate direction of transplant and re-transplant of unfiltered seawater (inoculum) to seawater media.

Seawater to be used as inoculum with natural bacterioplankton assemblages was collected simultaneously at the BAL and BOT sites on 15 July 2013, and was transported to the Linnaeus University within 12 h. This seawater for inocula remained unaltered (i.e., no manipulations such as filtrations were carried out). On both field samplings, measurements of temperature, salinity, and nutrient concentrations were taken. For the second field sampling, when water for bacterial inocula was obtained, nutrient limitation bioassays were carried out, and samples for determining *in situ* bacterial community composition were collected.

## Microcosm Setup

Water from each of the two stations was prepared for seawater culture media by sterile filtration (0.2 μm pore size; Sterivex cartridge; Millipore, USA), whereupon the filtrate was distributed into acid-washed Milli-Q rinsed 2 l polycarbonate bottles followed by autoclaving and subsequent storage in the dark at 16°C. Prior to inoculation, culture media had $<10^4$ cells $ml^{-1}$, as determined by flow cytometry. The experiment was made up of two parts: a transplant and a re-transplant part, running 5 and 4 days, respectively. In the transplant, unfiltered seawater was used to inoculate the sterile filtered and autoclaved seawater media in triplicates for each treatment at a ratio of 1:20. For the re-transplant, inoculum from transplant microcosms on day 5 were added to sterile filtered autoclaved seawater media in triplicates at a ratio of 1:20. This ratio was used based on our previous experience in obtaining clear bacterial growth responses in Baltic Sea microcosms (Gómez-Consarnau et al., 2012). Nomenclature of microcosms is as follows; $station_b \rightarrow station_{sw}$ where subscript "b" indicates bacteria and subscript "sw" indicates seawater medium. Thus, transplant microcosms consisted of native Baltic Proper bacteria growing in either Baltic Proper water ($BAL_b \rightarrow BAL_{sw}$) or Bothnian Sea water ($BAL_b \rightarrow BOT_{sw}$), and native Bothnian Sea bacteria incubated in either Bothnian Sea water ($BOT_b \rightarrow BOT_{sw}$) or Baltic Proper water ($BOT_b \rightarrow BAL_{sw}$). Re-transplant microcosms consist of Baltic Proper bacteria re-transferred to Baltic Proper water ($BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$) or with continued growth in Bothnian Sea water ($BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$), and Bothnian Sea bacteria re-transferred to Bothnian Sea water ($BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$) or continued incubation in Baltic Proper water ($BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$). All microcosms were incubated at 16°C in darkness. The microcosms were gently inverted manually twice a day and before sampling of biotic and abiotic parameters. The experimental setup is summarized in **Table 1** and detailed in Figure S1. In the transplant experiment, salinity was measured daily and total organic carbon (TOC) concentrations were measured on day 0, 2, and 5 (triplicates). Bacterial abundance was monitored daily by flow cytometry (duplicates) and heterotrophic production was determined on day 0, 2, and 4 (quadruplicates). Extracellular enzyme activities were measured on day 0, 2, and 4 (quadruplicates). In the re-transplant experiment, salinity was measured daily and TOC concentrations were measured on day 0, 2, and 4 (triplicates). Bacterial abundance (duplicates) and production (quadruplicates) were measured daily and extracellular enzyme activities were measured on day 0, 2, and 3 (quadruplicates).

**TABLE 1 | Simplified experimental setup of the microcosm experiment.**

| | *Seawater media* | | | |
|---|---|---|---|---|
| ***Bacterial source*** | **Transplant** | **Transplant control** | **Re-transplant** | **Re-transplant control** |
| $BAL_b$ | $BOT_{sw}$ | $BAL_{sw}$ | $BAL_{sw}$ | $BOT_{sw}$ |
| $BOT_b$ | $BAL_{sw}$ | $BOT_{sw}$ | $BOT_{sw}$ | $BAL_{sw}$ |

*Unfiltered water sampled in the Baltic Sea proper (BAL) and in the Bothnian Sea (BOT) was inoculated in triplicates at 1:20 ratio into sterile filtered autoclaved seawater media from these two stations. For re-transplant, samples from transplant microcosms were inoculated in triplicates at 1:20 ratio.*

## Nutrient Concentrations

*In situ* samples from the BAL and BOT stations for dissolved inorganic nutrient concentrations ($NH_4^+$, $NO_3^-$, $PO_4^{3-}$) were collected on the 15 July, when the water for the inocula was sampled, and were analyzed following the method of Valderrama (1995). For measuring TOC concentration, samples of 50 ml were filtered (0.2 μm Supor Membrane Syringe Filter, non-pyrogenic; Acrodisc®; Pall Life Sciences, USA), acidified with 0.67 ml of 1.2 M HCl and kept in acid rinsed 50 ml Falcon tubes at 4°C in the dark until processing. The samples were purged and measured using a Shimadzu TOC-5000 Analyzer (Shimadzu, Japan).

## Bacterial Abundance, Bacterial Heterotrophic Production, and Extracellular Enzyme Activity

Bacterial abundance samples of 900 μl were preserved with formaldehyde (2% final concentration) and stored at −80°C until processing. Bacterial abundance was measured by staining samples with SYTO 13 (5 μM final concentration: Molecular Probes, USA) and enumerated using a Cube 8 flow cytometer (Partec, Germany) according to the protocol described in del Giorgio et al. (1996). For bacterial heterotrophic production, 1.2 ml samples were collected with two killed controls and production was measured via the $^3$H-Leucine method according to Smith and Azam (1992). Extracellular enzymatic activities of beta-glucosidase, leucine aminopeptidase, and alkaline phosphatase were determined in technical quadruplicates according to the fluorometric enzyme assays described in Baltar et al. (2010).

## Nutrient Limitation Bioassays

Bacterial nutrient limitation was measured for *in situ* seawater by distributing 250 ml of seawater to acid-washed Milli-Q rinsed polycarbonate bottles adding 24 μM glucose ($C_6H_{12}O_6$), 4.2 μM ammonium ($NH_4Cl$), and 0.6 μM phosphate ($NaH_2PO_4$; final concentrations) in duplicate treatments incubating in the dark for 48 h at 16°C. Differential responses to nutrient addition were determined by measuring bacterial heterotrophic production.

## DNA and Illumina Miseq PCR

Collection of biomass for DNA extraction was done on day 5 for the transplant and day 4 for the re-transplant when 750 ml of water was filtered onto 0.2 μm 47 mm Supor filters (PALL Life Sciences) for all treatments except for the *in situ* samples for

which 4 l were Sterivex filtered (Millipore). Phenol-chloroform extraction of DNA was performed according to Riemann et al. (2000). Bacterial 16S rRNA was first amplified with HPLC purified bacterial primers 341F and 805R (Herlemann et al., 2011) following the PCR protocol of Hugerth et al. (2014) with some modifications; amplification was carried out in duplicates for each biological replicate and we used an annealing temperature of 58°C in the first PCR and 12 cycles in the second PCR. The resulting purified amplicons were sequenced on the Illumina Miseq (Illumina, USA) platform using the 300 bp paired-end setting at the Science for Life Laboratory, Sweden (www.scilifelab.se). Due to problems with either sampling or sequencing some treatments are only represented by duplicates or a single sample (Table S1).

### Sequence Processing and Analysis

Raw sequence data generated from Illumina Miseq were processed using the UPARSE pipeline (Edgar, 2013). Taxonomy was determined against the SINA/SILVA database (SILVA 115; Quast et al., 2013). After quality control, our data consisted of a total of 1.3 million reads, with an average of 40 086 ± 18 037 reads per sample. These sequences resulted in a final OTU table consisting of 3920 OTUs (excluding singletons) delineated at 97% 16S rRNA gene identity. For the OTU based analyses, chloroplast, mitochondrial, and eukaryotic sequences have been excluded from all analyses. The maximum likelihood tree was made using MEGA 5.2.1 and the Tamura-Nei model (Tamura et al., 2011) to examine the phylogenetic relationship between bacterioplankton responding in different microcosms and for Unifrac analysis. DNA sequences have been deposited in the National Center for Biotechnology Information (NCBI) Sequence Read Archive under accession number SRP048666.

### Statistical Analyses

For analysis of variance (ANOVA) statistics we tested the sample distribution for normality using Shapiro tests, and if the data was not normally distributed we log-transformed the data. ANOVA results were complemented with Tukey's *post hoc* test. To investigate patterns of bacterial community composition, non-metric multidimensional scaling (nMDS, Bray–Curtis distance) ordination and UPGMA (unweighted pair group arithmetic mean, UniFrac distance) dendrogram were used. Unifrac analysis was based on the average relative abundance of replicate microcosms. Differences in community composition between microcosms were tested using permutational analysis of variance (PERMANOVA) on Bray–Curtis distances. In our detailed OTU analyses (**Figure 7**; **Table 2**) we first selected the 200 most abundant OTUs that is OTUs with the highest total relative abundance across the experiments. These OTUs together represented 82% of total sequence reads. We further examined in detail the response in our transplant experiments of bacterial OTUs that typically represent abundant populations in the Baltic Sea (see, e.g., Herlemann et al., 2011; Lindh et al., 2015). Pronounced responses of particular OTUs were determined by comparing changes in relative sequence abundance between treatments and experiments. Correlations between community composition and enzymatic activity for different taxa were tested using PERMANOVA with Bray–Curtis distances. For testing the correlation between changes in community functioning and shifts in bacterioplankton community composition we performed MANTEL tests. We, therefore, combined the differential response of bacterial production and enzyme activities between microcosm treatments and constructed a distance matrix using the Canberra distance estimation. This community functioning matrix was compared with Bray–Curtis dissimilarity matrices of community composition at different cluster levels (99, 97, 95, 93, and 91% 16S rRNA gene identity). All statistical tests were performed in R 3.0.2 (R Core Team, 2014), using the packages Vegan (Oksanen et al., 2010) and Picante (Kembel et al., 2010). Graphical outputs were made using the package ggplot2 (Wickham, 2009).

## Results

### Initial Environmental Conditions and Nutrient Limitation Bioassay

When sampling the seawater for culture media, *in situ* temperature was 15.5 and 15.8°C and salinity was 7.2 and 3.6 for station BAL and BOT, respectively. When sampling the inoculum for initiating the transplant experiment, temperature was 14.8 and 16.7°C and salinity was 7.2 and 3.6, for station BOT and BAL, respectively. Nitrate and ammonium concentrations were about 1.5 times higher and phosphate around 2 times lower at BOT (0.19, 0.83, and 0.06 μM, respectively) compared to BAL (0.12, 0.56, and 0.11 μM, respectively). TOC concentrations were initially different between stations with 3.96 and 4.39 $mgL^{-1}$ for BAL and BOT, respectively. Although nutrient levels were different between BAL and BOT, bacterial nutrient limitation bioassays showed that bacterial growth was not limited by organic carbon or inorganic nutrients at any of the two stations within the time frame of the 48 h experiment (Figure S2).

### Transplant Experiment

In the transplant experiment, bacterial abundance increased in all microcosms until day 4 (**Figure 3A**). The $BOT_b \rightarrow BOT_{sw}$ treatment resulted in lower abundance ($0.6 \times 10^6$ cells $ml^{-1}$) on day 4 compared to $BAL_b \rightarrow BAL_{sw}$ ($1.3 \times 10^6$ cells $ml^{-1}$; **Figure 3A**). Bacteria in $BAL_b \rightarrow BOT_{sw}$ reached slightly higher abundance than in $BOT_b \rightarrow BOT_{sw}$, ($0.8 \times 10^6$ cells $ml^{-1}$) on day 4 (**Figure 3A**). TOC concentrations decreased in all microcosms from day 2 to 5 (**Figure 3B**). $BAL_b \rightarrow BOT_{sw}$ microcosms showed a steady decrease in TOC concentrations from 4.75 mg $L^{-1}$ at the start of the experiment to 3.9 mg $L^{-1}$ on day 5 (**Figure 3A**).

Bacterial production increased in all microcosms during the experiment and reached nearly twice as high levels in $BAL_b \rightarrow BOT_{sw}$ on day 4 (90 μg C $L^{-1}$ $d^{-1}$; **Figure 4A**) compared to the other microcosms (Tukey's test, $p = 0.001$, $n = 11$). Alkaline-phosphatase activity reached similar levels (10–15 nmol $L^{-1}$ $h^{-1}$) in all microcosms (**Figure 4B**). In contrast, beta-glucosidase activity remained low in the beginning of the experiment but on day 4 increases were observed, with three to sixfold higher responses for both $BAL_b \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BOT_{sw}$ compared to the other microcosms (**Figure 4C**;

**TABLE 2 | Relative abundances of important OTUs (delineated at 97% 16S rRNA gene identity) found during the experiments.**

| | OTU | Taxon (highest identified taxonomic rank) | Phyla/ Class | $BAL_b$ →$BAL_{sw}$ | $BOT_b$ →$BOT_{sw}$ | $BOT_b$ →$BAL_{sw}$ | $BAL_b$ →$BOT_{sw}$ | $BAL_b$ →$BOT_{sw}$ →$BAL_{sw}$ | $BAL_b$ →$BOT_{sw}$ →$BOT_{sw}$ § | $BOT_b$ →$BAL_{sw}$ →$BOT_{sw}$ | $BOT_b$ →$BAL_{sw}$ →$BAL_{sw}$ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *In situ* | TR_000038 | CL500-29 | Actino. | – | 0.3 (0.4) | 0.2 (0.3) | - | 0.1 (0.1) | - | – | – |
| | TR_000029 | hgcI clade | Actino. | 0.1 (0.3) | 0.3 (0.4) | 0.2 (0.3) | 0.4 (0.4) | – | – | – | – |
| | TR_000037 | SAR11 clade | Alpha. | <0.1 (0.1) | 0.3 (0.3) | 0.2 (0.3) | 0.1 (0.2) | – | – | – | – |
| | TR_000014 | *Roseobacter* clade | Alpha. | 5.2 (10.5) | 0.2 (0.3) | 0.3 (0.4) | 2.7 (3.0) | 0.3 (0.6) | – | 0.2 (0.3) | 0.4 (0.4) |
| | TR_000036 | NS3a | Bact. | <0.1 (0.1) | – | – | – | – | – | – | – |
| | TR_000133 | *Polaribacter* | Bact. | 0.2 (0.3) | – | 0.1 (0.1) | 0.1 (0.1) | <0.1 (0.1) | – | – | <0.1 (0.1) |
| | TR_000076 | BAL58 | Beta. | <0.1 (0.1) | – | <0.1 (0.1) | 0.1 (0.1) | – | – | – | – |
| | TR_000025 | *Synechococcus* | Cyano | <0.1 (0.1) | 0.1 (0.1) | <0.1 (0.1) | 0.1 (0.1) | – | – | – | – |
| | TR_000055 | SAR86 clade | Gamma | – | – | – | – | – | – | – | – |
| | TR_000020 | *LD29* | Verr. | 0.1 (0.1) | 0.1 (0.1) | – | 0.2 (0.3) | – | – | – | – |
| $BAL_b$ | TR_000018 | *Seohaeicola** | Alpha. | 3.6 (7.3) | <0.1 (0.1) | 0.4 (0.6) | 1.1 (1.2) | 5.4 (8.4) | 0.5 | 0.7 (0.9) | 2.7 (2.9) |
| | TR_000033 | *Brevundimonas** | Alpha. | – | – | – | – | 2.4 (4.8) | 4.5 | – | – |
| | TR_000200 | *Litoreibacter* | Alpha. | 5.7 (11.5) | 0.2 (0.2) | 0.4 (0.5) | 1.8 (2.2) | 0.3 (0.5) | 0.0 | 0.2 (0.3) | 0.7 (0.8) |
| | TR_000014 | see *in situ* above | | | | | | | | | |
| | TR_005668 | *Limnobacter** | Beta. | 0.1 (0.2) | – | – | 0.8 (1.2) | 7.0 (10.0) | 10.1 | 0.8 (1.6) | – |
| | TR_000005 | *Limnobacter** | Beta. | 0.1 (0.1) | – | – | 0.7 (1.1) | 6.5 (9.6) | 8.1 | 0.8 (1.6) | – |
| | TR_009032 | *Limnobacter** | Beta. | <0.1 (0.1) | – | – | 0.7 (1.1) | 5.0 (6.8) | 7.8 | 0.7 (1.5) | – |
| | TR_000010 | *Pseudomonas* | Gamma. | – | – | <0.1 (0.1) | 1.2 (2.2) | 15.2 (22.1) | 24.5 | 1.9 (3.7) | – |
| | TR_000006 | *Rheinheimera* | Gamma. | 26.8 (53.6) | 7.1 (8.4) | 4.9 (7.9) | 36.2 (37.5) | 15.0 (28.1) | 2.5 | 9.2 (14.3) | 2.0 (2.4) |
| | TR_002843 | *Rheinheimera* | Gamma. | 9.4 (18.8) | 1.2 (1.7) | 1.9 (2.5) | 2.7 (3.3) | 0.3 (0.4) | 0.1 | 0.8 (1.1) | 0.9 (1.0) |
| $BOT_b$ | TR_000003 | *Loktanella* | Alpha. | 1.5 (1.8) | 7.2 (14.4) | 5.6 (6.3) | 2.1 (2.4) | 0.4 (0.9) | – | 3.4 (5.5) | 10.6 (11.4) |
| | TR_000019 | *Rhodobacteraceae* | Alpha. | 2.4 (2.4) | 0.1 (0.3) | 2.3 (3.4) | 0.3 (0.4) | <0.1 (0.1) | – | 0.6 (0.7) | 1.3 (1.5) |
| | TR_000012 | *Alishewanella* | Gamma. | – | 0.1 (0.1) | 0.4 (1.2) | – | – | – | 2.1 (3.9) | 2.7 (2.9) |
| | TR_008541 | *Pseudomonas** | Gamma. | – | 2.3 (2.7) | 3.1 (4.1) | – | – | – | 2.8 (3.0) | 2.6 (2.7) |
| | TR_007801 | *Pseudomonas** | Gamma. | – | 2.0 (2.4) | 2.7 (3.6) | – | – | – | 2.5 (2.6) | 1.9 (1.9) |
| | TR_000001 | *Pseudomonas** | Gamma. | – | 1.5 (1.8) | 1.8 (2.4) | – | – | – | 1.7 (1.7) | 1.4 (1.4) |
| | TR_002653 | *Rheinheimera** | Gamma. | <0.1 (0.1) | 0.3 (0.6) | 4.3 (4.6) | – | <0.1 (0.1) | 0.1 | 4.7 (7.6) | 7.0 (8.3) |
| | TR_000007 | *Rheinheimera* | Gamma. | – | 0.3 (0.5) | 3.3 (3.4) | – | <0.1 (0.1) | 0.1 | 3.7 (6.3) | 5.5 (6.2) |
| | TR_001027 | *Rheinheimera** | Gamma. | – | 0.2 (0.4) | 2.1 (2.6) | – | – | – | 2.6 (4.4.) | 3.3 (4.2) |
| | TR_000820 | *Rheinheimera** | Gamma. | – | 0.2 (0.3) | 1.5 (1.8) | – | – | – | 1.4 (2.5) | 1.5 (1.7) |

*Responses of usually numerically abundant Baltic Sea OTUs that were also dominant in the in situ samples in this study, followed by the 10 most abundant Baltic Proper OTUs with the largest difference in relative abundance compared to Bothnian Sea populations and top 10 Bothnian Sea OTUs with the largest difference in relative abundance compared to Baltic Proper populations during the microcosm experiments. Average relative abundance in percentage of replicate microcosms together with maximum relative abundance in parenthesis is provided. OTUs recruited from being rare in situ (i.e., <0.1% in relative abundance) are indicated with asterisk (*). Alpha., Alphaproteobacteria; Actino., Actinobacteria; Bact., Bacteroidetes; Beta., Betaproteobacteria; Cyano, Cyanobacteria; Gamma., Gammaproteobacteria; Verr., Verrucomicrobia.*
*§ = Only a single replicate, see Table S1.*

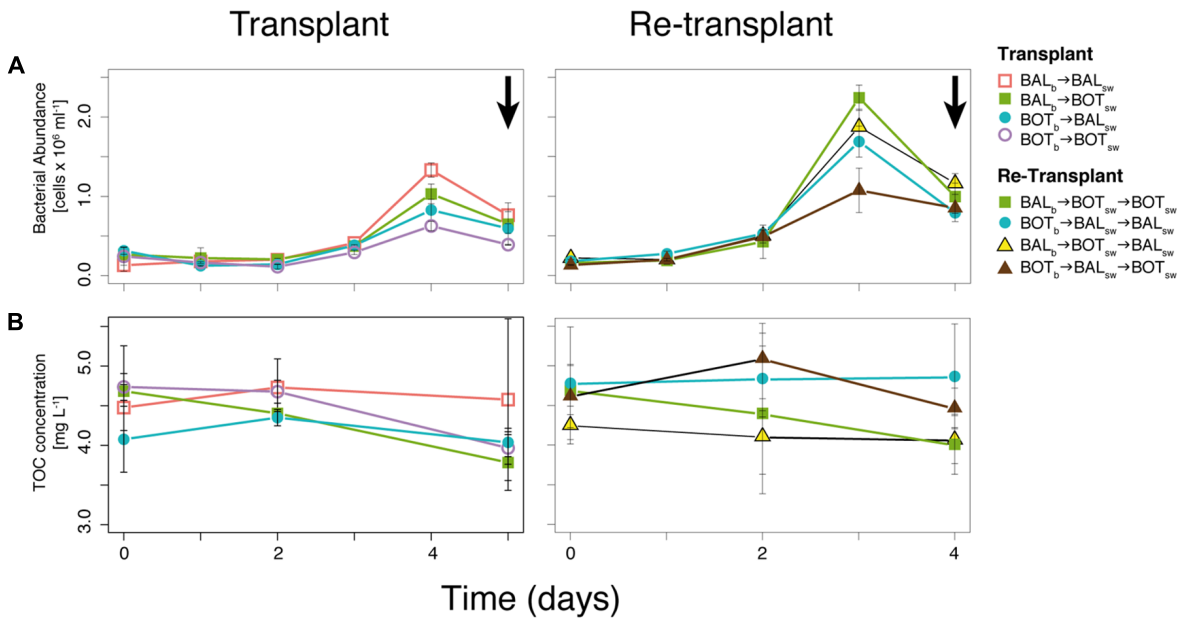


**FIGURE 3 | Bacterial abundance (A) and total organic carbon (TOC) concentrations (B) during transplant and re-transplant experiments.** Error bars denote SDs for replicate microcosms. Arrows in **(A)** indicate when samples for bacterioplankton community composition were collected.

Tukey's test, $p = 0.01$, $n = 11$). Leucine-aminopeptidase activity generally increased nearly fourfold during the experiment although levels were variable between treatments (**Figure 4D**).

Analysis of bacterial community composition by nMDS showed a visual clustering of samples largely determined by the source of the inoculum that is either Baltic Proper or Bothnian Sea bacteria (**Figure 5A**). Further, there was a pronounced differentiation between the *in situ* samples and the bacterial communities that developed in the microcosms, but also between communities growing in water from different geographical origin. Thus, $BAL_b \rightarrow BAL_{sw}$ or $BAL_b \rightarrow BOT_{sw}$ microcosm samples clustered separately from each other, and $BOT_b \rightarrow BOT_{sw}$ or $BOT_b \rightarrow BAL_{sw}$ clustered separately (**Figure 5A**). Unifrac analysis confirmed these general patterns, separating samples by the inoculum source and by origin of water used for growth medium (**Figure 5B**). The separation between bacterial inocula, i.e., $BAL_b$ vs. $BOT_b$, was statistically significant (PERMANOVA, $p = 0.001$, $n = 18$). Moreover, the *in situ* composition was significantly different from that in the microcosms (PERMANOVA, $p = 0.001$, $n = 20$), but there were no significant differences between microcosms in either the transplant and re-transplant experiments.

Nevertheless, there were marked changes in community composition between microcosms as seen from pronounced differences both in the presence/absence and in the relative abundance of a variety of bacterial taxa (**Figure 6**). At the phyla/class level, Gammaproteobacteria increased substantially in the experiment compared with their relative abundance in the *in situ* samples, to comprise nearly three quarters of the relative abundance in all microcosms (**Figure 6A**). Cyanobacteria almost disappeared in the microcosms compared to the *in situ* samples, likely resulting from the incubation of microcosms in the dark; accordingly, the diversity within this taxon was higher *in situ*. Among the Gammaproteobacteria, *Chromatiaceae* increased in all microcosms but on average displayed lower relative abundance in $BOT_b \rightarrow BAL_{sw}$ (**Figure 6B**). *Pseudomonadaceae* responded in most microcosms but not in $BAL_b \rightarrow BAL_{sw}$. Alphaproteobacteria had on average higher relative abundance in $BAL_b \rightarrow BAL_{sw}$ compared to the other microcosms. For example, *Rhodobacteraceae* were more abundant in $BAL_b \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw}$ microcosms but also in $BOT_b \rightarrow BAL_{sw}$ compared to $BOT_b \rightarrow BOT_{sw}$. In contrast, Betaproteobacteria reached higher abundance in $BAL_b \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BOT_{sw}$ than in Baltic Proper water, irrespective of the origin of the bacteria (**Figure 6A**). *Comamonadaceae* increased in all microcosms but were nearly absent in $BAL_b \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw}$ (**Figure 6B**). *Flavobacteriaceae* were predominant in $BOT_b \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BAL_{sw}$ microcosms but displayed overall low relative abundance in $BAL_b \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw}$ (**Figure 6B**).

**Figure 7** shows a summary of the response of the 200 most abundant individual populations (i.e., OTUs defined by 97% 16S rRNA gene identity), together representing 82% of total sequence reads. Further detail on particularly important OTUs is given in **Table 2**. Members of bacterial clades that typically are abundant in the Baltic Sea, such as SAR11 (TR_00037), SAR86 (TR_00055), *Synechococcus* (TR_00025), hgcI (TR_00029), and NS3a (TR_00036) were abundant (>1% relative abundance) or common (0.1–1% relative abundance) in our *in situ* samples and did not increase in relative abundance in any microcosms (**Figure 7**; **Table 2**). Nevertheless, among the OTUs that increased in relative abundance in the experiments, a majority (158 OTUs)

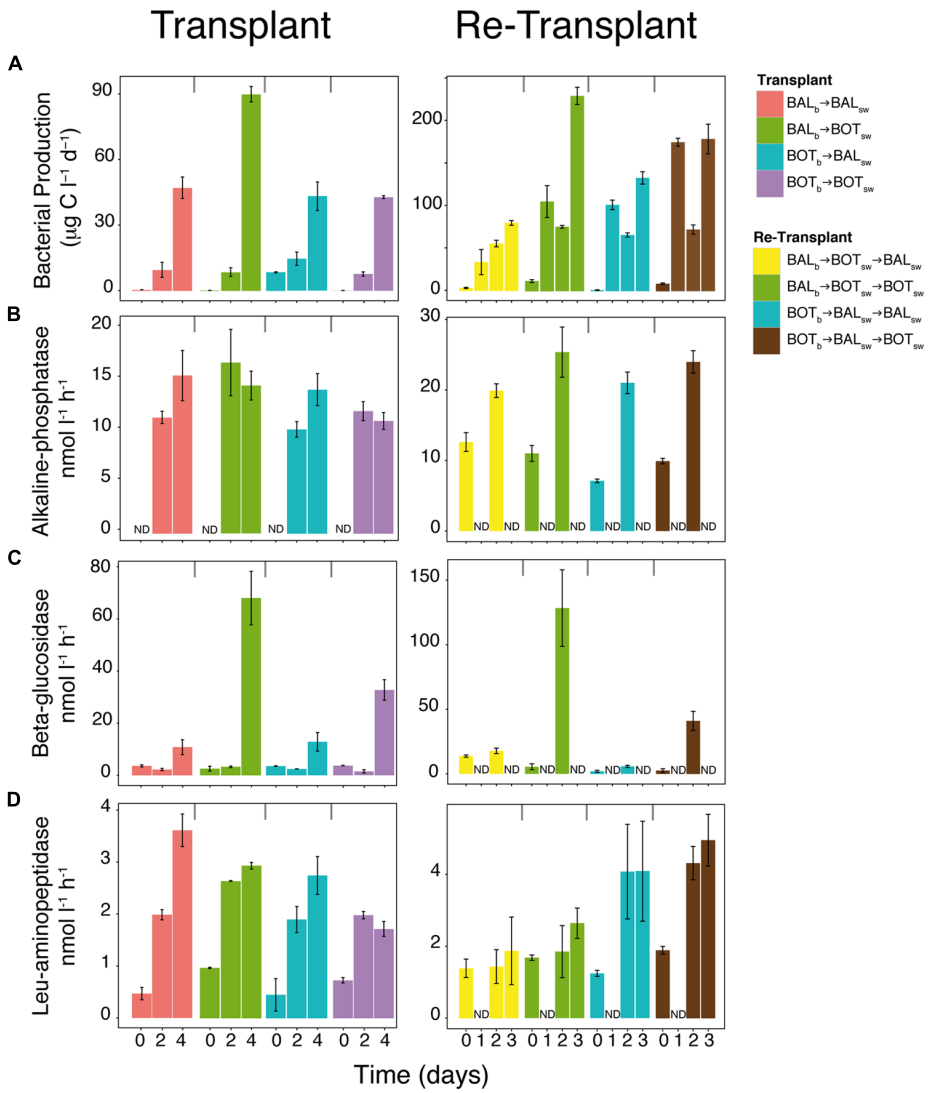


**FIGURE 4 | Measures of bacterial activity in the transplant and re-transplant experiments.** Bacterial heterotrophic production **(A)** and extracellular enzymatic activities **(B–D)**. Error bars denote SDs for quadruplicate technical replicates from biological triplicate microcosms. ND, not determined.

was found to be common and a few (5 OTUs) were even found to be abundant *in situ* (see OTUs indicated by larger blue filled circles in **Figure 7**). For example, among the alphaproteobacterial OTUs, an OTU affiliated with the *Roseobacter* clade that was abundant in the Baltic Proper *in situ* sample, responded in the transplant experiment. This *Roseobacter* OTU TR_000014 was abundant in $BAL_b \rightarrow BAL_{sw}$ microcosms at a relative abundance around 5.2% but reached an elevated relative abundance (2.7%) also in $BAL_b \rightarrow BOT_{sw}$ (**Figure 7**; **Table 2**). We also note that an unclassified *Rhodobacteraceae* OTU (TR_00019) was abundant in $BAL_b \rightarrow BAL_{sw}$, but that this OTU was low in $BOT_b \rightarrow BOT_{sw}$.

Regarding Gammaproteobacteria, three *Pseudomonas* OTUs (TR_08541, TR_07801, TR_00001) were absent in $BAL_b \rightarrow BAL_{sw}$ microcosms and rare *in situ* but became abundant in microcosms with Bothnian Sea bacteria (**Figure 7**; **Table 2**). Sixty populations affiliated with the *Rheinheimera* genus were found among the 200 most abundant OTUs and displayed highly variable patterns of occurrence in the experiments (**Figure 7**). These *Rheinheimera* populations were particularly abundant in transplanted communities and responded both in $BAL_b \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BAL_{sw}$ microcosms (**Figure 7**; **Table 2**). Thus, for example, *Rheinheimera* OTU TR_00006 was highly abundant in $BAL_b \rightarrow BAL_{sw}$ at 26.8% relative abundance and increased to 36.2% in $BAL_b \rightarrow BOT_{sw}$. At the other side of the spectrum, four *Rheinheimera* OTUs (TR_02653, TR_00007, TR_01027, TR_00820) were absent in $BAL_b \rightarrow BAL_{sw}$. These *Rheinheimera* OTUs had low abundance in $BOT_b \rightarrow BOT_{sw}$ but increased to a few percent in $BOT_b \rightarrow BAL_{sw}$ (**Figure 7**; **Table 2**).

## Re-Transplant

Bacterial abundance was higher in most re-transplant microcosms compared to transplant microcosms. However, bacteria

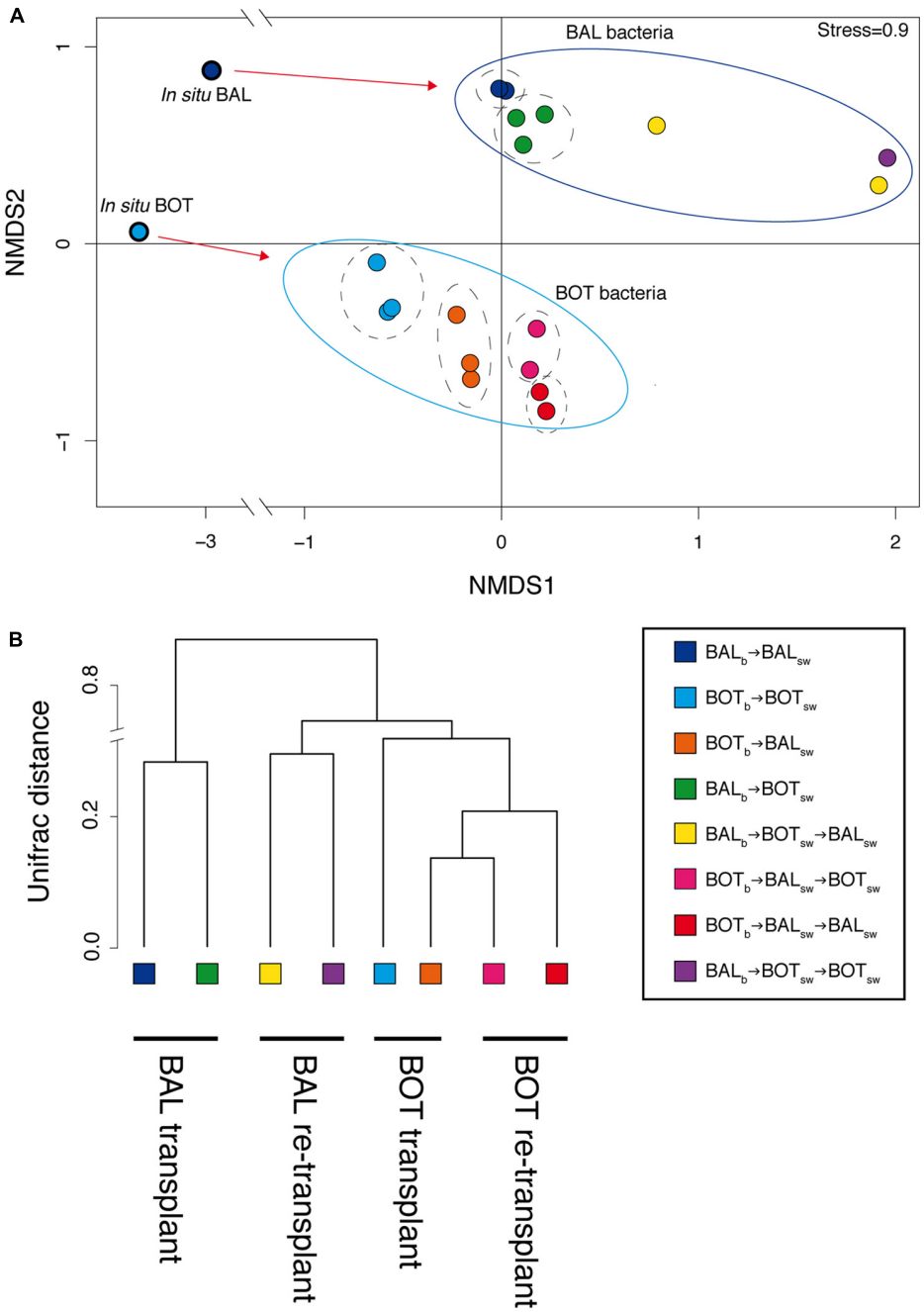


**FIGURE 5 | Comparison of beta-diversity between replicate microcosms with nMDS ordinance calculated from Bray–Curtis distance estimation (A) and a dendrogram visualizing Unifrac distances (B) using 97% 16S rRNA sequence similarity.**

in $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$ microcosms reached much lower abundance on day 3 ($1.0 \times 10^6$ cells $ml^{-1}$) compared to the highest peak ($2.2 \times 10^6$ cells $ml^{-1}$) for $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ (**Figure 3B**). TOC concentrations decreased in most re-transplant microcosms over time, except in $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$.

In the re-transplant experiment, bacterial production increased strongly in all microcosms and was generally about twice as high compared to the transplant experiment (**Figure 4A**). With some variability over time, the highest levels were reached in $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$ (230 μg C $L^{-1}$ $d^{-1}$ and 180 μg C $L^{-1}$ $d^{-1}$, respectively) compared with the other microcosms (**Figure 4A**; Tukey's test, $p = 0.001$, $n = 10$). Alkaline phosphatase increased over time from around 10 to 22 nmol $L^{-1}$ $h^{-1}$ over 2 days in all microcosms. As in the transplant experiment, beta-glucosidase activity

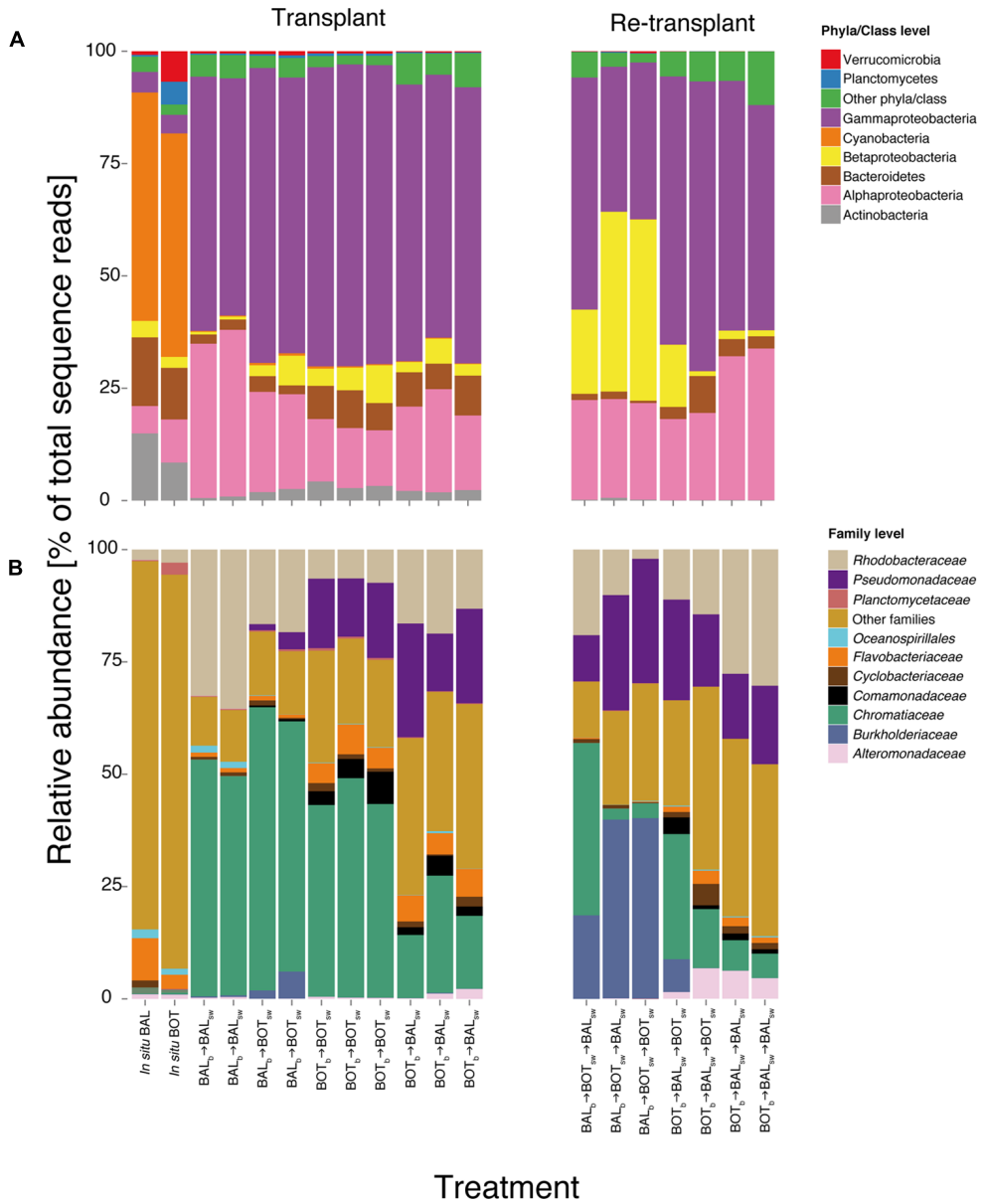


**FIGURE 6 | Bacterial community composition in microcosms at the phyla/class level (A) and family level (B).** Relative abundance is calculated from normalized reads, i.e., percent of total sequence reads.

increased more than 20-fold for both $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$ microcosms, while only small changes were observed in $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ (Tukey's test, $p = 0.001$, $n = 10$). For leucine-aminopeptidase, $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$ and $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ had twice as high activity, around 4 nmol $L^{-1}$ $h^{-1}$, compared to $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ on day 2 and 3 (**Figure 4A**).

Bacterial community composition analysis showed that re-transplants pushed the system further compared to the transplant experiment, while at the same time the visual clustering of samples became more variable (**Figure 5A**). When incorporating phylogenetic placement and average relative abundances between replicate microcosms, Unifrac analysis resolved the differentiation between microcosms by decreasing some of the variation observed in the nMDS analysis (**Figure 5B**).

In the re-transplant experiment, Betaproteobacteria reached overall higher relative abundance in $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ (**Figure 6A**). Concomitantly, Alphaproteobacteria were more important in $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ compared to the other microcosms. *Alteromonadaceae* became abundant in $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ and $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$, continuing an increase triggered already in $BOT_b \rightarrow BAL_{sw}$

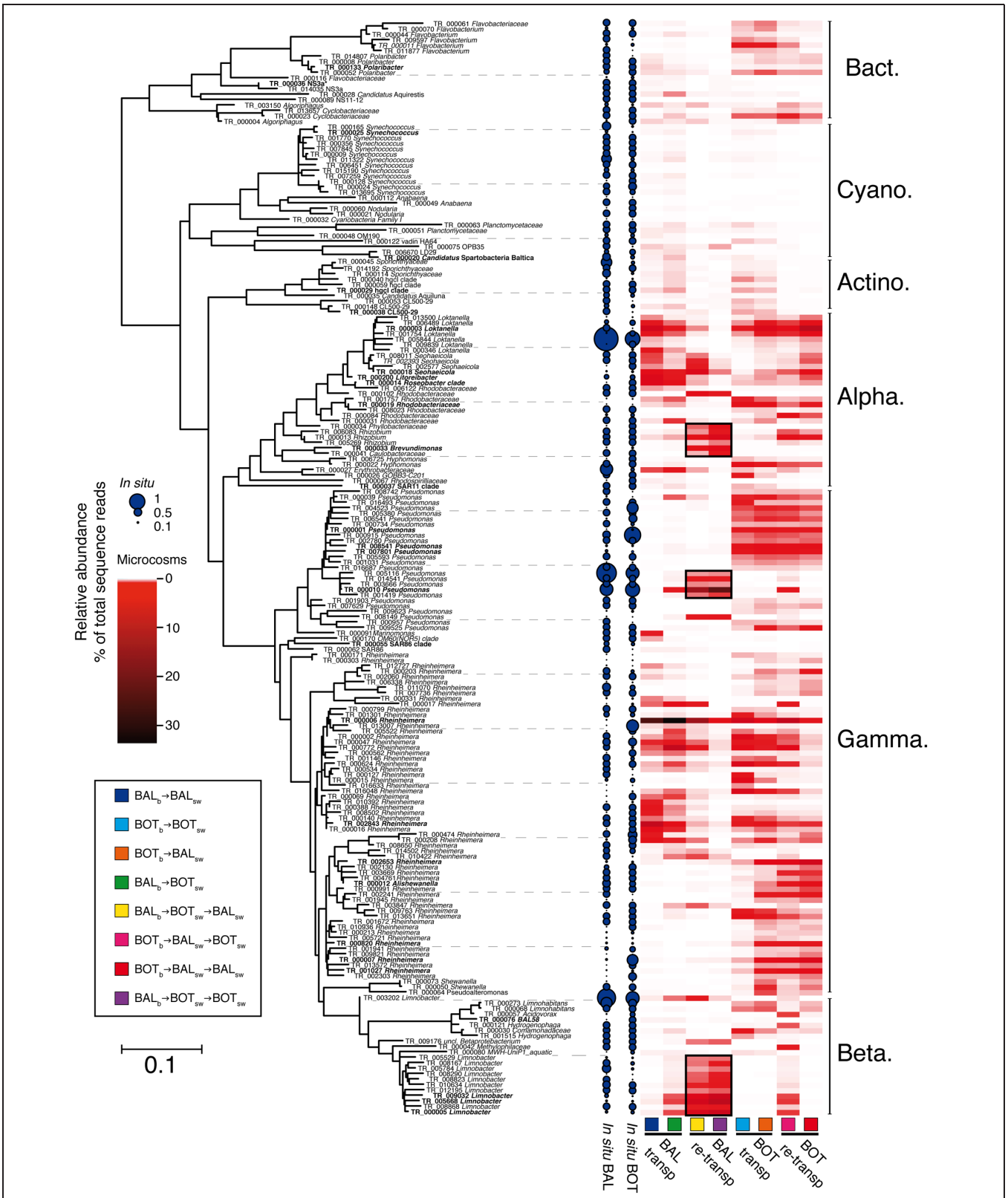


**FIGURE 7 | Maximum-Likelihood tree of 16S rRNA gene sequences obtained from illumina-sequencing.** Heatmaps indicate average relative abundance (percent of total sequence abundance) for the 200 most abundant OTUs in microcosm replicates. OTUs marked in bold are detailed in **Table 2** and in the text. Black squares in heatmap denote priming-effect, i.e., OTUs that were triggered in the transplant experiment with continued increases in relative abundance during the re-transplant experiment.

(**Figure 6B**). Similarly, *Burkholderiaceae* continued to increase in relative abundance in $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$, after being triggered upon growth in $BAL_b \rightarrow BOT_{sw}$ (**Figure 6B**). From here on we refer to this triggering of populations from transplant to re-transplant as a "priming effect."

Several of the OTUs that increased in the re-transplant experiment were not only rare *in situ* but also remained undetected or rare during the transplant experiment (**Figure 7**). For example, *Brevundimonas* OTU TR_000033 accounted for around 5% of the assemblage in the $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ microcosms (**Figure 7**; **Table 2**) but was below the detection limit in the other microcosms and during the transplant experiment. A priming effect was observed for three *Limnobacter* OTUs (TR_005668, TR_000005, TR_009032) that were rare *in situ* and virtually absent during the transplant experiment, except in $BAL_b \rightarrow BOT_{sw}$. These OTUs increased substantially in $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$. The *Pseudomonas* OTU TR_000010 was also primed already in $BAL_b \rightarrow BOT_{sw}$ microcosms and further increased over 10-fold in the re-transplant $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ microcosms. Similarly, priming effects were observed for three *Rhizobium* OTUs (TR_006083, TR_000013, and TR_005269) in $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ and $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$. Further, a *Loktanella* population (TR_000003) that responded in all microcosms during the transplant experiment was only found in $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ during the re-transplant experiment (**Table 2**). In the re-transplant experiment, much lower levels were observed of the *Roseobacter* OTU TR_000014 (0.6% in $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$) compared to the initial transplant (2.7%; **Figure 7**; **Table 2**).

## Diversity

Lower levels of Shannon and Chao1 indexes were detected in $BAL_b \rightarrow BOT_{sw}$ compared to $BAL_b \rightarrow BAL_{sw}$ microcosms (**Table 3**). On the other hand, Shannon diversity reached the highest value in $BOT_b \rightarrow BAL_{sw}$ compared to all other microcosms and the *in situ* samples. Alpha diversity levels remained relatively low in the $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ microcosms and decreased further in $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$.

**TABLE 3 | Shannon and Chao1 indexes ± SD "–" indicate lack of replicates.**

| Treatment | Shannon | Chao1 |
|---|---|---|
| *In situ* BAL | 4.59 – | 1247.03 – |
| *In situ* BOT | 4.97 – | 1296.72 – |
| $BAL_b \rightarrow BAL_{sw}$ | 4.60 ± 0.11 | 2352.21 ± 164.45 |
| $BOT_b \rightarrow BOT_{sw}$ | 4.68 ± 0.02 | 2221.73 ± 271.36 |
| $BOT_b \rightarrow BAL_{sw}$ | 5.06 ± 0.10 | 2195.57 ± 317.30 |
| $BAL_b \rightarrow BOT_{sw}$ | 3.55 ± 0.15 | 1383.66 ± 83.91 |
| $BAL_b \rightarrow BOT_{sw} \rightarrow BAL_{sw}$ | 3.62 ± 0.05 | 1248.68 ± 106.98 |
| $BOT_b \rightarrow BAL_{sw} \rightarrow BOT_{sw}$ | 4.65 ± 0.09 | 1843.28 ± 95.054 |
| $BOT_b \rightarrow BAL_{sw} \rightarrow BAL_{sw}$ | 4.68 ± 0.12 | 2045.08 ± 759.73 |
| $BAL_b \rightarrow BOT_{sw} \rightarrow BOT_{sw}$ | 3.41 – | 1222.18 – |

## Linking Bacterial Community Composition and Phylogeny with Bacterial Community Functioning

To determine if specific bacterial taxa could be associated with responses in enzymatic activities we performed PERMANOVA tests (Table S2). Although we found significant correlations between enzyme activities and, e.g., *Alteromonadaceae* (PERMANOVA, $p = 0.01$, $R^2 = 0.28$, $n = 18$), and *Chromatiaceae* (PERMANOVA, $p = 0.01$, $R^2 = 0.26$, $n = 18$), such correlations explained typically less than 20% of the variance (Table S2). Interestingly though, several taxa were significantly correlated with either beta-glucosidase or leucine-aminopeptidase but not with alkaline-phosphatase. Next, we analyzed bacterial community functioning (i.e., collective differences in bacterial production and enzyme activities) versus community composition clustered at different phylogenetic levels. In the transplant experiment, absolute shifts in community composition were significantly correlated with absolute shifts in bacterial community functioning, especially at the 97% 16S rRNA gene sequence cluster identity level (MANTEL, $p = 0.001$, Pearson $R^2 = 0.65$, $n = 17$; **Figure 8**). The correlation between bacterial community composition and bacterial community functioning was also strong at the 99 and 95% cluster level (MANTEL, $p < 0.01$, Pearson $R^2 = 0.59$–$61$, $n = 17$) but became weak and insignificant at lower taxonomic resolution (<95%). The absolute shifts in community composition and absolute shifts in bacterial community functioning in the re-transplant experiment were not significantly correlated (**Figure 8**).

# Discussion

## Bacterial Responses in Community Functioning

In our microcosm experiments we used transplants of bacterial assemblages to investigate connections between bacterioplankton community composition and metabolic plasticity in response to contrasting environmental conditions between the different Baltic Sea basins. Our experimental manipulations showed substantial differences between microcosms in terms of bacterial abundance (**Figure 3A**), organic carbon utilization (**Figure 3B**), bacterial heterotrophic production (**Figure 4A**), and beta-glucosidase activity (**Figure 4C**), indicating that bacterial community functioning changed when bacteria were exposed to water from different geographical origins. For example, when Baltic Proper bacteria were challenged by new environmental conditions found in Bothnian Sea water, we observed increased bacterial production and beta-glucosidase activity. Similar changes in bacterial production and enzyme activity occurred also in re-transplant experiments, reinforcing the role of Bothnian Sea water on the observed metabolic changes.

Our findings indicated that Baltic Proper bacteria show metabolic plasticity when transferred to Bothnian Sea water, as deduced from the observation that activity in these treatments actually increased. This effect was further promoted when transplanted Baltic Proper bacteria were allowed to resume growth in

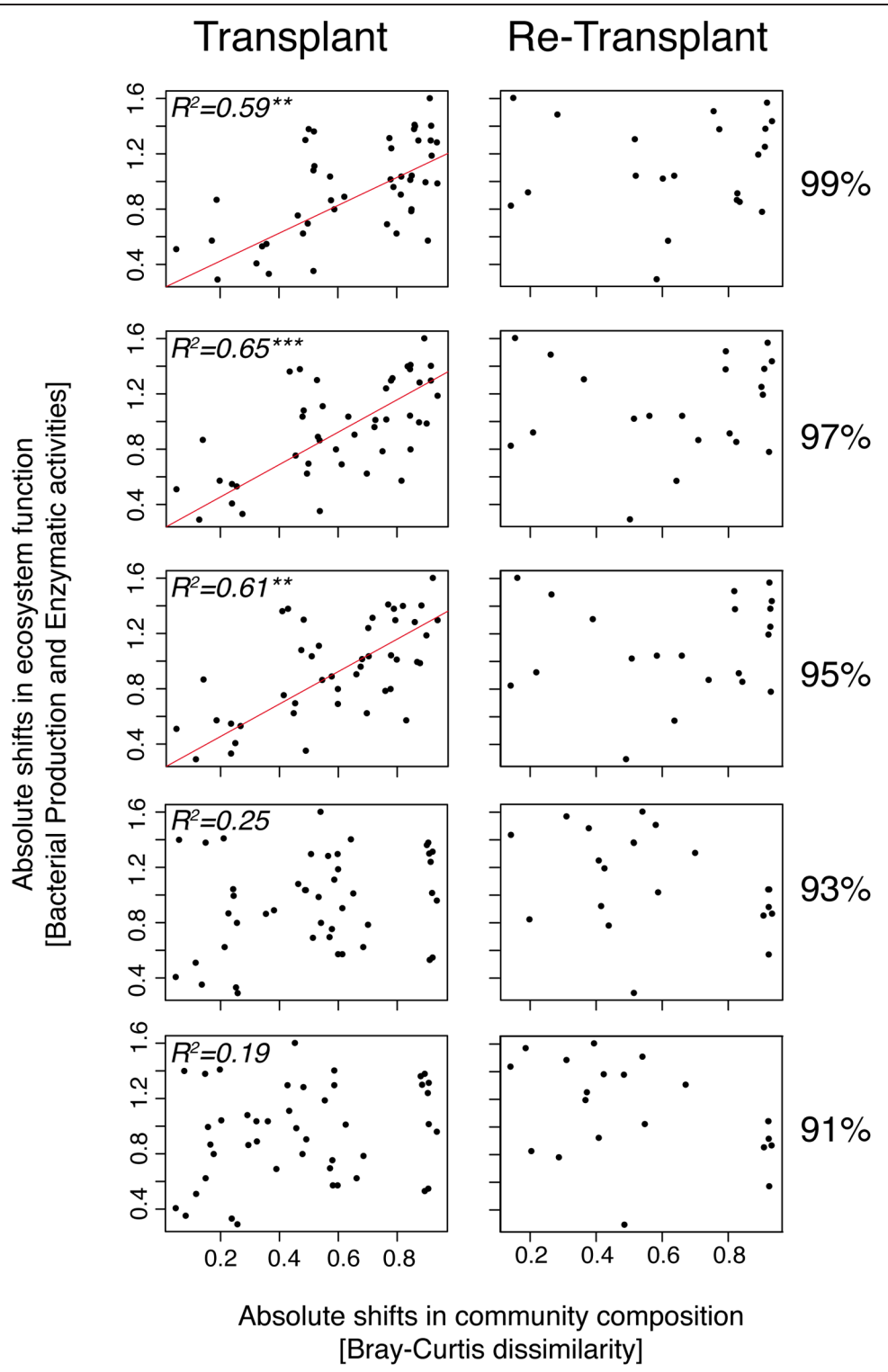


**FIGURE 8 | MANTEL tests and plots of distance matrices of absolute shifts in community composition measured by Bray–Curtis distance estimation versus absolute shifts in bacterial community functioning (pooled bacterial production and enzyme activities) measured by Canberra distance estimation.** Bray–Curtis dissimilarity matrices of community composition were performed at different cluster levels (99, 97, 95, 93, and 91%) of 16S rRNA gene identity. All microcosms are compared with each other. Red lines indicate significant correlations and $R^2$ values above 0.5. Asterisks denote the significance levels; $^{**}p < 0.001$, $^{***}p < 0.0001$.

Bothnian Sea water during the re-transplant experiment. The elevated levels of activity for Baltic Proper bacterial communities in changed environmental conditions compared to controls may suggest that higher bacterial activities could be expected in the Baltic Proper in response to climate change induced reductions in salinity and increased terrestrial DOM runoff. However, it is important to consider that results from this study are based on short-term responses to disturbances, whereas the effects of climate change implicate long-term changes in the water-chemistry of the Baltic Sea. Still, disturbance events that in part contribute to the long-term changes, for example massive river runoff events following heavy rainfall or storm induced upwelling of nutrient rich waters, are likely to be more frequent with anthropogenically induced changes in environmental conditions. These findings substantiate and support earlier model data and experimental results from the Baltic Sea, implicating changes from autotrophy toward microbial heterotrophy with increases in riverine outflow due to climate change (Sandberg et al., 2004; Wikner and Andersson, 2012; Degerman et al., 2013; Lefébure et al., 2013).

## Bacterioplankton Community Change

Concurrently with the changes in bacterial community functioning, transplants, and re-transplants of bacterial assemblages between water from different geographical origins also caused changes in the composition of bacterial communities. For example, shifts in composition were accompanied by increased bacterial production and beta-glucosidase activity in transplants with Baltic Proper bacteria growing in Bothnian Sea water. However, the shifts in environmental conditions did not completely transform the communities so that they all became the same, i.e., Baltic Proper communities did not converge to the same structure as Bothnian Sea communities and neither vice versa (**Figure 5**). In accordance with our results, substantial shifts in bacterial community composition also occurred when transplanting bacteria between Baltic Sea and Skagerrak Sea conditions, yet the communities did not become similar (Sjöstedt et al., 2012). These experimental approaches are short-term while *in situ* responses may look very different in the long run, emphasizing the need to carry out longer experiments and *in situ* time-series to elucidate the resistance, resilience, and sensitivity of bacterial communities responding to environmental disturbances. Nevertheless, a multitude of experimental and *in situ* approaches in coastal waters have established that bacterial community structure is sensitive to environmental disturbances, e.g., changes in terrestrial DOM (Kisand and Wikner, 2003; Rochelle-Newall et al., 2004; Kisand et al., 2008; Teira et al., 2009; Grubisic et al., 2012; Rocker et al., 2012) and salinity (Langenheder et al., 2003; Kaartokallio et al., 2005; Sjöstedt et al., 2012). Taken together, our data indicate distinct responses and links between bacterial community composition and community functioning resulting from exposure to seawater from the northern vs. southern Baltic Sea basins.

We have no immediate knowledge as to the specific chemical characteristics in the seawater from the Baltic Proper and Bothnian Sea that could have driven changes in bacterioplankton community structure in our experiments. Salinity is a critical factor in regulating bacterial community composition (Langenheder et al., 2003; Lozupone and Knight, 2007; Herlemann et al., 2011; Sjöstedt et al., 2012). A recent metagenomic analysis indicated genomic features that may contribute to such regulation (Dupont et al., 2014). Some of these features were indicative within the same narrow range of salinities (salinity 3.6–7.2) that characterize our studied waters. Moreover, salinity can regulate bacterial community functioning, and low salinity may have a negative influence on the growth and activity of marine bacterioplankton degrading terrigenous carbon (Langenheder et al., 2003; Kisand et al., 2008). Although bacteria can degrade allochthonous DOM (Rochelle-Newall et al., 2004; Rocker et al., 2012), autochthonously produced DOM is often more efficiently utilized due to its less refractory nature (Kritzberg et al., 2004). However, allochthonous DOM can lead to higher respiration and not be incorporated into biomass (Fasching et al., 2014). The discharge of allochthonous DOM is higher into the northern basins of the Baltic compared to the Baltic Proper (Omstedt et al., 2014). Furthermore, inorganic nutrient concentrations could have influenced the bacterial dynamics in our experiments. However, nutrient limitation bioassays with *in situ* samples indicated that the investigated communities were not directly limited by nutrient availability (Figure S2). Further, physicochemical factors, such as limitation of trace metals (Church et al., 2000), or top–down effects, such as protist grazing, or virus predation (Jürgens et al., 1999; Bouvy et al., 2011), may contribute to promoting changes in community structure and bacterial community functioning of transplanted bacterial communities. In addition, it is also important to consider that seasonal and inter-annual variation in environmental conditions, from, e.g., phytoplankton blooms, result in a succession of bacterioplankton populations and a wide spectrum of responses in abundances (Andersson et al., 2010; Lindh et al., 2015). Anthropogenically induced changes (in e.g., temperature) may influence such seasonal patterns, which could complicate interpretations of responses to precipitation patterns of bacterioplankton populations.

In our study, particular bacterial groups and populations showed distinct responses to water from different geographical origin in the experiments (**Figure 6**; **Table 2**). Thus, although there were pronounced changes in all microcosms, specific treatment effects resulted in communities that were distinct from one another at the end of the experiment. Community composition change due to environmental disturbances often results in the recruitment of rare OTUs that become abundant, as demonstrated both experimentally and *in situ* (Campbell et al., 2011; Sjöstedt et al., 2012; Alonso-Saez et al., 2014). However, it is noteworthy that among the 200 most abundant OTUs that responded in the microcosms at the end of the experiments, a few OTUs were actually abundant (>1% relative abundance, $n = 5$), while the grand majority were common (0.1–1% relative abundance, $n = 158$) *in situ*. In contrast, only 33 OTUs that responded in the experiments were initially rare (<0.1% relative abundance). These findings show that not all responsive OTUs represented initially rare copiotrophic populations stimulated by artificial "bottle-effects" but that common populations *in situ* are particularly responsive to environmental disturbances.

Among the initially rare populations several *Limnobacter* OTUs increased in abundance when Baltic bacteria were transferred to Bothnian Sea water, and several *Pseudomonas* OTUs

found among the Bothnian Sea bacteria proliferated in Baltic Proper water; this indicated replacement of populations. Also adjustment of bacterial populations to the experimental disturbances was observed among the bacterial populations (**Figure 7**; **Table 2**). In particular, one *Roseobacter* OTU was not only abundant *in situ* and in control microcosms, but also in transplants of Baltic proper bacteria to Bothnian Sea water. In addition, *Rheinheimera* populations were highly variable between microcosms, indicating population adjustment (**Figure 7**; **Table 2**). Collectively, our transplant and re-transplant experiments suggest a balance of adjustment and replacement effects when bacteria encounter distinct water conditions from different geographical origin.

## Priming Effect

Recruitment of rare bacteria as a response to changes in environmental conditions can result from proliferation of both specialist and generalist populations (Mills and Mallory, 1987; Atlas et al., 1991; Campbell et al., 2011; Lennon and Jones, 2011). Some bacterial taxa triggered in the transplant experiment, e.g., *Alteromonadaceae* and *Burkholderiaceae* OTUs, continued to increase in relative abundance during the re-transplant experiment in both types of seawater media, as a result of a "priming effect." Such priming seems to have resulted from the initial triggering of increases in abundance of a limited number of populations by exposure to water from a different location; and this initial growth stimulation then continued upon transfer also to waters from different basins. This response may result from challenging a bacterial community that is not immediately resilient but rather reward generalist OTUs that were successful in transplants. Therefore, it would be highly interesting to study the resilience potential of disturbed bacterial communities over longer time scales, either in long-term experiments or over several years *in situ* to elucidate the pace and frequency at which specific populations recover their abundances or the bacterial community returns to previous undisturbed structure.

## Diversity

An important ecological mechanism in nature is the insurance hypothesis or portfolio effect that balances negative (i.e., sensitive species) and positive effects (i.e., responsive species) simply by carrying a large number of taxa (Loreau, 2000; Allison and Martiny, 2008). This mechanism can result in a scenario, where bacterial community composition changes while maintained or even increased bacterial community functioning can be observed compared to the undisturbed community. The insurance hypothesis is intriguing; especially in relation to future climate change and the growing awareness of its substantial long term effects on biodiversity in all parts of the food-web in marine environments across the globe (Worm et al., 2006; Awasthi et al., 2014). Although richness effects on bacterial community functioning may be less important under current environmental conditions, they are likely to become important for handling future environmental disturbances (Loreau, 2000; Bell et al., 2005; Awasthi et al., 2014).

In our experiments, bacterial responses to experimental disturbances heavily influenced alpha diversity. Shannon and Chao1 levels were substantially lower in all microcosms with Baltic Proper bacteria except the controls (**Table 3**). Lower alpha diversity due to transplants and re-transplants with Baltic Proper bacteria could suggest that only few populations are able to cope with the changes in environmental conditions to which they were exposed. Alternatively, a few populations that were highly competitive under the new seawater conditions could increase in relative abundance to become dominant. In fact, lower alpha diversity was found when metabolic activity was high and community composition changed substantially, as exemplified by Baltic Proper bacteria growing in Bothnian Sea water. These data suggest that a portfolio effect likely aided the response of bacterial community composition and bacterial community functioning in the transplant experiment (Wittebolle et al., 2009; Awasthi et al., 2014). However, the resulting low alpha diversity due to transplants possibly led to a chaotic response in community composition and a more variable effect on metabolic activity during the re-transplant experiment, suggesting that environmental disturbances such as increased riverine discharge may render disturbed communities highly sensitive. Taken together, many OTUs in the Baltic Proper seem to be well suited for Bothnian sea-like environmental conditions; that is future predicted increases in terrigenous organic matter and lower salinity, but at the cost of overall lower alpha diversity and potentially a reduced responsiveness to added environmental change.

## Bacterial Community Functioning

The current debate of functionally redundant versus non-redundant bacterial communities is complex (Loreau, 2004; Wohl et al., 2004; Allison and Martiny, 2008; Comte and Del Giorgio, 2011; Miki et al., 2014). However, transplant and re-transplant experiments can be used to address some of the fundamental questions regarding the role of community composition for bacterial responses in metabolic activity (**Figure 8**; Table S2). In the transplant experiment, we observed a positive relationship between absolute shifts in community composition and absolute shifts in bacterial community functioning (explaining $>60\%$ of the variance, depending on phylogenetic scale). Interestingly this relationship was most prominent at 97% 16S rRNA gene sequence identity and only observed at $\geq 95\%$. At lower taxonomical resolution, community composition and bacterial community functioning were not correlated. The lack of correlation at lower taxonomic resolution thus resulted from the counterbalancing of differential responses among individual populations within the same major taxon and highlights the importance of analyzing specific responses to environmental disturbances at a detailed phylogenetic level.

In the re-transplant experiment this relationship was lacking regardless of phylogenetic scale, which would lead to the conclusion that bacterial assemblages were functionally redundant. However, it is important to note here that the relationship between community composition and bacterial community functioning breaks down in the experiment with continued experimental forcing (i.e., in the re-transplant experiment) in which the bacterial community had already gone through a pronounced succession from the original time zero. This could indicate that

successional progression temporarily offsets perceived relationships between bacterial community composition and functioning. In other words, interpretations of levels of redundancy, and hence the importance of species richness in the context of the insurance hypothesis/portfolio effect (Loreau, 2000; Allison and Martiny, 2008), could be heavily distorted both by the complexity of natural bacterial assemblages and by the inability to adequately determine successional stages of investigated communities. These findings indicate the efficacy of combining longer experiments with high taxonomical resolution (≥97% 16S rRNA gene identity) analyses for interpreting distribution patterns of individual bacterial populations in relation to environmental forcing. Ultimately, such analyses have the potential to identify causal relationships between bacterial community composition and functioning.

### Conclusion

According to our hypothesis, bacterial community composition and functioning would change after both transplantation and re-transplantation disturbances, following the replacement scenario. Indeed, this hypothesis was confirmed in the transplant experiment, such that the changes in community composition accounted for by responsive bacterial populations were reflected also in adjustment of bacterial activities. However, when adding a continued experimental forcing to the already disturbed community in the re-transplant experiment, the linkage between change in community composition and change in community functioning became disrupted. Rejection of our hypothesis in the re-transplant experiment implies that disturbances caused distinct responses of specialist or generalist bacteria in a manner that was dependent on the successional stage at which the disturbance took place. Our findings further indicate the potential of experimental manipulations to aid interpretations of the adaptability and metabolic plasticity of bacterioplankton communities responding to changes in environmental conditions. Notably, exposure of Baltic Proper bacteria to humic rich/low salinity Bothnian Sea water caused higher metabolic activity, while at the same time inducing shifts in bacterial community structure. This supports recent suggestions that climate change could lead to undesirable long-term shifts toward an increasingly net heterotrophic system in the Baltic Proper. Alterations in precipitation patterns across seasons or years or increased frequency of event driven river runoff episodes may at first result in only subtle changes in community composition or bacterial activities. However, such changes in runoff could have essential priming effects on bacterial community structure that subsequently translate into longer-term changes in bacterial community functioning and biogeochemical process rates.

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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.2015.00223/abstract

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# Distribution of iron- and sulfate-reducing bacteria across a coastal acid sulfate soil (CASS) environment: implications for passive bioremediation by tidal inundation

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Coastal acid sulfate soils (CASS) constitute a serious and global environmental problem. Oxidation of iron sulfide minerals exposed to air generates sulfuric acid with consequently negative impacts on coastal and estuarine ecosystems. Tidal inundation represents one current treatment strategy for CASS, with the aim of neutralizing acidity by triggering microbial iron- and sulfate-reduction and inducing the precipitation of iron-sulfides. Although well-known functional guilds of bacteria drive these processes, their distributions within CASS environments, as well as their relationships to tidal cycling and the availability of nutrients and electron acceptors, are poorly understood. These factors will determine the long-term efficacy of "passive" CASS remediation strategies. Here we studied microbial community structure and functional guild distribution in sediment cores obtained from 10 depths ranging from 0 to 20 cm in three sites located in the supra-, inter- and sub-tidal segments, respectively, of a CASS-affected salt marsh (East Trinity, Cairns, Australia). Whole community 16S rRNA gene diversity within each site was assessed by 454 pyrotag sequencing and bioinformatic analyses in the context of local hydrological, geochemical, and lithological factors. The results illustrate spatial overlap, or close association, of iron-, and sulfate-reducing bacteria (SRB) in an environment rich in organic matter and controlled by parameters such as acidity, redox potential, degree of water saturation, and mineralization. The observed spatial distribution implies the need for empirical understanding of the timing, relative to tidal cycling, of various terminal electron-accepting processes that control acid generation and biogeochemical iron and sulfur cycling.

**Keywords: acid sulfate, microbial community, iron-reducing, sulfate-reducing, iron sulfides, geomicrobiology, wetlands, remediation**

## Introduction

Coastal acid sulfate soils (CASS) constitute a major global environmental problem (Dent and Pons, 1995; White et al., 2007; Ljung et al., 2009). The resulting problems include fish kills (Powell and Martens, 2005; Stephens and Ingram, 2006), decreased rice yields (Bronswijk et al., 1995), release of greenhouse gases such as methane and dinitrogen oxide (Denmead et al., 2007), sulfur dioxide emissions (Macdonald et al., 2004), construction damage (Crammond, 2002), and changed mobility of toxic metals (Burton et al., 2008). In Australia, roughly $10 billion worth of acid sulfate soil "legacy" impacts remain (Fitzpatrick, 2003), and Australia contains only about 18% of acid sulfate soils worldwide (Ljung et al., 2009).

Although some CASS environments result from tectonic uplift (Åström and Björklund, 1995), anthropogenic drainage of wetlands generally accounts for most recent and modern CASS formation (Rosicky et al., 2004; Ljung et al., 2009). Drainage allows oxygen to penetrate further into the subsurface, resulting in oxidation of iron sulfides and release of protons and sulfuric acid. For instance, the oxidation of pyrite by molecular oxygen (Hicks et al., 1999):

$$FeS_2 + 3.75O_2 + 3.5H_2O \rightarrow Fe(OH)_3 + 4H^+ + 2SO_4^{2-} \quad (1)$$

releases 4 moles of acid and 2 moles of sulfate per mole of pyrite. Microbial intervention will expedite the reaction rate by co-oxidation of ferrous iron, with the product $Fe^{3+}$ acting as a strong oxidant of pyrite.

Low pH pore waters promote the mobility of toxic heavy metals such as aluminum and manganese (Willett et al., 1992; Sammut et al., 1996), which can be partitioned into metal-sulfides (Moreau et al., 2013). A pH decrease from 3.7 to 1.9 can result in a dissolved aluminum increase from 0.9 to 40 M (Van Breemen, 1973). Aluminum hydrolysis generates 3 moles of acid from one mole of aluminum and decreases pH further (Hicks et al., 1999):

$$Al^{3+} + H_2O \rightarrow Al(OH)^{2+} + H^+ \quad (2)$$

$$Al(OH)^{2+} + H_2O \rightarrow Al(OH)_2^+ + H^+ \quad (3)$$

$$Al(OH)_2^+ + H_2O \rightarrow Al(OH)_3 + H^+ \quad (4)$$

The most common treatments for CASS-related contamination are lime neutralization and seawater inundation (White et al., 1997; Johnston et al., 2009a,b). The primary disadvantage of lime treatment is the relatively high cost and need for extensive and continued maintenance. In the Great Barrier Reef catchments in Australia, it was estimated that $62 million AUD would be needed for lime treatment over $6.7 \times 10^5$ hectares of CASS-affected regions (Powell and Martens, 2005). However, lime addition accounts for less than 1% of the alkalinity in a lime-assisted tidal inundation treatment (Johnston et al., 2012). Re-flooding by seawater is hence a more cost effective treatment strategy, as marine bicarbonate accounts for 25∼40% of total alkalinity generation (Johnston et al., 2012). The processes of microbial sulfate and iron reduction triggered by seawater flooding contribute to more than 50% of total alkalinity (Johnston et al., 2012). Both iron and sulfate reduction consume protons:

$$Fe(OH)_3 + 0.25CH_2O + 2H^+ \rightarrow Fe^{2+} + 0.25CO_2 + 2.75H_2O \quad (5)$$

$$SO_4^{2-} + 2(CH_2O) \rightarrow 2HCO_3^- + H_2S \quad (6)$$

and can lead to formation of mackinawite (FeS) or pyrite ($FeS_2$), both of which can promote the immobilization of arsenic, a common toxic metalloid in CASS environments (Burton et al., 2011).

Microorganisms can also influence other biogeochemical cycles in natural and contaminated wetlands. However, little research has been conducted to provide detailed information about microbial processes and bioremediation efficiency specifically in CASS systems. Previous microbiological studies of CASS-like systems have discussed the vertical depth-distribution of sulfur- and iron-oxidizing bacteria in a paddy field (Ohba and Owa, 2005), revealed high microbial biomass and activity at the Baltic coast, Finland (Simek et al., 2013) and compared microbial communities from Ostrobothnian, Finland, with those found in acid mine drainage (AMD) (Wu et al., 2013). To understand the potential for microbial acid generation and biogeochemical cycling in CASS-impacted sediments, a comprehensive, spatially integrative resolution of microbial "functional guild" distribution is required. Specifically, we need to know more about the distribution and diversity of sulfur- and iron-cycling microorganisms. Previous studies have long established that iron reducing bacteria (IRB) can out-compete sulfate reducing bacteria (SRB) for limited electron donor when the environment is non-limiting in ferric iron (e.g., Lovley and Phillips, 1987; Chapelle and Lovley, 1992). However, we hypothesized that increases in the concentrations of organics in CASS systems decrease competition between IRB and SRB by increasing thermodynamic energy availability relative to enzyme kinetics. We present results and analyses from an investigation of whole community 16S rRNA genes amplified from CASS-impacted sediments from the East Trinity wetlands (Cairns, Australia), a tidally influenced wetland located on the northeast coast of Australia. Gene data are interpreted in the context of environmental and organic geochemical data acquired from the site. The results of this study were analyzed in the context of soil type and sediment lithology, degree of pore water saturation, tidal inundation and turbidity, acidity and organic carbon availability to understand the factors that shape microbial community structure and activity.

## Materials and Methods

### Field Site and Soil Sampling

The majority of CASS environments were formed during the last Holocene post-glacial period, as rising sea levels promoted the deposition of iron sulfide minerals (Dent, 1986). The East Trinity wetlands study site is characterized by abundant potential CASS in Holocene soil layers that formed when high rates of organic matter degradation under warm temperatures stimulated iron and sulfate reduction (White et al., 1997). In the 1970s,

large-scale drainage of seawater exposed CASS to air and resulting in soil acidification and heavy metal contamination problems in this area and nearby ecosystems. Since 2001, tidal exchange remediation is being practiced in this area (QASSIT, 2000).

The pH and Eh values of each core section were measured in the field with a portable pH/Eh meter by Thermo Scientific Orion 5-Star Portable Plus pH/ORP/ISE/Conductivity/DO meter with a Model 9678BN Pt-Ag/AgCl combination electrode. ORP measurements were calibrated to a standard hydrogen electrode at 20°C. The resolution and relative accuracy of pH and ORP are 0.01 and ±0.002, and 0.1 mV and ±0.2, or 0.05%, respectively. Soil samples were collected from a sub-catchment of Firewood Creek in the East Trinity Wetland (145°80'E, 16°94'S), northeast Australia (Burton et al., 2011; Johnston et al., 2012). Three 20 cm-deep sediment cores were collected along an upland to seawater transect (**Figure 1**, sampling sites A1 to A3, respectively) during a low tide period, and then sectioned into 2 cm intervals. Sediments were collected into 15 mL sterile bottles containing 3 mL of RNA*later*™ RNA stabilization reagent and then preserved at −80°C in a freezer until DNA extractions were performed. Chemical data were measured in the field by insertion of the electrode into soil/pore water at each of the sampling depths, with several rinses with nanopure water in between measurements. The sampling transects incorporated both the surface, or O horizon consisting of surficial organic deposits (Johnston et al., 2011b, 2012), and sulfuric horizon, consisting of actual acid sulfate soils characterized by low soil pH (Hicks et al., 1999).

## Organic Geochemistry Analyses

Organic geochemical analyses were performed on aliquots of the top 6 cm of sediment cores from the transect (A1–A3) and two control sites located (i) outside of the tidal bund-wall (not affected by drainage and CASS formation; "Mangrove Site") and (ii) at an CASS site not treated with tidal inundation ("Acidic Site"). The samples were freeze-dried, homogenized and Soxhlet-extracted (48 h) in a mixture of dichloromethane (90%) and methanol (10%). Elemental sulfur was removed with activated copper pellets. The extracts were separated into three fractions (aliphatics, aromatic and polar) by silica gel column chromatography using eluents of increasing polarity (e.g., Nabbefeld et al., 2010). Aliquots of the dried polar fractions were derivatised in a 3:2 mixture of bis(trimethylsilyl)-trifluoroacetamide (BSTFA) and anhydrous pyridine for (20 min) at 60–70°C and analyzed using GC-MS within a few hours.

Gas chromatography-mass spectrometry (GC-MS) analyses of aromatic and derivatised polar fractions were performed on an Agilent 6890/5973 GC-MS equipped with an Agilent 6890 autosampler and a 60 m × 0.25 mm i.d. × 0.25 μm film thickness DB5-MS column (J&W Scientific). Aromatic fractions were injected in splitless mode. Polar fractions were injected in splitless cool on column mode into an Alltech pre-column (2 m x 0.53 mm i.d.) fitted to the DB5-MS column. Helium was used as a carrier gas at a constant flow of 1.1 and 1.2 mL/min for aromatic and polar fractions, respectively. The GC oven was heated from 40 to 325°C at 3°C/min with initial and final hold times of 1 and 30 min for aromatic fractions and from 50 to 320°C at 6°C/min with initial and final hold times of 1 and 20 min for polar fractions. The MS was operated at 70 *eV* and acquired full scan mass spectra (50–550 Daltons and 50–750 Daltons for aromatic and polar fractions, respectively) at ∼3 scans/s and a source temperature of 230°C. Peak assignments were based on correlation of GC retention time and mass spectral data with reference compounds, library spectra or other published data.

## DNA Extraction and 454 Pyrosequencing

Each DNA extraction used ∼0.3 g of sediments (wet weight) with the PowerSoil DNA Isolation Kit (Mo Bio Laboratories, Inc. Carlsbad, CA) according to the manufacturer's protocol.

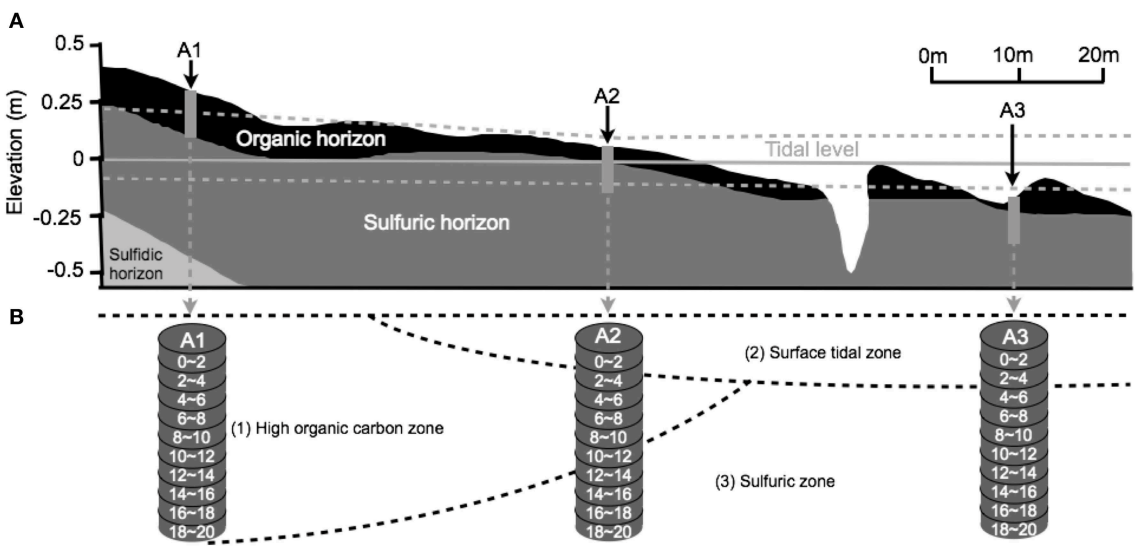


**FIGURE 1 | The distribution of soil layers, water saturation state, and β diversity. (A)** The *in situ* soil layers, water saturation state, and sampling sites. **(B)** Three zones at East Trinity site divided by β diversity. Figures are modified from Burton et al. (2011) and Johnston et al. (2011a,b).

DNA samples were sent for 454 pyrosequencing at the Australian Centre for Ecogenomics (ACE; Brisbane, Australia). A first round of PCR was conducted with SSU803F (combinations of 803Fa 5′-TTAAGATACCCTGGTAGTC-3′, 803Fb 5′-TTAGATACCCSG GTAGTC-3′, 803Fc 5′-TTAGATACCCTHGTAGTC-3′, 803Fd 5′-TTAGATACCCTGGTAGTC-3′ in a ratio of 2:1:1:1, *E. coli* position 2305–2322, Brosius et al., 1981) and SSU1392R (5′-ACG GGC GGT GWG TRC-3′, *E. coli* position 2908–2923, Brosius et al., 1981) primers used in the Fisher kit, with 1U Taq, dNTP at a final concentration of 0.2 mM, primers at a final concentration of 0.2 μM, $MgCl_2$ at a final concentration of 2 mM and BSA at a final concentration of 0.3 mg/mL. Thermal cycling had an annealing temperature of 55°C with 30 cycles. ACE has done extensive testing with this protocol and biases in amplication have been minimized. Then 2 μL of the first PCR product was used for a second PCR with no clean up to add barcodes (Multiplex Identifiers, MIDs), using the same reagents and conditions as for the first PCR but for 10 cycles. The PCR products were then quantified on the TapeStation and pooled at equal concentrations. The pooled DNA was gel extracted and amplified by emulsion PCR for sequencing. The Roche 454 sequencing (GS FLX Titanium chemistry) was performed with Roche 454 protocols.

## 16s rRNA Gene Sequences Analyses

DNA sequences were analyzed using the software environment Mothur (Schloss et al., 2009) v.1.32.1 following the Mothur 454 SOP (accessing date: Dec 2013) (Schloss et al., 2011). Raw sequence data were deposited to the Sequence Read Archive (SRA) of NCBI under the accession number: PRJNA275357. Sequences were removed for which the average quality dropped below 35. This step removed 7605 of a total 169,237 reads. Unique sequences were identified and the closest reference sequences were selected from SILVA bacterial and archaeal databases by the kmer search method, followed by a needleman alignment to make pairwise alignments between reference and candidate sequences (Schloss, 2010). Aligned sequences were checked to keep the most overlapping positions. Alignment results showed that 97.5% sequences had same ending position; thus we eliminated the 2.5% of sequences that ended before this position. Start positions were optimized to 85% sequence position equivalency, and sequences which started later were removed. Finally, columns in the alignments were filtered. The remaining 136,463 sequences were 206 bp in average length. Sequences were pre-clustered and 2052 reads were detected and removed as pyrosequencing errors and chimeras by the uchime program (Edgar et al., 2011). Taxonomy information was assigned to sequences with a cutoff of 50% (Claesson et al., 2009) by Naïve Bayesian classifier (Wang et al., 2007), with Ribosomal Database Project (RDP) references. Sequences with similarities higher than 97% were assigned to one OTU (operational taxonomic unit).

Samples were randomly resampled to 1498 reads for different calculations. The Good's coverage was calculated, which represents the ratio of OTUs that have been sampled once to the total number of sequences. The Chao 1 index was determined to estimate the richness of a sample based on the numbers of observed OTUs, singletons and doubletons. The inverse Simpson and Shannon indices were calculated to represent OTU diversity (alpha diversity) for each sample. The evenness values were used to evaluate the distribution evenness of relative OTU abundances. The beta diversity, which represents differentiation among each sample, was calculated and represented in two ways: a dendrogram which was calculated using the Jaccard index, then clustered using the UPGMA algorithm; and a principle coordinate analysis (PCoA) calculated using the Yue and Clayton index (Yue and Clayton, 2005). An ANOVA test was used to evaluate variability in diversity across sites.

## Kinetic Drive Evaluation

A combined thermodynamic-kinetic rate law was used to evaluate factors controlling microbial metabolic rates in the CASS system (Jin and Bethke, 2003, 2005):

$$v = \mathrm{k}\,[X]\;F_T\,F_K \tag{7}$$

Metabolic rate $v$ is the product of the rate constant $k$, microbial biomass concentration $[X]$, thermodynamic factor $F_T$ and the kinetic factor $F_K$. The range of values for kinetic and thermodynamic factors lies between 1 and 0. Larger factors (toward 1) represent less kinetic or thermodynamic limitations, or that the reaction is far from equilibrium and the forward direction overwhelms the reverse direction. If the reaction is close to equilibrium, the factors decrease toward to 0, which means little energy is available for microorganisms. This rate law is built on Monod and Michaelis-Menten kinetics (Monod, 1949; Michaelis et al., 2011), and is extended to consider the reverse reactions and include a thermodynamic potential factor. The consideration of reverse reactions and thermodynamic factors can be neglected where the environments contain abundant energy such that the forward reaction overcomes the reverse direction (Jin and Bethke, 2007). Thus, the model can be used to test the assumption that organic carbon substrates are non-limiting in the CASS environment under study.

Acetate is the most common organic substrate available for sulfate reduction in many environments (Lovley and Klug, 1982):

$$SO_4^{2-} + CH_3COO^- \leftrightarrow HS^- + 2HCO_3^- \tag{8}$$

The thermodynamic factor $F_T$ of acetotrophic sulfate reduction is:

$$F_T = 1 - \exp\left(\frac{\triangle G_A + \triangle G_C}{\chi RT}\right) \tag{9}$$

where $R$ and $T$ are the gas constant (8.314 J/Kmol) and absolute temperature (298 K was used in this study), $\chi$ is average stoichiometric number with a suggested value of 6 used in this study (Jin et al., 2013). $\triangle G_C$ is the energy conserved by SRB per mole of sulfate, which is estimated to be 33–47 kJ/mol (Jin and Bethke, 2009) with a value of 45 used in this study (Jin et al., 2013). The energy available in the environment $\triangle G_A$ is the Gibbs free energy of reaction (Equation 8):

$$\triangle G_A = \triangle G_T^0 + RT\ln\frac{\gamma_{HS^-}\left[HS^-\right]\cdot\gamma_{HCO_3^-}\left[HCO_3^-\right]^2}{\gamma_{SO_4^{2-}}\left[SO_4^{2-}\right]\cdot\gamma_{CH_3COO^-}\left[CH_3COO^-\right]} \tag{10}$$

where $\gamma_i$ is the activity coefficient, [i] is the concentration of reactant or product i, and the $\triangle G_T^0$ is the standard Gibbs free energy at absolute temperature, T°K. The activity coefficient $\gamma_i$used in this study was derived using the Geochemist's Workbench software (Bethke, 2007). The activity coefficient ranges for $SO_4^{2-}$, $CH_3COO^-$, $HS^-$, and $HCO_3^-$ are 0.1561–0.16508, 0.6825–0.6842, 0.6395–0.6426, and 0.6825–0.6842, respectively. The value of $\triangle G_T^0$ is −47.6 (kJ/mole) for acetotrophic sulfate reduction (Thauer et al., 1977; Sawadogo et al., 2013). The $SO_4^{2-}$ and $CH_3COO^-$ concentration profiles were modified from previous research at the same study site (**Figure 8**, Supplementary Table 2), the sulfate concentration range was 3–45 mM, the acetate concentration range was 0–95 mM, the sulfide value used the theoretically highest amount 2 μM since the sulfide concentration is below the detection limit (Supplementary Table 2) (Burton et al., 2011), and the bicarbonate concentrations used the highest values measured in the study site 1.6 mM (Johnston et al., 2011b). Other chemical concentrations used data reported from the same study site (Supplementary Table 2) (Ward et al., 2014).

The kinetic factor, $F_K$, derived from Monod (Monod, 1949) and Michaelis-Menten kinetic equations:

$$v = v_{max} \frac{\left[SO_4^{2-}\right]}{K_{SO_4^{2-}} + \left[SO_4^{2-}\right]} \frac{\left[CH_3COO^-\right]}{K_{CH_3COO^-} + \left[CH_3COO^-\right]} \quad (11)$$

where $v_{max}$ is the maximum metabolic rate, and the kinetic factor is described as:

$$F_K = \frac{\left[SO_4^{2-}\right]}{K_{SO_4^{2-}} + \left[SO_4^{2-}\right]} \frac{\left[CH_3COO^-\right]}{K_{CH_3COO^-} + \left[CH_3COO^-\right]} \quad (12)$$

where $K$ is the half-saturation constant; this study used values of $5.0 \times 10^{-6}$ M and $9.17 \times 10^{-4}$ M as $K_{CH_3COO^-}$ and$K_{SO_4^{2-}}$, respectively (Jin et al., 2013).

### Plotting Software

The software R (Statistical Package, 2009), R package gplots (Warnes et al., 2009), Microsoft Excel, iWork Numbers, and FigTree were used for generating plots.

## Results

### *In Situ* Geochemical Measurements

The pH of sediments generally decreased along the transect from the sea toward the upland site ("A1") for the upper portions (0–10 cm) of each core, with values from 3.29 to 6.13 at site A1, 4.38 to 5.92 at site A2, and 6.08 to 6.43 at site A3. In contrast, pH values were similar for all three sites for the lower portions (10–20 cm), ranging from 5.97 to 6.79. The Eh values decreased with depth within each core (from 51 to -127 mV at site A1, 20 to −207 mV at site A2, and 10 to −459 mV at site A3), but generally increased along the transect from the sea toward the upland site at each depth.

### Organic Geochemistry Analyses

The polar fractions from surface sediments of each transect site, as well as from the acidic control site, were dominated by a suite of plant-derived pentacyclic 3-oxy triterpenoids, including oleanolic, betulinic and ursolic acids, which showed a similar distribution in all samples. However, these compounds were absent in the polar fractions obtained from the mangrove control site, which was dominated by the triterpenoid taraxerol. In the aromatic fractions, pentacyclic triterpenoid derivatives were highly abundant (**Figure 2**). However, the aromatic fraction from the "Acidic Site" was dominated by a des-A-oleanane, whereas in the transect sites a des-A-lupane was the most abundant compound.

### DNA Sequences Analyses

The 95% confidence intervals of alpha diversity and richness were computed and the higher and lower boundaries were checked (Supplementary Table 2) to make sure the variations among samples were greater than the 95% confidence interval limitations. Average sampling coverage values showed significant increase along the transect moving away from the sea (72% for site A3, 81% for site A2, and 88% for site A1, $P < 0.001$, ANOVA). Chao richness estimates, which estimate the number of phylogenetically different OTUs (3% or more different in sequence composition), displayed a significant decrease from site A3 to A1 ($P < 0.001$, ANOVA), with the highest values in the upper part of site A3 (3668 reads), followed by sites A2 (1707 reads) and A1 (1128 reads), respectively. Both

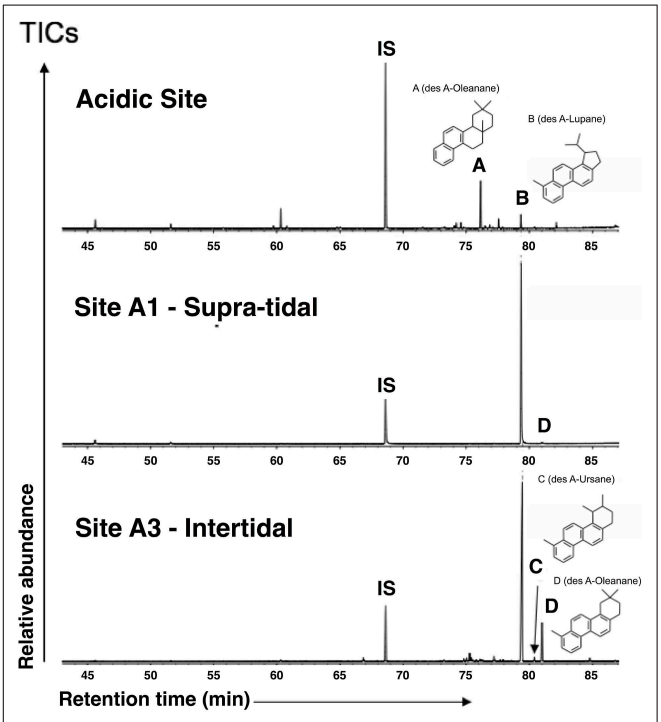


**FIGURE 2 | Total ion chromatograms from GC-MS analyses of aromatic fractions from surface sediments (study sites A1, A3, and an acidic control site not treated with tidal inundation).** Aromatic fractions from the study sites are dominated by a des A-Lupane whereas the aromatic fraction from the "Acidic Site" was dominated by a des A-Oleanane. IS, internal standard (c.f. Eiserbeck et al., 2012 and references therein).

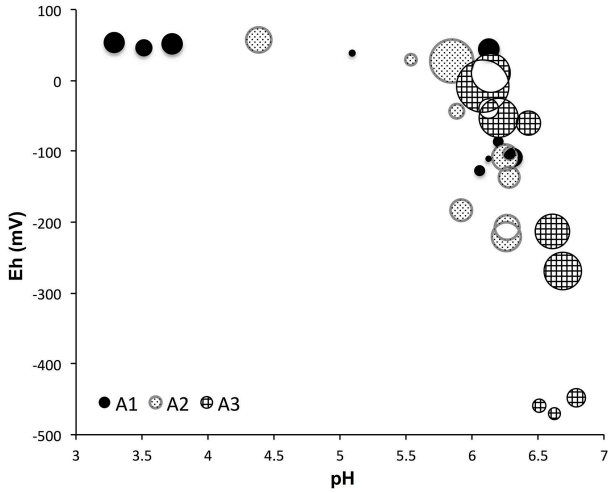


**FIGURE 3 | The alpha diversity (inverse Simpson) vs. pH and Eh values among three sampling sites, the diversity values are proportional to the area of bubbles.** The more positive Eh values indicate more oxidized environments. Diversity showed significant difference among three sites ($P < 0.01$, ANOVA).

Shannon and Simpson diversity indices showed a significant difference among the three sites ($P < 0.01$, ANOVA), which is shown on the pH and Eh diagram with the inverse Simpson index (**Figure 3**). The inverse Simpson index was in the range of 6–500 and Shannon index was in the range of 2.3–6.4 (Supplementary Table 2). Simpson's evenness indices were all below 0.37, which indicates a relatively even microbial distribution.

Beta diversity analysis illustrated the degree of similarity in microbial composition for each site and sample (**Figure 4**). Sequences tended to group into clusters consistent with major physico-chemical changes in soil profile, as well as with variations in the degree of soil moisture saturation (**Figure 1**). Relative percentage representations of microbial community structure, separated into domain, phylum and class for each zone are shown in **Figure 5**. The inner, middle and outer circles represent domain, phylum and class levels, respectively (**Figure 5**).

Bacteria comprised ∼89% and archaea ∼11% of the prokaryotic community structure, taken across all sites within the East Trinity wetlands. In total 30 bacterial and three archaeal phyla (*nanoarchaeota* occupied only 0.04% and therefore did not show observable area in **Figure 5**) were identified. *Proteobacteria* was the most abundant phylum detected at any site, contributing ∼39% of the total 16S rRNA gene sequences. The nine most abundant phyla recovered accounted for ∼93% of these sequences. The relative abundance of each shown class or phylum differed between zonations. For example, the abundance of *deltaproteobacteria* had the highest abundance at Zone 2 (19%, compared to 13% at Zone 1 and 10% at Zone 3). The *gamma-, beta-,* and *alpha-proteobacteria* exhibited higher sequence abundances at Zone 1 (38% totally, compared to 30% at site Zone 2 and 12% at site Zone 3). The *Chloroflexi*, *Bacteroidetes*,

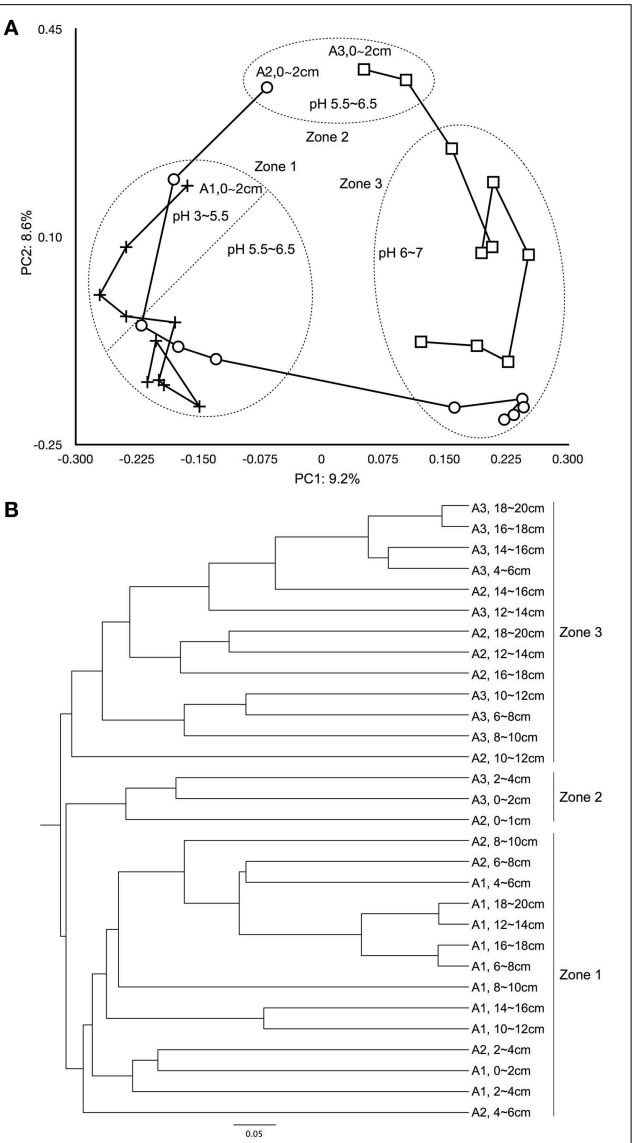


**FIGURE 4 | Beta diversity analysis comparing microbial structure similarity among samples. (A)** Principal coordination analysis among samples from the Jaccard calculator (PC1 = 9.2%, PC2 = 8.6%). The "+" represents samples from site A1, the "○" represents samples from site A2, and the "□" represents samples from site A3. **(B)** Yue and Clayton measure of samples similarity.

*Firmicutes, Euryarcheota*, and *Crenarchaeota* showed greater abundances in Zone 2 (22%) and Zone 3 (39%), compared to Zone 1 (8%).

Classes *delta-*, *gamma-*, *alpha-proteobacteria,* and *Acidobacteria* were selected to compare their relative abundances among different zonations and other environments, such as marine sediments and AMD systems (**Figure 6**). Several genera, which have been reported by previous researchers to have iron reducing ability, were picked from this study to represent the abundance of IRB (**Table 1**). The iron-reducing bacterial reads were proportional to those of *deltaproteobacteria*, and iron-oxidizing bacterial reads were proportional to those of

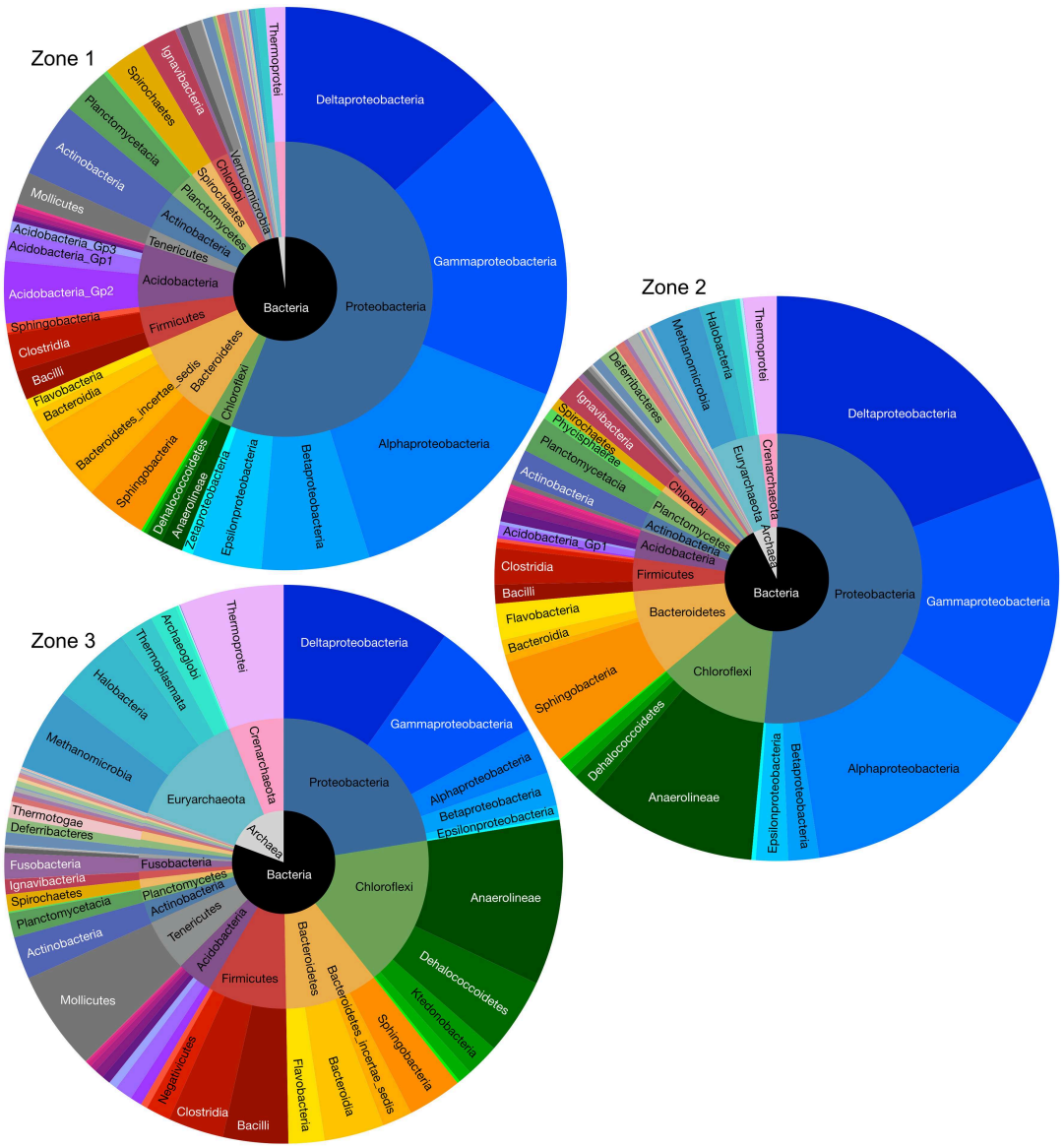


**FIGURE 5 | Microbial abundance and compositions of Zone 1, Zone 2, and Zone 3 in kingdom (inner circle), phylum (middle circle), and class (outer lever) taxonomic levels.**

sulfur-oxidizing bacteria, in the organic and sulfuric horizons (**Figure 7**). The kinetic drives of heterotrophic sulfate and iron reductions were calculated to be close to unity in the study area (top 20 cm, **Figure 8** and Supplementary Table 3), which supports the lack of kinetic inhibition for metabolisms in this CASS system.

## Discussion

### Organic Source and Preservation

There is more than 20% (by weight) organic carbon in the organic horizon at East Trinity wetlands (Hicks et al., 1999). This relatively high organic matter content most likely results from a mode of origin and preservation uniquely associated with re-flooded CASS environments. Mangroves can slow surface water flow rates and reduce wave scour, which favors fine particle trapping and organic matter accumulation (Young and Harvey, 1996; Alongi, 2008). Sediments in mangrove swamps usually contain a large amount of organic matter (Kristensen et al., 2008). Mineralogy may play a role in preserving organic carbon in CASS systems; the oxidation of iron sulfides generates secondary iron minerals such as ferrihydrite and goethite in the study site (Hicks et al., 1999; Johnston et al., 2010), which have been shown to preserve mineral-bound organic carbon in subsoils (Kogel-Knabner et al., 2008). Environmental factors including salinity, soil pH or the tidal-inundation level at the sites control primary

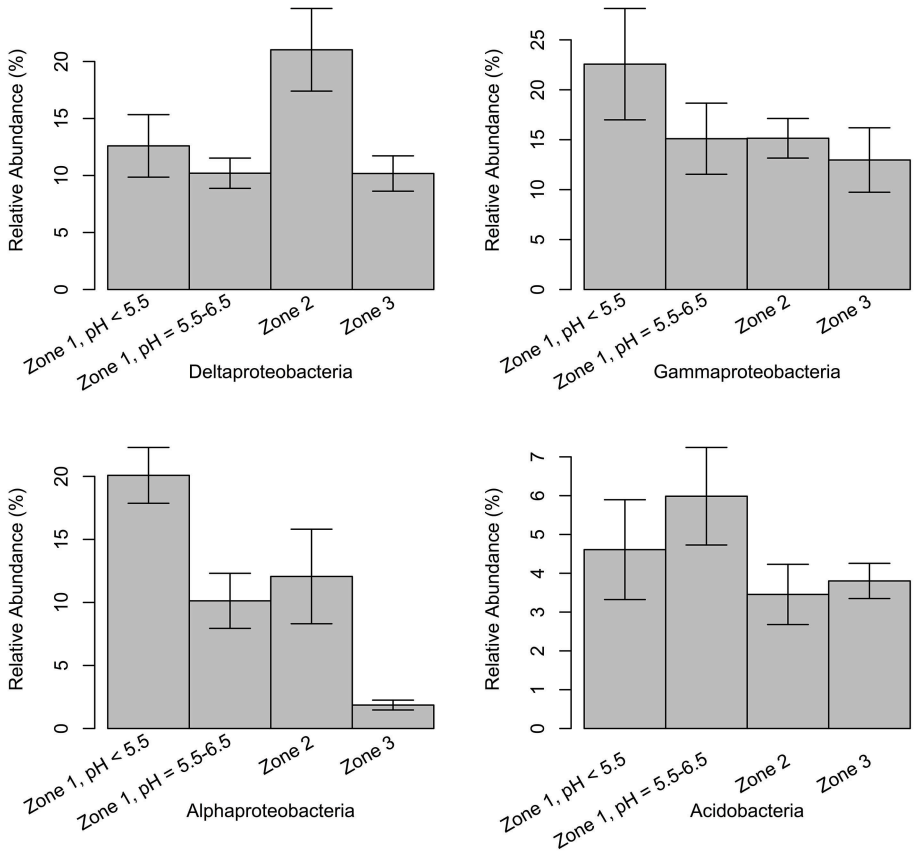


**FIGURE 6 | Relative abundance of classes *delta-*, *gamma-*, *alpha-proteobacteria,* and *Acidobacteria* from Zone 1 to Zone 3.** Samples in Zone 1 are separated into two parts based on the pH values and structure similarity (**Figure 3A**). The error bars show the 95% higher and lower confidence intervals around mean values.

production; in particular the plant type will shape the types of organics available for microbial degradation.

Changes in plant types due to tidal inundation treatment contributed a large amount of organic matter input, while organo-mineral interactions resulted in unusual preservation of organic acids. Combined with invertebrate decay in the mangrove area, these factors contributed to the high abundance of organic matter in the system, and hence influenced microbial structures and distributions. In our wetland site, *Melaleuca* trees (mostly *Melaleuca leucadendra*) became the predominant plant species in the drained lands, but at locations with re-introduced tidal flows, such as our study site, they died off and the original mangrove vegetation (*Avicennia marina, Aegiceras corniculatum,* and *Excoecaria agallocha,* etc…) returned (Newton et al., 2014). There have been several reports of various pentacyclic triterpenoid acids in *Melaleuca* species (Lee, 1998; Lee and Chang, 1998, 1999; Abdel Bar et al., 2008), the ursolic and oleanolic acids have also been found in mangrove leaves (Ghosh et al., 1985). However, due to their high reactivity, the abundance of these compounds in sediments is rare. The suite of compounds in the polar fractions of sites A1–A3 and the "Acidic Site" presented here may have been preserved by organo-mineral interactions with iron oxyhydroxides (Kogel-Knabner et al., 2008). Furthermore, previous research suggests that triterpenoids play an important role in salt adaptation for plants, and therefore the abundance of triterpenoids in mangrove species increases with salinity (Oku et al., 2003). Among the pentacyclic triterpenoids, betulin is more easily degraded and is thought to be a marker of mangrove *Avicennia* (Koch et al., 2005), which is widespread in the East Trinity study site (Department of Agriculture, Fisheries and Forestry, Queensland Government), consistent with our finding of abundant betulinic acid across our sites. Under reducing/anoxic conditions in sediments, pentacyclic triterpenoids are transformed by microbially-mediated A-ring degradation and progressive aromatization reactions during (early) diagenesis (e.g., Trendel et al., 1989; Le Métayer et al., 2005; Melendez et al., 2013; Schnell et al., 2014; **Figure 2**).

In addition to the type and abundance of organic matter, the existence of plants would also influence microbial distributions. In the rhizosphere area of treated wetlands, microbial diversity and activity are typically enhanced (Münch et al., 2005; Faulwetter et al., 2009). *Phragmites australis*, also called Common Reed, is distributed throughout the study site (Johns, 2010) with

**TABLE 1 | Genera of iron-reducing bacteria.**

| Genus name | References |
|---|---|
| **IRON-REDUCING** | |
| *Paraferrimonas* | Khan and Harayama, 2007 |
| *Acidiferrimonas* | Itoh et al., 2011 |
| *Desulfuromonas* | Coates et al., 1995 |
| *Bacillus* | Pollock et al., 2007 |
| *Pelobacter* | Lovley et al., 1995 |
| *Desulfuromusa* | Vandieken, 2006 |
| *Desulfitobacterium* | Finneran et al., 2002 |
| *Thiobacillus* | Temple and Colmer, 1951 |
| *Geobacter* | Lovley et al., 1993; Caccavo et al., 1994 |
| *Desulfosporosinus* | Robertson et al., 2001 |
| *Ferroplasma* | Golyshina et al., 2000 |
| *Geothrix* | Coates et al., 1999 |
| *Shewanella* | Roh et al., 2006 |
| *Ferribacterium* | Cummings et al., 1999 |
| *Ferrimonas* | Rosselló-Mora et al., 1995 |

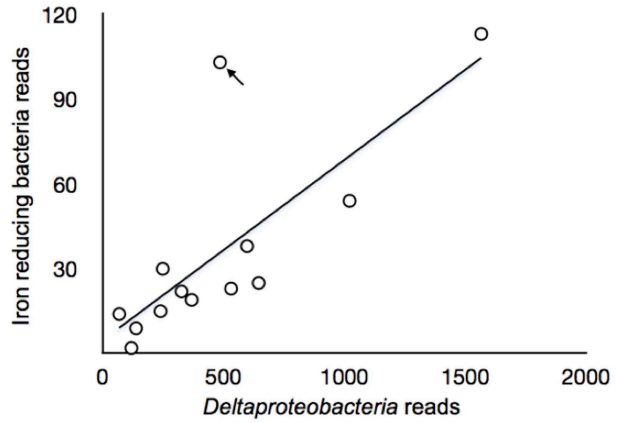


**FIGURE 7 | Correlations between sequence reads of samples collected of sulfate-reducing *deltaproteobacteria* and iron reducing bacteria in Zone 3.** The arrow points to the A3 4–6 cm, where the sample differed from all other samples and was discussed in the text.

roots down to a depth of 20–30 cm (Stottmeister et al., 2003). It is reported that these roots improve nitrification and denitrification 20–50 mm away from the roots (Münch et al., 2005), and have a higher efficiency of transporting oxygen into the rhizosphere than diffusion alone (Armstrong and Armstrong, 1990). A higher redox potential gradient was observed from ∼500 mV near root surface to ∼ −250 mV in 1–20 mm from the roots (Faulwetter et al., 2009). The roots likely increased soil heterogeneity in the subsurface, which could enhance microbial diversity since aerobic or microaerophilic microorganisms could survive in niches throughout otherwise anaerobic zones (Lamers et al., 2012).

## Alpha Diversity Controlled by Organic Matter, pH, and Eh Values

Much research has shown that pH (Fierer and Jackson, 2006; Hartman et al., 2008; Lauber et al., 2009), Eh (DeAngelis et al., 2010), and organic matter content and type (Zhou et al., 2002) have strong influences on microbial diversity. In this study, a large range of alpha diversity indices was observed in the East Trinity wetland (Supplementary Table 1). The highest diversity was observed at A3 0–4 cm, and is comparable to that of a coral ecosystem (Chen et al., 2010; Gaidos et al., 2010). The lowest diversity was observed at A1 16–18 cm and is comparable with an AMD contaminated lake (Laplante and Derome, 2011). Both Eh and pH values led to differentiation of alpha diversity across the sampling sites.

Higher diversities were observed with more natural pH and higher Eh (more oxidizing) values (**Figure 3**). Site A1 contained much lower diversity (Supplementary Table 1, Supplementary Figure 1) when compared to sites A2 and A3, as a result of local pH and organic carbon content (**Figure 8**). The 0–8 cm depths of site A1 showed the lowest pH values (3–5) across all the samples, and site A1 contained the lowest organic carbon content among all 3 sites (**Figure 8**, Supplementary Table 1) (Burton et al., 2011). These conditions likely prevented colonization and growth by less acid-tolerant microbial groups. Site A3 showed pH > 6 for all depths (**Figure 3**), and microbial diversity decreased with depth in response to Eh (Supplementary Table 1, Supplementary Figure 1). For site A2, diversity appears to be influenced by both pH and Eh. From 0 to 10 cm depths in site A2, the diversity decreased with Eh/depth, and the pH (from 4 to ∼6) did not show an effect on decreasing diversity. But for depths 10–20 cm in site A2, pH increased to >6, and the degree of diversity also increased, even though the environment was more reduced (i.e., deeper). Compared to site A1, which mostly experiences exposure to air, and site A3, which is mostly tidal-inundated, site A2 cut through two different soil layers (**Figure 1A**) and experiences the most oscillatory redox fluctuations, and also contained the highest amount of dissolved organic matter (Burton et al., 2011). The Eh values we measured represent the most oxidized potential since sediments were collected during the low tide period. The fluctuating redox potential results in higher diversity than would otherwise be present under more static chemical conditions (DeAngelis et al., 2010). Previous study of the same site showed sulfate reduction has the highest rate at site A2, which is controlled by dissolved organic matter content (Burton et al., 2011). The more neutral pH, high organic matter content, and oscillatory redox fluctuations therefore likely resulted in the increased microbial diversity in site A2.

## Beta Diversity Shaped by Soil Layering, Water Saturation, and pH

The soil in the organic horizon contains high concentrations of organic carbon, while the sulfuric horizon contains the actual acid sulfate soil, and the sulfidic horizon consists of potential acid sulfate soils (Hicks et al., 1999). Both Jaccard and the Yue and Clayton indices showed that microbial community similarity changed spatially across the East Trinity field site (**Figure 4**). Based on principal coordinate scaling and hierarchical clustering,

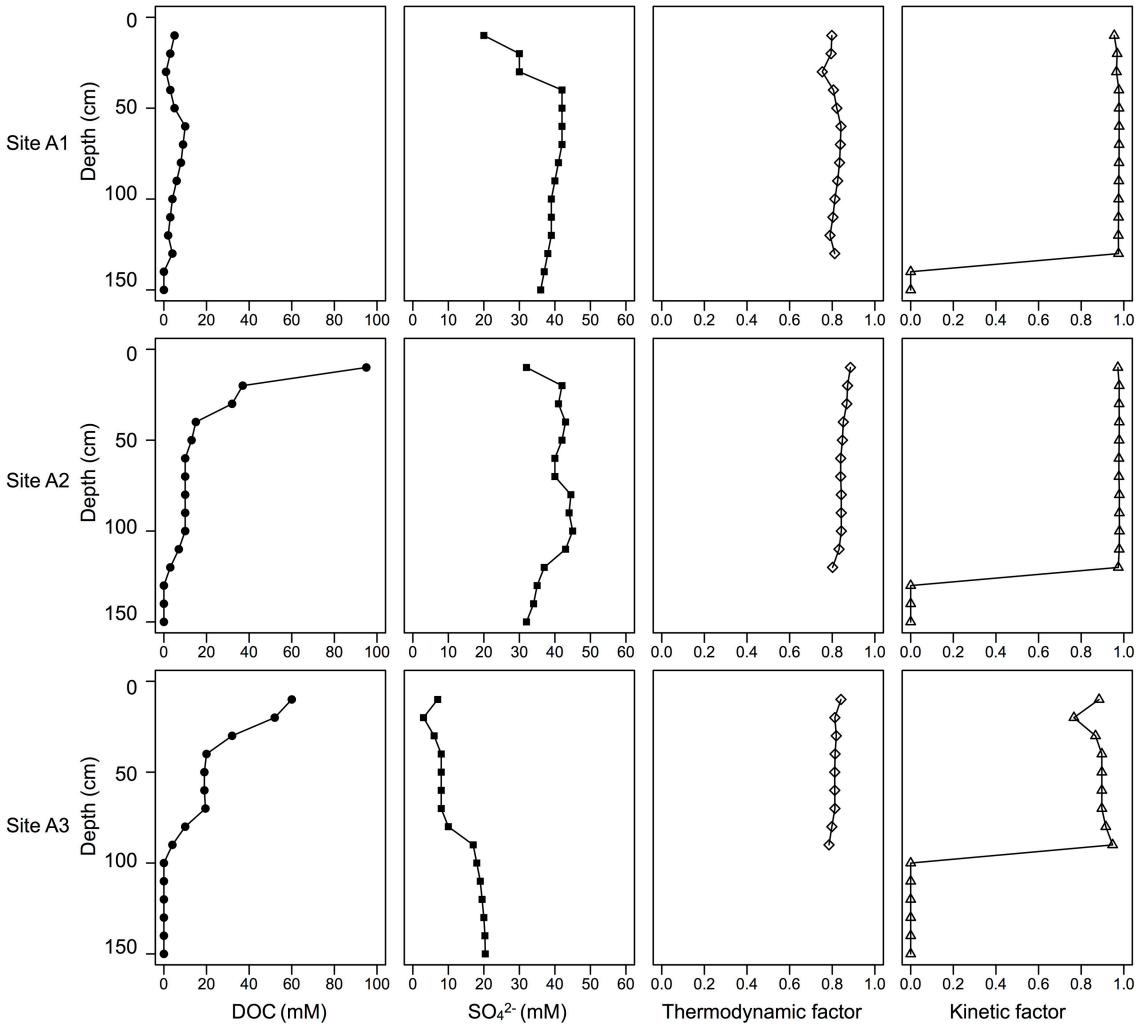


**FIGURE 8 | Depth profile of DOC, $SO_4^{2-}$, labile Fe(III), crystalline Fe(III), soil density, and kinetic drives of heterotrophic sulfate and iron reductions.** The values of DOC, $SO_4^{2-}$, labile Fe(III), crystalline Fe(III), and soil density are modified from Burton et al. (2011).

three zones were distinguishable. Zone 1 included all of sites A1 and A2 from 2 to 10 cm in depth. Zone 2 consisted of site A3, 0–4 cm, and site A2, 0–2 cm, in depth. Site A2, depths 10–20 cm, and site A3, depths 4–20 cm, made up Zone 3. When we compare microbial diversity with *in situ* soil layering, water saturation, and pH values, the boundaries of these three zones were consistent with major variations in these environmental parameters (**Figure 1**). Zone 1 was located in the organic horizon; the upper part of Zone 1 (A1, 0–8 cm, and A2, 2–4 cm) had the lowest pH values at 3.0–5.5, and the remaining lower part of Zone 1 had pH values between 5.5 and 6.5 (**Figure 4A**). Zone 2 experienced the most disturbance from tidal activity, with pH values between 5.5 and 6.5. Below the organic horizon is the sulfuric horizon, which hosts Zone 3 in which all samples had pH values > 6 (**Figure 4A**), reflecting more than a decade of tidal inundation treatment (QASSIT, 2000). In this zone, the pH values have shown a increase from 3–4 to 3–8 and pyrite has accumulated up to 30 μmol/g (Johnston et al., 2011a).

## Geochemical Parameters Influence Specific Functional Guilds

*Proteobacteria* comprised the most abundant phylum at East Trinity (**Figure 5**). The most abundant classes (in decreasing order) were *delta-*, *gamma-*, *alpha-*, and *beta-proteobacteria*. This ordering differs from a more typical soil community structure, which exhibits the ordering *alpha-*, *delta-*, *beta-*, and then *gamma-proteobacteria* (Spain et al., 2009). The *delta-* and *gamma-* classes showed much higher abundances in East Trinity, suggesting marine and acidity influences. The *deltaproteobacteria* were more abundant in marine-influenced sediments [**Figure 6**, Zone 2, corresponding to the upper parts of sites A2 (0–10 cm) and A3 (0–14 cm)], which is consistent with seawater as a source of sulfate for bacterial sulfate reduction and the predominance of sulfate-reducing bacteria (SRB) within the *delta* class (Rabus et al., 2013). In these samples, >45% of *deltaproteobacteria* were most closely related to members of order *Desulfobacterales*. The observed distribution of SRB at higher

abundances in the upper depths at these sites also suggests a certain degree of oxygen tolerance to periods between tidal inundation (Canfield and Des Marais, 1991; Baumgartner et al., 2006), and/or possibly rapid changes in SRB activity with tidal fluctuation. The relative higher abundances of *gamma-* and *alpha-proteobacteria* in Zone 1 (**Figure 6**) are consistent with the microbial community composition observed in some AMD systems (Edwards et al., 2006; Brantner et al., 2014; Kamika and Momba, 2014), which are comparable to some CASS systems in terms of extreme acidification. Previous studies revealed that gamma-proteobacteria are more abundant at lower pH (Kuang et al., 2013; Fabisch et al., 2013). *Acidobacteria* (Lauber et al., 2009), which favors a low pH environment, was also present in relatively high abundance in Zone 1 but only in the higher pH area (**Figure 6**).

When the abundance of organic carbon exceeds the rate at which microorganisms can consume this resource (e.g., the maximum rate of enzymatic activity), microbes may not need to compete for electrons and carbon (Ling et al., 2012) and diversity can increase (Zhou et al., 2002). High organic matter can also increase soil aggregation by decreasing wetability (Chenu et al., 2000), which in turn further promotes physical heterogeneity and microbial diversity. The high concentration and multiple types of organic matter present at East Trinity could facilitate co-habitation of different metabolic guilds in close proximity within redox gradients throughout our sampling sites. For example, in Zone 1 (**Figure 5**), 49% of *beta-proteobacteria* were derived from the genus *Delftia* (**Figure 5**), which is known to possess nitrate reduction ability (Wen et al., 1999; Shigematsu et al., 2003) and was isolated from biofilm of common reed *P. australis* (Borsodi et al., 2007), a plant species common to the study area (Johns, 2010). Roughly 64% of *epsilon-proteobacteria* were closely related to *Sulfurimonas*, which possesses sulfur and thiosulfate oxidation abilities (Inagaki et al., 2003; Takai, 2006). Methanogenic *Methanomicrobia* showed increased abundance toward site A3 and *deltaproteobacteria*, typically associated with IRB and/or SRB, was a dominant class in all three sites. These metabolic guilds usually compete with each other for a limited energy source by maintaining the concentration of that source at the lowest threshold therefore establish a well resolved redox zonation which can be predicted thermodynamically (Hoehler et al., 1998).

The number of sequences representative of *deltaproteobacteria* was proportional (linear regression, $r^2 = 0.59$, $p = 0.002$) to those representing known iron-reducing bacteria or close relatives for Zone 3 (at the genus level, **Table 1**), which represents the sulfuric horizon (**Figure 7**). Zone 1 did not show this proportionality ($r^2 = 0.17$, $p = 0.1427$), and there are not enough samples in Zone 2 to demonstrate correlation convincingly. Site A3, depths 4–6 cm, differed from all other samples and can be explained by the observation that the sulfuric horizon is much closer to the tidal zone in site A3 than in site A2 (**Figure 7**). We infer that high organic content allowed the two metabolic guilds effectively to co-exist in Zone 3 (**Figure 8**). The (re)precipitation of iron sulfide minerals will thermodynamically favor iron, sulfate and elemental sulfur reduction, by removing the products of these metabolic reactions. In addition, tidal activity can potentially drive reductive dissolution of crystalline iron(III) minerals (e.g., jarosite) from the lower soil profile, redistributing the iron as poorly crystalline iron(III) minerals in the upper organic horizon (Johnston et al., 2011a). These poorly crystalline iron(III) minerals can act as a relatively labile electron acceptor for iron-reducing bacteria. This observation suggests that microorganisms exhibit a relatively rapid response to tidally generated redox fluctuations.

To test the central hypothesis of this study, a thermodynamic-kinetic model was used to evaluate factors that control microbial metabolic rates. Microorganisms conserve energy from redox changes between the reactant and the product to form adenosine triphosphate (ATP). When the energy available in the environment is in excess of energy conserved by microbial metabolism, the thermodynamic factor moves toward a greater value, which means the reaction is far from equilibrium and the reaction has a greater tendency to move in the forward direction. Taking acetotrophic sulfate reduction as an example, the concentrations of sulfate and organic carbon (reactants) are higher than the concentrations of sulfide and bicarbonate (products) in the study site, and higher thermodynamic factors (0.75–0.89) were observed (**Figure 8**). In the study site, sulfide was removed by deposition of iron-sulfide minerals and bicarbonate is removed by titrating acidity (Johnston et al., 2011b); both mechanisms favor sulfate reduction thermodynamically. In this situation, thermodynamic limitation can be ignored. The microbial metabolic rate law then becomes:

$$v = k\,[X]\,\frac{\left[SO_4^{2-}\right]}{K_{SO_4^{2-}} + \left[SO_4^{2-}\right]}\,\frac{\left[CH_3COO^-\right]}{K_{CH_3COO^-} + \left[CH_3COO^-\right]} \quad (13)$$

In this case, the concentrations of sulfate and acetate were greater than the half-reaction constant $K_{SO_4^{2-}}$ and $K_{CH_3COO^-}$ by at least one order of magnitude, and therefore the kinetic factor moves toward unity (**Figure 8**, Supplementary Table 2). This phenomenon was observed by Jin and Bethke (2003) for the initial stage of an incubation experiment when all substrates were present at high level. Both thermodynamic and kinetic factors showed high values in the study site, suggesting that the energy available was higher than the equilibrium state. The "snapshot" data used in this study support the hypothesis that organic matter content was higher than thermodynamic maintainance concentrations. Therefore, we infer that IRB and SRB did not need to compete for energy in the study site.

Microbial metabolic rates depend on a the rate constant $k$ and microbial biomass concentrations. Biomass in the thermodynamic-kinetic model and other kinetic models does not account for dormant microbial cells (Jin et al., 2013). However, dormancy has been reported to help maintain biodiversity (Jones and Lennon, 2010), and was suggested as a survival strategy in highly dynamic environments (Lennon and Jones, 2011).

## Microbial Ecology and CASS System Evaluation

Microbial distributions in the East Trinity wetlands support the paradigm of community selection by from a homogenous population (De Wit and Bouvier, 2006), which is controlled by environmental heterogeneities (Bowen et al., 2009) associated

with increased acid or salinity in this site. High concentrations and multiple types of organic matter would further increase similarity across samples. The low axis loading in our principal coordinate analysis (**Figure 4A**) confirmed that all sites were similar in terms of microbial community structure. Zones of similar dominant microbial guilds were defined across sites on the basis of environmental parameters such as pH, Eh, soil layering, and water saturation. This research revealed that vertically stratified models linking redox zonation and microbial guild distribution are not useful for predicting biogeochemical cycling at East Trinity.

Tidal re-inundation is being tested as an effective means for natural remediation of CASS systems. However, tidal fluctuations can make CASS systems highly dynamic environments with respect to redox states and the flux of nutrients and electron donors or acceptors. Correspondingly, microbial communities living in tidal zones experience both static and fluctuating environmental conditions that, in turn, are modulated by lithological compositions and hydrological connectivity. For researchers attempting to construct complete biogeochemical process models, these factors must also be considered.

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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.2015.00624

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# Microbial and biogeochemical responses to projected future nitrate enrichment in the California upwelling system

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Coastal California is a dynamic upwelling region where nitrogen (N) and iron (Fe) can both limit productivity and influence biogeochemistry over different spatial and temporal scales. With global change, the flux of nitrate from upwelling is expected to increase over the next century, potentially driving additional oceanic regions toward Fe limitation. In this study we explored the effect of changes in Fe/N ratio on native phytoplankton from five currently Fe-replete sites near the major California upwelling centers at Bodega Bay and Monterey Bay using nutrient addition incubation experiments. Despite the high nitrate levels (13–30 μM) in the upwelled water, phytoplankton at three of the five sites showed increased growth when 10 μM nitrate was added. None of the sites showed enhanced growth following addition of 10 nM Fe. Nitrate additions favored slow sinking single-celled diatoms over faster sinking chain-forming diatoms, suggesting that future increases in nitrate flux could affect carbon and silicate export and alter grazer populations. In particular, solitary cells of *Cylindrotheca* were more abundant than the toxin-producing genus *Pseudonitzschia* following nitrate addition. These responses suggest the biogeochemistry of coastal California could change in response to future increases in nitrate, and multiple stressors like ocean acidification and hypoxia may further result in ecosystem shifts.

**Keywords: global change, phytoplankton, upwelling, nutrient cycling, Iron limitation, nitrogen limitation, diatom**

## INTRODUCTION

Coastal upwelling regions along eastern boundary currents are the most productive marine ecosystems, supporting complex ecological networks and economically important fisheries. These systems experience a high degree of natural spatial and temporal variability with respect to biological, chemical, and physical characteristics. Upwelling is typically a seasonal phenomenon, where alongshore winds drive sub-surface, nutrient rich waters toward the sunlit surface layers, enriching them with the macronutrients nitrogen (N) and phosphorus (P) (Pennington and Chavez, 2000; Chavez and Messie, 2009). Other factors, such as the width and depth of the continental shelf at the upwelling site and internal cycling of elements play a role in determining the flux of trace metals to surface waters (Bruland et al., 2001; Biller and Bruland, 2013; Biller et al., 2013).

Coastal upwelling regions face threats from anthropogenic global change, including changes in stratification patterns due to sea surface temperature (SST) warming, altered nutrient chemistry, increased hypoxia, and ocean acidification (as reviewed in Capone and Hutchins, 2013). For example, decreased ventilation of the Pacific Ocean due to increased stratification in the gyres is expected to alter seawater chemistry, increasing the nitrate inventory and decreasing the oxygen content in waters that are upwelled (Rykaczewski and Dunne, 2010). As a result, the flux of nitrate in the coastal California upwelling system is expected to be 64% greater in the year 2100 compared to preindustrial times (or 28% over modern day values; Rykaczewski and Dunne, 2010).

Iron (Fe) availability governs nitrate drawdown in many coastal upwelling systems and is strongly influenced by the physical and bathymetric characteristics of each site. Along the California coast, biomass at locations with narrow continental shelves can become Fe-limited due to the low suspended sediment levels and high nitrate concentrations from upwelling (Hutchins and Bruland, 1998; Firme et al., 2003). Recent work by Biller and Bruland (2014) expanded these regions to include the coastal California transition zone (TZ, **Figure 1C**), which is an offshore region with high nitrate from upwelled waters advected offshore. As the water moves offshore, labile Fe is consumed leading to Fe limitation and excess nitrate. These Fe-limited regions typically have iron-to-nitrogen (Fe/N) ratios below 0.2 nmol Fe/1 μmol N, and have been designated as high nutrient low chlorophyll (HNLC) regions due to the relatively high residual nitrate and lower than expected chlorophyll levels. Other studies in the region have shown that despite high nitrate concentrations in the water, phytoplankton biomass remains nitrogen-limited (Kudela and Dugdale, 2000; Mackey et al., 2010), with other nutrients like phosphorus influencing physiology and competition between taxonomic groups (Nicholson et al., 2006; Mackey et al., 2012).

Iron availability in coastal California and other HNLC regions is also known to influence phytoplankton community structure and biogeochemistry. Large scale iron addition experiments conducted in the Southern Ocean and equatorial Pacific HNLC regions favored blooms of diatoms over other types of

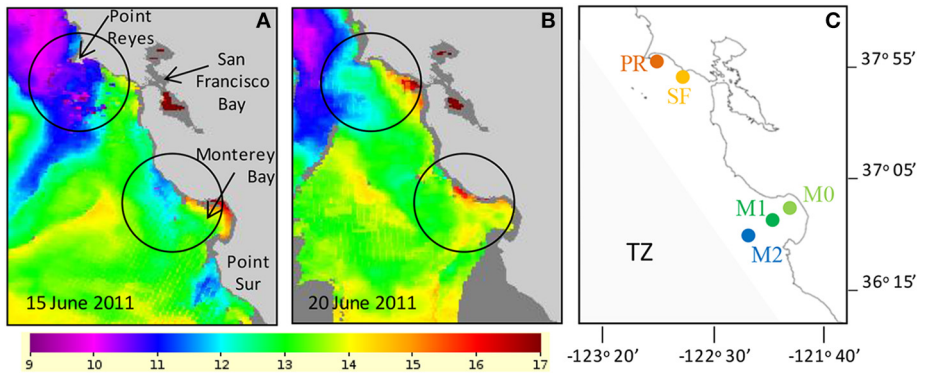


**FIGURE 1 | Sea surface temperature indicated that colder upwelled water was present on June 15 (A) and that upwelling had relaxed and surface water began to warm by June 20 (B).** Temperature scale is in °C. Areas of intense upwelling in this study are circled on each map. **(C)** Map showing the locations where incubation water was collected (see methods for latitude and longitude). Shaded region in **(C)** indicates the approximate location of the "transition zone" (TZ) described by Biller and Bruland (2014).

phytoplankton (Coale et al., 1996; Boyd et al., 2000; Smetacek et al., 2012), affected diatom speciation (Tsuda et al., 2003, 2005; Assmy et al., 2007), and altered grazing rates (Tsuda et al., 2006). Iron additions have also been shown to stimulate the toxin producing diatom *Pseudo-nitzschia* spp. in the equatorial Pacific Ocean (Silver et al., 2010; Trick et al., 2010). In Fe-limited areas of coastal California, selective enrichment of chain-forming diatoms occurred following iron addition, and diatoms formed more heavily silicified cells (Hutchins and Bruland, 1998). Iron availability therefore has the potential to influence the type of phytoplankton that dominate blooms following upwelling, affect the uptake and cycling of other nutrients, and alter the relative proportions of silica and carbon sequestration by the biological pump. In addition blooms with different types of phytoplankton (size, density, TEP production etc.) have different sinking velocities, also impacting carbon sequestration by the biological pump (De La Rocha and Passow, 2007). Larger cells or chain-forming cells have faster sinking rates and therefore sequester carbon more efficiently than smaller cells.

A number of factors sensitive to global change influence the supply and bioavailability of Fe in coastal upwelling regions. Biological ligand production, ocean acidification, hypoxia, rainfall, groundwater discharge, and deposition of anthropogenic aerosols influence Fe supply and biogeochemistry in coastal waters. The predicted increased supply of nitrate in the future (Rykaczewski and Dunne, 2010) could likewise shift Fe limitation regimes for biomass by changing the Fe/N ratio in the water. For example, a 50% increase in nitrate supply could drive certain Fe-replete California waters below the Fe/N limitation threshold (Capone and Hutchins, 2013), potentially expanding Fe limitation in the California upwelling region.

To understand how Fe-replete waters in coastal California could respond to changing nitrate fluxes (and hence changing Fe/N ratios) in the future, we conducted nutrient addition incubation experiments with water collected at five Fe replete sites from Monterey Bay to Point Reyes several days after upwelling when Fe and nitrate levels remained elevated. The goal of the study was to expand our understanding of how N and Fe availability and ratios could influence phytoplankton growth and physiology in modern-day and future coastal California waters and to assess the spatial variability in this response. These coastal sites were selected to encompass various distances from shore and represent major upwelling centers such that a range of nutrient conditions could be tested. We used phytoplankton cell counts to determine if there is a phytoplankton community shift toward faster sinking chain-forming diatoms as observed by Hutchins and Bruland (1998) and Silver et al. (2010) in Fe-limited regions following Fe fertilization. Finally, based on nutrient measurements, we show that only in some cases do Fe and N cause shifts in the drawdown ratios of N:P and Si:N. We discuss the implications of these findings to the export of C and Si.

## MATERIALS AND METHODS

### REMOTE SENSING AND MOORING DATA

*In situ* wind speed, wind direction, SST and nitrate concentrations at station M1 were obtained from http://www.mbari.org/oasis/. Satellite images of SST were obtained from NOAA POES AVHRR, LAC, 0.0125°, day and night, courtesy of NOAA NWS Monterey and NOAA CoastWatch. Oceanographic and atmospheric conditions were monitored prior to the experiment to identify a period of upwelling followed by relaxation. The incubation experiments were conducted several days after upwelling occurred when upwelling-favorable winds had relaxed and SST values indicated surface waters were warming (**Figures 1A,B**).

### INCUBATION SETUP AND SAMPLING

Incubation experiments were conducted to determine the effect of N and Fe on phytoplankton using seawater collected from five sites as shown in **Figure 1C** a few days after the relaxation of upwelling. Water temperature and salinity were used to identify water masses by assuming that upwelled water warms ~0.5–1°C per day (K. Bruland, personal communication) and is more saline than surface water from the North Pacific. Water from Drakes Bay near Point Reyes (PR; 37°59′23.2″N, 122°58′52.2″W) and north of the mouth of San Francisco Bay (SF; 37°55′30.7″,

122°49′41.7″W) was collected on June 20, 2011 (**Figure 1C**) aboard the R/V Shana Rae. These two sites are located to the south and downstream of the major upwelling center located at Bodega Bay. Water was collected from the Monterey Bay moorings M0 (36°49.442N 121°56.967W) and M1 (36°45.190N 122°01.525W) and offshore of Monterey Bay near mooring M2 on June 22, 2011 using a small motor boat. In this study we refer to the offshore site as "M2," although its actual location (36°42.382N 122°13.798W) differs slightly from the official location of the offshore mooring. Station M0 is located within Monterey Bay. Station M1 is situated directly downstream of the Monterey Bay upwelling center.

Surface (5 m) seawater was collected at each site into trace metal clean, seawater rinsed carboys. Water was transported in the dark back to Long Marine Laboratory in Santa Cruz, CA where the experiments were conducted. The following protocol was followed while setting up the experiments for each site. Three baseline samples to characterize the collected seawater were immediately collected and processed for each of the measurements described below. Water was then dispensed into acid cleaned, sample rinsed, transparent polycarbonate bottles (500 mL per bottle, 9 bottles per treatment). All materials used in the experiment were rendered trace metal clean prior to use. Plastic ware (including incubation bottles, carboys, and sample bottles) was soaked overnight in heated, ultrapure 10% hydrochloroic acid (HCl; Optima), rinsed several times with MilliQ water, and stored individually inside clean plastic bags prior to use. During sampling, bottles were opened within a laminar flow hood to minimize contamination from airborne particles.

The Fe/N ratio of the seawater was manipulated by making additions of Fe or nitrate. Different treatments used in the experiments included control (no addition), 10 μM sodium nitrate (hereafter "nitrate"), or 10 nM iron prepared from atomic absorption standard stock in HCl (Sigma). The nitrate addition was intended to mimic the increase in nitrate projected for the California upwelling system in the year 2100 (Rykaczewski and Dunne, 2010), and the Fe was intended to at least double ambient concentrations of Fe in this area (Bruland et al., 2001). Following the addition of nutrient spikes (time zero), three bottles were immediately sampled for each of the measurements described below. The remaining bottles (6 per treatment) were placed in a large pool through which ocean water was continually circulated. A neutral density shade cloth was placed over the pool to decrease the irradiance by half, and the shading does not alter the spectral quality of the light. Three bottles from each treatment were collected 48 and 96 h after time zero and were processed as described below.

Bottle incubation experiments are useful for examining short term changes in phytoplankton community composition and physiology, particularly with respect to changing water chemistry including nutrients. The impact of grazers is more difficult to assess from bottle experiments, particularly when the incubation volume is low. This is particularly true for large grazers, like copepods, that are far less abundant than phytoplankton and may not survive in the bottles over time. Bottle experiments also have difficulty capturing variability in light due to mixing of surface water. These factors must be taken into consideration when interpreting bottle experiment results, where grazing and other factors do not perfectly mimic the environment.

### WATER CHEMISTRY

Nutrient samples were 0.45 μm filtered and frozen until analysis. Nutrient analyses for nitrate (with nitrite), soluble reactive phosphorus (SRP), and silicate were carried out on a Lachat Autoanalyzer (FIA, Lachat Instruments Model QuickChem 8000). Total dissolved Fe was measured in the seawater from each site. Samples were 0.2 μm filtered in a laminar flow hood and acidified to pH <2.0 with concentrated trace metal grade nitric acid (final concentration 0.02 M) at least 48 h before analysis. The pH was adjusted to 6 with ammonium acetate and ammonium hydroxide, and samples were concentrated using Nobias Chelate-PA1 resin (HITACHI High Technologies, Japan) to remove the seawater matrix (Sohrin et al., 2008; Biller and Bruland, 2012). The Fe was eluted with 1 M trace metal grade nitric acid and analyzed for by HR-ICPMS (Thermo Element XR). The detection limit for Fe was 0.91 nM.

### PHYTOPLANKTON GROWTH

Phytoplankton growth was assessed based on measurements of chlorophyll *a* (chl *a*) and direct enumeration of cells using flow cytometry and microscope cell counts. For chl *a* measurement, 50 mL of incubation water was filtered through GFF filters (Whatman) and the filters were flash frozen and stored at −80°C until analysis. Filters were extracted in 5 mL 90% acetone (Optima) in the dark at −20°C for 24 h. Fluorescence was measured on a Turner Designs AU10 and converted to concentration via a standard curve calibration.

Flow cytometry samples were collected and fixed with a final concentration of 0.1% glutaraldehyde and flash frozen and stored at −80°C until analysis. Samples were thawed on ice and run on an Influx flow cytometer (Becton Dickinson). Using FlowJo software, phytoplankton cells were identified from their red (692 nm) chlorophyll autofluorescence signal. *Synechococcus* cells were identified by their phycoerythrin signal (572 nm).

Samples for microscope analysis were fixed with a final concentration of 2% formalin and stored in the dark at 4°C in glass bottles. A settling chamber was used to concentrate 2–10 mL of sample, and cells were viewed on an inverted microscope. Typically ∼1000 cells were counted per sample, but never less than 300, and replicate samples were randomly included to check for accuracy. Cells >4 μm in diameter were identified and enumerated as pennate diatoms, centric diatoms, or "others" (Supplemental Figures 1–8). Pennate and centric diatoms were further grouped according to whether they occurred as single cells or in chains of two or more cells. In the text their relative abundances are categorized as rare (<1%), present (1–9%), common (10–49%) or abundant (>50%). Statistical significance was determined using ANOVA followed by pairwise comparisons using a Bonferroni correction. Cell counts in the Fe and N treatments at each site were compared to the control (two comparisons per site).

## RESULTS

### REMOTE MONITORING

Wind velocity recorded at the M1 mooring indicated that upwelling favorable winds reached speeds up to ~8 m $s^{-1}$ during the period of June 10–16. The winds consistently blew from the northwest to the southeast during this period (**Figure 2A**). Cold water masses were observed from the AVHRR satellite during these dates along the California coast, indicating upwelling of deep water was occurring. By June 15, a large, cold (9–10°C) upwelled water mass was located surrounding Point Reyes and was centered offshore of Bodega Bay, and smaller upwelled water masses were located north of Monterey Bay and at Point Sur (**Figure 1A**). Upwelling favorable winds relaxed from June 17 to 23, and the wind direction became more variable (**Figure 2A**). By June 20 the amount of cold water being upwelled had declined and was more restricted to the coastline north of Point Reyes (**Figure 1B**). The water masses south of Point Reyes and near Monterey Bay continued to warm once upwelling ceased (**Figure 1B**).

Temperature, salinity, and nitrate data from the mooring at M1confirmed the pattern of upwelling and relaxation observed from the satellite images (**Figure 2**, Supplemental

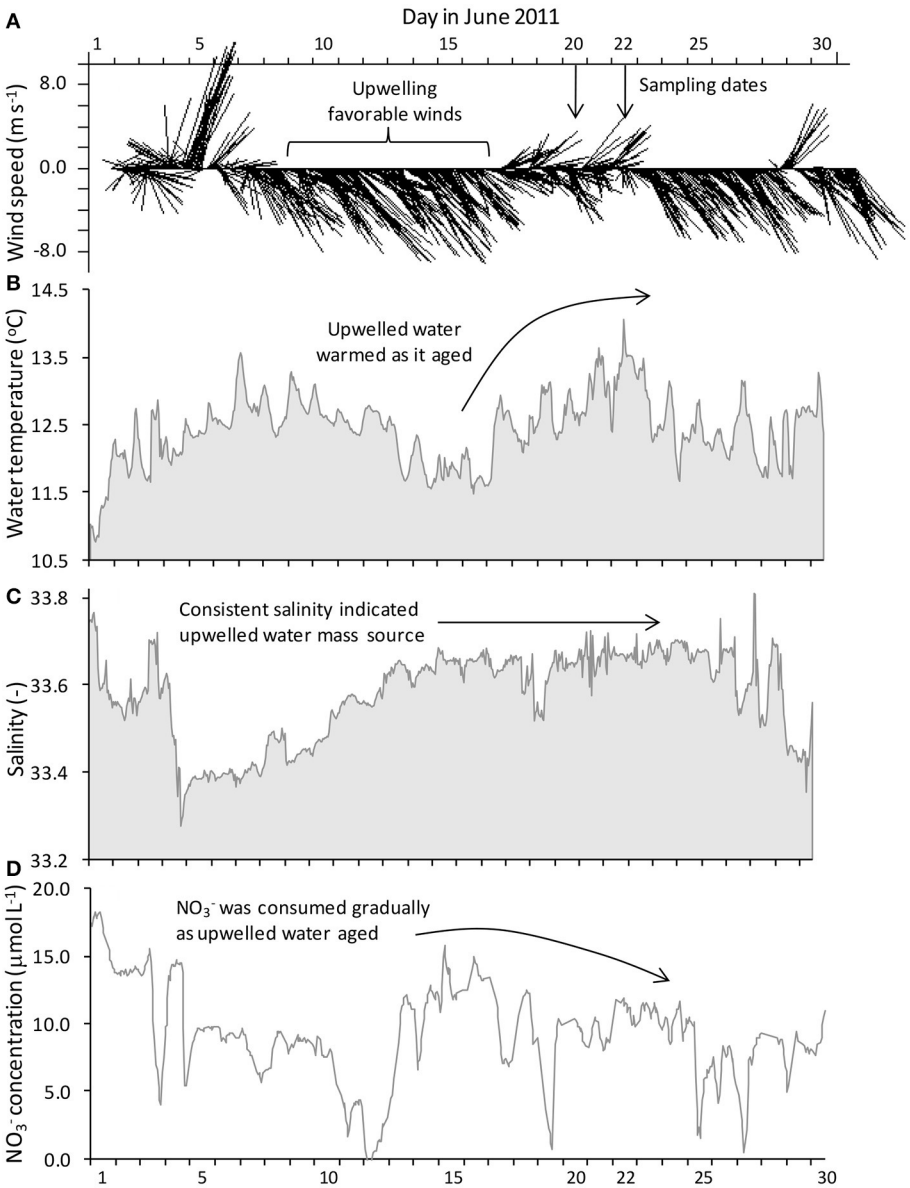


**FIGURE 2 | *In situ* mooring data for station M1 showing (A) hourly gridded wind velocity, (B) sea surface temperature, (C) salinity, and (D) nitrate concentration.** Upwelling favorable winds occurred in the days prior to sampling and were accompanied by cold, saline water with high nitrate levels. Winds relaxed beginning June 17, causing upwelling to cease and allowing surface waters to warm as nitrate was drawn down. Incubation water was collected on June 20 and 22.

Figure 9). At M1, surface waters became progressively colder and more saline from June 10 to 16 while upwelling favorable winds prevailed (**Figures 2B,C**, Supplemental Figure 9). By June 15 a cold (~11.5°C), saline (~33.6) water mass was present at the mooring. Upon relaxation of upwelling favorable winds (June 17–23), the water mass began to warm. Relatively stable salinity indicated that the warmer temperatures were associated with the same upwelled water mass rather than from intrusion of waters from the less saline California current.

The nitrate concentration at mooring M1 reached its highest level (~15 μmol $L^{-1}$; **Figure 2D**) in surface waters by the end of the upwelling period (June 15–16). Nitrate declined gradually by ~5 μmol $L^{-1}$ from June 17 to 23 once upwelling favorable winds relaxed; however, nitrate levels were still relatively high (~10 μmol $L^{-1}$) and had not been consumed entirely by June 23.

Water was collected for the incubation experiments once upwelling favorable winds had relaxed for several days and surface water had warmed 1–2°C based on remote monitoring data as described above (e.g., June 20 for stations PR and SF, and June 22 for stations M0, M1, and M2). Nutrient and Fe levels were lower at stations M0, M1, and M2 than at stations PR or SF (**Figures 3A–D**). The ratio of N/P was similar among all five sites (**Figures 4A,B**), whereas the ratio of Si/N was higher at stations PR and SF than at M0, M1, or M2 (**Figures 4E,F**). The Fe/N ratio was not statistically different among the five sites at $p < 0.05$ (**Figures 4C,D**). The incubation results for each of the stations are discussed below.

## INCUBATION EXPERIMENTS

### *Drakes Bay at Point Reyes (station PR)*

Initial nutrient concentrations at station PR were 29.5 ± 3.3 μmol $L^{-1}$ nitrate, 2.2 ± 0.0 μmol $L^{-1}$ SRP, 40.8 ± 0.1 μmol $L^{-1}$ silicate, and 9.2 ± 0.65 nmol $L^{-1}$ Fe, with a N/P ratio of 13.4 ± 1.5, a Si/N ratio of 1.4 ± 0.1, and a Fe/N ratio of 0.33 ± 0.0 nM/μM (**Figures 3**, **4**). The range of N/P drawdown ratios for all treatments was 13–19, and the range for Si/N was 0.8–1.2 (**Figure 5**).

The initial chl *a* concentration was 1.9 ± 0.1 mg $m^{-3}$, and the range of final concentrations for all treatments was 32.2–43.0 mg $m^{-3}$. The N treatment resulted in a higher chl *a* increase than other treatments (**Figure 6**). The phytoplankton population at Point Reyes was dominated by several species of *Thalassiosira*, and the majority of these were chain-forming in all treatments by the end of the incubation. Pennate diatoms were present at this site across all treatments and between 40 and 57% of them were chain-forming (**Figures 7**, **8**; cell concentration data is given in Supplemental Table 1). Among the picophytoplankton, picoeukaryotes (range across treatments = 78–104 $\times 10^3$ cells $mL^{-1}$) outnumbered *Synechococcus* (range across treatments = 5–7 $\times 10^3$ cells $mL^{-1}$) (**Figure 7**).

### *Coastline north of San Francisco Bay (Station SF)*

Initial nutrient concentrations at station SF were 27.9 ± 3.8 μmol $L^{-1}$ nitrate, 2.1 ± 0.0 μmol $L^{-1}$ SRP, 49.5 ± 0.2 μmol $L^{-1}$ silicate, and 6.2 ± 0.61 nmol $L^{-1}$ Fe with a N/P ratio of 13.3 ± 1.8, a Si/N ratio of 1.8 ± 0.2, and a Fe/N ratio of 0.24 ± 0.04 nM/μM (**Figures 3**, **4**). The range of N/P drawdown ratios for all treatments was 13–18, and the range for Si/N was 0.7–1.0 (**Figure 5**).

The highest initial chl *a* concentration (3.3 ± 0.0 mg $m^{-3}$) was observed at this site. By the end of the experiment chl

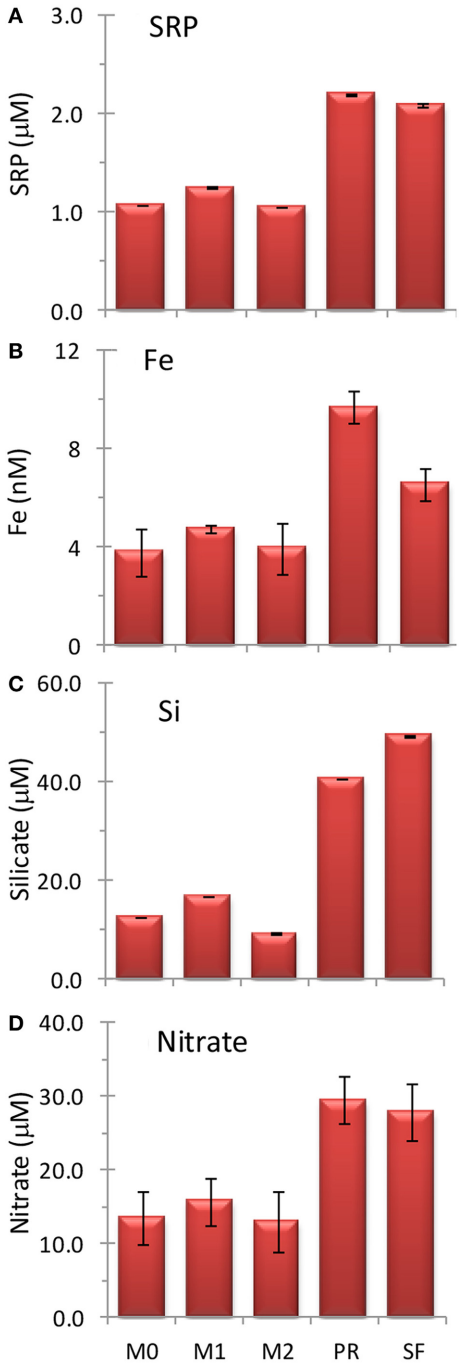


**FIGURE 3 | Initial concentrations of (A) soluble reactive phosphorus, SRP, (B) Fe, (C) silicate, and (D) nitrate at the five sampling locations.** Error bars show standard deviation.

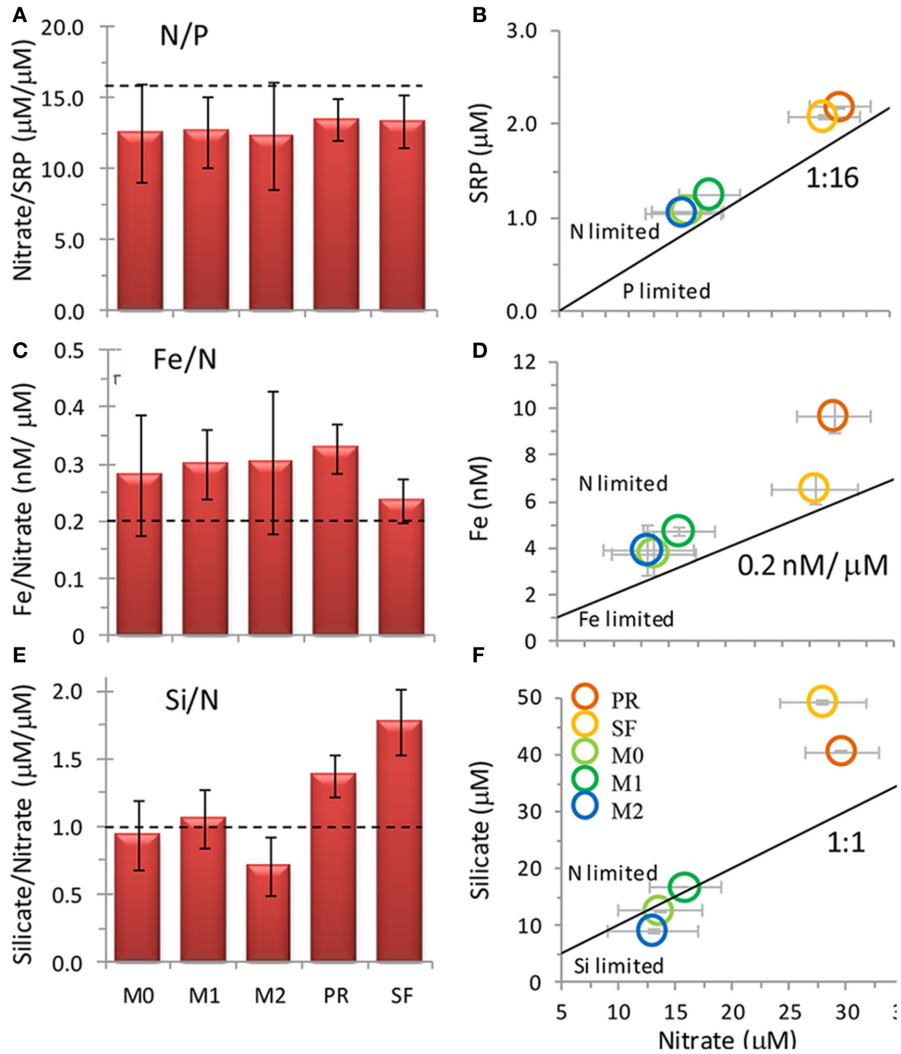


**FIGURE 4 | Nutrient ratios of (A,B) SRP: nitrate, (C,D) Fe: nitrate, and (E,F) silicate: nitrate at the five sampling locations when the incubation water was collected.** Legend for (**B,D,F** shown in **F**). Error bars show standard deviation. Broken lines in **(A,C,E)** and solid lines in **(B,D,E)** show typical ratios of diatom cellular nutrient quotas.

*a* levels in the Fe treatments clustered with the control (16.0-18.4 mg $m^{-3}$), and N caused an increase in chl *a* (29.4 ± 1.1 mg $m^{-3}$) above the control (**Figure 6**). The highest picophytoplankton abundances were observed at this site, where picoeukaryote abundances ranged from 198 to 276 × $10^3$ cells $mL^{-1}$, and *Synechococcus* abundances ranged from 6 to 9 × $10^3$ cells $mL^{-1}$ (**Figure 7**). Among phytoplankton larger than 4 μm, small centric diatoms were abundant and the majority existed as single cells (**Figures 7**, **8**). Some of these small centric cells fell below the 4 μm size category and are instead included in the picoeukaryote category (enumerated by flow cytometry). Treatment with Fe caused an increase in chain-forming centric diatoms relative to the control (**Figures 7**, **8**). *Coscinodiscus* was also observed at this site, though rare. Pennate diatoms were observed, and chain-forming pennate cells were very rare (<<1%). The majority were *Cylindrotheca* spp.

### *Inner Monterey Bay (Station M0)*

Initial nutrient concentrations at station M0 were 13.5 ± 3.7 μmol $L^{-1}$ nitrate, 1.1 ± 0.0 μmol $L^{-1}$ SRP, 12.7 ± 0.0 μmol $L^{-1}$ silicate, and 3.6 ± 0.93 nmol $L^{-1}$ Fe, with a N/P ratio of 12.5 ± 3.4, a Si/N ratio of 0.94 ± 0.3, and a Fe/N ratio of 0.28 ± 0.11 nM/μM (**Figure 4**). The range of N/P drawdown ratios for all treatments was 17–19, and the range for Si/N was 0.1–0.2 (**Figure 5**).

The initial chl *a* concentration was 1.6 ± 0.1 mg $m^{-3}$, and all treatments had similar final concentrations as the control (8.2–9.3 mg $m^{-3}$; **Figure 6**). The phytoplankton population at M0 was dominated by *Pseudonitzschia* spp., and the majority of cells were chain-forming in all treatments. Bottles receiving N additions had a lower percentage (60%) of chain-forming pennate diatoms compared to the control and Fe addition treatments (78–82%, **Figure 7**). Centric diatoms were common at this site across

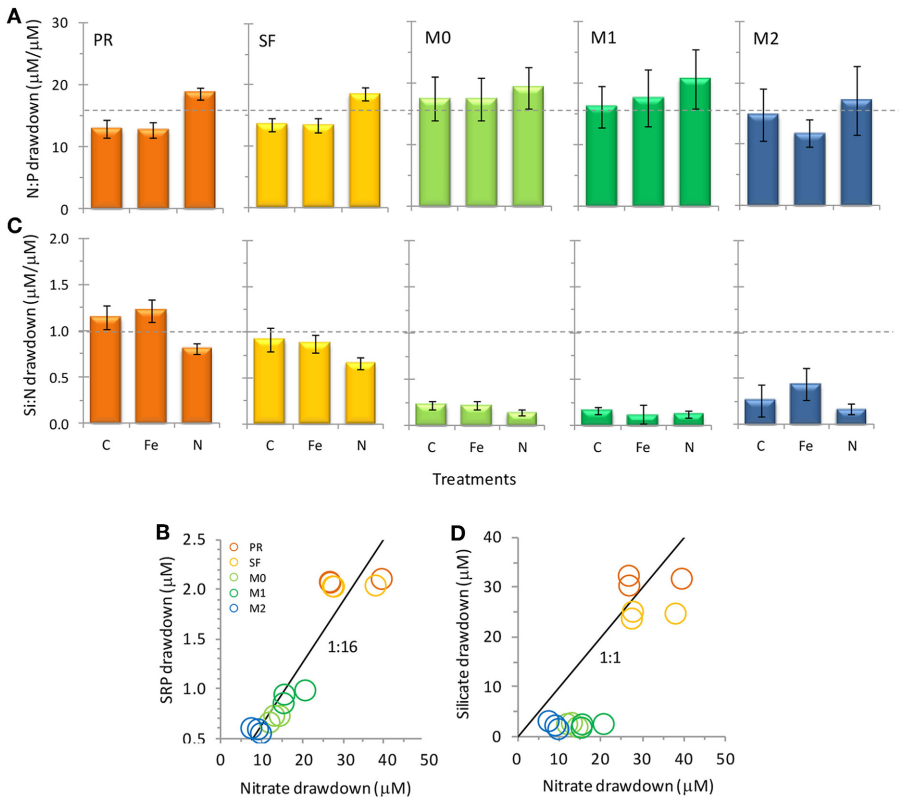


**FIGURE 5 | Nutrient drawdown ratios of (A,B) N/P and (C,D) Si/N for the five stations in this study.** For **(B,D)** values are shown for all three treatments for each station. Values are calculated as the ratios of final minus initial nutrient concentrations. Error bars show standard error. Broken lines in **(A,C)** and solid lines in **(B,D)** show typical ratios of diatom cellular nutrient quotas.

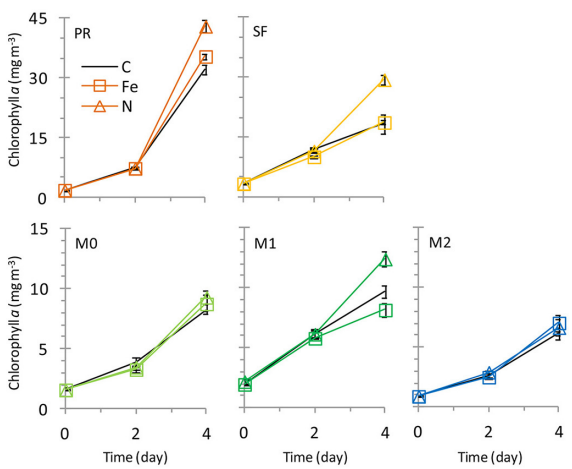


**FIGURE 6 | Growth curves showing chlorophyll *a* over time.** Nitrogen additions increased growth at stations PR, SF, and M1. Error Bars show standard error.

all treatments, and between 34 and 57% were chain-forming (**Figures 7**, **8**). Picoeukaryotes (range across treatments = 49–57 $\times 10^3$ cells $mL^{-1}$) outnumbered *Synechococcus* (range across treatments = 6–7 $\times 10^3$ cells $mL^{-1}$) (**Figure 7**).

### *Outer Monterey Bay (Station M1)*

Initial nutrient concentrations at station M1 were 15.8 ± 3.2 μmol $L^{-1}$ nitrate, 1.3 ± 0.0 μmol $L^{-1}$ SRP, 16.8 ± 0.0 μmol $L^{-1}$ silicate, and 4.5 ± 0.17 nmol $L^{-1}$ Fe, with a N/P ratio of 12.6 ± 2.5, a Si/N ratio of 1.1 ± 0.2, and a Fe/N ratio of 0.30 ± 0.06 nM/μM (**Figures 3**, **4**). The range of N/P drawdown ratios for all treatments was 16–21, and the range for Si/N was 0.1–0.2 (**Figure 5**).

The initial chl *a* concentration was 1.9 ± 0.0 mg $m^{-3}$, and the range of final concentrations for all treatments was 8.2–12.4 mg $m^{-3}$. Nitrogen additions caused the greatest increase in chl *a* (12.4 mg $m^{-3}$, **Figure 6**). The phytoplankton population at M1 was dominated by *Pseudonitzschia* spp., and the majority of cells were chain-forming. Bottles receiving N additions had a lower percentage (56%) of chain-forming pennate diatoms compared to the control and Fe addition treatments (64–70%, **Figure 7**). Centric diatoms were common at this site across all treatments, and between 64 and 72% were chain-forming (**Figures 7**, **8**). Picoeukaryotes (range across treatments = 61–65 $\times 10^3$ cells $mL^{-1}$) outnumbered *Synechococcus* (range across treatments = 6–9 $\times 10^3$ cells $mL^{-1}$) (**Figure 7**). Treatment with Fe additions caused a decline in *Synechococcus* abundance relative to the control, whereas picoeukaryote abundances were not sensitive to any of the treatments (**Figure 7**).

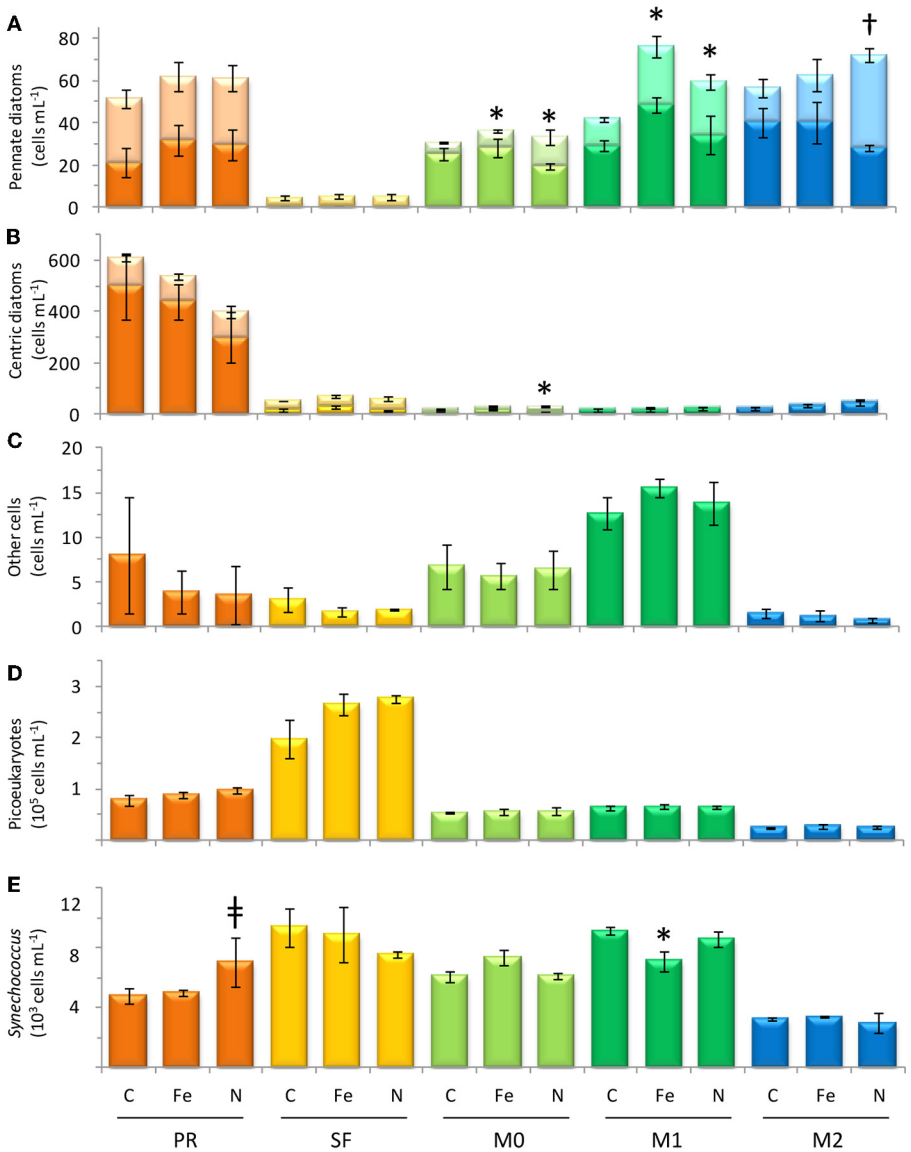


**FIGURE 7 | Cell count data from the final time point of each incubation, showing concentrations of (A) pennate diatoms, (B) centric diatoms, (C) dinoflagellates, and other cells > 4μm, (D) picoeukaryotes, and (E) *Synechococcus*.** Error bars show standard error. Light-colored stacked bars in **(A,B)** show single celled diatoms and dark-colored stacked bars show chain-forming cells. Symbol † denotes $p < 0.05$; symbol *denotes $p < 0.10$; symbol ‡ denotes $p < 0.15$.

### *Offshore of Monterey Bay (Station M2)*

Initial nutrient concentrations at station M2 were 13.0 ± 4.0 μmol $L^{-1}$ nitrate, 1.1 ± 0.0 μmol $L^{-1}$ SRP, 9.2 ± 0.2 μmol $L^{-1}$ silicate, and 3.7 ± 1.0 nmol $L^{-1}$ Fe, with a N/P ratio of 12.3 ± 3.8, a Si/N ratio of 0.7 ± 0.2, and a Fe/N ratio of 0.30 ± 0.12 nM/μM (**Figures 3**, **4**). The range of N/P drawdown ratios for all treatments was 16–21, and the range for Si/N was 0.2–0.4 (**Figure 5**).

The initial chl *a* concentration was 0.92 ± 0.0 mg $m^{-3}$. All treatments had final concentrations similar to the control (6.1–7.2 mg $m^{-3}$, **Figure 6**). The phytoplankton population at M2 was dominated by *Pseudonitzschia* spp. Bottles receiving N additions had a lower percentage (39%) of chain-forming pennate diatoms compared to the control and Fe addition treatments (69–71%, **Figures 7**, **8**). Centric diatoms were common at this site across all treatments, and the majority (77–87%) were chain-forming (**Figures 7**, **8**). Picoeukaryotes (range across treatments = 49–57 $\times 10^3$ cells $mL^{-1}$) outnumbered *Synechococcus* (range across treatments = 6–7 $\times 10^3$ cells $mL^{-1}$) (**Figure 7**).

## DISCUSSION

Coastal California nutrient availability creates a mosaic of nutrient limitation patterns that vary with shelf width, distance from shore, and timing and extent of upwelling. In this study we

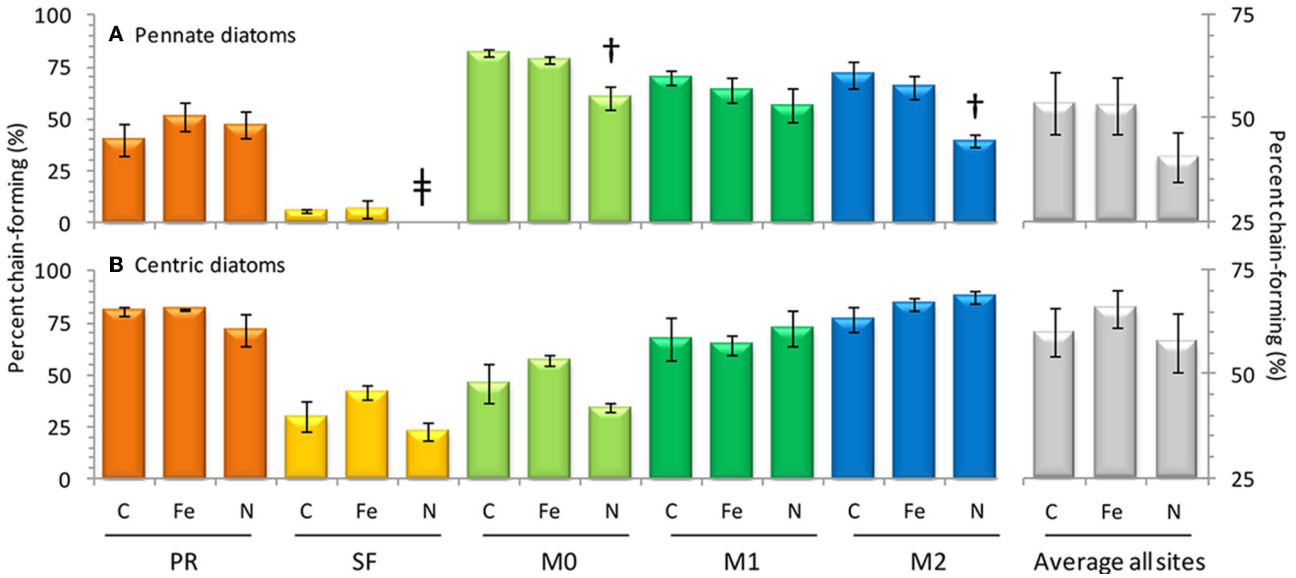


**FIGURE 8 | Observations of chain-forming behavior for pennate diatoms (above) and centric diatoms (below).** At right data from all sites has been (gray bars, $n = 15$ for each treatment). Error bars show standard error. Symbol † denotes $p < 0.05$; symbol *denotes $p < 0.10$; symbol ‡ denotes $p < 0.15$.

examined five Fe replete sites close to the central California coastline as upwelling relaxed (**Figure 2**), when N and Fe levels were both elevated (**Figures 2**, **3**). Even within this relatively small geographical area, the waters in this study showed a high degree of spatial heterogeneity with respect to nutrient levels, phytoplankton populations, and biomass nutrient limitation characteristics. Concentrations of nitrate, SRP, silicate, and Fe were approximately twice as high at stations PR and SF than at stations M0, M1, and M2 (**Figures 2**, **3**), likely because they are influenced by the very strong upwelling center at Bodega Bay (**Figure 1**). Stations PR and SF also had higher ratios of Si:N, although the ratios of N:P were similar among all five sites (**Figure 4**).

The plankton community compositions differed considerably among sites. The phytoplankton community near Monterey Bay at stations M0, M1, and M2 was dominated by *Pseudonitzschia* spp. (**Figure 9**). Picophytoplankton comprised the vast majority of cells in station SF, whereas station PR was populated by *Thalassiosira* spp., and virtually no pennate diatoms were observed (see Supplemental Figures). Grazer populations also varied by location; stations M0, M1, and M2 supported diverse populations of tintinnids (see Supplemental Figure 7), whereas station SF samples contained copepods and copepod fragments, as well as *Protoperidinium* sp. No grazers were observed in samples from station PR. The patchy distribution of populations is consistent with prior observations in Monterey Bay, where phytoplankton species trade off dominance over small spatial scales and time periods (Ryan et al., 2011; Mackey et al., 2012).

Nutrient limitation patterns for biomass also varied by location. All sites had N:P ratios of ∼12; this would typically suggest N limitation (because it is lower than the Redfield Ratio of 16:1), however, only three stations (PR, SF, and M1) showed evidence of N limitation based on chl *a* responses in the incubation experiments (**Figure 6**). At those sites, more N tended to be preferentially drawn down relative to P in bottles receiving additional N (higher N:P drawdown ratio, **Figure 5**). This suggests that N uptake rates were not saturated even though ambient nitrate levels were high, because added nitrate increased the N uptake rate. This could indicate luxury uptake of N by the diatoms, many of which store nitrate in large vacuoles (Lomas and Gilbert, 2000). In contrast, at stations M0 and M2 all treatments led to similar chl *a* increases, suggesting that nutrient levels were high enough to saturate phytoplankton at these sites and biomass was not nutrient limited.

As expected for the broad shelf regions tested in this study, phytoplankton were not Fe-limited in our experiments. This differs from the nearby California transition zone, where Fe levels are routinely below 1 nM (Biller and Bruland, 2014). The Fe levels at sites in this study ranged from ∼4 to 10 nM (**Figure 3**) comparable to the range of total dissolved Fe reported previously for this part of the California coast (0.3–10 nM; Bruland et al., 2001). Additionally, Fe/N ratios in the transition zone were generally below 0.05 nM/μM (Biller and Bruland, 2014), which is well below the threshold for Fe limitation in oceanic (0.07) and coastal (0.20) diatoms (Sunda and Huntsman, 1995). Only in the stations closest to shore did Biller and Bruland report Fe/N ratios exceeding the Fe-replete threshold, where values reached up to 0.20, similar to ratios we observed in this study (0.24–0.33; **Figures 4C,D**). Moreover, natural populations of diatoms can take up excess iron to go through a number of cell divisions when Fe becomes scarce (Yoshida et al., 2006; Sugie et al., 2011), which could further explain the lack of biomass Fe limitation in our bottles.

Changes in the chain forming behavior of diatoms has been linked to Fe and N availability (Hutchins and Bruland, 1998) and temperature (Takabayashi et al., 2006). In this study Fe addition did not cause deviations from the control with respect to chain

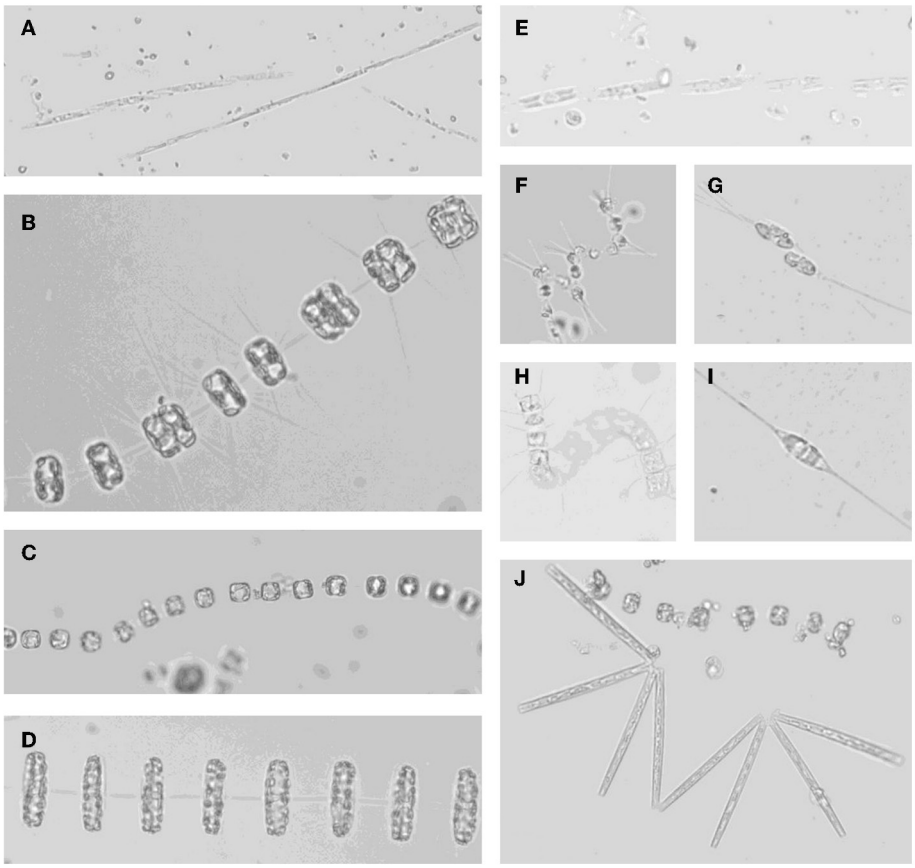


**FIGURE 9 | Micrographs of (A) *Pseudonitzschia* spp.; (B) *Thalassiosira* sp. with dividing cells; (C) *Thalassiosira* sp.; (D) *Thalassiosira rotula*; (E) *Pseudonitzschia* sp.; (F) *Asterionellopsis glacialis*; (G) *Cylindrotheca closterium* dividing cells; (H) unknown chain forming centric diatom; (I) *Cylindrotheca closterium*; (J) *Thalassionema* sp. with *Thalassiosira* sp.**

forming behavior in pennate diatoms (**Figure 8**). However, Fe additions did appear to enhance chain forming morphology in centric diatoms slightly, though the effect was not significant at $p < 0.05$ (**Figure 8**). This behavior is consistent with the findings of Hutchins and Bruland (1998), where Fe additions caused more fast sinking, chain forming centric diatoms to bloom. The incubation results suggest that the Fe/N ratios at sites in this study were already high enough (0.24–0.33 nM/μM) to support chain forming behavior in pennate diatoms in the control and Fe addition samples, thus further Fe additions did not cause any change (**Figures 7**, **8**).

Major differences in the drawdown of Si relative to N were observed among the sites (**Figures 5C,D**). Stations M0, M1, and M2 all had Si/N drawdown ratios well below the 1:1 Si/N drawdown ratio expected for diatoms (even though these sites were dominated by the diatom *Pseudo-nitzschia* spp.), while stations SF and PR had drawdown ratios close to 1:1. Therefore, less Si than expected was taken up in Monterey Bay compared to the other sites. Interestingly, Si/N drawdown did not show a consistent response to either Fe or N additions (and therefore Fe/N ratios). This response differs from Fe-limited sites along the CA coast where Fe additions caused diatoms to incorporate up to 50% less Si into their frustules relative to N during incubation experiments (Hutchins and Bruland, 1998). The discrepancy in Si/N drawdown among sites suggests factors other than Fe availability influence the drawdown of Si relative to N. One possibility could be differences in Si drawdown between different diatom species, as stations M0, M1 and M2 were dominated by pennate diatoms, whereas SF and PR had more centric diatoms. Alternately, as Si was depleted at M0, M1, and M2, cells may have begun taking up Si and N at different rates, where luxury N uptake could drive lower Si:N drawdown.

Global change presents the possibility for a number of alterations to the biogeochemistry of coastal California (Capone and Hutchins, 2013 and references therein). The flux of nitrate is expected to increase over the next century, and areas that are currently Fe replete could transition toward Fe limitation as the Fe/N ratio decreases (Rykaczewski and Dunne, 2010). In this study, the transition toward higher nitrate flux (lower Fe/N ratios) was simulated by nitrate additions in the incubation experiment. These additions of 10 μmol/L nitrate are within the range of predicted future N increases, and brought Fe/N ratios into Fe-limited range (range of Fe/N = 0.16–0.18 nmol/μmol) at each site except for station PR (Fe/N = 0.25 nmol/ μmol).

We compared our data to published values of Fe and N for the region spanning from Monterey Bay to Point Reyes (Biller et al., 2013), and calculated the potential shift in Fe/N ratio assuming a 50% increase in nitrate at each site as projected by Rykaczewski and Dunne (2010) (**Figure 10**). While biomass at many of the locations in this region are currently Fe replete, others are closer to becoming Fe-limited. Coastal diatoms become Fe-limited below a Fe/N ratio of 0.20 nmol/μmol, and oceanic species have a slightly lower threshold ratio (0.07 nmol/μmol; Sunda and Huntsman, 1995). Thirteen of the 23 sites we compared would be considered Fe replete under present day conditions (>0.2 nmol Fe/μmol N); however, biomass at five of these locations (e.g., within Monterey Bay, south of Half Moon Bay, and along the coast near San Francisco Bay) would cross into the Fe-limited range in the future if nitrate flux increases. The ratios of the other 10 sites that are presently Fe-limited would of course also decline further. These shifts could lead to succession of phytoplankton species better adapted to dealing with Fe limitation, as well as changes in chain forming behavior as observed in this study.

Increased nitrate flux is also anticipated to cause a concomitant increase in productivity in coastal California (Rykaczewski and Dunne, 2010). Addition of nitrate increased phytoplankton biomass in three of the five sites assayed in this study, leading to a 28–60% increase in chl *a* (**Figure 6**) and increasing the drawdown of N relative to P (**Figures 5A,B**). Nitrogen addition also affected the chain-forming behavior of phytoplankton by encouraging the growth of slower sinking, single-celled pennate diatoms in all of the sites (**Figure 8A**). This was due to both a physiological shift in *Pseudonitzschia* spp. toward a single-celled growth habit, as well as a community shift toward species like *Cylindrotheca* spp. that naturally tend to exist as solitary cells.

These changes in phytoplankton physiology and community structure suggest that enhanced N flux could lead to ecological and biogeochemical shifts in the California upwelling system in the future. First, the sinking rate of cells, and hence the export of C, Si, and other elements could decline with the shift to smaller chains of cells with slower sinking rates. Second, these shifts have the potential to propagate up the food web because different, potentially smaller grazers could be favored by the increase in single cells. And finally, if increased N flux favors pennate species other than *Pseudonitzschia* spp. as our experiments suggest, then blooms of this potentially toxin-producing genus could become less prevalent in the future. Indeed, the toxin producing species *Pseudonitszchia pungens* is more competitive at low N:P ratios of ∼10:1 compared to more N rich waters (Hu et al., 2008). The N:P ratios in our unamended water ranged from 12.3 to 13.4, whereas the nitrate-treated water ranged from 18.0 to 21.8. It is therefore not surprising that the slightly N limited conditions and low N/P ratios in the present-day water would favor *Pseudonitszchia,* which leaves open the possibility that *Pseudonitszchia* could become less competitive due to excess N in the future.

Changes in ecosystem services, which are the benefits people derive from marine ecosystems, could also be affected by the changes described here. In California, a major ecosystem service provided by coastal waters is fishery yield. Many studies have shown a link between the amount of upwelling that occurs in a given year and the production of fisheries (Gunsolus, 1978; Nickelson, 1986). The relationship is also apparent in comparing fishery production along the west coast of North and South America during El Nino (low upwelling) and La Nina (high upwelling) years. If future primary productivity increases affect fisheries in a similar manner to natural increases in upwelling, it is possible that CA fisheries could become more productive in the future. The phytoplankton population shift toward smaller cells, which decreases export production due to a decrease in sinking rates, could likewise increase fisheries yields by providing more carbon biomass to grazers.

The projected increase in N flux by the year 2100 is expected to coincide with an 18% decrease in oxygen concentration (Rykaczewski and Dunne, 2010) and a decrease in pH by 0.5 units due to anthropogenic ocean acidification (Doney et al., 2009). These multiple stressors could exacerbate or mitigate the effects observed in this study. In particular, the effect of changing

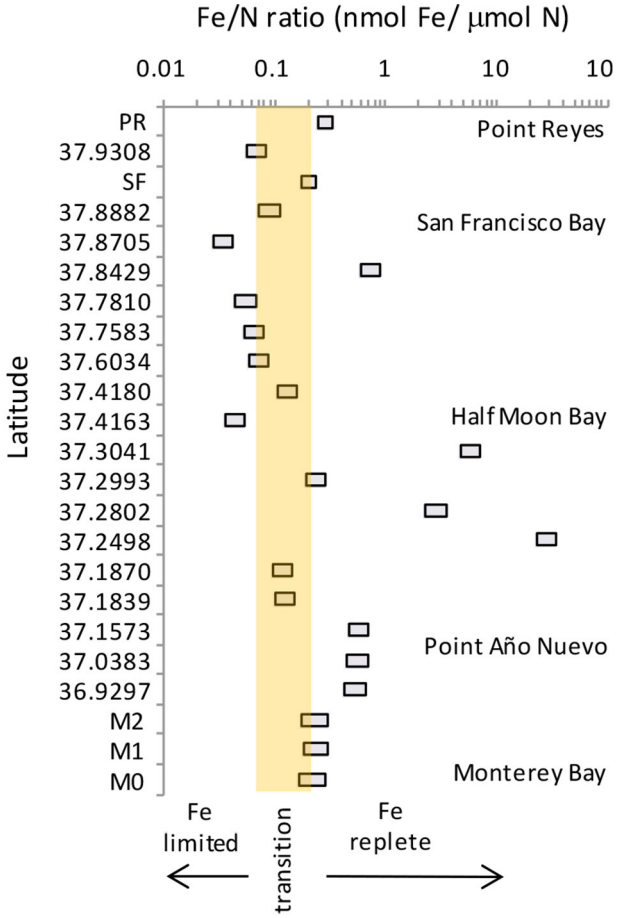


**FIGURE 10 | Range of Fe/N ratios for the region between Monterey Bay and Point Reyes.** Boxes show the present day (right hand side of box) and projected future Fe/N ratio (left hand side of box). Shaded region denoted the range of Fe/N ratios (0.07–0.2) over which oceanic and coastal diatoms become Fe-limited. Data are from this study for stations PR, SF, M0, M1, and M2, and from table 1 of Biller et al. (2013) for all other latitudes. Projected future ratios were calculated assuming a 50% increase in nitrate as predicted by Rykaczewski and Dunne (2010).

seawater chemistry on Fe solubility is difficult to predict. Ocean acidification has the potential to reduce Fe bioavailability by protonating Fe ligands, causing them to retain Fe ions (Shi et al., 2010). In contrast, expansion and shoaling of hypoxic zones would serve to increase Fe solubility, because the reduced Fe(II) in hypoxic waters is more soluble than the Fe(III) in oxygenated waters. In this study Fe additions caused little change in the phytoplankton community because cells were already Fe replete; however, future shifts toward Fe limitation could enhance the importance of soluble Fe supply in these regions (**Figure 10**). Moreover, large blooms of phytoplankton increase the pH of seawater, which could partially or wholly offset the local effects of ocean acidification on Fe availability. It is therefore not clear whether Fe availability will increase or decrease in the future, or what effect, if any, this will have on marine biota in upwelling regions. The Canary and Benguela upwelling systems, which are more Fe replete than the California and Peru systems, are likely to be less sensitive to these changes in Fe biogeochemistry, and changes in N flux may be more important.

Upwelling systems are naturally variable regions inhabited by organisms that thrive under changing conditions. In regions like coastal California where phytoplankton biomass is already on the cusp between N and Fe limitation, modest shifts in nutrient supply ratios could elicit important changes in cell physiology, community composition, and nutrient uptake. This study shows that increased N availability in recently upwelled water leads to faster cell growth, greater nitrate drawdown, and favors solitary cells over chain-formers. Upwelling centers throughout the world's oceans are morphologically and biogeochemically diverse, and future studies should help determine if similar responses could occur in other upwelling regions.

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## SUPPLEMENTARY MATERIAL

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# Polar front associated variation in prokaryotic community structure in Arctic shelf seafloor

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Spatial variations in composition of marine microbial communities and its causes have largely been disclosed in studies comprising rather large environmental and spatial differences. In the present study, we explored if a moderate but temporally permanent climatic division within a contiguous arctic shelf seafloor was traceable in the diversity patterns of its bacterial and archaeal communities. Soft bottom sediment samples were collected at 10 geographical locations, spanning spatial distances of up to 640 km, transecting the oceanic polar front in the Barents Sea. The northern sampling sites were generally colder, less saline, shallower, and showed higher concentrations of freshly sedimented phytopigments compared to the southern study locations. Sampling sites depicted low variation in relative abundances of taxa at class level, with persistent numerical dominance by lineages of Gamma- and Deltaproteobacteria (57–66% of bacterial sequence reads). The Archaea, which constituted 0.7–1.8% of 16S rRNA gene copy numbers in the sediment, were overwhelmingly (85.8%) affiliated with the Thaumarchaeota. Beta-diversity analyses showed the environmental variations throughout the sampling range to have a stronger impact on the structuring of both the bacterial and archaeal communities than spatial effects. While bacterial communities were significantly influenced by the combined effect of several weakly selective environmental differences, including temperature, archaeal communities appeared to be more uniquely structured by the level of freshly sedimented phytopigments.

**Keywords: archaea, bacteria, Barents Sea, beta-diversity, sediment, 16S rRNA gene**

## INTRODUCTION

Microbial community similarities tend to show a distance decay relationship, implying that the phylogenetic composition of communities becomes increasingly dissimilar with increasing geographical distance. It is now generally accepted that both contemporary environmental parameters and historical contingencies, maintained by dispersal limitation, may contribute to this beta-diversity. Hence, the classical Baas Becking statement "Everything is everywhere, but the environment selects" is questioned as a universal model for explaining the observed variation in microbial community composition (Hedlund and Staley, 2003; Martiny et al., 2006; Hanson et al., 2012). In the conceptual framework of metacommunity ecology (Leibold et al., 2004; Logue and Lindström, 2008) this emphasis on local environmental factors vs. spatial (regional) effects largely coincides with the distinction between species sorting and mass effects as the two models best explaining microbial community assembly dynamics (Lindström and Langenheder, 2012). The disentanglement of these different effects is, however, not trivial in many systems due to spatial autocorrelation or co-variations among environmental variables (Horner-Devine et al., 2004; Böer et al., 2009; Zinger et al., 2011; Bienhold et al., 2012; Jacob et al., 2013; Wang et al., 2013).

The microbial communities in the upper sediment layers in marine environments show a steeper decay in similarity with distance than assemblies of the pelagic water masses, which may be attributed to more pronounced environmental gradients within the sediments and more restricted dispersal of sediment microorganisms. Additionally, the more heterogeneous environments in coastal areas have been found to generate steeper gradients than such found in the open ocean both in the seawater and sediments (Zinger et al., 2014). The environmental conditions on the continental shelf seafloors may in several respects be characterized as intermediate between those of the deep ocean and the shallow coastal areas. Due to the combination of less water depth and frequently much higher primary production than in the open oceans, the shelf sediments will receive higher influxes of sedimentary material (Suess, 1980) that sustain stronger heterotrophic activity. The bottom-dwelling fauna, including bioturbating animals (Bertics and Ziebis, 2009) and demersal fishes, contributes to resuspension of sediment particles into the water column, as anthropogenic influences (e.g., from bottom trawling) may do as well. Moreover, because ocean currents, including tidal currents, have often been found to be of great importance at the shelf seafloors, microorganisms are likely dispersed quite efficiently, thereby making mass effects a potentially import factor

in the establishment of microbial community assemblies in this habitat type.

The Barents Sea (1.4 mill km$^2$) is part of the circumpolar Arctic Continental Shelf. It extends northwards from the northern coasts of Norway and Russia to the Arctic Ocean, and is delimited by the Novaya Zemlya and the Norwegian Sea along the east-west axis. With an average depth of 230 m, it is the deepest of the Arctic shelf seas. It is also characterized by less coastal erosion and river water inflow than other Arctic shelf seas (Vetrov and Romankevich, 2004). The most distinctive oceanographic feature of the Barents Sea is, however, the influx of temperate and salty Atlantic water from the southwest. These water masses meet and mix with sub-zero, less saline Arctic Ocean water from the north, resulting in a coarse division of the Barents Sea into a northern and a southern region separated by a transition zone named the polar front (Ingvaldsen and Loeng, 2009). The temperature differences are most pronounced in the surface waters, resulting in winter sea ice covering the northern regions, while the southern parts of the Barents Sea are ice-free throughout the entire year. Near the seafloor, the temperature difference is modest, i.e., about 2°C, and it has even shown a diminishing trend in recent years (Lind and Ingvaldsen, 2012). The overall primary production is highest in the southern parts (Sakshaug et al., 2009) but the deposition of organic material shows a more patchy pattern, caused by additional factors like water depth, bottom topography and local currents (Vetrov and Romankevich, 2004).

On this background, the primary aim of present study was to explore if significant community variations could be detected in a sampling area, which encompassed the moderate environmental variations of the Barents Sea polar front. And if such variations were detectable, should they be attributed to environmental or spatial effects, or both. Beta-diversity analyses were based on 16S rRNA gene sequence data obtained by 454 pyrosequencing. Additionally, the study provided a comprehensive picture of the prokaryotic alpha-diversities in the upper centimeters of this kind of arctic shelf seafloor.

## MATERIALS AND METHODS

### SAMPLING

Sediment samples were taken from 10 locations in the western Barents Sea separated by up to 640 km. Sampling was carried out over the course of 3 days from 20th to 23rd May 2009. The sampling was done along a curved transect that followed the gradually more shallow Bear Island – Hopen channel from close to the continental slope to east of the Svalbard archipelago (**Figure 1**). Seawater temperature and salinity, as measured within 10 m of the seafloor by a CTD instrument, were used as proxies for seafloor values. The upper 4 cm sediment cores of van Veen grab samples were pressed into sterile plastic tubes. The content of each core was homogenized by mixing and stored frozen at -80°C until processing in the laboratory.

### SEDIMENT CHARACTERISTICS

Grain size distribution was determined by dry sieving. The sediment samples were separated into two grain size classes, i.e., clay/silt (<63 μm) and sand/gravel (>63 μm). Total organic carbon (TOC) content was analyzed by a LECO CS-200 Analyzer

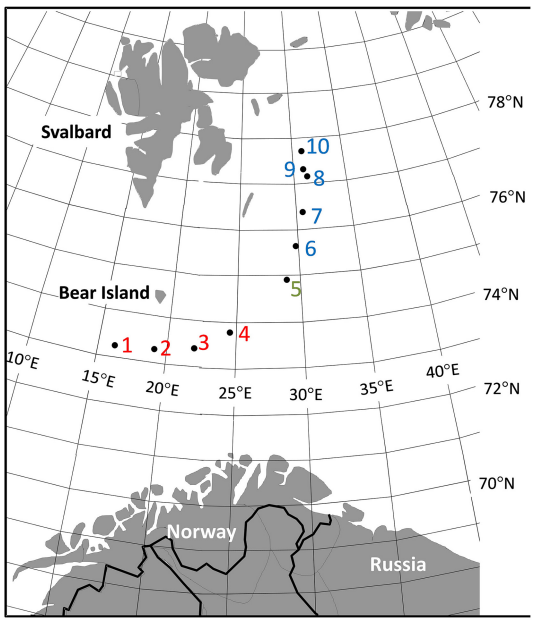


**FIGURE 1 | The western Barents Sea with geographical positions of sampling stations.** Red color (southern part); blue color (northern part), green color (transition temperature zone).

(LECO Corporation, St. Joseph, MI, USA). Sediment chlorophyll *a* (Chl *a*) and phaeophytin were determined by a Turner 7000 fluorometer (Turner Designs Inc., Synnyvale, CA, USA) from readings at 665 nm in ethanol extracts before and after treatment with 1 M acetic acid (Pápista et al., 2002).

### DNA EXTRACTION

Total DNA was extracted from duplicate 0.5 g samples of each site using the PowerSoil™ DNA Isolation kit (Mo Bio Labs, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. The concentration and quality of extracted DNA were determined by a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

### BACTERIAL AND ARCHAEAL ABUNDANCES

Quantification of 16S ribosomal RNA genes was used for the estimation of prokaryotic cell densities. Quantitative real-time PCR (qPCR) was performed on an ABI 7500Fast real-time PCR system (Applied Biosystems, NYSE, Waltham, MA, USA) using primers 27F/338R for Bacteria and A571F/915R for Archaea (see Supplementary Table S1). The environmental DNA samples were run in duplicate with three dilutions of the primary extract ($10^{-1}$ to $10^{-3}$). Standard curves for threshold cycle (Ct) vs. logarithm of the start concentration of 16S rRNA gene copies, from $10^6$ to $10^1$, were established with *Escherichia coli* K10 for Bacteria and *Methanoplanus petrolearius* DSM11571 for Archaea. This corresponded to *E. coli* genomic DNA being serially diluted from 0.76 to $0.76 \times 10^{-5}$ ng and *M. petrolearius* diluted from 1.56 to $1.56 \times 10^{-5}$ ng. Genomic standards were included in each qPCR run to ensure linearity and expected slope values of the Ct/log[gDNA] curves.

### AMPLIFICATION AND MULTIPLEX PYROSEQUENCING OF 16S rRNA GENES

Tagged PCR primers for each sampling station were constructed by adding unique oligonucleotides to the universal forward primers 27F for Bacteria and 571F for Archaea (Supplementary Table S1). The 25 μL PCR reaction mixtures contained 1X PCR buffer (Invitrogen, Waltham, MA, USA), 0.2 mM dNTPs (Invitrogen), 0.5 μM of each primer (Eurofins MWG, Ebersberg, Germany), 1.25 U of *Taq* polymerase (Invitrogen), and 10 ng of genomic DNA template. The thermocycler (Applied Biosystems) conditions were: initial denaturation step at 94°C for 5 min; 30 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min; a final extension at 72°C for 5 min. To minimize potential random PCR biases, each sample was amplified in sextuplicate (triplicates of each DNA isolation). Correctly sized amplification products were extracted from the gel by use of the QIAquick Gel Extraction kit (Qiagen, Hilden, Germany), and replicate samples were pooled and purified one more time with Agencourt AMPure XP beads (Beckman Coulter, Brea, CA, USA). Equal amounts of amplicons from each PCR run were pooled and subjected to multiplex pyrosequencing using a 454/Roche GS-FLX Titanium instrument (454 Life Sciences, Branford, CT, UAS) installed at the Norwegian High Throughput Sequencing Centre (NSC, Oslo, Norway; http://www.sequencing.uio.no). The bacterial (BM) and archaeal (AM) amplicons were sequenced separately, as was a second bacterial preparation from sampling station 6 (D6). The latter was subjected to a deeper sequencing effort than used in the multiplex analysis. The raw sequence data have been submitted to the EMBL database under the accession numbers ERP003605 (BM dataset), ERP003606 (AM dataset), and ERP003607 (D6 dataset).

### SEQUENCE ANALYSES

Quality checks, OTU clusterings and phylogenetic annotations of the sequences were all done by the Quantitative Insights Into Microbial Ecology (QIIME v.1.8.0) pipeline (Caporaso et al., 2010b). In brief, low quality sequences were removed, including sequences shorter than 150 bp or with a quality score below 25. Furthermore, sequences containing ambiguous nucleotides or homopolymers longer than six nucleotides were removed (Huse et al., 2007) using Denoiser software (v.0.91) (Reeder and Knight, 2010). Putative chimeras were identified by ChimeraSlayer and discarded (Haas et al., 2011). The overall numbers of pyrotags were reduced by 26.0% for Bacteria and 12.9% for Archaea by removing low-quality, chimeric and chloroplast-affiliated reads. The qualified sequences were clustered into Operational Taxonomic Units (OTUs) based on 97% sequence similarity by the UCLUST algorithm (Edgar, 2010), and representative sequences from each OTU were aligned to the GreenGenes (version May 2013) public database (http://greengenes.lbl.gov) using the PyNAST tool, as integrated in the QIIME package (DeSantis et al., 2006; Caporaso et al., 2010a). Taxon assignments were obtained with 80% bootstrap cutoffs for both Bacteria and Archaea.

Singletons, i.e., OTUs with only one sequence, were removed as putative sequencing errors or PCR amplification artifacts to prevent artificial diversity inflation (Huse et al., 2010; Kunin et al., 2010). The singletons constituted 62.8 and 38.2% of the primary bacterial and archaeal datasets, respectively. OTU richness was calculated by the non-parametric Chao1 estimator (Chao, 1984) after normalization of the sequence numbers in each sample to 4000 for the Bacteria and 9000 for the Archaea.

### STATISTICAL ANALYSES

A geographical distance matrix was calculated from the latitude and longitude coordinates obtained by the Global Positioning System by use of the package *fossil* (Vavrek, 2011) in the R statistical software (R Development Core Team, 2008). The community beta-diversities were determined by the Bray-Curtis, Sørensen and phylogenetic distance based unweighted UniFrac indices, as implemented in the QIIME and R software packages (Lozupone et al., 2006; R Development Core Team, 2008). The community distance matrices were based on jackknifing (100 permutations) with 75% of the sequence number in the sample with the lowest number of sequences.

To visualize the grouping patterns of the samples based on community distances, non-metric multidimensional scaling (NMDS) based on the Hellinger transformed Bray-Curtis distance metric was used (Legendre and Gallagher, 2001). Vector fitting was employed to identify directions and strengths of the effects of environmental factors and geographical distance in relation to the community-based ordination of samples, in accordance with the procedure of Monier et al. (2014). This included the use of the *envfit* function of the *vegan* package in R (Oksanen et al., 2012).

The combinations of environmental variables that best explained community variation among the sampling stations were obtained as the ones generating maximum rank correlations between the environmental and community distance matrices (Clarke and Ainsworth, 1993) by employing the *bioenv* procedure in the *vegan* R package. Generalized linear models (GLM) were subsequently constructed in R from the standardized environmental variables to quantify their relative importance and test the significance of the individual environmental factors by using the *glm* function. To partition the possible community structuring effects of geography and environmental factors, partial Mantel tests were used (Legendre and Legendre, 1998; Martiny et al., 2011). To test if southern and northern communities were significantly different, a multivariate generalized linear models approach (Warton et al., 2012) was employed as implemented in the R package *mvabund* (Wang et al., 2012). The model that was fitted is log-linear and assumes a negative binomial distribution of data. To determine which taxa contributed the most to the differences between the two regions, the univariate ANOVA function with adjusted *p*-values for multiple testing in *mvabund* was used. Community distance decays were calculated by regressing the community distance matrices on the geographical distance matrices. The significance of these decays was determined by simple Mantel tests based on Spearman rank correlation coefficients ($\rho$) with $10^4$ Monte Carlo permutations. The same procedure was used for testing the relationships between geographical and environmental distances. Tests for correlations between bacterial and archaeal abundances and environmental variables were also based on Spearman rank correlation coefficients.

## RESULTS

### ENVIRONMENTAL VARIATION AND PROKARYOTIC ABUNDANCES

Sediment samples from 10 stations separated by up to 640 km were collected during a time period of 3 days, implying that the impact of temporal changes due to the length of the sampling period was minimized. The temperature recordings through the sampling area confirmed a consistent drop of roughly 1.6°C at the seafloor, when moving from the southern stations (1–4) to the northern ones (6–10), while station 5 was in a transitional temperature zone (**Table 1**). The temperature variation showed significant spatial autocorrelation (Spearman $\rho = 0.87$; $p = 0.001$), as did the additional environmental factors water depth (Spearman $\rho = 0.87$; $p = 0.001$), salinity (Spearman $\rho = 0.39$; $p = 0.03$), and Chl *a*/phaeophytin ratio (Spearman $\rho = 0.41$; $p = 0.02$), the latter being used as indicator of freshly sedimented phytopigment material. On the other hand, the grain size distribution and organic content of the sediment showed a more random variation between the sampling stations. Principal component ordination, based on the environmental data, separated the sampling stations in accordance with the south-north dichotomy along PC1 (Figure S1 in the Supplementary Information). Noticeably, the peak phytopigment concentration at station 6 was reflected in the fraction of the putative chloroplast 16S rRNA gene sequence reads to the total sequence reads, which also showed a distinct maximum at station 6 (**Table 1**).

Bacterial 16S rRNA gene copy numbers varied in the range of $3.1 \times 10^9$ to $1.7 \times 10^{10}$ per g dry sediment, and the Archaea constituted 0.7 to 1.8% of total 16S rRNA copy numbers in the corresponding samples (**Table 1**). If employing the empirical average rRNA operon numbers of 3.9 for Bacteria and 1.8 for Archaea (Lee et al., 2009), the quantitative PCR figures corresponded to $7.9 \times 10^8$ to $4.4 \times 10^9$ bacterial cells per g and $3.2 \times 10^7$ to $1.5 \times 10^8$ archaeal cells per g, respectively. A positive correlation was observed between the bacterial and archaeal copy number log abundance values (Spearman $\rho = 0.75$; $p = 0.01$). Furthermore, the bacterial gene abundance showed significant relationships with temperature (Spearman $\rho = 0.60$; $p = 0.04$), and phytopigment ratio (Spearman $\rho = 0.66$; $p = 0.04$), while no correlations were found between the abundance and environmental data for the Archaea.

### PROKARYOTIC DIVERSITY

The sequence datasets comprised 65 904, 139 590, and 164 880 qualified reads (excluding reads representing singletons) from the sequencing of the bacterial (BM) and archaeal (AM) multiplex amplicons of the transect and a deeper bacterial sequencing of station 6 (D6). A high bacterial diversity was confirmed in this Barents Sea sediment sample as the numbers of unique OTUs obtained both from the multiplex BM and the single station D6 material exceeded 5500 at ≥97% sequence identity (**Table 2**). The 21-fold deeper D6 sequencing of station 6, as compared with the BM data, led to a more than

**Table 2 | Pyrosequencing statistics, number of operational taxonomic units at 97% similarity level and richness estimates.**

| St | Bacteria | | | Archaea | | |
|---|---|---|---|---|---|---|
| | Qualified reads | OTUs | Chao1* | Qualified reads | OTUs | Chao1* |
| 1 | 6148 | 1799 | 2842 | 12,023 | 253 | 369 |
| 2 | 6148 | 1856 | 2728 | 13,613 | 266 | 343 |
| 3 | 4033 | 1583 | 3593 | 18,502 | 695 | 883 |
| 4 | 11,417 | 2872 | 3542 | 18,510 | 585 | 594 |
| 5 | 5387 | 1604 | 2963 | 9006 | 209 | 353 |
| 6 | 7761 | 2116 | 3339 | 11,821 | 805 | 1044 |
| 7 | 7049 | 2096 | 3392 | 18,271 | 972 | 1184 |
| 8 | 5157 | 1578 | 2918 | 10,704 | 492 | 659 |
| 9 | 8238 | 2263 | 3064 | 14,410 | 578 | 696 |
| 10 | 4566 | 1578 | 3369 | 12,730 | 650 | 876 |
| D6 | 164,880 | 9072 | 14,016 | | | |

*Abbreviations: St, Station; OTUs, operational taxonomic units. D6; Deeper sequence at station 6.*

**Computed on quality read subsampled at an even depth of 4000 sequences for bacteria and 9000 sequences for archaea.*

**Table 1 | Geographical locations and environmental characteristics of samples.**

| St | Latitude (N) | Longitude (E) | Depth (m) | Temp (°C) | TOC (%) | Clay/silt (%) | Salinity (%) | Chl*a* ($\mu g/gdw^{-1}$) | Chl*a*:Phae ratio | Chl-16S (%) | 16S gene copies/g | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | | | Bacteria ($\times 10^9$) | Archaea ($\times 10^7$) |
| 1 | 73°13′52″ | 16°20′55″ | 474 | 2.7 | 0.73 | 67.2 | 35.01 | 0.96 | 0.33 | 0.1 | 5.4 ± 0.3 | 9.7 ± 3.4 |
| 2 | 73°17′74″ | 19°15′59″ | 460 | 2.8 | 2.24 | 38.7 | 35.05 | 0.55 | 0.42 | 0.1 | 3.1 ± 0.5 | 6.9 ± 2.4 |
| 3 | 73°23′99″ | 22°03′13″ | 450 | 2.6 | 2.44 | 77.0 | 35.06 | 1.04 | 0.32 | 0.0 | 4.5 ± 0.2 | 5.6 ± 0.9 |
| 4 | 73°47′55″ | 24°35′38″ | 442 | 2.5 | 1.61 | 86.0 | 35.05 | 1.13 | 0.32 | 0.2 | 10.7 ± 3.2 | 19.0 ± 6.6 |
| 5 | 74°55′01″ | 28°54′52″ | 364 | 1.7 | 1.96 | 86.9 | 35.05 | 3.24 | 0.54 | 1.0 | 10.8 ± 1.0 | 7.9 ± 0.7 |
| 6 | 75°38′81″ | 29°44′48″ | 330 | 1.1 | 2.21 | 83.5 | 35.04 | 8.10 | 0.96 | 8.3 | 13.7 ± 0.8 | 12.8 ± 5.3 |
| 7 | 76°24′12″ | 30°37′13″ | 290 | 1.2 | 1.83 | 86.8 | 34.98 | 3.91 | 0.77 | 2.5 | 17.0 ± 0.5 | 26.7 ± 2.5 |
| 8 | 77°08′92″ | 31°16′67″ | 189 | 1.1 | 1.21 | 63.5 | 34.99 | 2.00 | 0.53 | 2.0 | 10.2 ± 0.9 | 8.2 ± 3.5 |
| 9 | 77°20′48″ | 30°58′81″ | 194 | 1.2 | 1.21 | 63.5 | 34.97 | 1.92 | 0.64 | 0.3 | 11.5 ± 1.6 | 18.5 ± 1.4 |
| 10 | 77°43′10″ | 30°56′30″ | 230 | 0.9 | 1.26 | 66.1 | 34.97 | 3.67 | 0.97 | 1.8 | 8.8 ± 0.4 | 9.4 ± 0.0 |

*Abbreviations: St, station; Temp, temperature; TOC, total organic carbon in % of dry weight; Chla, chlorophyll a; Phae, phaeophytin; Chl-16S, chloroplast 16S rRNA genes as % of total sequence reads in each sample.*

four-fold increase in the OTU richness estimate for this station by the Chao1 estimator. This suggested that deeper sequencing of all stations would result in corresponding increases in richness estimates as observed for station 6. Proteobacteria were shown to be dominant in the Barents Sea seafloor by comprising an average of 73.8% of bacterial sequence tags (**Figure 2A**). Gammaproteobacteria and Deltaproteobacteria accounted for 41.1 and 23.2% of the reads, respectively. The taxonomic assignment pointed to the Piscirickettsiaceae as the most prominent sub-group of the Gammaproteobacteria (43.6% of sequence reads), while a substantial fraction of the deltaproteobacterial reads (31.9%) were affiliated with the orders Desulfobacterales and Desulfuromonadales.

Despite the more than two-fold deeper sequencing of the AM than the BM dataset, archaeal OTU numbers were, on average, 29% of the bacterial figures for the same stations. The archaeal communities were highly dominated by a few phylotypes, as the three most prevalent OTUs constituted 60 to 89% of total sequence reads in the different samples. The other striking feature of the archaeal communities was the overwhelming quantitative dominance by the class Thaumarchaeota, which averaged 85.8% of archaeal sequence reads in the samples (**Figure 2B**). A substantial fraction (33.9%) of the thaumarchaeotal reads was affiliated with the marine, ammonia-oxidizing genus *Nitrosopumilus*. Besides the Thaumarchaeota, phylotypes representing the Miscellaneous Crenarchaeotal Group (Inagaki et al., 2003), the Marine benthic group B (Knittel et al., 2005) and the candidate phylum Parvarchaeota (Rinke et al., 2013; Hedlund et al., 2014) constituted significant groups, while less than 1% of the archaeal sequence reads showed euryarchaeotal affiliation.

## COMMUNITY STRUCTURE VARIATION

The overall stable distribution of phylotypes (**Figure 2**) and congruent ranking of abundant OTUs (data not shown) at the different sampling stations for both the Bacteria and Archaea weighed against strong community structuring forces within this range of arctic seafloor. However, NMDS ordination based on the complete sequence information (singletons not included) indicated some level of clustering of the prokaryotic communities in accordance with the separation by environmental factors and spatial distance. For both Bacteria and Archaea, the communities of stations 1–4 tended to be associated with the slightly warmer, deeper and more saline conditions in the southern part of the sampling range, while the communities from stations 6–10 were associated with the observed higher levels of the phytopigment indicators in that region (**Figure 3**). Statistical comparisons between the southern (1–4) and northern (6–10) communities showed significant differences by the multivariate generalized linear models approach for Bacteria and Archaea (ANOVA, $p = 0.009$ for both groups). The five bacterial taxa that generated most explained difference between the two regions were the proteobacterial orders Nitrosomonadales ($p = 0.006$), Rhodospirillales ($p = 0.009$), Marinicellales ($p = 0.009$), Desulfuromonadales ($p = 0.010$) and the uncultured proteobacterial group Sva0853 ($p = 0.010$). For the Archaea, just the variation in the Parvarchaeota ($p = 0.001$) and Thaumarchaeota ($p = 0.021$) tag abundances gave significant contributions to explained regional difference (Supplementary Figures S2, S3). Distance decays of community similarity were confirmed both by the Bray-Curtis index (**Figures 4A,B**) and the phylogeny-based unweighted UniFrac dissimilarity index ($\rho = 0.34$; $p = 0.02$ for Bacteria; $\rho = 0.54$; $p = 0.01$ for Archaea). The zero-distance bacterial Bray-Curtis index value of 0.65 (**Figure 4A**) represents the similarity between the BM and the D6 sequence pools of station 6. They constituted separate, independently analyzed DNA extracts from the same, well-mixed sediment sample material. Hence, the deviation of this value from unity reflects the stochastic beta-diversity associated with non-exhaustive sequencing within

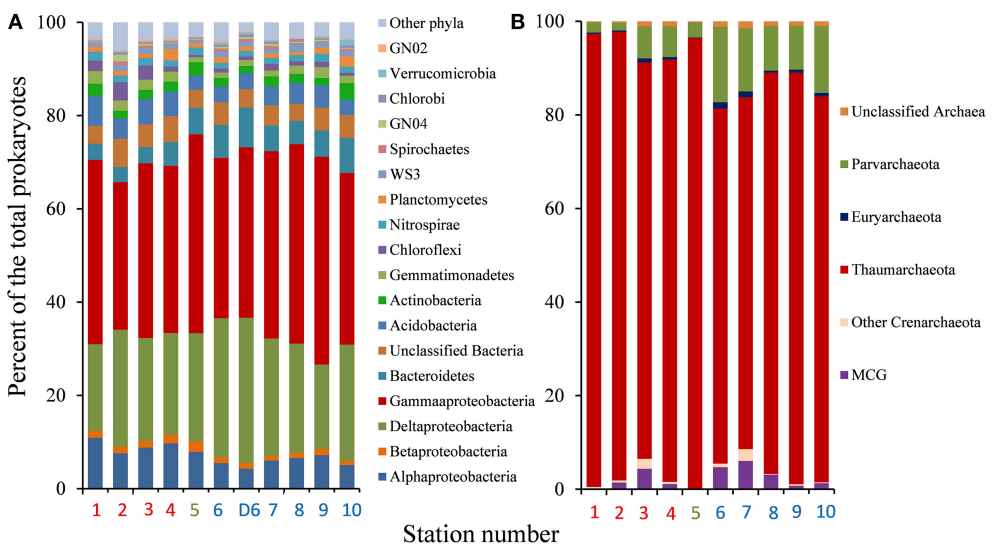


**FIGURE 2 | Distribution of major phylogenetic groups of Bacteria (A) and Archaea (B) at each sampling station.** The analyses of both the multiplex (BM) and the deeper D6 pyrotag datasets are presented for Bacteria at station 6. Abbreviations: MCG, Miscellaneous Crenarchaeotal Group. "Other Crenarchaeota" include Marine benthic group A, Marine benthic group B and Marine Hydrothermal Vent group. Red color (southern part), blue color (northern part), green color (transition temperature zone).

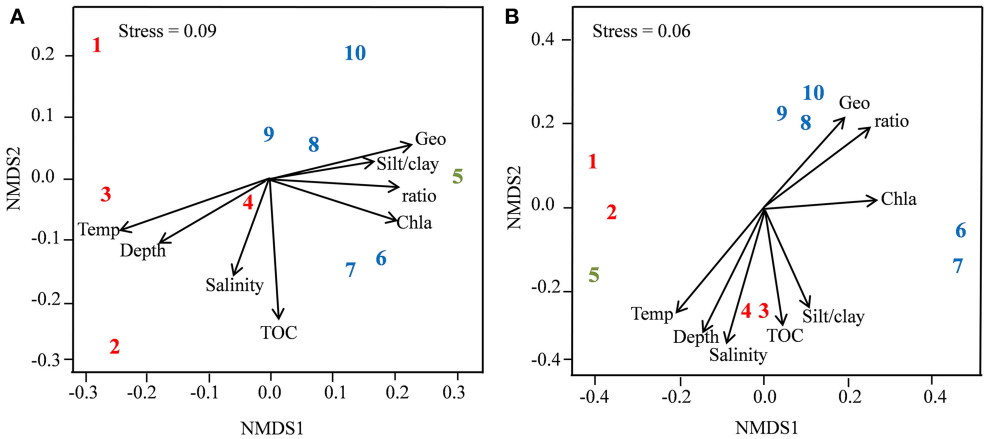


**FIGURE 3 | Non-metric multidimensional scaling based on Bray Curtis community distances for Bacteria (A) and Archaea (B).** Numbers represent sampling stations and arrows show vector fitting of the environmental variables. Abbreviations: Temp, Temperature; Depth, water depth; TOC, total organic carbon; Chl *a*, chlorophyll *a*; ratio, chlorophyll a/phaeophytin ratio; Geo, spatial distance between sampling stations. Red color (southern part), blue color (northern part), green color (transition temperature zone).

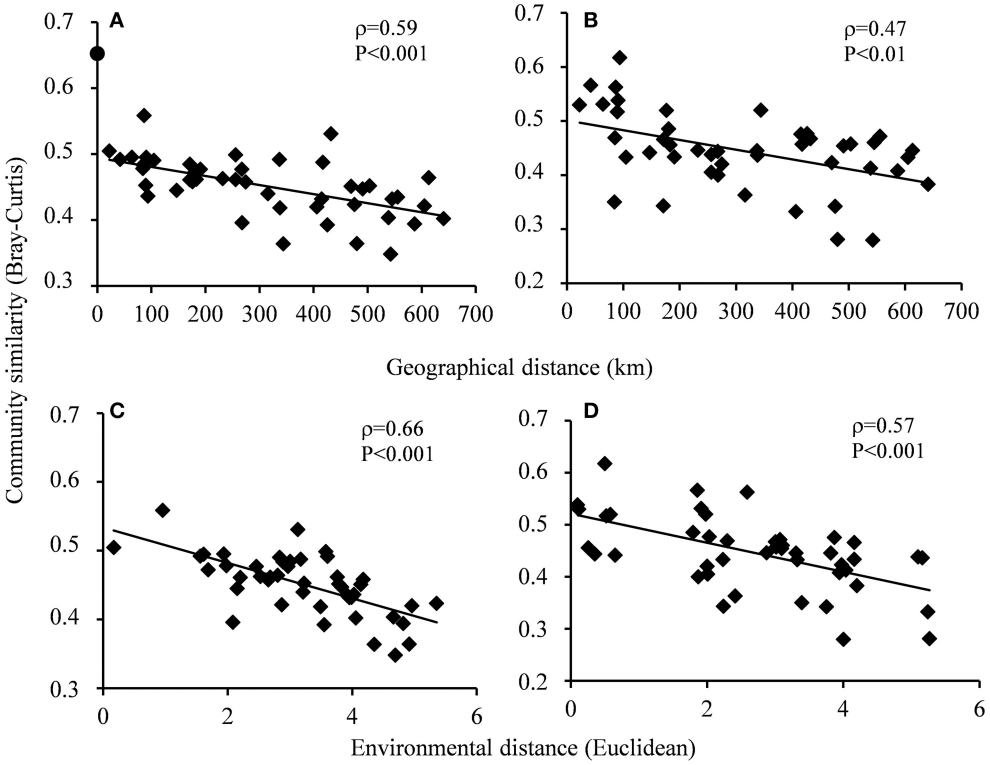


**FIGURE 4 | Relationships between community similarity (1-Bray Curtis index) and spatial distance (A,B) and between community similarity and environmental distance (C,D) for Bacteria (A,C) and Archaea (B,D).** The beta-diversity of the two bacterial station 6 datasets (filled circle in **A**) is not included in the regression line or correlation analyses. The significance of the correlations were assessed by Mantel tests based on Spearman's rank correlation with $10^4$ Monte Carlo permutations.

a single community. The |β|coefficients, i.e., the absolute values of the linear regression coefficients based on the Sørensen similarity index in a double logarithmic plot (Zinger et al., 2014) were $0.056 \pm 0.013$ for the Bacteria and $0.153 \pm 0.041$ for the Archaea.

Mantel tests showed that the independence of possible influential factors on the community structuring was obscured both by significant collinearities between several of the individual environmental factors, i.e., temperature, depth, salinity and

phytopigment ratio ($p = 0.001$ for all combinations) and by spatial autocorrelations of the same environmental factors ($p = 0.001$). Hence, relationships were optimized between combinations of environmental parameters and community variation by the *bioenv* procedure, and significant relationships were found between these combinations of environmental factors and community distances (**Figures 4C,D**). For the Bacteria, the four variables temperature, phytopigment ratio, %silt/clay and TOC were maintained in the model, while depth, phytopigment ratio and Chl *a* gave positive contributions for the Archaea.

Partial Mantel tests were employed to assess the independent effects of space and environment on the community structuring. Significant relationships between community and environmental variation were confirmed when controlling for spatial distance (**Table 3**). For the reciprocal tests, i.e., spatial effects when controlling for environmental distance, the null hypothesis could not be rejected, but the similar magnitudes of the correlation coefficients for the two bacterial tests indicated comparable contributions to explained variation by environment and space. To quantitate the relative contributions to explained community variation by the different environmental factors, general linear models were established. These models retained the independently varying factors temperature, %silt + clay and TOC as statistically significant contributors for the Bacteria, while the phytopigment ratio alone showed significance for the Archaea. The contribution to overall community variation explained by environmental variables was 42.1% for the bacterial communities and 31.0% for the archaeal communities (**Table 4**).

## DISCUSSION

### PROKARYOTIC COMMUNITIES IN THE BARENTS SEA SEDIMENT

Our data confirmed the initial finding of Torsvik et al. (1996) that upper marine sediments harbor one of nature's most diverse microbiotas. High 16S rRNA gene diversity estimates for sediments have previously been obtained from rarefaction analyses of clone libraries (Ravenschlag et al., 2001; Pedrós-Alió, 2006) and, more recently, by massive parallel sequencing efforts (Zinger et al., 2011; Bowen et al., 2012; Hamdan et al., 2013). The four-time increase in the bacterial richness estimate for station 6 when comparing the one based on the roughly 7700 reads of the BM with the 21-fold deeper D6 dataset confirmed the strong dependency on sequencing depth that has previously been documented for the Chao1 estimator (Lemos et al., 2011). As compared with near full-length amplicons of the bacterial 16S rRNA gene, our sequence reads of the V1–V2 region may have produced up to 30% overestimations of OTU richness due to a higher fraction of hypervariable basepairs than in the complete gene (Youssef et al., 2009).

The bacterial taxa composition of the Barents Sea samples was similar to recent reports for marine seafloor upper sediments, i.e., distinctly higher fractions of Deltaproteobacteria, but lower abundances of Alphaproteobacteria than commonly found in the pelagic bacterial communities. This main feature has been observed from deep ocean seabeds with low influx of water-column derived sedimenting material to more shallow coastal areas, where benthic–pelagic coupling likely is strong. It therefore seems to reflect a universal environmental adaptation of the marine sediment bacterial communities (Li et al., 2009; Teske et al., 2011; Zinger et al., 2011; Bienhold et al., 2012). Hence, the uppermost centimeters of the sediment appeared to be dominated by autochtonous bacterial assemblies throughout the sampling area.

The less than 2% of total 16S rRNA genes affiliated with Archaea seems characteristic of the uppermost layer of marine sediments. Comparably low presence of Archaea have been found in other Arctic and Antarctic sediments (Sahm and Berninger, 1998; Ravenschlag et al., 2001; Bowman and McCuaig, 2003). The archaeal communities showed a noticeable skewness in phylotype distribution, as the three top-ranking OTUs constituted more than two-thirds of total archaeal sequence reads and there was an absolute dominance by representatives of the recently established group Thaumarchaeota (Brochier-Armanet et al., 2008). The Thaumarchaeota comprise the phylotypes

**Table 4 | General linear model analyses of the effect of individual environmental variables on bacterial and archaeal communities.**

| | Coefficient | *p*-Value |
|---|---|---|
| **BACTERIA** | | |
| Temperature | 0.020 | **0.009** |
| TOC | 0.016 | **0.016** |
| %(Silt/Clay) | 0.014 | **0.019** |
| Phytopigment | 0.007 | 0.432 |
| **ARCHAEA** | | |
| Phytopigment | 0.033 | **0.024** |
| Chl-*a* | 0.014 | 0.201 |
| Depth | 0.017 | 0.199 |

*Statistically significant relationships are indicated by bold letters; TOC, total organic carbon in % of dry weight; Chl a, chlorophyll a; Phytopigment, chlorophyll a/phaeophytin ratio. $R^2$ adj = 0.42 for bacteria, $R^2$ adj = 0.31 for archaea.*

**Table 3 | Partial Mantel tests of Spearman's rank correlations between prokaryotic community distance and either geographical or environmental distance.**

| Correlation between Prokaryotic community and | Controlling for | Bacteria | | Archaea | |
|---|---|---|---|---|---|
| | | ρ-Value | *p*-Value | ρ-Value | *p*-Value |
| Geographic distance | Environmental distance | 0.28 | 0.06 | 0.1 | 0.27 |
| Environmental distance | Geographic distance | 0.3 | **0.04** | 0.43 | **0.005** |

*Statistically significant relationships are indicated by bold letters.*

that were previously classified as Crenarchaeotal Group 1.1a (Schleper and Nicol, 2010), which have been identified as major archaeal constituents in marine pelagic waters and sediments, including polar and other cold regions (Bano et al., 2004; Galand et al., 2009; Dang et al., 2010; Alonso-Sáez et al., 2011; Durbin and Teske, 2011). In contrast, Hamdan et al. (2013) did not identify Thaumarchaeota in the sediment of the Alaska Beaufort Sea shelf. The Thaumarchaeota are associated with an autotrophic ammonia-oxidizing energy metabolism with the capacity to utilize very low substrate concentrations (Könneke et al., 2005; Herfort et al., 2007; Pester et al., 2011). As established ammonia-oxidizing bacterial phylogenetic groups, like Nitrosomonadales, were very poorly represented among the Bacteria, the Thaumarchaeota appeared to be the predominant ammonia oxidizers in this cold shelf sediment. With some reservations regarding seasonal variations or primer bias in the 16S rRNA gene amplification, the virtual absence of relevant groups of Euryarchaeota in our material excluded methanogenesis or anaerobic methane oxidation as significant processes in the top centimeters of this seafloor.

#### SOURCES OF COMMUNITY VARIATION

The dissimilarity between spatially separated microbial communities is established in the balance between neutral factors, the rate of dispersal of the organisms and the strength of local selective forces (Sloan et al., 2006; Lindström and Langenheder, 2012; Wang et al., 2013). The Barents Sea comprises a contiguous shelf seafloor, where minor differences in the prokaryotic assemblies were expected due to moderate environmental variations in combination with an anticipated substantial dispersal effected by re-suspension of fine-grained sediment particles. The stability in higher taxa composition throughout the sampling range consolidated this presumption. Additionally, allochtonous influx of microorganisms via particulate pelagic material may have promoted the high community similarity, as the bacterioplankton is more weakly biogeographically structured than the benthic microbiotas across similar distances (Zinger et al., 2014). However, our phylogenetic data gave no basis to conclude that bacterial groups that are associable with sedimenting planktonic material constituted a significant fraction of the seafloor microbiota. The frequently cultivable, copiotrophic lineages of Gammaproteobacteria, principally members of the Alteromonadales, Oceanospirallales, Vibrionales and Pseudomonadales, are pointed out as characteristic of particle-bound planktonic Bacteria (Zhang et al., 2007; Lauro et al., 2011; Teske et al., 2011; Crespo et al., 2013; D'Ambrosio et al., 2014). These groups constituted minor proportions of the Gammaproteobacteria in our sediment material, while representatives of the dominating Piscirickettsiaceae family have not, to our knowledge, been associated with pelagic particulate material.

A main objective of our study was, however, to elucidate if even these small environmental differences across the more than 600 km sampling area transecting the Barents Sea polar front, were reflected in non-random community variations if analyzed by a next-generation sequencing approach. The NMDS ordination patterns of the assemblies of both Bacteria and Archaea suggested some degree of community structuring in accordance with the south-north spatial and environmental separation of the sampling range. However, estimates of the importance of the factors that gave rise to this structuring was complicated both by the extensive collinearity between several environmental factors, i.e., temperature, phytopigment ratio, water depth and salinity, and the just as strong spatial autocorrelation of the same factors. These phenomena weakened the possibility to disentangle the contributions by the various factors to the overall beta-diversity and made general linear models labile, with coefficient estimates that were sensitive to minor changes in the data or the optimization criteria (Legendre, 1993; Dormann et al., 2013).

The spatial separations of sampling sites in the present study varied from 23 to 640 km, thereby falling into the intermediate range (10–1200 km) in which Schauer et al. (2010) have found dispersal limitation and contemporary environmental selective forces to show comparable contributions to biogeographic patterning in deep-sea sediments. We found the impact of spatial effects to be subordinate to the one of environmental factors, although this ranking was less evident for the Bacteria than the Archaea. Hence, the data did not exclude our initial assumption that dispersal is substantial in this kind of shelf sediment, but dispersal was evidently not strong enough to blur the community structuring effects of the moderate environmental differences along the sampling area. The partitioning of the various environmental factors that contributed to explained community variation was based on the criterion of Clarke and Ainsworth (1993) of optimized fit between community and environmentally based distance matrices, in combination with a general linear model. This approach picked two different, covarying environmental variables, i.e., temperature for the Bacteria and phytopigment ratio for the Archaea, as the most influential community structuring factors, with some additional contribution to explained variation by the independently varying factors organic content and grain size distribution for the Bacteria. Published studies in the field or additional data acquired through the present study did not give robust grounds to conclude whether this difference had a true ecological basis or rather was a consequence of model lability caused by collinearity.

The temperature difference between the southern and northern sampling stations constitutes a stable oceanographic feature of the western Barents Sea (Ingvaldsen and Loeng, 2009) but the observed difference appears to be at best marginal with regards to leaving a detectable footprint in the prokaryotic assemblies. Previous documentations of temperature effects have largely been associated with markedly wider ranges (Fuhrman et al., 2008; Gilbert et al., 2009; Wietz et al., 2010; Agogué et al., 2011), while Hamdan et al. (2013) found no contribution to beta-diversity by a $\leq 2.4°C$ temperature variation in arctic marine sediment.

At the time of our sampling effort in late May, winter sea ice had retracted from around sampling stations 5–6 to about station 8 and was partly disintegrated even further north. The spring bloom, which is particularly intensive in the 20–50 km marginal zone south of the ice edge (Sakshaug et al., 2009), was well-under way and sedimentation from this bloom may explain the distinctly higher phytopigment and chloroplast-associated 16S rRNA gene levels in the northern part of the sampling range. In

addition, the greater water depth in the southern part, with less sedimented material reaching the seafloor, may have contributed in the same direction. Both the Chl *a*/phaeophytin ratio and the concentration of Chl *a* have been used as estimators of freshness of sedimented phytoplanktonic material in e.g., the western Barents Sea. Positive relationships between the content of sediment phytopigments and bacterial growth and production has been demonstrated (Jørgensen and Boetius, 2007; Morata and Renaud, 2008) and pigment content has been used as a proxy for available energy to benthic bacteria in arctic marine sediment (Bienhold et al., 2012). The abundance of Thaumarchaeota in pelagic marine waters has also been shown to correlate positively with Chl *a* (Robidart et al., 2012) but in the present study, the candidate phylum Parvarchaeota rather was the group that showed a marked increase in the northern region. This recently identified group (Rinke et al., 2013) of very small cells with correspondingly small genomes has as yet only been genetically characterized through an acid mine drainage single-cell sequencing project (Hedlund et al., 2014) and these data do not give any hint to its ecological adaptation in marine sediment.

No significant community structuring effects of water depth or salinity were observed. Previous studies documenting effects of water depth are founded on substantially wider depth ranges than the less than 300 m in the present study. In two studies based on pyrosequence data comprising sampling sites from surface level to the deep ocean floor, up to 3.0% of the sediment bacterial community variation was found to be explained by water depth (Zinger et al., 2011; Bienhold et al., 2012). Although consistent, the shift in salinity close to the seafloor between the southern and northern parts of the sampling range was below 0.1%, and we anticipate it generates a negligible structuring effect on the prokaryotic communities.

There is the possibility that unmeasured environmental variables contributed significantly to community variation through the sampling range. Possible unaccounted variables include the levels of inorganic nutrients (Wu et al., 2008; Böer et al., 2009) and the degree of oxygen penetration into the sediment (Durbin and Teske, 2012). However, no variations in abiotic composition have been reported along this well-characterized extent of contiguous soft bottom seafloor, which will likely overshadow the influences by the variables that were included in the study. In addition, the moderate variation in the fraction of taxonomic groups associated with anaerobic sulfur compound metabolisms, e.g., the orders Desulfobacterales and Desulfuromonadales, was not indicative of major changes in oxgen profiles within the upper 4 cm of the seafloor (data not shown).

Several studies have confirmed microbial community distance decay relationships in marine habitat types like pelagic water (Monier et al., 2014; Zinger et al., 2014), salt marshes (Horner-Devine et al., 2004; Martiny et al., 2011), and oceanic sediments (Schauer et al., 2010; Zinger et al., 2014). Sapp et al. (2010) represent an exception as they were unable to detect significant spatially induced variation of bacterial and archaeal communities in North Sea sediment by a denaturing gradient gel electrophoresis approach. The actual magnitudes of the distance decays are, however, difficult to compare due to differences in diversity indices, organismal target groups, genetic entity compared, etc. A recent global sampling study employing an analytical approach highly similar to the one used by us (Zinger et al., 2014) consolidates our estimate of 0.056 for the absolute value of the double-logarithmic distance decay regression coefficient on shelf seafloor. Our figure was in-between the values of for deep-sea and coastal sediments estimated by Zinger et al. (2014). On the other hand, the corresponding coefficient estimated by Schauer et al. (2010) for South Atlantic deep-sea sediments was just 0.003, i.e., at least an order of magnitude smaller than our shelf sea figure, and the authors associate this low distance decay with high dispersal rates and low extinction rates of the vast bacterial populations in this kind of environment. The archaeal coefficient of 0.15 estimated in our study corresponded to the upper extreme bacterial values recorded by Zinger et al. (2014). Together with the above-mentioned assignment of the explained archaeal community variation solely to environmental factors, the archaeal beta-diversity appeared as more sensitive to environmental variation than the one of the Bacteria in this shelf seafloor environment. To our knowledge, this kind of comparative beta-diversity observations between Bacteria and Archaea in the same environment has not previously been reported.

In conclusion, our data consolidated previous findings regarding the bacterial alpha-diversities of marine shelf seafloor sediments but reinforced the significance of the Thaumarchaeota as the principal archaeal group in this type of environment. Furthermore, the study confirmed that biogeographical structures are detectable in marine sediment prokaryotic communities by deep 16S rRNA gene sequencing, even where high dispersal rates combined with weak environmental filtration counteract the build-up of beta-diversity patterns. This may have implications for the practicality of employing such approaches to monitoring microbial effects of e.g., the predicted rise in air and water temperatures in the polar regions, including the Barents Sea, in the years to come. This climate change is expected to be manifested in the microbial communities (Kirchman et al., 2009). On the other hand, the study also emphasized the importance of sufficient prior knowledge of the environmental variations within the sampling area to avoid complications caused by extensive co-variations among the spatial and environmental variables.

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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.2015.00017/abstract

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# Denitrifying and diazotrophic community responses to artificial warming in permafrost and tallgrass prairie soils

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Increasing temperatures have been shown to impact soil biogeochemical processes, although the corresponding changes to the underlying microbial functional communities are not well understood. Alterations in the nitrogen (N) cycling functional component are particularly important as N availability can affect microbial decomposition rates of soil organic matter and influence plant productivity. To assess changes in the microbial component responsible for these changes, the composition of the N-fixing (*nifH*), and denitrifying (*nirS, nirK, nosZ*) soil microbial communities was assessed by targeted pyrosequencing of functional genes involved in N cycling in two major biomes where the experimental effect of climate warming is under investigation, a tallgrass prairie in Oklahoma (OK) and the active layer above permafrost in Alaska (AK). Raw reads were processed for quality, translated with frameshift correction, and a total of 313,842 amino acid sequences were clustered and linked to a nearest neighbor using reference datasets. The number of OTUs recovered ranged from 231 (NifH) to 862 (NirK). The N functional microbial communities of the prairie, which had experienced a decade of experimental warming were the most affected with changes in the richness and/or overall structure of NifH, NirS, NirK and NosZ. In contrast, the AK permafrost communities, which had experienced only 1 year of warming, showed decreased richness and a structural change only with the *nirK*-harboring bacterial community. A highly divergent *nirK*-harboring bacterial community was identified in the permafrost soils, suggesting much novelty, while other N functional communities exhibited similar relatedness to the reference databases, regardless of site. Prairie and permafrost soils also harbored highly divergent communities due mostly to differing major populations.

**Keywords: nifH, nirK, nirS, nosZ, denitrification, climate change, permafrost, warming**

## Introduction

Microbial communities mediate positive feedback mechanisms to warming of the Earth's climate due to their decomposition of organic carbon ($CO_2$, $CH_4$) and nitrogen cycling ($N_2O$). Community responses to warming such as changes in diversity, population size and shifts in functional groups ultimately impact carbon (C) and nitrogen (N) cycling pathways and rates. Microbially-mediated decomposition rates are impacted not only by climate but substrate quality and the composition of the microbial community (Cornelissen, 1996; Aerts, 1997; Berg and McClaugherty, 2007; Parton et al., 2007). For substrate quality, litter lignin and nitrogen content have strong effects on decomposition rates locally (Melillo et al., 1982; Taylor et al., 1989) and on the global scale (Cornwell et al., 2008), along with climate (Meentemeyer, 1978). However, the impact of exogenous N addition on decomposition has produced mixed results that are often ecosystem-dependent (Keller et al., 2010; Norris et al., 2012) with the general consensus that, particularly in sites with low N availability, decomposition is limited by nitrogen in the early phases (Hobbie et al., 2012). However, changes in plant community composition and thus species-specific litter chemistry (Norris et al., 2012), as a result of climate change (precipitation/temperature) or nutrient availability, may also impact the decomposer community and its responses to the addition or removal of N from the system. Lastly, by employing different methodological approaches such as qPCR and microbial community profiling techniques, temperature has been shown to significantly impact the size and structure of the denitrifying (Yergeau et al., 2007; Braker et al., 2010; Jung et al., 2011) and diazotrophic (Deslippe et al., 2005) communities.

In this study we utilized high-throughput targeted amplicon sequencing to assess changes in microbial communities involved in N cycling in two ecosystems, Alaskan permafrost and an Oklahoma tallgrass prairie, sites with a range of past and on-going complementary studies. The Carbon in Permafrost Experiment Heating Research (CiPEHR) project was established in Alaska in 2008 to examine the effects of experimental warming on permafrost biogeochemical cycling, and changes to the underlying microbial community. Winter warming (1.5–2.3°C to 40 cm depth) has resulted in a 10% increase in thaw depth and a 20% increase in gross primary productivity (GPP). Though no significant change in soil respiration was found during the growing season, a doubling of net $CO_2$ loss was observed during the winter months (Natali et al., 2011). Winter warming also increased total plant N and since soil N mineralization is tightly coupled to plant N uptake, it was hypothesized that soil N availability was enhanced under warming (Natali et al., 2012). Experimental warming has also resulted in a shallower water table, increases in soil moisture and enhanced cellulose decomposition in the 0 to −10 cm soil depths (Natali et al., 2015). Fungal community composition was also shown to significantly differ between the soils positioned in the active layer, where seasonal thawing and freezing occurs, and the (frozen) permafrost layer that was first formed in the Pleistocene era (Penton et al., 2013b).

The tallgrass prairie ecosystem site was established in 1999. Experimental warming using infrared heaters has resulted in compositional and metabolic shifts within the soil microbial community, assessed through metagenomics, with significant enrichment of pathways relating to C degradation, $CO_2$ production and N cycling (esp. denitrification). These changes were further linked to changes in aboveground plant productivity (Luo et al., 2014). Both GeoChip (Zhou et al., 2012) and shotgun metagenomic analyses (Luo et al., 2014) revealed significant differences in phylogenetic or functional gene richness and diversity with warming in addition to enrichment of metabolic pathways involved in N cycling. In contrast, the composition and diversity of fungal communities did not differ with warming (Penton et al., 2013b). Warming has also been shown to decrease plant tissue N concentration though total plant biomass N increased, due to higher biomass production (An et al., 2005). This was also linked to higher labile C and N, microbial biomass C and N, and indicated an increase in microbial C- and N- use efficiency (Belay-Tedla et al., 2009). Soil $CO_2$ flux also increased with warming (Zhou et al., 2007).

Here we assessed changes in the diversity and composition of the N-cycling genes and their host microbial communities in these Alaskan permafrost and Oklahoma tallgrass prairie sites where experimental warming was underway for 1 year and one decade at the time of sampling, respectively. The overall goal of this project was to discern whether molecular tools are able to provide insight into the sensitivity of N-cycling communities to warming, regardless of site. The observed changes in N availability and cycling at these sites with warming led us to hypothesize that significant alterations in the N cycling community compositions would be driving these process-level differences. In order to address this question, targeted high-throughput amplicon sequencing of portions of the *nirK*, *nirS*, and *nosZ* genes, functional gene markers for denitrifying bacteria as well as of *nifH* which encodes a subunit of the $N_2$-fixing nitrogenase complex in diazotrophic bacteria, was performed. Our results suggested that N functional gene-harboring bacterial richness and overall composition were most affected in the tallgrass prairie soils after 10 years of warming. After only 1 year of warming the Alaskan permafrost N processing communities were less affected. The *nirK* harboring bacterial community: (i) was found to be the most sensitive to warming, (ii) was the only N functional gene community significantly altered by warming in the permafrost samples, and (iii) contained highly divergent NirK sequences in the permafrost samples.

## Methods

### Site Description

The two experimental warming sites in this study are subjects of ongoing studies examining changes in biogeochemical pools and fluxes; plant, fungal and bacterial community structures; soil metagenomes and metatranscriptomes and other impacts related to short-term and long-term experimental warming. The Oklahoma (OK) samples originated from the unclipped plots at the Great Plains Apiaries site (34°58′54″N, 97°31′14″W). Initiated in November 1999, its purpose was to determine

ecosystem (plant and soil) responses to experimental warming and is a subject of continuing research (Luo et al., 2001, 2014; Wan et al., 2002a,b; Zhang et al., 2005; Zhou et al., 2007, 2012; Jia et al., 2014; Xu et al., 2014). The grassland is dominated by $C_3$ forbs *Xanthocephalum texanum* and *Ambrosia psilostachyia* and the $C_4$ grasses *Sorghastrum nutans*, *Schizachyrium scoparium* and *Eragrostis curvula*. The control plot (C) contained a dummy heater to simulate the shading effect while experimental warming (T) was performed with an infrared heater. Mean soil temperatures in the warming plot were increased by 1.8–2.7°C to a depth of 10 cm. Soil cores were collected in October 2009 from the surface layer (0–15 cm) of six replicates in control and six in warming plots.

Alaskan (AK) permafrost samples originated from the Carbon in Permafrost Experimental Heating Research project (CiPEHR). Located near Denali National Park and Preserve near Eight Mile Lake, Alaska (63°52′59″N, 149°13′32″W), the site was established in September 2008. The overarching goal of this site is to determine the effects of soil and permafrost warming on tundra ecosystems (Natali et al., 2011) and is a subject of ongoing research, as noted prior. Accumulating snow due to snow fences passively increased the winter soil temperatures by 1.5°C (over a depth of 5–40 cm) (Natali et al., 2011) and resulted in thawing of the near-surface permafrost layers. Accumulated snow was removed in the spring to reduce the impact of excess snowmelt at the site. The sites are dominated by *Eriophorum vaginatum* and *Vaccinium uliginosum* along with other vascular plants (Luo et al., 2001; Schuur et al., 2007, 2009; Natali et al., 2011). Soil cores were taken in May 2009 up to a 65 cm depth from control (C) and winter warming (T) plots after 1 year of warming. The cores were sliced into 10 cm depth increments while the active layer was still frozen and the depth to the permafrost layer was determined in the field. For both sites samples remained frozen until DNA extraction using the mechanical lysis method (Zhou et al., 1996) without the removal of plant roots. In total, 12 samples per treatment were analyzed from the 0 to −45 cm depths, corresponding to soils above the permanently frozen permafrost depth (−50 cm) and average water table depth (−25 cm) (Natali et al., 2011).

### Amplification and Sequencing

All PCR amplifications for *nifH*, *nirS*, *nirK*, and *nosZ* were performed in quadruplicate using primers tagged with multiplex identifier (MID) sequences. Primer sequences and conditions are noted (Supplementary Table 1). Amplicons were sequenced using the 454 Titanium pyrosequencing platform at the Utah State University Center for Integrated Biosystems. Raw reads were deposited in the European Nucleotide Short Read Archive (SRA) under study accession PRJEB8005, sample accession numbers ERS629288-ERS629367. Nucleotide sequences were processed using the Ribosomal Database Project (RDP) functional gene (FunGene) pipeline (Fish et al., 2013; http://fungene.cme.msu.edu). Chimeric sequences were removed using UCHIME 6.0 in de novo mode. The filtered sequences were translated to protein and frameshift-corrected using the RDP DNA-protein alignment tool FrameBot (min length=80 a.a., identity cutoff=80%) (Wang et al., 2013) and the corresponding FunGene database as a reference. Amino acid sequences were then aligned and clustered using complete linkage clustering at the respective amino acid dissimilarity threshold determined for each gene (NifH, NirS 5%; NirK NosZ 3%) (Supplementary Figure 1). Sequences were randomly re-sampled for each gene to between 1053 and 3800 sequences per sample (Supplementary Table 2). Representative minimum sum of square distances sequences for each cluster were generated and used for BLASTp (closest-match) against reference databases generated from the RDP Fungene database. For *nifH,* representative sequences were subjected to BLASTp against a *nifH* database where the extracted protein region that corresponds to that amplified by the primers was used (augmented Zehr-set; Wang et al., 2013).

### Statistical Analyses

Raw sequence abundances were normalized by Hellinger transformation (square root of relative abundance) and Bray-Curtis (+1) dissimilarity matrices were constructed using the PRIMER-6 package (Clarke and Warwick, 2001) (Primer-E Ltd, 239 Plymouth, U.K.). Ordinations were produced using permutated non-metric multidimensional scaling (nMDS) and significant differences among treatments tested using permutational analysis of variance (PERMANOVA) (Anderson, 2001). The effect of replicate dispersion on PERMANOVA results was tested using permutational analysis of multivariate dispersions (PERMDISP) and the individual OTUs that contributed to the majority of the Bray-Curtis dissimilarity were obtained using similarity percentage analysis (SIMPER) (Warwick et al., 1990). Margalef's richness (d) and Pielou's Evenness were calculated using PRIMER-E and significant differences among treatment groups tested using ANOVA analyses (Minitab 16, Minitab Inc., USA). Comparative tests on environmental and community similarity matrices were performed using the function RELATE. Gene-based resemblance matrices were compared to those created from log-transformed, normalized Euclidean distance matrices using the function BEST.

## Results

The amino acid dissimilarity value at which to cluster was determined by analyzing the number of OTUs recovered at 1–15% amino acid dissimilarity for an inflection point for each gene (Supplementary Figure 1), with the exception of NifH that was pre-determined at 5% dissimilarity (Wang et al., 2013). Cluster analysis was based on 3% amino acid dissimilarity for NirK and NosZ while NirS was based on 5%. Overall, the fraction of sequences with similarity to a reference protein sequence above the FrameBot cutoff was lower in the AK vs. Oklahoma (OK) samples for all functional genes (Supplementary Table 2). For NirS, NirK and NosZ the forward and reverse translated reads were clustered separately and the resulting OTU tables were combined into one data matrix for downstream analyses while NifH were unidirectional reads. The following gene-specific results involve two comparisons: (i) the effect of warming within each site and (ii) the differences between sites (AK and OK), regardless of treatment (both control and warming treatments are combined for these comparisons).

### NifH

For *nifH*, a total of 232 OTUs were generated at 5% amino acid dissimilarity that represented closest BLASTp matches to 81 unique taxa from the reference dataset. Both gene richness and evenness were significantly increased in the warming treatment in OK, though the increase was not significant in AK (**Table 1**). PERMANOVA results showed no significant warming effect on the diazotroph community structure in both OK and AK (**Table 2**). Two samples were identified as outliers in OK, each within the warming treatment. Both were distant from both AK and OK, with an average of 25.1% similarity to all other samples. PERMANOVA was not possible after the removal of these samples although ANOSIM results showed a non-significant differentiation with warming (Global $R = 0.1$, $p = 0.21$). PERMANOVA revealed no significant community difference between the organic (surface) and mineral (variable depth) layers in AK ($F = 2.25$, $p = 0.072$), but with significant differences between the samples taken above and below the average water table depth ($F = 6.01$, $p = 0.004$).

Significant overall community differences were identified according to site (AK vs. OK), without regard to treatment (**Table 2**). According to NMDS ordination, OK samples were tightly grouped with a contrasting large dispersion of AK samples (**Figure 1A**). Both AK and OK diazotrophic communities were weighted heavily toward a few OTUs, which contributed to the lowest evenness of all functional genes. Average BLASTp identities to the reference database were 89.4 ± 7.3% in OK and 89.0 ± 5.0% in AK. One OTU (7V1S) that grouped with Alpha- and Betaproteobacteria was present in high abundance in both AK and OK, with an average of 33.7 and 17.7% relative abundance, respectively (Supplementary Figure 2). This OTU was most closely matched to *Rhizobium* sp. at 93.5% amino acid identity. A second OTU (6U2R), also assigned to *Rhizobium* sp., was most associated with AK at 19.4% relative abundance but only 1.5% in OK. In total, more OK than AK NifH sequences exhibited similarities to the Deltaproteobacteria while there appeared to be a somewhat larger diazotrophic population in AK most closely identified to the Verrucomicrobia at 85.5–96.3% amino acid similarity. Overall, OTUs with a relative abundance >1.0% had significantly higher percent identities (92.2 ± 0.5%) to the reference database (*t*-test, $p = 0.01$) than the more rare OTUs (88.8 ± 0.7%).

### NirK

For *nirK*, FrameBot translation and frameshift correction required a decrease in the threshold identity cutoff to the FrameBot reference database to 20%. This was due to the exclusion of *Bradyrhizobium*-like sequences identified through BLASTn using the NCBI non-redundant (nr) database. These sequences were <40% amino acid identity to the FrameBot reference training set that consisted of 77 reference sequences. The nirK reference dataset for best-match analysis using BLASTp was created using the FunGene nirK database set at a minimum score of 220, HMM coverage of 80 and filtered to 381 sequences. At 3% amino acid dissimilarity, a total of 1690 OTUs (828 forward, 862 reverse) that represented 126 unique closest-match taxa were identified (Supplementary Table 2). Among all genes, NirK richness was highest in AK, and differed significantly between the control and warming sites in AK but not OK (**Table 1**). PERMANOVA showed no significant community difference between the organic and mineral layers in AK ($F = 1.13$, $p = 0.317$) as well as between samples originating

**TABLE 1 | Margalef's species richness (J') and Pielou's evenness in Oklahoma (OK) and Alaska (AK) control (C) and artificial warming (T) samples.**

| | Margalef richness (d) | | | | Pielou's evenness (J') | | | |
|---|---|---|---|---|---|---|---|---|
| | **OK-C** | **OK-T** | **AK-C** | **AK-T** | **OK-C** | **OK-T** | **AK-C** | **AK-T** |
| *nifH* | **8.31$^{b}$** | **10.26$^{a}$** | 8.58$^{c}$ | 9.00$^{c}$ | **0.552$^{b}$** | **0.581$^{a}$** | 0.516$^{c}$ | 0.575$^{b}$ |
| *nirS* | **38.59$^{b}$** | **51.73$^{a}$** | 22.83$^{c}$ | 21.36$^{c}$ | **0.759$^{b}$** | **0.848$^{a}$** | 0.729$^{c}$ | 0.709$^{c}$ |
| *nirK* | 53.38$^{ab}$ | 57.44$^{a}$ | **65.68$^{a}$** | **41.82$^{b}$** | 0.968$^{a}$ | 0.966$^{a}$ | 0.932$^{b}$ | 0.924$^{b}$ |
| *nosZ* | 51.41$^{ab}$ | 64.55$^{a}$ | 30.71$^{c}$ | 33.81$^{bc}$ | 0.958$^{ab}$ | 0.967$^{a}$ | 0.935$^{c}$ | 0.945$^{bc}$ |

*Bolded pairs highlight significant differences between control and artificial warming samples. Superscript letters indicate ANOVA grouping. nd, not determined.*

**TABLE 2 | Overview of PERMANOVA and permutational dispersion (PERMDISP) results for the indicated functional gene containing bacterial community addressing warming treatment effects of the Oklahoma (OK) and Alaska (AK) sites and the comparison of AK vs. OK.**

| | Oklahoma | | | Alaska | | | AK-OK | | |
|---|---|---|---|---|---|---|---|---|---|
| | **F** | **P** | **Disp-P** | **F** | **P** | **Disp-P** | **F** | **P** | **Disp-P** |
| *nifH* | 2.00 | 0.209 | 0.06 | 0.652 | 0.629 | 0.67 | **2.56** | **0.001** | 0.37 |
| *nirS* | 0.995 | 0.270 | 0.30 | 0.633 | 0.841 | 0.53 | **8.87** | **0.001** | 0.74 |
| *nirK* | **50.50** | **0.001** | 0.50 | **2.05** | **0.048** | 0.47 | **8.91** | **0.001** | 0.58 |
| *nosZ* | **2.30** | **0.004** | 0.64 | 0.580 | 0.857 | 0.68 | **6.89** | **0.001** | **0.01** |

*Significant comparisons are bolded to highlight.*

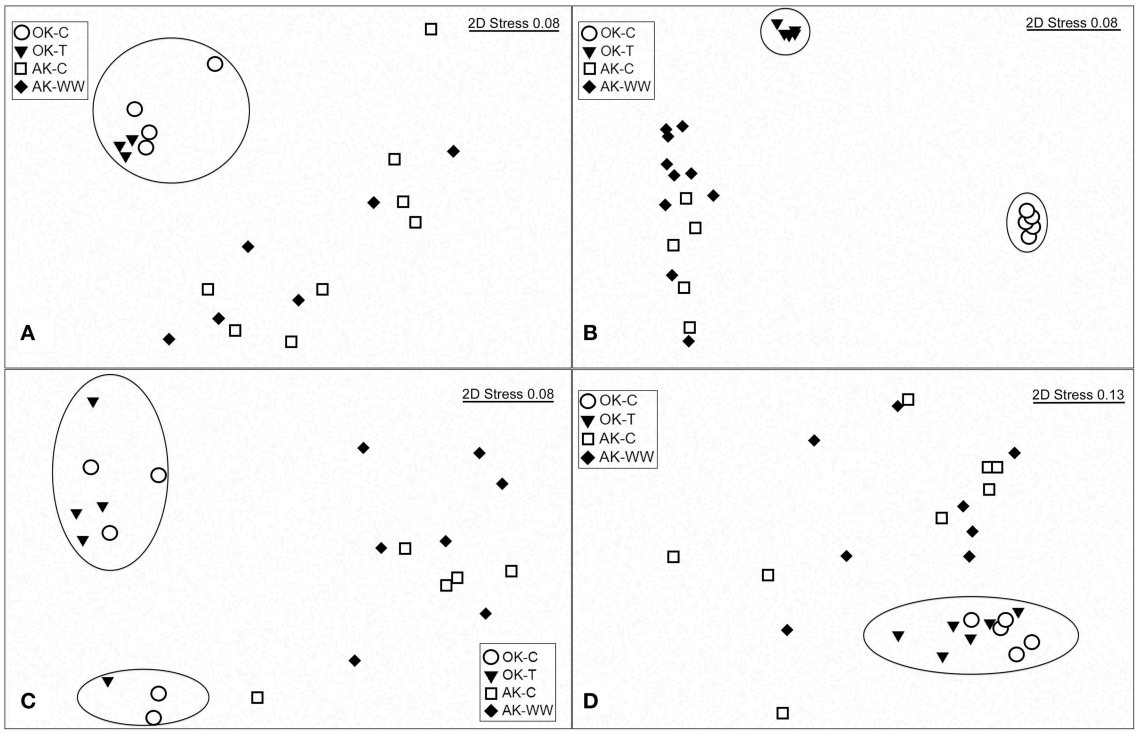


**FIGURE 1 | nMDS ordinations based on Bray-Curtis dissimilarity matrices for (A) NifH, (B) NirK, (C) NirS and (D) NosZ for the Oklahoma control (OK-C) and warming (OK-T) and Alaska control (AK-C) and warming (AK-WW) sites.** For NifH, the two outliers were removed for visualization of site relationships.

from above and below the water table depth ($F = 1.41$, $p = 0.143$).

The overall bacterial *nirK* gene-containing community structure was significantly different with warming in both OK and AK (**Table 2**). NMDS ordination showed a clear distinction in OK, less so in the AK samples (**Figure 1B**). Similarity percentage analysis (SIMPER) for the warming effect revealed that 69 OTUs contributed to 50% of the Bray-Curtis dissimilarity in OK while 28 OTUs contributed in AK. Trees illustrating the significant change in the overall *nirK*-containing bacterial community with warming showed that the difference was mostly due to changes in relative abundance, not presence-absence (**Figures 2**, **3**). In relation to OK warming, there were a few exceptions including OTU BVZU6 (98.1% a.a. identity to *Mesorhizobium amorphae*) at an average of 8.6% (OK-C) and 0.0% (OK-T) relative abundance and OTU A5JSC (distantly related to *Rhodopseudomonas palustris* at 78.3% identity) at 0.0% (OK-C) and 7.4% (OK-T) relative abundance (**Figure 2**). A deep branching clade consisting of 7 OTUs (OTUs JQMFD-JK0V4) in the AK warming comparison (**Figure 3**) exhibited <33% amino acid similarity to a nearest uncultured bacterium in the dataset, indicating a potentially abundant unknown component in these soils.

For AK vs. OK, a significant site influence on total community structure was identified without regard to treatment (**Table 2**) (**Figure 1B**). Average percent identities of the OTU representative sequences to the reference database indicated that the *nirK*-harboring communities unique to AK are largely unknown (36.5 ± 16.3% ID), compared to the higher identifies in OK (81.7 ± 7.3% ID). Within the AK site, 26.2% of sequences in 27 OTUs had no best match in the reference database and thus identity was not reported. In comparison, only one sequence in OK did not match to the database. A total of 148 OTUs accounted for 50% of the Bray-Curtis dissimilarity. In OK, these OTUs exhibited the highest amino acid similarity to *Mesorhizobium*, *Rhodopseudomonas*, *Nitratireductor*, and *Methylocystis*. The low identities in AK present an obstacle to establishing any relationship to a closest database match. Sites were differentiated in a large part due to presence/absence and were grouped in specific clades specific to each site (Supplementary Figure 3).

## *NirS*

For *nirS*, 34.7 and 41.4% of NirS sequences failed FrameBot (length 80, identity = 40) in the OK and AK samples, respectively. This was due to low amino acid identity (21–39%) to the reference database consisting of 210 sequences retrieved from FunGene with a minimum HMM coverage of 80% and minimum score of 500. A subset of the FrameBot failed sequences was subjected to BLASTn against the nr NCBI database. The majority of hits were to non-contiguous *nirS* gene regions and non-target genes. The non-target near-full length hits include those with putative functions to pyruvate dikinases, hypothetical proteins, phosphoenolpyruvate carboxykinases, cytochrome p460, CoA substrate specific enzyme activases and quinolinate synthetases,

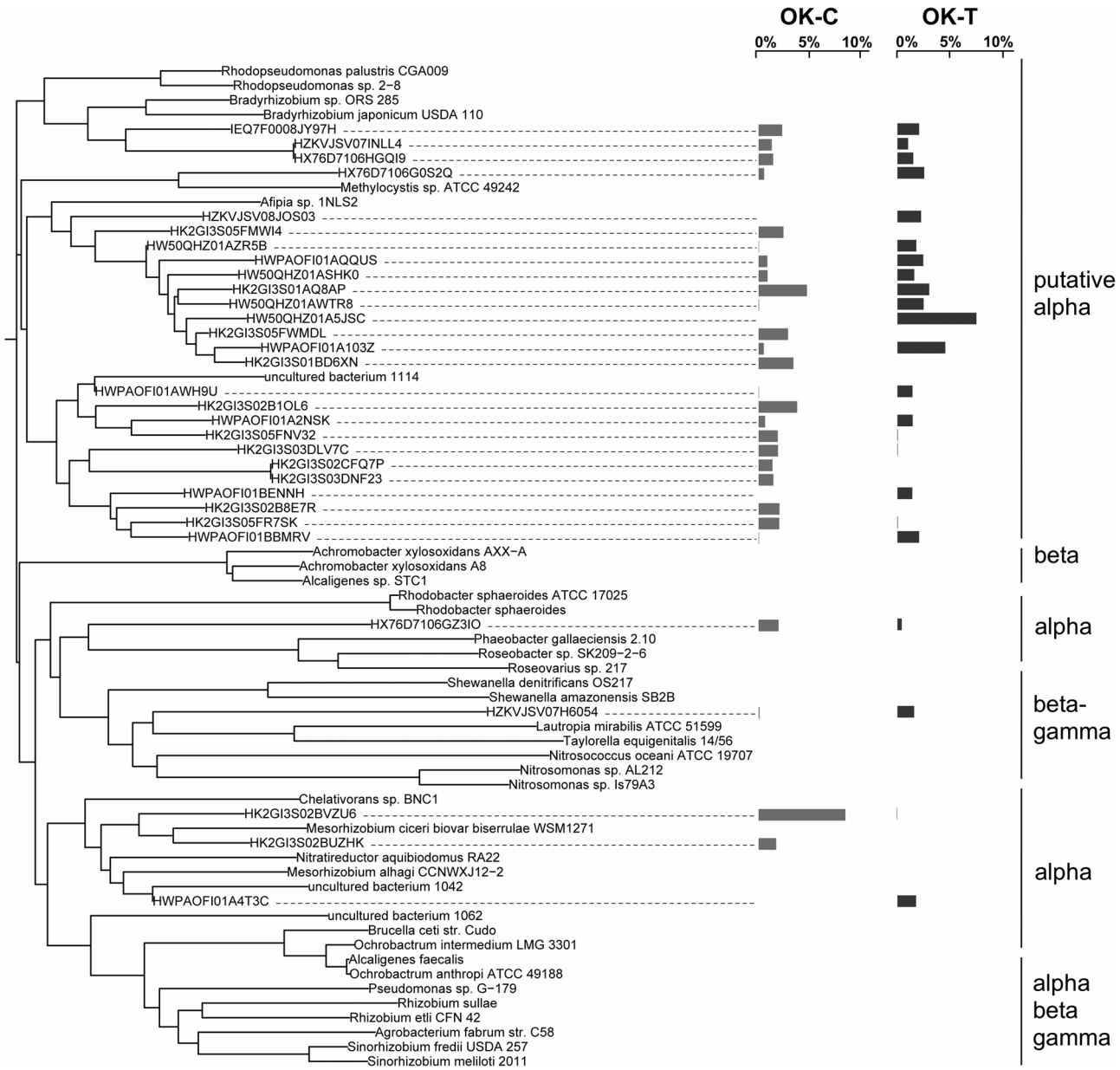


**FIGURE 2 | NirK neighbor-joining tree based on amino acid sequences showing the most abundant 32 OTU representative sequences comparing Oklahoma control (OK-C) to warming (OK-T) samples with reference sequences.** The OTUs shown contain 48.3% of sequences in OK-C and 43.4% of the sequences in OK-T. The average relative abundances are represented by bar graphs to the right of the OTU. The first column corresponds to OK-C, the second to OK-T.

among others. As such, the FrameBot filter was set to 40% amino acid identity and the failed sequences were not considered for random re-sampling.

A total of 1245 OTUs were identified at 5% amino acid dissimilarity from a total of 52,580 passing reads (Supplementary Table 2). *NirS*-harboring bacterial community richness and evenness were significantly higher in the OK warming vs. control samples with no significant change observed in AK. The overall *nirS*-harboring bacterial community structure was not altered significantly with warming in either OK or AK (**Table 2**). PERMANOVA showed no significant community difference between the organic and mineral layers in AK ($F = 0.938$, $p = 0.545$) or with position above or below the water table depth ($F = 1.23$, $p = 0.242$). Comparing sites, OK NirS richness and evenness at OK were significantly higher than those at AK samples and a significant difference in community structure was observed between sites (**Table 1**). Compared to other genes, OK samples were the least tightly grouped but were clearly distinct

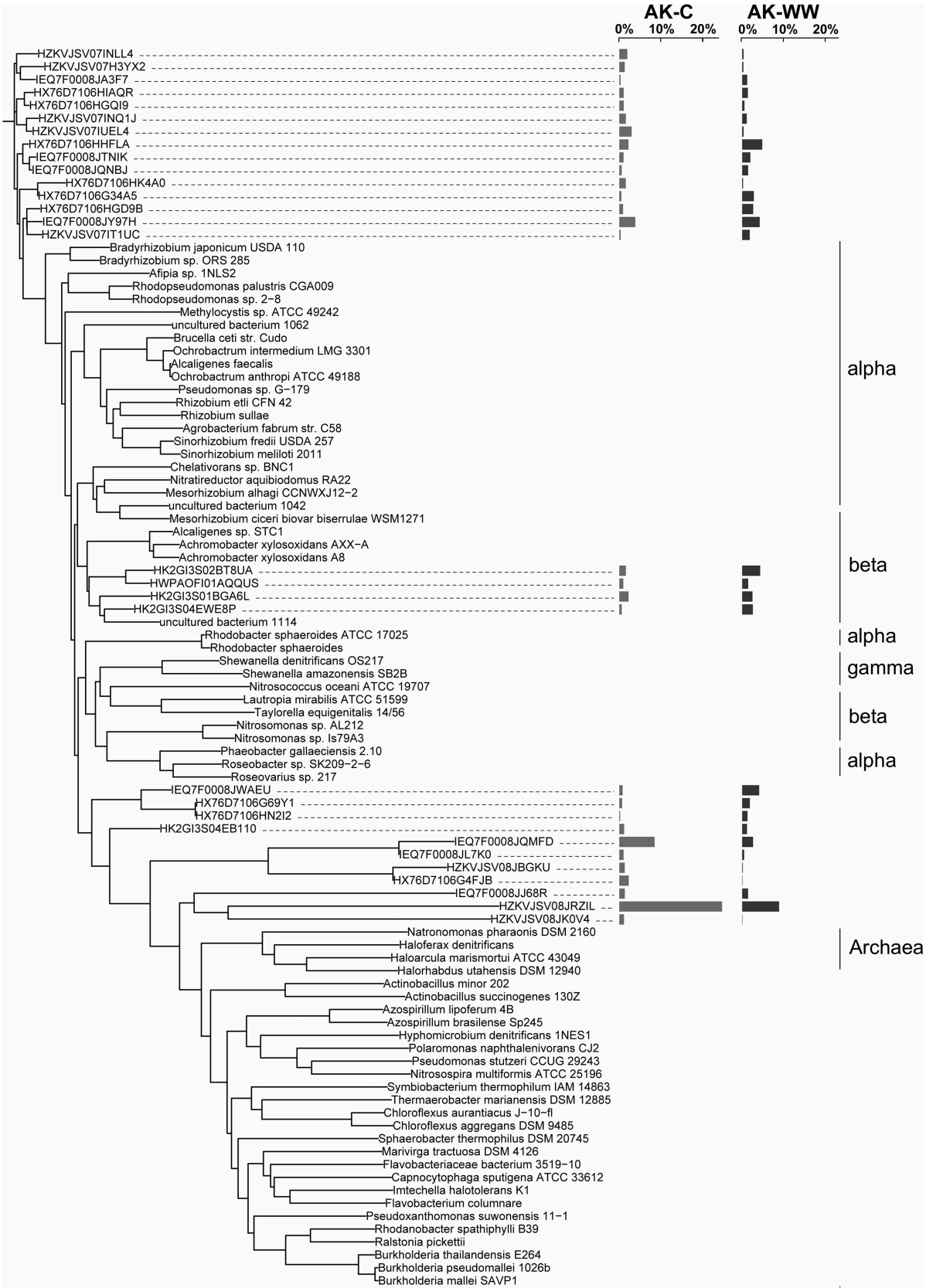


**FIGURE 3 | Continued**

**FIGURE 3 | Continued**
**NirK neighbor-joining tree based on amino acid sequences showing the most abundant 33 OTU representative sequences from Alaska control (AK-C) and warming (AK-WW) with reference sequences.** The OTUs shown contain 78.0% of sequences in AK-C and 72.0% of the sequences in AK-WW. The average relative abundances are represented by bar graphs to the right of the OTU. The first column corresponds to AK-C, the second to AK-WW.

from the AK samples (**Figure 1C**). Average BLASTp identities of OTU representative sequences to the reference database were nearly identical in both OK (76.2 ± 6.3%) and AK (76.0 ± 7.3%). The mostly low percent identities in AK do not allow for a reasonable relationship to the closest reference database match for any particular OTU. Differences in community structure between sites were attributed to changes in relative abundances in the most abundant OTUs (Supplementary Figure 4). OTUs tended to group together with no anchored reference sequence, indicating potentially novel clades.

### *NosZ*

*NosZ* sequences were translated through FrameBot at a minimum length of 80 amino acids at a 40% minimum similarity using a reference dataset of 160 NosZ sequences. A total of 1144 NosZ clusters were identified following clustering at 3% amino acid dissimilarity. The NosZ reference database consisted of 426 amino acid sequences parsed from FunGene with a minimum HMM coverage of 80 and minimum score of 360. A total of 52 unique closest-match bacteria were identified after BLASTp analysis of OTU representative sequences. Although there was a trend of increasing richness and evenness with warming in both OK and AK, these differences were not significant (**Table 1**). Warming significantly affected the overall *nosZ*-harboring bacterial community structure in OK but not in AK. Tree analyses of the most abundant OTUs showed that changes in relative abundances were responsible for the observed community differences (**Figure 4**) with a large, independent clade accounting for the majority of sequences (OTUs 1HJ3–FQWS). PERMANOVA showed no significant community difference between the organic and mineral layers in AK ($F = 1.53$, $p = 0.162$) though a significant difference based on position in relation to water table depth was identified ($F = 3.56, p = 0.002$).

The *nosZ*-harboring communities were different between OK and AK (**Table 2**), regardless of treatment, though a significant difference in dispersion (PERMDISP) may have influenced the PERMANOVA results. OK sites were grouped tightly and independent of the AK sites (**Figure 1D**). Percent amino acid identities to the reference database were low and nearly identical in both OK (53.3 ± 6.0%) and AK (54.0 ± 5.2%), indicating numerous, potentially novel bacteria. As in the OK warming contrast, the difference in *nosZ*-harboring communities between AK and OK were due to changes in relative abundances with large clades distant from reference sequences (Supplementary Figure 5).

## Edaphic Factors and Community Structure

Bulk density (BD), percent nitrogen (%N) and percent carbon (%C) did not differ significantly with the warming treatment in Alaska (ANOVA, $p > 0.05$). However, there was a significant difference between the organic and mineral layers (Supplementary Table 3) with the organic layers having lower BD and higher %N and %C. In the tallgrass prairie (OK) there were no significant differences with warming for moisture, pH, $NO_3^-$, $NH_4^+$, total nitrogen, total carbon or percent organic matter. The PRIMER function RELATE, using all environmental data, showed that within the Alaskan permafrost, *nifH* and *nirK* harboring bacterial community structures were significantly correlated to the environmental matrices containing bulk density while *nosZ* was marginally correlated to percent carbon (**Table 3**). In the AK samples, bulk density was the highest contributor to BEST Spearman rank correlations for most genes. However, correlation was only substantial for NirK (BD, $\rho = 0.205$, $p = 0.05$). NosZ was again best correlated to %C, though not significantly ($\rho = 0.213$, $p = 0.15$). In the OK samples only NifH had a significant correlation to pH and total nitrogen (TN) combined ($\rho = 0.723$, $p = 0.05$) whereas NirS was correlated to the combination of $NH_4^+$, TN, and total carbon (TC) ($\rho = 0.593$, $p = 0.05$).

# Discussion

## Warming Effects on Diversity and Structure

We examined the diversity and composition of denitrifying and nitrogen-fixing microbial communities under experimental warming in Alaskan permafrost and a tallgrass prairie after 1 and 10 years of warming, respectively. Experimental warming in the Oklahoma tallgrass prairie surface soils (0 to −10 cm), where warming was initiated over a decade ago, resulted in significantly higher richness and evenness of the *nifH* and *nirS* harboring communities and significant shifts in the overall *nirK* and *nosZ* community structures (**Tables 1, 2**). This increase in richness differs from the results of Sheik et al. (2011) where decreased overall microbial community diversity was associated with warming at the same tallgrass prairie site (under normal precipitation) after 4 years of warming. In addition, fungal communities were found not to differ significantly with warming after a decade of warming (Penton et al., 2013b). However, GeoChip (Zhou et al., 2012) and shotgun metagenomic analyses (Luo et al., 2014) at these identical sites after 10 years of warming detected significant phylogenetic shifts, differences in functional gene richness and diversity and enrichment of metabolic pathways involved in N cycling and $CO_2$ production.

After only 1 year of warming in Alaska, only the *nirK* harboring community was significantly altered with a corresponding decrease in richness (**Tables 1**, **2**). In agreement with our overall trends within the permafrost samples, lower *nirK* and *nirS* and higher *nosZ* diversity have been identified in cryoturbated peat vs. unturbated peat soils (Palmer and Horn, 2012). It has been proposed that *nirS* (heme) bearing denitrifiers are better adapted to waterlogged soils (Kim et al.,

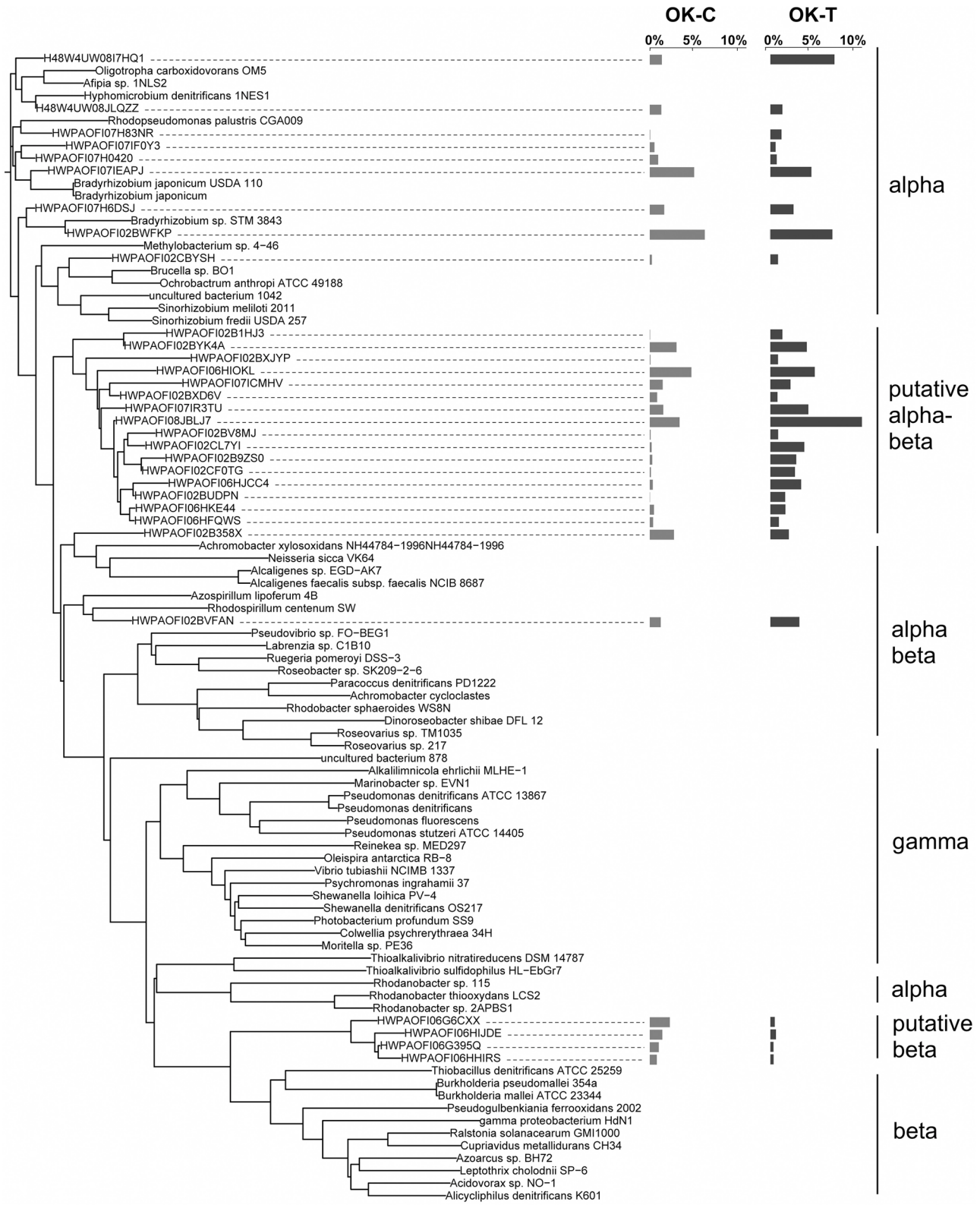


**FIGURE 4 | NosZ neighbor-joining tree based on amino acid sequences showing the most abundant 31 OTU representative sequences from Oklahoma control (OK-C) and warming (OK-T) with reference sequences.** Tree is based on forward reads only. The OTUs shown contain 41.6% of sequences in OK-C and 86.9% of the sequences in OK-T. The average relative abundances are represented by bar graphs to the right of the OTU. The first column corresponds to OK, the second to AK.

**TABLE 3 | RELATE statistics (rho and *p*-values) from comparing resemblance matrices between each gene and environmental variables in the tallgrass prairie (OK) and permafrost samples (AK).**

| | | OK | | | | | | | AK | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | **Moisture** | **pH** | **$NO_3^-$** | **$NH_4^+$** | **TN** | **TC** | **OM** | **BD** | **%N** | **%C** |
| NifH | rho (ρ) | −0.13 | 0.30 | 0.26 | 0.13 | **0.54** | 0.49 | 0.16 | **0.24** | 0.05 | 0.17 |
| | P | 0.72 | 0.17 | 0.17 | 0.26 | **0.03** | 0.43 | 0.24 | **0.04** | 0.30 | 0.11 |
| NirK | rho (ρ) | −0.06 | 0.05 | 0.03 | 0.10 | 0.04 | −0.06 | −0.06 | **0.21** | 0.07 | 0.13 |
| | P | 0.66 | 0.34 | 0.37 | 0.23 | 0.40 | 0.64 | 0.71 | **0.04** | 0.21 | 0.11 |
| NirS | rho (ρ) | 0.08 | 0.54 | 0.17 | **0.43** | 0.04 | 0.26 | 0.25 | −0.13 | 0.13 | −0.04 |
| | P | 0.26 | 0.07 | 0.81 | **0.01** | 0.32 | 0.08 | 0.10 | 0.78 | 0.13 | 0.61 |
| NosZ | rho (ρ) | 0.06 | 0.19 | 0.12 | −0.02 | −0.22 | 0.05 | 0.05 | 0.09 | 0.11 | 0.21 |
| | P | 0.35 | 0.19 | 0.26 | 0.49 | 0.88 | 0.37 | 0.39 | 0.25 | 0.16 | 0.07 |

*P-values less than 0.10 are bolded. TN, total nitrogen; TC, total carbon; OM, organic matter; BD, bulk density.*

2008; Petersen et al., 2012) while *nirK* (Cu) denitrifiers appear to be more associated with drier soils (Dandie et al., 2008; Bremer et al., 2009). While *nirK* was the most impacted by warming, the question remains as to whether they are the dominant denitrifiers in these soils and thus community changes would more likely be reflected through the changes in process rates.

Thus, overall, the *nirK*-containing denitrifiers were the most sensitive to warming, with significant community shifts at both sites. Linking these changes in diversity or functional community structure to process-level outcomes is difficult across systems, though correlations between qPCR quantities of genes for nitrite reduction (*nirK*) and $N_2O$ reduction (*nosZ*) and potential denitrification rates was observed across Alaskan ecosystem types (Petersen et al., 2012). In support of our findings in Alaska, *nirK* was found to be the most sensitive N cycling gene to warming and N addition, with increased abundances with warming in Antarctic soils (Jung et al., 2011).

## Differences among Sites

As a proxy for contrasting soil physicochemical properties, climate and other factors (e.g., different plant chemical and physical characteristics, soil micro- macrofauna), the Oklahoma tallgrass prairie and Alaskan permafrost soils harbored distinctly different N-gene-harboring bacterial communities, regardless of warming treatment (both control and warming were combined for these comparisons) or length of treatment. In general there was lower gene richness in the permafrost, perhaps due to the selective pressures of low temperatures and anaerobiosis. With our targeted sequencing approach we found that presence-absence contributed more to the significant denitrifying community differences between these two sites, as compared to the observed within-site differences that were driven by differences in relative abundances. However, there were instances where major populations were shared among sites, possibly indicative of generalists that are viable in both locations. Other studies targeting denitrification genes have shown that few genotypes are shared between disparate locations (Braker et al., 2000, 2001; Scala and Kerkhof, 2000).

The tallgrass prairie diazotrophic (N-fixing) communities exhibited the lowest within-site variability of any N cycling genes. This could perhaps be due to less robust differences in small-scale soil and plant cover heterogeneity, some of the multiple factors that have been found to influence diazotrophic communities. These tend to be habitat-specific and related to plant cover, soil texture and chemistry, which in turn influence community composition across scales (Zehr et al., 1998; Shaffer et al., 2000; Poly et al., 2001). Conversely, the large within-site variability observed in the permafrost samples is likely due to the range of sampled depths and thus additional influences such as water availability, redox status and past spatiotemporal isolation (due to the frozen state in the deeper samples).

## Sequence Divergence from Known Functional Genes

Our current understanding of functional gene diversity is largely limited by constraints on current primer coverage (Penton et al., 2013a). In most cases this artificially limits the retrieved diversity of the N-gene-harboring microbial community. As an example, until recently and due to the lack of coverage of typical *nosZ* primers, an important atypical *nosZ* clade that potentially mediates soil $N_2O$ sink capacity was left undiscovered (Sanford et al., 2012; Jones et al., 2013, 2014). Additionally, increased recovery of more diverse (or even non-target) sequences is due to deeper sequencing projects that reveal the rare, but targeted taxa and shotgun metagenomics that results in a smaller number, but potentially more diverse sets of target genes. This is supported in that, for all N functional genes except NirK in Alaska, OTUs with a relative abundance >1.0% had significantly higher percent identities (92.2 ± 0.5%) to the reference database (*t*-test, $p = 0.01$) than the more rare OTUs (88.8 ± 0.7%).

While other genes shared similar OTU identities to their respective databases in both AK and OK, NirK identities were markedly lower in the permafrost at an average 36.5% amino acid identity. Also, 26.2% of AK NirK sequences could not be matched to the reference database that contained only known NirK proteins. The high divergence of *nirK* compared explicitly to *nirS* was first shown using antibodies against $cd_1$ *nirS* and Cu *nirK*. It was postulated that *nirK* originated earlier than *nirS*,

with the resulting variation from a higher cumulative divergence (Coyne et al., 1989). The unmatched sequences are possibly due to (a) the presence of novel sequences not yet identified through metagenomic or targeted sequencing (b) reference database coverage limitation, or (c) mis-priming leading to the capture of non-nirK sequences. Mis-priming is unlikely as the permafrost samples were amplified in concert with the tallgrass prairie samples whose sequences averaged 81.7% identity to the reference database. To further investigate we BLAST'd the reference protein sequences against the NCBI reference databases. We found that, in some cases, sequences more closely matched putative environmental nirK sequences with as high as 79% identity (e.g., nirK sequences obtained from soils in Finland, Braker et al., 2012). When compared against an archaeal nirK variant (54d9) (Treusch et al., 2005), alignment showed a maximum 29% identity to our representative sequences. As with our sequences, the Treusch archaeal nirK variant also exhibited low identity (25–32%) to reference nirK sequences. Overall, this indicates the likely presence of a poorly-understood *nirK* harboring community in the permafrost samples, possibly from spatial and temporal isolation due to the frozen state maintained from the Pleistocene era.

Methodology is a central issue to many molecular microbial ecology studies as all current methods have their limitations. Primer based studies like this one have their advantages (sensitivity and a focus on functions of interest) but also disadvantages (primer bias and representative sampling, Zhou et al., 2011, 2013). In this study we employed widely used N-cycle primers, but with the expanding sequence database, it is apparent that primer coverage of diversity is limiting (Penton et al., 2013a). However, by keeping conditions constant, comparisons among treatments or conditions can be made reflecting information for the sampled group though it is not comprehensive for the guild. It is time though for the development of new, broader coverage primers for both sequencing and quantitation. Ideally, qPCR primer design should target amplification at the clade level to enable a higher resolution analysis of microbial functional groups. This would allow for response comparisons among specific clades, thus providing data regarding the sensitivity of sub-groups to experimental warming.

In conclusion, our study demonstrated that a decade of warming in a tallgrass prairie resulted in significant shifts in *nirK* and *nosZ* harboring bacterial community structures as well as significantly higher richness and evenness of the diazotrophic and *nirS* harboring communities. As might be expected the 1 year of permafrost soil warming and with colder temperatures limiting growth rates, there was not much shift in N-processing communities. The fact that the *nirK* harboring permafrost community was significantly impacted with an accompanying decrease in richness is interesting, and perhaps relates to the very novel sequences and by inference novel populations that may be adapted to lower temperature responses. It is also notable that across sites, the *nirK* harboring communities were the most impacted by warming. As these sites are part of long-term studies, it will be important to compare the types and rates of response after equal time periods, and if these can be related to process changes. As such, the AK information here provides a beginning reference for such a long-term comparison.

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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.2015.00746

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# Microbial food web components, bulk metabolism, and single-cell physiology of piconeuston in surface microlayers of high-altitude lakes

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Sharp boundaries in the physical environment are usually associated with abrupt shifts in organism abundance, activity, and diversity. Aquatic surface microlayers (SML) form a steep gradient between two contrasted environments, the atmosphere and surface waters, where they regulate the gas exchange between both environments. They usually harbor an abundant and active microbial life: the neuston. Few ecosystems are subjected to such a high UVR regime as high altitude lakes during summer. Here, we measured bulk estimates of heterotrophic activity, community structure and single-cell physiological properties by flow cytometry in 19 high-altitude remote Pyrenean lakes and compared the biological processes in the SML with those in the underlying surface waters. Phototrophic picoplankton (PPP) populations, were generally present in high abundances and in those lakes containing PPP populations with phycoerythrin (PE), total PPP abundance was higher at the SML. Heterotrophic nanoflagellates (HNF) were also more abundant in the SML. Bacteria in the SML had lower leucine incorporation rates, lower percentages of "live" cells, and higher numbers of highly-respiring cells, likely resulting in a lower growth efficiency. No simple and direct linear relationships could be found between microbial abundances or activities and environmental variables, but factor analysis revealed that, despite their physical proximity, microbial life in SML and underlying waters was governed by different and independent processes. Overall, we demonstrate that piconeuston in high altitude lakes has specific features different from those of the picoplankton, and that they are highly affected by potential stressful environmental factors, such as high UVR radiation.

**Keywords: picoplankton, leucine incorporation, bacterioplankton, flagellates, flow cytometry, NADS, CTC, ultraviolet radiation**

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## Introduction

The upper micrometers of the water column of aquatic ecosystems form a substantially different environment than that of the underlying surface water, despite its close proximity as reviewed by

Cunliffe et al. (2011). The group of organisms living at the water-air interface, the surface microlayer (SML), was named neuston (Naumann, 1917), and the smallest organisms (<2 μm) living there can be defined as piconeuston. The SML is the boundary between two totally different environments and the site where fundamental biological and chemical processes, such as gas exchanges between atmosphere and water occur (e.g., Conrad and Seiler, 1988). The SML is known to concentrate substances, which may enhance or inhibit microbial activities. Previous studies showed that the marine SML was enriched with amino acids and bacteria and, in most instances, virus-like particles (Kuznetsova et al., 2004), and that heterotrophic piconeuston had enhanced extracellular enzymatic peptide hydrolysis rates (Kuznetsova and Lee, 2001), compared to subsurface waters.

However, and at least in marine/estuarine environments, early reports indicated that heterotrophic piconeuston has lower metabolic activity than surface picoplankton, (Dietz et al., 1976). This difference in metabolism was explained as a community response to different environmental stress factors occurring preferentially in the SML, such as high incident solar UVR, and more variable water temperature and salinity, as well as potentially higher concentrations of toxic substances (Maki, 1993). In aquatic environments naturally subjected to high UVR doses, such as high altitude lakes in summer, a difference in activity should therefore be expected.

The differences in metabolism, however, might also be attributed to differences in community compositions between the SML and the surface. Auguet and Casamayor (2008), using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) and 16S rRNA gene sequence analysis, reported a higher abundance of archaeal communities in the SML of Pyrenean oligotrophic high-mountain lakes as compared to surface communities. These Archaea populations were composed mainly of Crenarchaeota, whereas surface populations were mainly comprised of Euryarchaeota. Similarly, Vila-Costa et al. (2014) found distinctive populations of both archaea and bacteria inhabiting SML and surface waters of the same Pyrenean lakes using 454 pyrosequencing, and the differences were exacerbated under atmospheric loadings that stimulated microbial activities. A less clear pattern was observed in a set of six Alpine lakes located across an altitude gradient (Hörtnagl et al., 2010), where Betaproteobacteria (enumerated by CARD-FISH) dominated in both SML and underlying water, and the differences observed among lakes were attributed to lake-specific intrinsic factors.

Living in the SML is rather challenging, mainly due to the harsh prevailing conditions resulting from summer extreme UVR levels (Sommaruga, 2001). Previous reports indicating that UVR negatively affects bacterial activity (i.e., Ruiz-González et al., 2013), HNF growth, and bacterial consumption rates (Sommaruga et al., 1999) suggest that microorganisms living in the neuston should experience heavy environmental stress. Independently of the peculiarity of SML's prokaryotic taxonomic composition described in the studies cited above, there is little information available on the *in situ* microbial food web structure (i.e., both abundance and composition of heterotrophic prokaryotes, phototrophic picoplankton [PPP], and heterotrophic nanoflagellates [HNF]) and of bacterial single-cell activity and physiology, which could illustrate the ecological processes shaping life in the SML.

The aim of this study was to study microbial community structure, metabolism, and physiology of piconeuston of SML compared to underlying water in high mountain lakes. Our working hypothesis is that microbial communities living in the SML of high altitude lakes are subjected to environmental harshness that affects their composition, community structure, activity, and physiology in a different way than that of surface waters communities. In order to achieve this goal, we carried out a comprehensive flow-cytometry measurement of (i) microbial community structure, (ii) prokaryotic bulk and single-cell activity, and (iii) physiological status in 19 remote high altitude lakes sampled under summer high solar radiation conditions, in order to determine the variability of these parameters in the SML as compared to surface waters. To the best of our knowledge, most of the variables studied, such as detailed microbial community structure by flow cytometry and bacterial single-cell activity, had never before been measured in the SML.

## Materials and Methods

### Sampling Sites and Limnological Parameters

A set of 19 high mountain lakes from the Central Pyrenees were sampled from 17th to 24th, June 2008 at 3 depths: in the first ~400 μm of the water column, here defined as the SML; at 0.5 m depth—which we label as "surface"; and at the depth equivalent to 1.5-fold Secchi disk value, usually corresponding to the depth of the summer deep chlorophyll maximum (DCM) (Catalan et al., 2006), which ranged from 2 to 30 m depth, depending on the lake. In this report the DCM values of Chlorophyll-*a* (Chla) were only used to characterize the lakes according to their nutrient and trophic status. In these clear water mountain lakes Chla at the surface does not reflect the trophic status of the lake because most primary production is located at the DCM (Catalan et al., 2006). The lakes were selected in order to maximize variability in chemical and morphological characteristics and were accessed by foot as they are located in uninhabited remote locations.

SML samples were collected from the upper ~400 μm water with a nylon screen sampler (Agogué et al., 2004, 2005) near the deepest point of each lake. Surface (0.5 m depth) and deeper samples were taken using a 3-litre sampler (either Ruttner or Patalas bottles). Samples were pre-screened through a 40 μm pore-size net to remove large plankton components.

Water transparency was measured with a Secchi disk. Temperature profiles were measured *in situ* using a PT10 type thermistor. In the lab, pH was measured with an Orion instrument equipped with a probe for low ionic strength samples (Crison). Conductivity was measured with conductivimeter of the brand WTW. Total phosphorus (TP) was extracted with an oxidative persulfate digestion, and analyzed by spectrophotometry using the malachite green method (Camarero, 1994). Total nitrogen (TN) was extracted with an oxidative persulfate digestion in the autoclave, and measured by UV spectrometry. Dissolved organic carbon (DOC) was assessed on Whatman GF/F filtered lake-water with a Shimadzu TOC5000 analyzer. Chla concentrations were measured in 90% acetone extracts (1 L sample filtered on Whatman GF/F filter and

extracted by 3 min sonication in 4 mL 90% acetone) according to Jeffrey and Humphrey (1975).

## Abundances of Microbial Food Web Components by Flow Cytometry

### Sample Collection

Microbial food web components (cell abundance of heterotrophic prokaryotes, PPP, and HNF) were quantified by flow cytometry. Three subsamples were taken for separate counts of heterotrophic prokaryotes, PPP, and heterotrophic flagellates. The last two samples were kept in water baths with *in situ* water and analyzed as soon as possible (3 h on average after collection). For heterotrophic prokaryotes counts, 4 ml of lake water were collected from each depth, fixed immediately with cold glutaraldehyde 10% (final concentration 1%), left in the dark for 10 min at *in situ* temperature, placed in liquid nitrogen and then stored at −80°C. Samples were analyzed 2 months after sampling. An aliquot of these frozen samples were used to quantify PPP and results were compared to the fresh analysis done *in situ* (see below).

### Heterotrophic Prokaryote Abundances

Stored samples were thawed at room temperature and 400 μl were mixed with DMSO-diluted SYTO-13 (Molecular Probes Inc., Eugene, OR, USA) at 2.5 μmol $l^{-1}$ final concentration. The mixture was left for about 10 min in the dark for complete staining and was run in a FACSCalibur (BectonDickinson) flow cytometer equipped with a 15 mW Argon-ion laser (488 nm emission). At least 30,000 events were acquired for each subsample. Heterotrophic prokaryotes were detected by their signature in a plot of side scatter (SSC) vs. FL1 (green fluorescence) following del Giorgio et al. (1996) as discussed in Gasol and del Giorgio (2000).

### Phototrophic Plankton Abundances

The fixed samples were thawed and run without addition of any stain in a FACSCalibur flow cytometer equipped with a blue laser (488 nm, 15 mW) and a red laser diode (∼635 nm). Small algae were identified in plots of SSC vs. FL3 (red fluorescence of Chla), FL2 (orange fluorescence of phycoerithrine, PE) vs FL3 (Olson et al., 1993), and FL3 vs. FL4 (far red fluorescence after red-light excitation, indicative of phycocyanine, PC). Samples were run twice with different settings (different voltages for scatter and fluorescence), in order to maximize the "sampling window" and cover cells sizes below 1 μm to ca. 20 μm, thus including pico- and nanophototrophs. Data analysis was performed with the CellQuest software (BectonDickinson), and we combined the information collected with the two settings into one set of populations. Examples of cytograms with distinct populations are presented in Schiaffino et al. (2013) and Sarmento et al. (2008). While the data presented in this study corresponds to samples analyzed a few months after collection and from fixed replicates, we also analyzed fresh samples the same day of collection, ∼3 h after sampling. Since we did not have the red laser for these live samples, the numbers presented are those of the fixed sampled. The fresh samples were used to confirm that no populations were negatively affected by fixation and storage.

### Small Heterotrophic Eukaryotes Abundances

In this study, "small heterotrophic eukaryotes" included pico and nano eukaryotes, and refers mainly to small HNF. Small eukaryote abundances were estimated following the protocol by Rose et al. (2004). From a stock solution of 1 mM Lysotracker Green (Molecular Probes), 1 μl was added to 99 μl of <0.2 μm MilliQ, and 3.8 μl of this diluted Lysotracker were added to 0.5 ml of the sample, ending at 75 nM Lysotracker final concentration. We analyzed the samples as in Rose et al. (2004), using a combination of light scatter (SSC) and green (FL1) and red (FL3) fluorescence. Samples were run alive, less than 3 h after sample collection, and at high (ca. 100 μl $min^{-1}$) speed. Concentrations were obtained from weight measurement of the volume analyzed.

## Bacterial Bulk and Single-Cell Activity

Bacterial bulk heterotrophic activity was estimated using the $^{3}$H-leucine incorporation method (Kirchman et al., 1985). Quadruplicate aliquots of 1.2 ml and 2 trichloroacetic acid (TCA) killed controls were taken immediately after sample collection. The samples were incubated with 40 nM $^{3}$H-leucine final concentration added immediately and for about 2 h in the dark in a water bath with lake water and at *in situ* temperature. The incorporation was stopped with the addition of 120 μl of cold TCA 50% to each replicate and the samples were kept frozen at −20°C until processing, which was carried out by the centrifugation method described by Smith and Azam (1992).

We used two physiological probes to test the metabolic and physiological single-cell status of prokaryotic microbes, respectively (i.e., Del Giorgio and Gasol, 2008). The abundance of respiring bacteria was determined using the probe 5-cyano-2,3-ditolyl tetrazolium chloride (CTC, Polysciences), an indicator of electron transport system respiratory activity. Highly respiring prokaryotes were considered those able to reduce CTC. Reduced CTC turns into a red fluorescent formazan that is detectable by epifluorescence and flow cytometry (Sherr et al., 1999; Sieracki et al., 1999). A stock solution of 50 mmol $L^{-1}$ CTC (Polysciences) was prepared daily, filtered through 0.1 μm, and kept in the dark at 4°C until use. The CTC stock solution was then added to 0.45 mL of sample (5 mmol $L^{-1}$ final CTC concentration) and incubated for 3 h at room temperature in the dark. The red fluorescence of CTC (FL3) and SSC were used to discriminate the CTC positive cells from other particles and an FL2-FL3 plot to exclude picoautotrophs. The percentage of CTC positive cells was calculated relative to the total bacterial counts obtained as mentioned above.

Cells with intact membranes were enumerated using the Nucleic-Acid-Double-Staining (NADS) viability protocol, which uses a combination of the cell-permanent nucleic acid strain SybrGreen I (SG1, Molecular Probes, Eugene, OR) and the cell-impermeant propidium iodine (PI, Sigma Chemical Co.) fluorescent probe. We used a 1:10 SG1 and 10 μg $ml^{-1}$ PI concentrations that were added to fresh samples, less than 2 h after sampling. After simultaneous addition of each stain, the samples were incubated for 20 min in the dark at room temperature and then analyzed by flow cytometry. SG1 and PI fluorescence were detected in the green (FL1) and orange-red (FL3) cytometric channels, respectively. A dot plot of red vs.

green fluorescence allowed distinction of the "live" cells (i.e., cells with intact membranes and DNA present) from the "dead" cells (i.e., with compromised membranes, (Grégori et al., 2001; Falcioni et al., 2008).

### Statistical Analyses

We computed a neuston enrichment factor as the ratio between abundance or activity in the SML divided by values measured at the surface to quantify the magnitude of the differences between layers. We tested for differences between layers using paired Student's *t*-tests. In order to explore possible relationships between environmental variables and microbial abundance or activity obtained for the different lakes and lake layers, we run a Factor Analysis (FA) using SPSS 20 Statistics and the stats package in R software (R Core Team, 2014). We separated physical and chemical variables and performed two different FA for each set of environmental variables. We included lake and catchment areas, altitude, maximum depth, surface temperature, and Secchi depth as physical variables. As for chemical variables, we included alkalinity, conductivity, DOC, total dissolved phosphorus (TDP), $NH_4^+$, $NO_2^-$, $NO_3^-$, $SO_4^{2-}$, $Ca^{2+}$-dissolved reactive silica (DRSi), Chla. We had measured other parameters (pH, soluble reactive phosphorus, total dissolved nitrogen, DIC, $Cl^-$, $Na^+$, $K^+$, $Mg^{2+}$) that were excluded from the FA because they were highly correlated or were a lineal combination of some of the parameters included and violated the requirement of uniqueness. All variables were previously log transformed to normalize their distribution. The Kolmogorov–Smirnoff test was applied to check that there were no strong departures from normality. The abundance or activity variables at the surface and the SML, and the enrichment factor were tested for correlation against the scores obtained for the physical and chemical factors.

## Results

### *In situ* Physical and Chemical Conditions

The formation of a true SML requires stable conditions of wind and temperature for a certain amount of time. We sampled 19 lakes in a window of 9 days in which stable and sunny conditions occurred in the mountain. In order to detect the presence of a true SML, we first scanned our database, comparing temperature in the first millimeters of surface water (temperature profiles in Supplementary Material). We considered that there was no true SML in lakes in which water temperature was equal (at the 0.01°C resolution, the smallest reliable according to the resolution of the temperature probe used) in the lake skin and few millimeters below the surface, and we discarded these lakes from the analysis (indicated with "∗" in **Table 1**), by considering that a true SML did not exist in those lakes. From here on, we refer only to the results obtained in the 16 selected lakes, which developed a true SML at the moment they were sampled.

Most of the lakes sampled were located at >2000 m above sea level and can be considered as high altitude lakes. This set of lakes includes a large range of maximal depths (from 5 to 113 m) and geochemical types. As an example, the pH among the 16 lakes ranged from 4.6 to 9.2, and conductivity ranged from 6 to 133 $\mu S\ cm^{-1}$ (**Table 1**). All the lakes were oligotrophic except Muntanyó d'Àrreu, which was mesotrophic (**Table 1**). Average daily-integrated long-wave solar radiation during the sampling period (17th–24th July 2008) was 26.4 ± 8.3 MJ $m^{-2}$. The UV doses corresponding to this radiation were 1.4 ± 0.4 kJ $m^{-2}$ at 305 nm, 8.2 ± 2.5 kJ $m^{-2}$ at 320 nm, 15.3 ± 4.5 kJ $m^{-2}$ at 340 nm, and 20.7 ± 6.3 kJ $m^{-2}$ at 380 nm.

Chla (measured at the DCM) ranged from 0.2 $\mu g\ l^{-1}$ in Lake Certascan to 12.0 $\mu g\ l^{-1}$ in Lake Muntanyó d'Àrreu. Accordingly, water transparency ranged between 1.3 and 19.6 m of Secchi disk depth. Chla was correlated to total phosphorus ($n = 17$, $p < 0.001$, $r^2 = 0.70$) and nitrogen ($n = 17$, $p < 0.01$, $r^2 = 0.58$). Dissolved organic carbon (DOC) was relatively low, ranging from 0.2 to 1.4 mg $l^{-1}$ in Lake Certascan and Lake Plan, respectively.

### Abundances of Microbial Food Web Components

#### Phototrophic Components

Flow cytometry-determined abundances of PPP ranged two orders of magnitude from $2.5 \times 10^2$ to $1.8 \times 10^4$ $ml^{-1}$, with slightly higher average abundances in the SML than in the surface waters, although not significant (**Table 2**). We observed a high diversity of populations in the cytograms obtained. The number of identified populations was on average 4.1 (range 1–7). In general we identified higher diversity of small phototrophic eukaryotes (red-fluorescing cells) than of phycoerythrin (PE)-rich (picocyanobacteria). None of the considered lakes had red-fluorescing (phycocyanin-rich) cyanobacteria. While all lakes had small phototrophic eukaryotes, some did not have cyanobacteria. In lakes with no populations of cyanobacteria, total PPP abundances were higher in the surface than in the SML. Lakes harboring PE-rich populations had higher total PPP abundance in the SML. Moreover, PE-rich (*Synechococcus*-like) cells were more abundant in the SML than in the surface, in those lakes where such populations were present (**Figure 1**). In most lakes, the number of different populations observed by flow cytometry (cytometric diversity) was higher at the surface than in the SML (**Table 2**). In a general comparison, the diversity of populations was higher at the surface but abundances were higher at the SML, although most comparisons were not significant or at the significance borderline (**Table 2**).

#### Heterotrophic Components

We observed significantly more heterotrophic prokaryotes and HNF in the SML than in surface waters (**Table 2**, **Figure 2D**). The total heterotrophic prokaryote:HNF ratio was relatively low (∼480–490 bacteria per HNF, **Table 2**), and no significant differences were found between layers.

### Bulk and Single-Cell Prokaryote Heterotrophic Activities

We measured several indices of bacterial activity, some at the bulk level, such as leucine incorporation, and others at the single-cell level, such as abundance of bacterial cells presenting high levels of respiration (i.e., CTC positive). We used plots of surface against SML samples for the different parameters to discriminate

**TABLE 1 | Location, general features, and some limnological parameters of the 21 samples from Pyrenean high-altitude lakes (DOC, dissolved organic carbon; TP, total phosphorous; TN, total nitrogen).**

| Lake | Latitude | Longitude | Altitude (m asl) | Max. depth (m) | pH units | Conductivity (mS cm$^{-1}$) | DOC (mg l$^{-1}$) | TP (nM) | TN ($\mu$M) | Chl *a* ($\mu$g l$^{-1}$) | Secchi depth (m) |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Aixeus | 42°36′40″ | 1°22′18″ | 2370 | 15.5 | 4.97 | 49.9 | 0.2 | 74 | 18 | 0.6 | 15.5 |
| *Bassa de les Granotes | 42°34′24″ | 0°58′16″ | 2330 | 5 | 6.45 | 9.8 | 2.3 | 119 | 26 | 4.6 | 2.4 |
| Botornàs | 42°35′35″ | 0°40′52″ | 2340 | 22 | 7.23 | 24.1 | 0.3 | 168 | 26 | 2.0 | 12.4 |
| Certascan | 42°42′40″ | 1°18′12″ | 2335 | 113 | 5.67 | 9.1 | 0.2 | 78 | 35 | 0.2 | 19.6 |
| Filià | 42°27′4″ | 0°57′12″ | 2140 | 5.5 | 7.79 | 133.3 | not available | 94 | 21 | 1.7 | 1.3 |
| Gerber | 42°37′50″ | 0°59′41″ | 2170 | 63 | 7.13 | 23.4 | 0.6 | 88 | 9 | 1.3 | 11.8 |
| Ibonet Perramó | 42°38′34″ | 0°29′49″ | 2293 | 5 | 7.49 | 33.3 | 0.4 | 150 | 17 | 0.6 | 5.2 |
| Illa | 42°37′6″ | 0°59′37″ | 2452 | 18 | 6.68 | 13.3 | 0.4 | 70 | 7 | 1.3 | 12.0 |
| Llauset | 42°39′22″ | 0°41′11″ | 2190 | 90 | 7.52 | 58.7 | not available | 113 | 18 | 1.1 | 10.0 |
| Llebreta | 42°33′3″ | 0°53′25″ | 1620 | 11.5 | 7.55 | 30.6 | 1.1 | 203 | not available | 1.8 | 7.9 |
| Long de Liat | 42°48′24″ | 0°52′26″ | 2140 | 32 | 7.27 | 20.8 | 0.7 | 68 | 5 | 1.0 | 11.8 |
| Muntanyó d′Àrreu | 42°40′26″ | 1°00′29″ | 2210 | 29 | 9.18 | 74.3 | 0.8 | 674 | 9 | 12.0 | 10.1 |
| Pica Palomèra | 42°47′38″ | 0°52′8″ | 2308 | 10 | 4.61 | 29.6 | not available | 50 | 9 | 0.5 | 8.0 |
| Plan | 42°37′21″ | 0°55′51″ | 2188 | 11 | 7.04 | 16.7 | 1.4 | 63 | 7 | 0.6 | 9.0 |
| Pòdo | 42°36′11″ | 0°56′21″ | 2450 | 20 | 6.41 | 9.4 | 0.3 | 273 | 79 | 4.2 | 9.6 |
| Pois | 42°39′20″ | 0°42′27″ | 2056 | 19.5 | 8.19 | 67.2 | 0.2 | 82 | 14 | 2.9 | 8.6 |
| *Redon | 42°38′33″ | 0°46′13″ | 2240 | 73 | 6.67 | 10.5 | 0.4 | n.a. | n.a. | 1.5 | n.a. |
| Roi | 42°34′29″ | 0°48′12″ | 2310 | 10 | 7.06 | 25.8 | 0.5 | 126 | 18 | 5.2 | 5.9 |
| Romedo de Dalt | 42°42′22″ | 1°19′29″ | 2114 | 40 | 6.31 | 5.8 | 0.8 | 103 | 13 | 2.8 | 14.4 |

*Chemical and Chla values measured at the depth of the deep chlorophyll maxima. (*Lakes discarded from the analysis, see text).*

**TABLE 2 | Average and range of values of the different variables measured in the SML and surface plankton of the studied lakes.**

| Variable | SML | Surface | *P* (paired *t*-test) |
|---|---|---|---|
| **PHOTOTROPHIC PICOPLANKTON** | | | |
| Total PPP abundance | 7.1 $10^3$ (0.3–18.9) | 6.3 $10^3$ (0.5–12.6) | NS |
| Picocyanobacteria | 2.4 $10^3$ (0–13.0) | 1.3 $10^3$ (0–9.5) | 0.02 |
| Small eukaryotes | 4.7 $10^3$ (0.3–13.7) | 5.0 $10^3$ (0.2–10.8) | NS |
| Ratio small eukaryotes:cyanobacteria | 150 (0-650) | 238 (0–800) | 0.03 |
| Number of diff. PPP populations | 4.1 (1–7) | 4.5 (2–7) | NS |
| Number of diff. picocyanobacterial pop. | 0.8 (0–2) | 0.6 (0–2) | NS |
| Number of diff. small eukaryotes populations | 3.3 (1–5) | 3.9 (2–6) | 0.04 |
| **HETEROTROPHIC PICOPLANKTON** | | | |
| Total heterotrophic prokaryotes | 1.0 $10^6$ (0.3–2.0) | 0.8 $10^6$ (0.3–1.8) | 0.03 |
| NADS-Live prokaryotes | 5.0 $10^5$ (0.7–9.9) | 4.9 $10^5$ (1.1–12.1) | NS |
| NADS-Dead prokaryotes | 5.1 $10^5$ (1.8–11.2) | 3.2 $10^5$ (0.8–5.4) | 0.01 |
| HNF abundance | 3.0 $10^3$ (0.9–8.8) | 2.0 $10^3$ (0.9–5.7) | 0.02 |
| Ratio Total heterotrophic prokaryotes:HNF | 482 (63–1297) | 491 (135–1122) | NS |
| CTC positive prokaryotes | 3.7 $10^4$ (2.1–6.1) | 2.8 $10^4$ (1.6–5.8) | 0.01 |
| Prokaryotic heterotrophic act. (leucine inc.) | 9.5 (1.4–29.0) | 27.9 (7.4–61.4) | <0.01 |
| Cell-specific prokaryotic activity | 9.9 (3–31) | 36.0 (16–64) | <0.01 |
| % Live prokaryotes | 49 (22–70) | 59 (44–82) | <0.01 |

*Abundances in cells ml$^{-1}$. Bacterial activity in pmol Leu ml$^{-1}$ h$^{-1}$ and cell-specific activity in amol cell$^{-1}$ h$^{-1}$. Averages and the statistical comparison of the picophytoplankton (PPP) abundance data do not include Bassa de les Granotes, an obvious outlier. All variables were log transformed to achieve normality prior to the paired t-test.*

the variables that did not randomly lay around the 1:1 ratio (**Figure 2**).

Leucine incorporation varied in a large range, from 1.4 to 121 pmol Leu l$^{-1}$ d$^{-1}$ and was systematically higher in the surface than in the SML with the exception of lakes Pois and Roi (**Figure 2A**). A similar plot for CTC indicated that the number of heterotrophic prokaryotic cells with high respiration rates was higher in the SML than at the surface, as most values were above

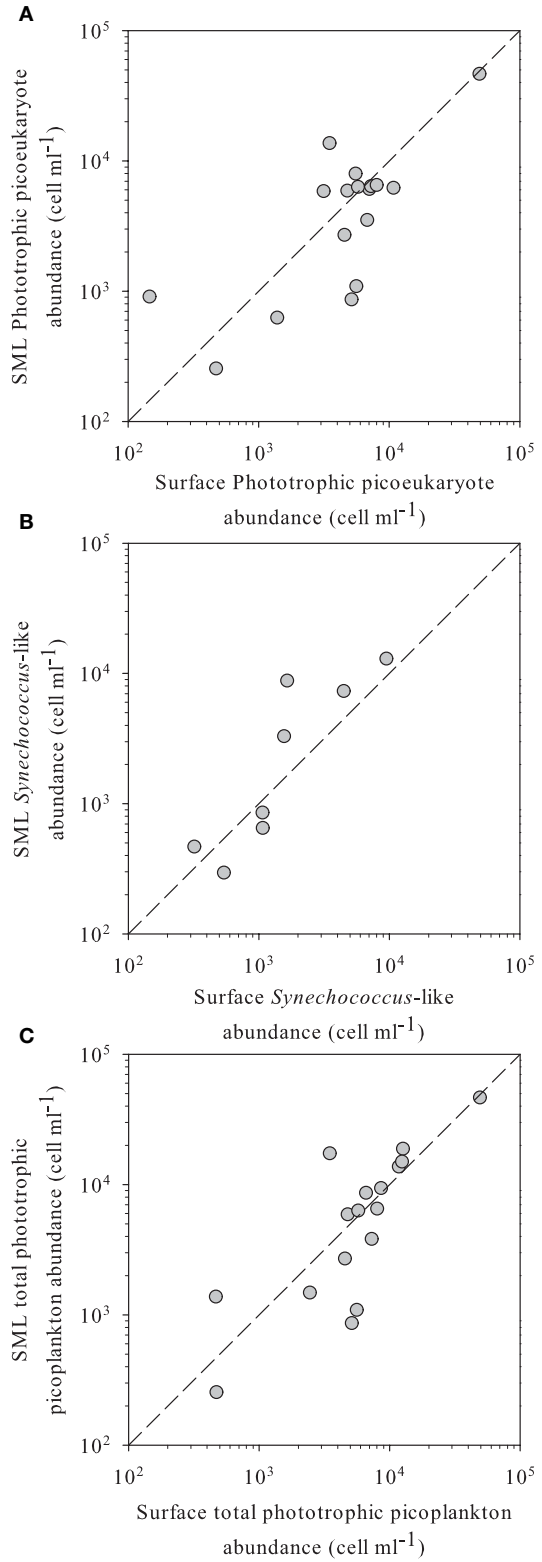


**FIGURE 1 | (A)** Phototrophic small eukaryotes, **(B)** *Synechococcus*-like, and **(C)** total phototrophic picoplankton abundance in SML vs. surface waters of the different high altitude lakes studied, as determined by flow cytometry. In all panels, the dashed line indicates the 1:1 ratio.

the 1:1 ratio (**Figure 2B**), Overall, there were more respiring prokaryotic cells at the SML, while they produced more in surface waters, both trends being highly significant (**Table 2**).

The NADS staining protocol (that measures the relationships between membrane-intact and membrane-damaged heterotrophic prokaryotic cells) showed healthier heterotrophic prokaryotes in surface plankton than at the neuston (**Figure 2C**). In fact, damaged cells accumulated at the SML while intact cells had similar abundances in both layers (**Table 2**).

## Enrichment Factors

Enrichment factors were calculated as the ratio between the values of abundance or activity in the SML divided by those in surface waters. The results of enrichment factors indicated different degrees of enrichment in the neuston for CTC positive, and bacterial, HNF and PPP abundances. On the contrary, leucine incorporation and the percentage of "live" bacterial cells were lower in the neuston (**Figure 3**).

## Factor Analysis

FA simplifies the interpretation of the many environmental variables measured in the 16 lakes. We performed separated FA for physical and chemical variables, and the three first factors explained 85.3–64.5% of the variance, respectively (**Table 3**). The first three physical factors reflected lake morphometry (Factor F1), altitude (Factor F2), and water temperature (Factor F3). Regarding chemical factors, the FA analysis revealed productivity (Factor C1) as the main factor, then DOC, silica, sulfate, and nitrate concentration (Factor C2), and finally conductivity and calcium concentration (Factor C3), which would capture the lithology as reflected in water chemistry. A fourth factor was obtained on which $NH_4^+$ (negatively) and $NO_2^-$ and alkalinity (positively) loaded. However, this factor was forced by a single outlier lake. It did not show any correlation with any biological parameter and therefore it is not discussed here. Most factors were easily interpretable with exception of factor C2 (DOC, silica, sulfate, and nitrate). In this factor, DOC, silica, and sulfate were the strongest variables (higher loadings), and nitrate, to a lesser extend. Silica and sulfate are usually related to the metamorphic nature of rocks in the Pyrenees (Catalan et al., 1993). This factor C2 could be interpreted as a "watershed DOC factor," representing a gradient from a rockier watershed with low DOC production (high silica and sulfate from metamorphic rock weathering), to higher soil coverage with higher DOC production (less silica and sulfate). An alternative explanation is that the covariation of these three parameters could be related to the landscape position of the lakes (Sadro et al., 2011).

We tested for correlation of the scores of the physical factors against those of the chemical factors and no significant correlations were found, indicating that the factors were independent (and so were the variables that laid behind them). One exception was the correlation between factors F3 (water temperature) and C2 (watershed DOC), which were positively correlated ($p < 0.05$, Pearson coefficient = 0.518).

In a second step, the abundance or activity variables in the two layers (surface and SML), and the enrichment factors were

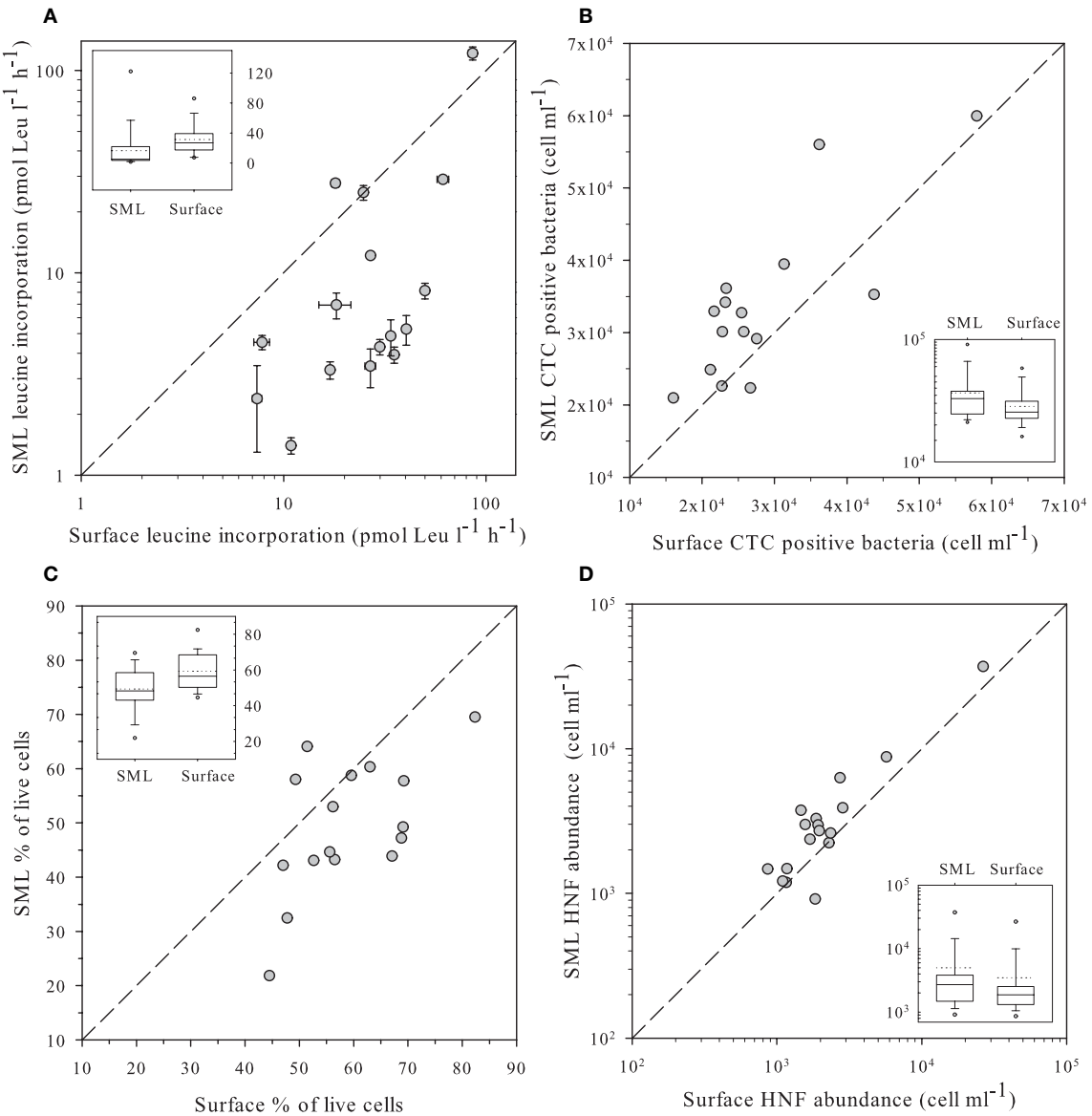


**FIGURE 2 | (A)** Leucine incorporation in SML samples vs. values observed in the surface; **(B)** Number of heterotrophic prokaryote cells with high respiration rates (CTC+) in SML samples vs. those observed in the surface as determined by flow cytometry and the CTC staining protocol. **(C)** Percentage of "live" cells in the SML samples vs. the value observed at the surface as determined by flow cytometry and the NADS staining protocol. **(D)** Abundance of HNF in SML samples vs. values observed in the surface measured by flow cytometry with the Lysotracker staining protocol. In all panels, the dashed line indicates the 1:1 ratio. Insets in each panel indicate the distribution of values for surface and the SML. The central line indicates the median value, boxes indicate the lower and upper quartiles, and vertical lines indicate the 10th and 90th percentiles.

tested for correlation against the scores of the main chemical and physical factors (**Table 4**). In general, biological variables in the SML were more related to physical factors, especially lake morphometry (Factor F1) and temperature (Factor F3) and to the nature of the rock (Factor C3). Conversely, surface biological variables were frequently correlated with the Factor C2 (watershed DOC), but also with water temperature (Factor F3). Concerning enrichment factors, the same pattern was observed: stronger and more correlations were found with the Factor C2 (watershed DOC).

## Discussion

Consistent differences were observed for picoplankton abundance, activity, and single cell physiological status between the SML and surface waters for the great majority of the 16 high mountain lakes that had developed a SML. In most lakes, PPP abundances were higher in the SML, particularly when PE-rich populations were present, whereas leucine incorporation rates in the SML were clearly below the values found in underlying waters, and these differences were probably related to the *in*

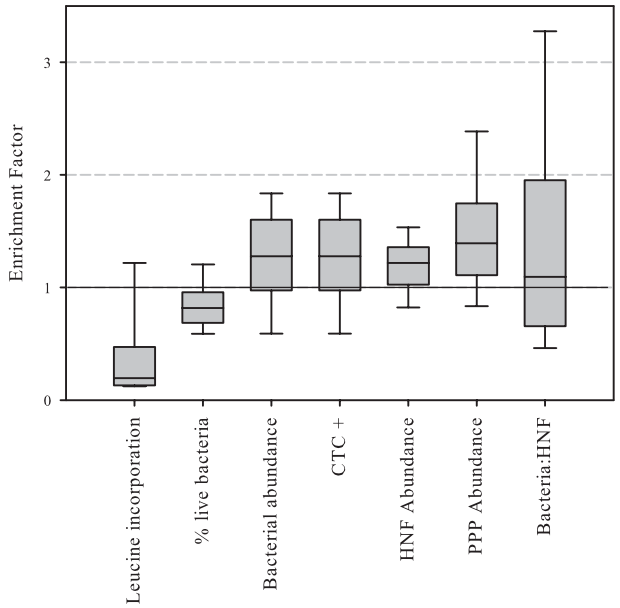


**FIGURE 3 | Boxplot of abundance or activity enrichment factors in the piconeuston (i.e., SML:surface).** Central line indicates median value, boxes the lower and upper quartiles, and vertical lines indicate 10th and 90th percentiles.

**TABLE 3 | Factor loadings and cumulative variance explained obtained in the factor analysis of chemical and physical variables of the 17 high altitude lakes studied.**

| **Physical variables** | **Factor F1** | **Factor F2** | **Factor F3** |
|---|---|---|---|
| | **Lake morphometry** | **–Altitude** | **–Temperature** |
| Zmax | 0.946 | | |
| Lake area | 0.874 | | |
| Secchi depth | 0.685 | | |
| Watershed area | | 0.926 | |
| Altitude | | −0.884 | |
| Water temperature | | | −0.911 |
| Cumulative Variance | 37.8% | 67.4% | 85.3% |
| **Chemical variables** | **Factor C1** | **Factor C2** | **Factor C3** |
| | **Productivity** | **–Watershed DOC** | **Lithology** |
| TP | 0.922 | | |
| Chla | 0.776 | | |
| DOC | | −0.769 | |
| DRSi | | 0.765 | |
| $SO_4$ | | 0.678 | |
| $NO_3$ | | 0.526 | |
| Conductivity | | | 0.951 |
| Ca2+ | | | 0.937 |
| Cumulative Variance | 21.8% | 43.4% | 64.5% |

*situ* stressful environmental conditions experienced by the neuston.

All the lakes studied had several PPP populations, and roughly half of them had picocyanobacteria. As far as we are aware, no other study had reported such a widespread presence of PPP in this type of environments, i.e., lakes located above 2000 m, although a large genetic diversity of planktonic eukaryotic microorganisms (3–40 microns sized) has been recently reported from the same Pyrenean lakes dataset (Triadó-Margarit and Casamayor, 2012).

Concerning heterotrophic prokaryotes, leucine incorporation was clearly lower in the SML compared to surface waters. A possible explanation for this observation is the direct effect of sunlight, particularly UVR, on the prokaryotic community. Several studies have demonstrated the inhibitory effect of solar radiation on substrate (thymidine, leucine, glucose, and acetate) incorporation rates on exposed prokaryotic communities, compared to dark or UVR-free incubations (Herndl et al., 1993; Sommaruga et al., 1997; Santos et al., 2012b; Ruiz-González et al., 2013). Actually, these processes are known to be by far more complex, as different bacterial groups have different sensitivities to UVR exposure, some of them being strongly inhibited (such as the SAR11 clade, at least in marine systems), some resistant (such as *Gammaproteobacteria* and *Bacteroidetes*), and some stimulated (such as *Roseobacter*) by natural sunlight (Alonso-Sáez et al., 2006; Santos et al., 2012a). The observed bulk community response could therefore be the sum of the positive and negative effects on the different prokaryotic populations.

The theoretical framework outlined by Carlson et al. (2007) postulates that increased environmental hostility (lack of resources - organic matter or nutrients, salinity, extreme temperature, UVR, extreme pH values, or a combination of several of these) should result in an increase in the proportion of the total flux of energy that is devoted to cell maintenance. Associated with this increase in cell maintenance, cell-specific respiration should increase to fuel the increased maintenance and the repair mechanisms. Thus, bacterial growth efficiency (BGE) would be expected to decrease with increasing environmental hostility. Our results provide experimental evidence that validate this hypothesis, at least in high-mountain lakes with high levels of UVR: not only bulk bacterial activity was lower in the SML (ca. four times lower on average), but the number of heterotrophic prokaryote cells with high respiration rates (CTC positives) was higher in the SML than at the surface (1.5x). We also observed a higher percentage of membrane-damaged heterotrophic prokaryotes in the SML than in the surface, probably as a consequence of the postulated environmental hostility. The number of CTC-positive cells has been found to correlate with total bacterial respiration in several previous studies (Cook and Garland, 1997; Smith, 1998; Berman et al., 2001).

It is unlikely that virus-induced lysogeny could be the main cause for the differences observed between layers, as it is known that solar radiation, particularly UV-B radiation (290–320 nm), is an important factor contributing to the decay of aquatic viruses (Suttle and Chen, 1992). Moreover, a previous study in a high mountain lake reported a negative correlation between viral abundances and solar radiation (Hofer and

**TABLE 4 | Pearson correlation coefficient (r) of the statistically significant correlations (*$p < 0.05$ or **$p < 0.01$) between the variables measured in the 17 high altitude lakes and the chemical and physical scores (obtained in the factor analyses, see Table 3).**

| | Factor F1 | Factor F2 | Factor F3 | Factor C1 | Factor C2 | Factor C3 |
|---|---|---|---|---|---|---|
| | Lake morphometry | –Altitude | –Temperature | Productivity | –Watershed DOC | Lithology |
| **SML** | | | | | | |
| Total heterotrophic prokaryotes | −0.654** | | | | | 0.558* |
| % Live prokaryotes | −0.536* | | −0.523* | | | |
| Total PPP abundance | | | −0.652** | | | |
| Prokaryotic heterotrophic activity (leucine incorporation) | | 0.540* | | | | |
| **SURFACE** | | | | | | |
| % Live prokaryotes | −0.498* | | | | | |
| CTC positive prokaryotes | | 0.535* | | | | |
| Ratio Total heterotrophic prokaryotes:HNF | | −0.531* | | | 0.488* | −0.544* |
| Total heterotrophic prokaryotes | | | −0.628** | | −0.533* | |
| Total PPP abundance | | | | 0.530* | −0.540* | |
| Prokaryotic heterotrophic activity (leucine incorporation) | | | | | −0.567* | |
| **ENRICHMENT FACTOR** | | | | | | |
| Total PPP abundance | | | −0.506* | | | |
| Ratio Total heterotrophic prokaryotes:HNF | | | | 0.677** | | |
| Prokaryotic heterotrophic activity (leucine incorporation) | | | | | 0.713** | |

Sommaruga, 2001). In addition to HNF pressure, higher number of membrane-damaged heterotrophic prokaryotes cells in the SML comparing to surface, can also be explained by the fact that many airborne bacteria can be trapped in the air-water interface (Hervàs and Casamayor, 2009).

Our observations contrast with a recent study in 6 alpine lakes situated in an altitude gradient, which could not find a consistent pattern in the comparison of bacterial production between layers, and attributed the variation observed to lake-specific communities and environmental conditions (Hörtnagl et al., 2010). However, in such study, 3 out of 4 lakes located above 1500 m a.s.l. had lower bacterial production values in the SML than at the surface. In our study, most lakes were above 2000 m a.s.l.. As UVR increases with altitude (Blumthaler et al., 1997), this might explain the discrepancies between our study and that of Hörtnagl et al. Additionally, in our case, we only sampled under very stable weather conditions, sunny and calm, something that might have facilitated the detection of a well-formed SML with a microbiota strongly affected by solar radiation.

To the best of our knowledge, this is the first report of HNF abundance in freshwater SML and our results show that the neuston holds generally more HNF than surface lake water. The enrichment factor of HNF in the SML was, in average, slightly higher (1.47) than that of bacteria (1.27). These observations are somehow unexpected, as previous studies had shown that UVR affects negatively HNF growth and grazing on bacteria (Sommaruga et al., 1999). The total heterotrophic prokaryote:HNF ratio might provide some hints on this issue, and although not significantly different, this ratio was slightly higher in the SML. In addition, absolute values (∼480–490 bacteria per HNF) were relatively low compared to literature data (Gasol, 1994; Fermani et al., 2013). This might indicate relatively high grazing pressure of HNF on bacteria, which might have to do with specific features of food web structure in high altitude lakes. *Daphnia,* and large cladocerans in general, are particularly sensitive to UVR reviewed by Sommaruga (2001). The dominance of large filter feeding cladocerans reduces HNF abundance, and affects the microbial food web (Gasol et al., 1995; Zöllner et al., 2003; Sarmento, 2012). Thus, HNF from high altitude lakes are likely to undergo low predation pressure, and this would result in higher grazing pressure on bacteria, providing an explanation for the relatively low heterotrophic prokaryote:HNF ratio observed in this study.

No simple and direct linear relationships could be found between microbial abundances or activities and raw environmental variables. Nevertheless, factors derived from the FA did correlate in some cases with biological variables. Both physical and chemical FA captured a fairly good percentage of the total variance (85.3–64.5% respectively), which means that the extracted factors successfully explained most of the variability observed in our dataset. It has to be noted that our data do not account of course for all aspects of environmental variability. Our discussion includes only those aspects captured by our measurements, and further research may seed light on other aspects not considered here.

The physical and chemical multivariate factors identified in the FA were not correlated between them, reinforcing the independency of the factors, with the exception of factors F3 (–temperature) and C2 (–watershed DOC), which were positively correlated ($p < 0.05$, $r = 0.518$). It is possible that higher temperatures promote higher DOC production in the watershed increasing allochthonous DOC inputs to the water. Two alternative hypotheses are that lakes with higher DOC are warmer because of solar radiation absorption, or that snowmelt increases stream DOC inputs into lakes.

Interestingly, the factors that influenced heterotrophic prokaryote abundance in the different layers were different. On one hand, prokaryote abundance in SML was negatively correlated with lake morphometry (factor F1). On the other hand, surface abundance was negatively correlated with factor F3 (–temperature). Larger lakes have greater fetch and turbulence, and probably present less well-defined SML and present them less often than smaller lakes. Larger lakes might thus lack some taxa well-adapted to SML conditions (environmental filtering), while surface prokaryote abundance depends on water temperature, regardless of the size of the lake.

Prokaryote abundance and activity in the surface co-varied with watershed DOC (factor C1) but not in the SML (**Table 4**). This suggests that surface and piconeuston microbial communities rely on different energy and/or nutrient sources. Pyrenean high mountain lakes receive considerable amounts of atmospheric deposition, namely from the Sahara desert (Mladenov et al., 2011; Barberán et al., 2014) that might be the main source of nutrients and organic matter for the piconeuston.

Another interesting outcome of the FA is the fact that altitude (factor F2) correlated negatively with prokaryotic activity in the SML. In other words, leucine incorporation in the SML decreased with altitude. UVR increases with altitude, but part of the radiation is reflected and absorbed in the first millimeters of the water column, and the UVR that reaches subsurface water is not as high as that reaching the SML (Sommaruga and Psenner, 1997), This probably explains why leucine incorporation in subsurface waters had no relationship with altitude. However, in the SML exposure levels are of such magnitude that anabolic processes are affected according to the UVR dose (i.e., directly correlated to altitude).

Finally, the prokaryotic activity enrichment factor (which was always lower than one, **Figure 3**) was correlated to factor C2 (–watershed DOC), meaning that in rocky watersheds with low DOC production, neuston and surface prokaryotic activity had relatively small differences, and these differences increased in DOC enriched watersheds. Basically, prokaryotic activity increased in DOC-rich surface waters, but not in neuston, indicating that despite the physical proximity between these two habitats, the processes shaping microbial metabolism were substantially different.

In conclusion, we found evidence that heterotrophic prokaryotic communities in the piconeuston of high altitude lakes had lower percentage of healthy cells, lower leucine incorporation rates and higher number of cells with high respiration rates (CTC positive), likely resulting in lower BGE as compared to those of the underlying surface water, evidencing a high degree of environmental harshness. PPP were widespread in these high mountain lakes and PE-rich picocyanobacteria were particularly prominent in the SML. Piconeuston was also enriched in HNF and heterotrophic prokaryotes. Overall, we demonstrate that steep gradients in the upper millimeters of surface waters of high mountain lakes are common in stable conditions, and that different mechanisms drive microbial processes in the SML and the underlying surface water.

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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.2015.00361/abstract

Detailed temperature profiles in the high mountain lakes studied in the sampling day.

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# Cropping systems modulate the rate and magnitude of soil microbial autotrophic $CO_2$ fixation in soil

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The effect of different cropping systems on $CO_2$ fixation by soil microorganisms was studied by comparing soils from three exemplary cropping systems after 10 years of agricultural practice. Studied cropping systems included: continuous cropping of paddy rice (rice-rice), rotation of paddy rice and rapeseed (rice-rapeseed), and rotated cropping of rapeseed and corn (rapeseed-corn). Soils from different cropping systems were incubated with continuous $^{14}C$-$CO_2$ labeling for 110 days. The $CO_2$-fixing bacterial communities were investigated by analyzing the *cbbL* gene encoding ribulose-1,5-bisphosphate carboxylase oxygenase (RubisCO). Abundance, diversity and activity of *cbbL*-carrying bacteria were analyzed by quantitative PCR, *cbbL* clone libraries and enzyme assays. After 110 days incubation, substantial amounts of $^{14}C$-$CO_2$ were incorporated into soil organic carbon ($^{14}C$-SOC) and microbial biomass carbon ($^{14}C$-MBC). Rice-rice rotated soil showed stronger incorporation rates when looking at $^{14}C$-SOC and $^{14}C$-MBC contents. These differences in incorporation rates were also reflected by determined RubisCO activities. $^{14}C$-MBC, *cbbL* gene abundances and RubisCO activity were found to correlate significantly with $^{14}C$-SOC, indicating *cbbL*-carrying bacteria to be key players for $CO_2$ fixation in these soils. The analysis of clone libraries revealed distinct *cbbL*-carrying bacterial communities for the individual soils analyzed. Most of the identified operational taxonomic units (OTU) were related to *Nitrobacter hamburgensis*, *Methylibium petroleiphilum*, *Rhodoblastus acidophilus*, *Bradyrhizobium*, *Cupriavidus metallidurans*, *Rubrivivax, Burkholderia, Stappia, and Thiobacillus thiophilus*. OTUs related to *Rubrivivax gelatinosus* were specific for rice-rice soil. OTUs linked to *Methylibium petroleiphilum* were exclusively found in rice-rapeseed soil. Observed differences could be linked to differences in soil parameters such as SOC. We conclude that the long-term application of cropping systems alters underlying soil parameters, which in turn selects for distinct autotrophic communities.

**Keywords: cropping systems, autotrophic bacteria $CO_2$ fixation, RubisCO, *cbbL* genes, $^{14}C$ continuous labeling, $^{14}C$-SOC, soil depth**

## Introduction

Autotrophic bacteria with the capacity to fix $CO_2$ are widespread in extreme terrestrial ecosystems (Giri et al., 2004; Nanba et al., 2004; Nakai et al., 2012). Recently, an isotope incubation experiment revealed high $CO_2$ assimilation rates by autotrophic bacteria in agricultural soils, which represented a potential carbon sequestration of 0.6–4.9 Pg C $year^{-1}$ (Yuan et al., 2012a; Ge et al., 2013). Autotrophic bacteria evolved six pathways for $CO_2$ fixation: (1) the Calvin-Benson-Bassham cycle, (2) the reductive tricarboxylic acid cycle, (3) the reductive acetyl-CoA pathway, (4) the 3-hydroxypropionate cycle, (5) the 3-hydroxypropionate/4-hydroxybutyrate pathway, and (6) the dicarboxylate/4-hydroxybutyrate cycle (Fuchs, 2011). The predominant pathway for autotrophic bacteria to assimilate $CO_2$ is the Calvin-Benson-Bassham cycle (CBB) (Selesi et al., 2005). Ribulose-1,5-bisphosphate carboxylase oxygenase (RuBisCO), the enzyme which catalyzes the rate-limiting step in the CBB cycle, exists in four distinct holoenzyme forms (I, II, III, and IV). These forms differ in structure, catalytic activity, and $O_2$ sensitivity (Tabita, 1999). Form I RubisCO, composed of eight large subunits and eight small subunits, is the most abundant among the four forms (Tabita et al., 2008). Four clades of the *cbbL* gene that encodes the large subunits of form I RubisCO are known, namely IA to ID (Tabita, 1999). The presence of the *cbbL* gene has been documented in diverse phylogenetic groups from obligate autotrophic bacteria (form IA) to facultative autotrophic bacteria (form IC) (Kusian and Bowien, 1997; Kong et al., 2012).

In recent years, the *cbbL* gene has been widely used as a functional marker to analyze the diversity of autotrophic bacteria in diverse environments. Based on *cbbL* gene analysis, an unexpected level of *cbbL* diversity has been reported in agricultural soils (Selesi et al., 2005; Tolli and King, 2005; Yuan et al., 2012b; Xiao et al., 2014a). Phylogenetic analysis showed that *Azospirillum lipoferum, Rhodopseudomonas palustris, Bradyrhizobium japonicum, Ralstonia eutropha* are the dominant autotrophic bacteria in these soils (Yuan et al., 2012a). Different management practices including fertilizer treatments, land use alterations and different plant covers showed effects on the diversity and abundance of autotrophic bacterial communities in soils (Selesi et al., 2005; Tolli and King, 2005; Yuan et al., 2012b; Xiao et al., 2014a). Moreover, a link between microbial autotrophy and edaphic factors such as soil organic carbon (SOC), pH and clay content was identified (Selesi et al., 2007; Yuan et al., 2012a,b; Xiao et al., 2014a,b). A recent study revealed that differences in community composition, abundance and activity of autotrophic bacteria affect microbial carbon fixation across soil depth (Wu et al., 2014). A large proportion of the fixed C was restricted to surface soil (0–1 cm), and the assimilated $^{14}C$ was mainly aliphatically stabilized in the humin fraction of agricultural soils (Hart et al., 2013a,b; Jian et al., 2014). Relating to agricultural soils, previous studies focused on the process of $CO_2$ fixation by autotrophic bacteria in continuous cropping systems. However, information on autotrophic bacteria involved in $CO_2$ fixation in rotated cropping systems is limited.

The rotation of paddy rice and upland crop is a common agricultural practice in the subtropical area of China (Zhu et al., 2010). Field studies indicated that paddy-upland rotated soils are characterized by different physical and chemical properties in comparison to paddy and upland soils (Nishida et al., 2013; Liu et al., 2014). The different soil condition in paddy-upland rotated soil was shown to affect soil microbial communities, especially functional guilds like purple phototrophic bacteria and methanogens (Feng et al., 2011; Bernard et al., 2012). The effect of paddy-upland rotation on autotrophic bacteria remains unclear. Therefore, three cropping systems: (i) double cropping of paddy rice, (ii) rotation of paddy rice and rapeseed, and (iii) double cropping of rapeseed and corn in an experimental field with a known cultivation record of continuous paddy rice farming were examined to study how different cropping systems affect $CO_2$ fixation by autotrophic bacteria. Continuous $^{14}C$-$CO_2$ labeling was applied to quantify the incorporation of microbial fixed C to the soil organic matter pool ($^{14}C$-SOC) and soil microbial biomass ($^{14}C$-MBC) at different depths (0–1, 1–5, 5–17 cm). Based on *cbbL* gene analysis, the abundance, diversity and composition of autotrophic bacterial communities in different cropping systems were investigated.

## Materials and Methods

### Soils and Sampling

The sampling was conducted in a long term agricultural management experiment site at Pantang in subtropical China (29° 10′ − 29° 18′ N, 111° 18′ − 111° 33′ E). The experimental site was characterized by a typical subtropical climate with an annual mean precipitation of approximately 1400 mm and an average annual temperature of 16.8°C. Three cropping systems, namely double cropping of paddy rice (rice-rice), rotation of paddy rice and rapeseed (rice-rapeseed), and double cropping of rapeseed and corn (rapeseed-corn) were established in 2000. Four replicate plots of each cropping system were randomly arranged in the fields. Soils at the field site were developed from quaternary red earth and were used for rice farming for decades prior to the implementation of the cropping systems. For rice-rice, the field was permanently flooded during the spring and autumn rice growing seasons. For rice-rapeseed, the field was flooded in the spring rice growing season while it was drained in the rapeseed season. For rapeseed-corn, crops were planted in rapeseed-corn sequence in a drained paddy field. These treatments were maintained for more than 10 years when we conducted this study. After the harvest of the late crop, one soil core was retrieved from each field by inserting a PVC column (10 cm diameter, 20 cm height) to 17 cm depth at a random location within each 33 $m^2$ plot. Visible crops or grass at the surface were removed. Basic geochemical parameters for all soils are given in **Table 1**.

### Incubation with $^{14}C$-$CO_2$

All PVC columns were incubated in a growth chamber (80 × 250 cm, height 120 cm) for 110 days with continuous $^{14}C$-$CO_2$ labeling as described previously (Ge et al., 2012; Yuan et al., 2012a; Wu et al., 2014). The $^{14}C$-$CO_2$ was generated by forcing a $Na_2^{14}CO_3$ solution (1.0M, a radioactivity of $1.68 \times 10^4$ Bqμg$^{-1}$ C) into an acid bath (HCl, 2M) and the gas concentration ($^{14}C$-$CO_2$) was maintained at approximately 350 μL $L^{-1}$. During

**TABLE 1 | Characteristics of soils from different cropping systems.**

| Cropping system | pH | SOC (g $kg^{-1}$) | Total N (g $kg^{-1}$) | Total P (g $kg^{-1}$) | Clay content (%) | CEC (cmol $kg^{-1}$) |
|---|---|---|---|---|---|---|
| Rice-rice | 5.66 ± 0.01 | 20.93 ± 0.72 | 2.81 ± 0.01 | 0.70 ± 0.00 | 33.19 ± 0.43 | 13.16 ± 0.23 |
| Rice-rapeseed | 5.79 ± 0.01 | 6.64 ± 0.24 | 1.44 ± 0.00 | 0.82 ± 0.03 | 46.19 ± 0.41 | 7.96 ± 0.13 |
| Rapeseed-corn | 4.40 ± 0.03 | 6.19 ± 0.04 | 1.39 ± 0.01 | 0.75 ± 0.02 | 31.38 ± 0.55 | 11.05 ± 0.01 |

*Cropping systems were established in 2000.*

the incubation period, all soils were illuminated by a parabolic aluminum reflector lamp with an intensity of about 500 mmol photons $m^{-2}$ $s^{-1}$ for 12 h each day (8:00 a.m.–8:00 p.m.). The day/night air temperature inside the chamber was maintained at 31 ± 1°C/24 ± 1°C and the relative humidity was kept at 80–90%. Soils from rice-rice plots remained flooded with a 1–2 cm water layer while those from rapeseed-corn plots were drained. Rice-rapeseed soils were also incubated under the waterlogged condition, due to rice being the crop plant for the following growing season in this cropping system. At the end of the 110 day incubation, the flooded water was removed and soils from 0 to 1, 1 to 5, and 5 to 17 cm depth intervals were sampled. The sectioned soil layers, each with four replicates, were divided into two sub-samples. One sub-sample was stored at 4°C for biochemical analysis while the other was kept at −70°C for molecular analysis. For each sectioned soil sample, the soil moisture content was measured immediately after sampling.

## Determination of Soil Properties

Soil pH was determined using a pH meter (Delta 320, Mettler-Toledo Instruments Ltd., China) at a 1:2.5 (w:v) soil-to-$H_2O$ ratio. Soil organic carbon (SOC) and total nitrogen (TN) contents were measured by dry combustion with a macro elemental analyzer (Vario MAX C/N, Elementar Analyse Systeme, Germany). Total phosphorus (TP) was determined using the Mo-Sb colorimeteric method (Lu, 2000). Clay content was measured using the pipette method and cation exchange capacity (CEC) was determined by titration (Rhoades, 1982; Müller and Höper, 2004). $^{14}$C-SOC (mg $kg^{-1}$) was determined according to Wu and O'Donnell (1997), and$^{14}$C-MBC (mg $kg^{-1}$) was analyzed using the fumigation-extraction method (Wu et al., 1990). The amounts of $^{14}$C-SOC and $^{14}$C-MBC were calculated using the following formulas:

$$^{14}C - SOC = F_1 R_s / R_p W$$
$$^{14}C - MBC = F_2 (R_f - R_{uf}) / R_p W\, kc$$

where $F_1$ and $F_2$ represent the factors to convert the counting volume (1 ml from 40 ml plus soil water volume in ml for $F_1$ and 1 ml from 80 ml plus soil water volume in ml for $F_2$); $R_s$ and $R_p$,radioactivity (Bq $ml^{-1}$; blank counts omitted) for the trap solution and that for $Na_2^{14}CO_3$ (Bq $mg^{-1}$ C $l^{-1}$) used to produce $^{14}$C-$CO_2$ in the growth chamber; $R_f$ and $R_{uf}$, radioactivity ($B_q$ $l^{-1}$; blank counts subtracted for the extractants of the fumigated soil and unfumigated soil, respectively); W, the weight (kg) of digested soil on an oven-dry basis; Kc, the factor (0.45) converting measured $^{14}$C into biomass $^{14}$C (Wu et al., 1990).

## DNA Extraction, Clone Library Construction, and Phylogenetic Analysis

DNA was extracted in triplicate from 500 mg (fresh weight) soil from each independent replicate, using the FastDNA Spin Kit (BIO101, Qbiogene Inc., Carlsbad, CA) according to the manufacturer's protocol. The integrity and quantity of the extracted DNA were evaluated by standard agarose gel electrophoresis and a spectrophotometer (Nanodrop ND-1000, PeqLab, Germany). The *cbbL* gene fragments from one randomly chosen replicate of rice-rice and rapeseed-corn cropping systems were amplified using the same thermal profile as previously described by Wu et al. (2014). PCR reactions were set up as follows: 12.5 µl 2 × PCR MasterMix (Tiangen, China), approximately 50 ng soil DNA, and 0.1 µM of each *cbbL* primer, modified by Tolli and King (2005) per reaction. In order to show the reproducibility of our approach, the *cbbL* gene fragments from two replicate samples originating from the rice-rapeseed rotation were generated separately. Subsequently, PCR products were purified with an agarose gel DNA purification kit (Tiangen, China) and ligated into the pGEM-T Easy Vector System (Promega, Mannheim, Germany), and then transformed into *E. coli* DH5α-competent cells. Positive clones were sequenced at the Beijing Genome Institute (Beijing, China).

Clone sequences were grouped into operational taxonomic units (OTUs) based on 95% nucleotide sequence similarity using Mothur (Schloss et al., 2009). The OTUs primarily responsible for the differences in *cbbL*-carrying bacterial community among samples were identified based on the similarity percentage analysis (SIMPER) using PAST (Hammer et al., 2001). The representative nucleotide sequences of these OTUs were subsequently translated into amino acid sequences and aligned with closely related known sequences in GenBank using Clustal W (http://www.ebi.ac.uk/clustalw). If necessary, alignments were manually refined. The resulting alignment was used to construct a neighbor-joining tree using MEGA 5.0 (Tamura et al., 2011). Bootstrap analysis of 1000 replicates was conducted to estimate the robustness of the tree topologies.

## Community Diversity Analysis

Rarefaction curves were generated by the Analytic Rarefaction program (http://strata.uga.edu/software/Software.html) to assess the sampling effort. Shannon indices were computed using Mothur to compare the diversity of the *cbbL*-bearing bacterial communities in three cropping systems (Schloss and Handelsman, 2008). The coverage rate was computed as C = [1− (n/N)] × 100, where n represents the number of OTUs containing one individual sequence and N is the total number of sequences.

### Real-time PCR

The *cbbL* gene abundance was quantified using an ABI 7900 real-time PCR system (ABI 7900, Foster City, CA, USA) using SYBR Green I based assays. Quantitative PCR was performed in 10 μL reaction mixtures containing 5 μL 1 × SYBR Premix Extaq (Takara Bio Inc., Shiga, Japan), 5 ng of template DNA, 0.1 μM of primers with the following thermal profile: 30 s at 95°C, followed by 5 cycles of 5 s at 95°C, 45 s annealing temperature decreased from 66 to 62°C and an extension at 72°C for 30 s. In addition, another 35 cycles at 95°C for 5 s, 62°C for 45 s, and 72°C for 30 s. A final melting curve was generated to evaluate the amplification specificity. Ten-fold serial dilutions of plasmid DNA extracted from positive clones were used to establish a standard curve. The real-time PCR assays were performed in triplicate for each replicate sample. The copy number of the *cbbL* gene was calculated directly using SDS 2.3 software.

### RubisCO Activity

RubisCO enzyme activity was assayed according to Yuan et al. (2012a). Briefly, 2 g soil (four replicates) were thoroughly homogenized by an ultrasonic cell mixer (JY92-II Scientz, China) in an extraction buffer containing Tris-HCl (100 mM, pH 7.8) and Dithioreitol (DTT, 1 mM). The supernatant was collected by centrifugation and was precipitated with solid ammonium sulfate to reach 80% saturation. The resulting pellets were collected and dissolved in Tris-HCl/DTT. RubisCO activity was measured at 30°C using spectrophotometry (UV-2450, Shimadzu, Japan) and calculated according to Takai et al. (2005).

### Statistical Analysis

Canonical correspondence analysis (CCA) was performed using CANOCO 5.0 for Windows (Microcomputer Power, Ithaca, NY, USA) to characterize the effect of measured soil properties on the composition of bacteria communities carrying *cbbL* gene. Significant differences in community composition were tested by permutational Two-Way analysis of variance or multivariate analysis of variance (MANOVA) implemented in PAST (Hammer et al., 2001). PERMANOVA is a distance-based non-parametric MANOVA that allows the analysis of multivariate (or univariate) data in response to treatments in an experimental design. Statistical significant differences between data sets based on metadata (soil parameters, *cbbL* copy numbers, RubisCO activities) were identified by two-way analysis of variance (ANOVA) and differences were considered significant at $P < 0.05$. A multiple regression model was built by stepwise regression with significance being defined as $P < 0.05$. ANOVA and multiple regression analyses were carried out using SPSS (version 16.0, SPSS Inc., USA). The reproducibility of the carried out clone library analysis was tested using the aforementioned replicated clone libraries originating from the rice-rapeseed rotation. The robustness of clone library analysis was assessed based on calculated unweighted UniFrac distances (Lozupone and Knight, 2005).

### Nucleotide Sequence Accession Numbers

Nucleotide sequences were deposited in the EMBL European Nucleotide Database (http://www.ebi.ac.uk/ena/data/view/) under accession numbers HG940678–HG941631.

## Results

### Incorporation of Labeled $^{14}C$ into Soil Organic Matter and Soil Microbial Biomass

The $^{14}C$-SOC and $^{14}C$-MBC concentrations were significantly different in the three cropping systems according to Two-Way ANOVA analysis. In 0–1 cm, the maximum $^{14}C$-SOC and $^{14}C$-MBC concentrations were detected in rice-rice rotated soil, reaching 935 mg $kg^{-1}$ and 375 mg $kg^{-1}$ respectively. The $^{14}C$-SOC and $^{14}C$-MBC concentrations in corresponding depths of rice-rapeseed rotated soil were 1.6 and 4.0 times lower than those in rice-rice rotated soil (**Table 2**). In 1–5 cm, the radioactivity, in terms of SOC and MBC was higher in rice-rice rotated soil than in rice-rapeseed rotated soil (**Table 2**). The amount of $^{14}C$ incorporated in rapeseed-corn rotated soil was much lower compared to all other soils when investigating the 0–1 and 1–5 cm soil layers (**Table 2**). In addition, the $^{14}C$-SOC and $^{14}C$-MBC contents decreased with increasing depth, irrespective of the cropping systems. No radioactivity was detected in the 5–17 cm

**TABLE 2 | Amounts of $^{14}C$-SOC, $^{14}C$-MBC, abundance and activity of *cbbL*-carrying bacteria in three cropping systems.**

| Cropping system | Depth (cm) | $^{14}C$-SOC concentration (mg $kg^{-1}$ soil) | $^{14}C$-MBC concentration (mg $kg^{-1}$ soil) | Abundance ($10^9$copies $g^{-1}$ dry soil) | Activity (nmol $CO_2$ $g^{-1}$ soil $min^{-1}$) |
|---|---|---|---|---|---|
| Rice-rice | 0–1 | 934.97 ± 17.54Aa | 375.22 ± 5.19Aa | 2.59 ± 0.31Aa | 55.18 ± 7.82Aa |
| | 1–5 | 66.57 ± 3.05Ab | 27.88 ± 1.20Ab | 1.16 ± 0.19Ab | 48.84 ± 2.71Aab |
| | 5–17 | ND | ND | 0.66 ± 0.08Ab | 40.56 ± 1.02Ab |
| Rice-rapeseed | 0–1 | 363.16 ± 117.64Ba | 73.03 ± 5.86Ba | 0.63 ± 0.11Ba | 43.64 ± 1.50Ba |
| | 1–5 | 12.45 ± 1.12Bb | 4.37 ± 0.34Bb | 0.44 ± 0.06Bab | 30.69 ± 0.92Bb |
| | 5–17 | 3.71 ± 0.94Bb | ND | 0.25 ± 0.05Bb | 26.92 ± 0.68Bc |
| Rapeseed-corn | 0–1 | 9.87 ± 0.41Ba | 3.91 ± 0.30Ca | 0.30 ± 0.13Ba | 29.13 ± 2.37Ca |
| | 1–5 | 5.29 ± 0.44Ba | 1.70 ± 0.37Ca | 0.39 ± 0.14Ba | 27.55 ± 0.91Cab |
| | 5–17 | ND | ND | 0.36 ± 0.04Ba | 24.76 ± 0.55Cb |

*ND, not determined. Values are mean ± standard error in four replicates, different capital and low-case letters in the same column indicate significant differences ($P < 0.05$) among cropping systems and soil depths.*

layer with the exception of rice-rapeseed rotated soil (**Table 2**). A statistically significant correlation was observed between $^{14}$C-SOC and $^{14}$C-MBC concentration ($P < 0.05$). The *cbbL* gene abundance and RubisCO activity were significantly related to $^{14}$C-SOC concentration ($P < 0.05$).

### Diversity Analysis of *cbbL*-Carrying Bacterial Communities

Nine clone libraries of 106 clones each were obtained from different depth intervals for the three cropping systems. Overall, sequences were grouped into 148, 155, and 111 OTUs for rice-rice, rice-rapeseed and rapeseed-corn rotated soils, respectively (**Table 3**). High levels of diversity were observed based on the number of OTUs as well as calculated Shannon indices. Differences in diversity between cropping systems were small (**Table 3**). Rarefaction analyses suggested that additional sequencing effort is needed to cover the full diversity of *cbbL* sequences in our systems (**Figure 1**). The underestimated diversity was also reflected by the coverage of libraries, which ranged from 52 to 71% (**Table 3**). Determined Pearson coefficients revealed no significant correlations between diversity and monitored soil properties ($P > 0.05$).

### *cbbL*-Carrying Bacterial Community Structures

The comparison of *cbbL*-bearing bacterial communities in two rice-rapeseed rotated soils revealed no statistically significant differences in community structures for replicated samples (Table S1), suggesting that our sampling approach is robust enough to draw reliable conclusions. The *cbbL*-carrying bacterial communities within the three cropping systems clustered into different groups, as revealed by the CCA analysis (**Figure 2**). Samples from different depth layers of the same cropping system formed relatively tight clusters (**Figure 2**). PERMANOVA analysis showed that the individual cropping system was a statistically significant determinant of community composition ($P < 0.05$), whereas the community structure did not change markedly at different sampling depths ($P > 0.05$). CCA analysis revealed that the SOC content ($P < 0.05$) was the main environmental driver for changes in the *cbbL*-bearing bacterial communities.

### *cbbL* Gene Abundance and RubisCO Activity

The *cbbL* gene abundances in rice-rice rotated soil were significantly higher than those determined in rice-rapeseed and rapeseed-corn rotated soils (**Table 2**). Gene copy numbers were four times higher in the 0–1 cm layer, when compared to the 5–17 cm layer in rice-rice rotated soil (**Table 2**). Similar vertical trends were found for rice-rapeseed rotated soil, where copy numbers decreased by 63% in the 5–17 cm layer compared to the 0–1 cm layer (**Table 2**). *cbbL* copy numbers changed only slightly with depth in rapeseed-corn soil (**Table 2**). The abundance of *cbbL*-carrying bacteria was significantly correlated with DOC and MBC ($P < 0.05$).

RubisCO activity differed in the individual cropping systems. Highest activities were seen in rice-rice rotated soil (**Table 2**). Activities were generally found to decrease with soil depth. Multiple regression analysis based on stepwise showed that MBC was the main factor affecting RubisCO activity ($P < 0.05$).

### Phylogenetic Affiliations of Abundant OTUs

A total of 57 OTUs were identified as main phylotypes responsible for observed differences in community structure, cumulatively contributing 50% of the community variation (**Figure 3**). Sequences from these OTUs were dominated by facultative *cbbL*-carrying bacterial communities (Form IC), and they varied in their relative abundances in relation to cropping system and soil depth. Sequences from rapeseed-corn soil mainly formed four clades, with two clusters relating to *Nitrobacter hamburgensis* and *Nocardia asteroides* respectively and two novel clades without known representatives (**Figure 4**). Sequences from rice-rapeseed rotated soil were phylogenetically diverse, but closely related to sequences from *Methylibium petroleiphilum*, *Rhodoblastus acidophilus*, *Bradyrhizobium,* and *Cupriavidus metallidurans* (**Figure 4**). Sequences retrieved from rice-rice soil were widely distributed, grouping with various facultative and obligate autotrophic groups such as *Rubrivivax, Burkholderia, Bradyrhizobium, Stappia, and Thiobacillus thiophilus* (**Figure 4**).

## Discussion

According to our previous microcosm experiments, autotrophic bacteria contribute significantly to $CO_2$ fixation in agricultural soils (Yuan et al., 2012a; Ge et al., 2013; Wu et al., 2014). We

**TABLE 3 | Diversity of *cbbL*-containing bacterial community in sectioned soil depths from different cropping systems.**

| Cropping system | Depth (cm) | No. of clones | No. of OTUs | Shannon-Weiner (H) | Evenness | Coverage (%) |
|---|---|---|---|---|---|---|
| Rice-rice | 0–1 | 106 | 61 | 3.93 | 0.96 | 66 |
| | 1–5 | 106 | 59 | 3.76 | 0.93 | 63 |
| | 5–17 | 106 | 66 | 3.66 | 0.89 | 57 |
| Rice-rapeseed | 0–1 | 106 | 51 | 3.18 | 0.81 | 63 |
| | 1–5 | 106 | 67 | 3.95 | 0.94 | 52 |
| | 5–17 | 106 | 66 | 4.01 | 0.96 | 58 |
| Rapeseed-corn | 0–1 | 106 | 55 | 3.56 | 0.89 | 64 |
| | 1–5 | 106 | 49 | 3.40 | 0.89 | 73 |
| | 5–17 | 106 | 57 | 3.86 | 0.95 | 71 |

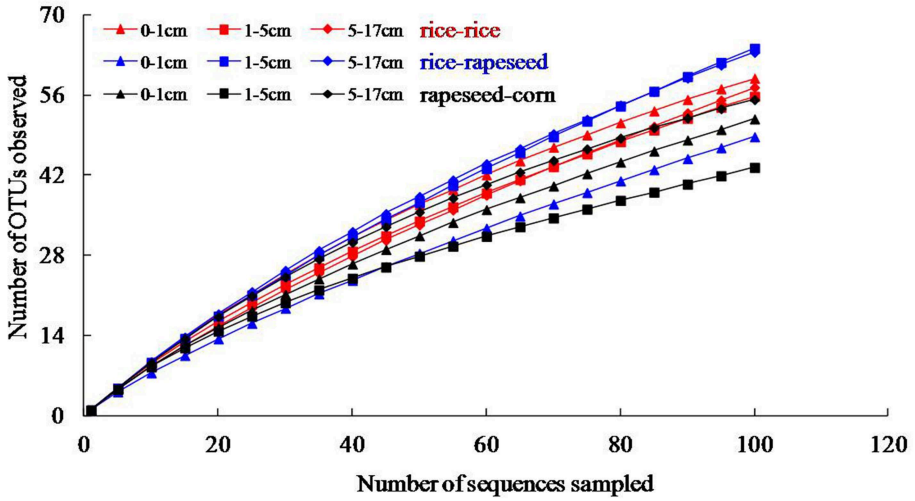


**FIGURE 1 | Rarefaction analysis of *cbbL* clone libraries based on 95% nucleotide sequences similarity.**

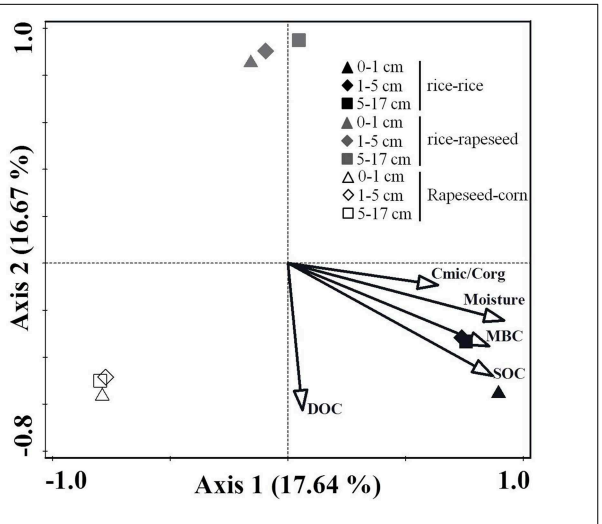


**FIGURE 2 | Changes in the *cbbL*-containing bacterial communities in response to different cropping systems.** Cmic/Corg describes the ratio of microbial biomass carbon to total organic carbon.

previously ascertained that the $^{14}C$ incorporation, as a measure for the autotrophic soil carbon sink mediated by autotrophic bacteria, was 2–13-folds larger in continuous paddy rice soils than upland crop soils (Yuan et al., 2012a; Ge et al., 2013). Here we extended previous work to gain insights into the effect of different cropping systems on microbial $CO_2$ fixation processes, using three different cropping systems, including rice-rice, rice-rapeseed and rapeseed-corn rotated soils. The significant linear correlation between $^{14}C$-SOC and $^{14}C$-MBC concentrations indicated that the fixed $^{14}C$ in three cropping systems was derived from microbial fixation (Yuan et al., 2012a; Ge et al., 2013; Wu et al., 2014). Autotrophic bacteria, as revealed by the positive relationships between *cbbL* gene abundance, RubisCO activity and $^{14}C$-SOC concentration, were the major microbial players behind $^{14}CO_2$ incorporation into SOC. The *cbbL*-carrying bacteria recovered in these soils were dominated by sequences related to facultative autotrophs like phototrophic, nitrogen fixing, nitrifying and CO and hydrogen oxidizing bacteria. However, some members of the *cbbL*-carrying bacterial communities were exclusive in one cropping system. For example, clone sequences in OTUs specific to rice-rice soil were closely related to *Rubrivivax gelatinosus*, which is a phototrophic bacterium with two *cbbL* gene copies surviving in aquatic ecosystems and moist soils (Kuske et al., 1997; Badger and Bek, 2008). While sequences closely affiliated to methylotrophic bacterium *Methylibium petroleiphilum* PM1 were exclusive to rice-rapeseed soil, whose presence has been documented in aquatic systems previously (Chen et al., 2009). Although facultative chemoautotrophy has been identified as an alternative metabolism in the methylotrophic bacterium *Beijerinckia mobilis*, a potential autotrophic metabolism of *Methylibium petroleiphilum* PM1 has not yet been demonstrated (Dedysh et al., 2005; Kane et al., 2007).

The diversity of *cbbL*-carrying bacterial communities suggested the presence of metabolically versatile autotrophic bacteria in the three cropping systems under study here. Much lower diversity patterns were reported in previous work regarding different managed agricultural soils using T-RFLP analysis (Selesi et al., 2005; Yuan et al., 2013; Xiao et al., 2014a). Applying clone library analyses improved the resolution of *cbbL* sequence analysis in comparison to previous studies (Marsh, 1999). Observed high diversities are presumably a consequence of changing underlying soil properties due to the applied cropping systems. Numerous studies have established a link between the *cbbL* diversity and soil properties (Nanba et al., 2004; Selesi et al., 2005; Yuan et al., 2012a,b, 2013; Xiao et al., 2014a). In our study, all the tested soils were developed from the quaternary red earth, which is characterized by a high clay content. The high amount of available nutrients in soil clay fractions were reported to favor the development of diverse

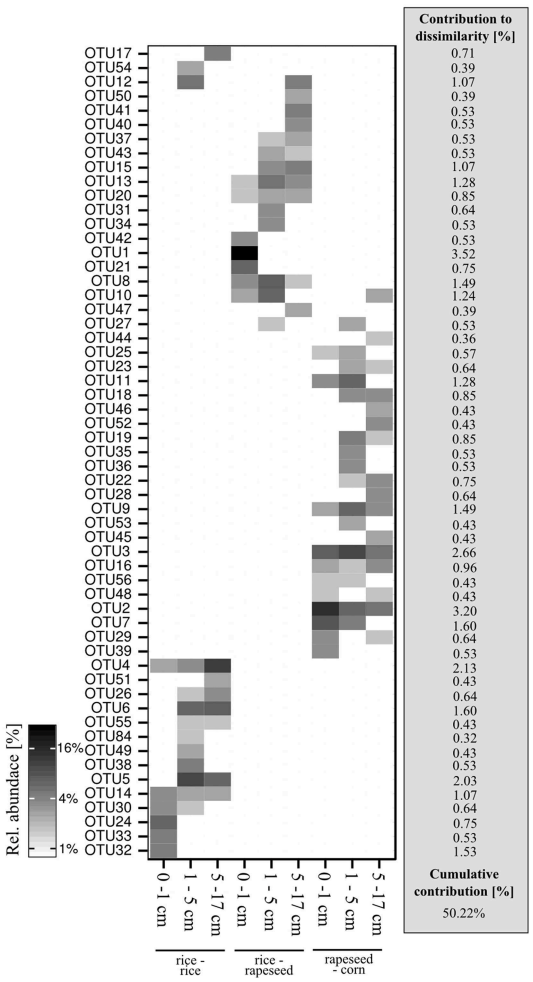


**FIGURE 3 | Heatmap of the 57 OTUs primarily responsible for community differences.** Respective cumulative contributions to community dissimilarities are given in addition.

*cbbL*-carrying bacterial communities (Paul et al., 2001; Selesi et al., 2007). This likely explains higher diversities in our soils in comparison to those observed in coastal barren saline soils based on clone library analysis (Yousuf et al., 2012).

The contributions of autotrophic bacteria to $CO_2$ fixation in the three cropping systems were different, with the highest $^{14}C$ incorporation rate observed in rice-rice, followed by rice-rapeseed, with the lowest value detected in rapeseed-corn rotated soil (**Table 1**). Paddy-upland rotation cropping systems differ from normal paddy rice and upland crop systems, where various water regimes are practiced in different crop growth seasons (Nishimura et al., 2008; Liu et al., 2010). During the submerged period of paddy rice cultivation, the anoxic conditions restrict the mineralization processes in soil, whereas the drainage of paddy fields for upland crop cultivation resulted in an oxic condition which enhances the decomposition processes within the soil (Chang Chien et al., 2006; Iqbal et al., 2009). As a result, physicochemical properties (e.g., SOC) are significantly altered in paddy-upland rotated systems when compared to paddy and upland soil traditional cultivation methods (Wang and Yang, 2003; Liu et al., 2010; Zhu et al., 2010). In this study, all soils were collected from the same climatic condition, and have the same origin and cultivation history. Soil properties such as SOC and TN were changing in response to the three cropping systems (**Table 1**), due to the differences in field management and crop regime over the 10 year period. The variations in soil properties (SOC, TN, MBC, and DOC) caused by the applied cropping systems affected the abundance, activity and composition of *cbbL*-carrying bacteria (Selesi et al., 2005; Xiao et al., 2014a), and thus resulted in the differences in $^{14}C$ incorporation rates in soils. We observed an almost two orders of magnitude difference in $^{14}C$ assimilation rates between rotation systems but only two- to four-fold differences in *cbbL* gene abundance and RubisCO activity. Considering that only *cbbL* copy numbers have been determined, differences on the level of transcription and translation cannot be ruled out. It appears reasonable to assume that $^{14}C$ was at least partially assimilated by alternative $CO_2$ fixation pathways, such as the reductive acetyl-CoA cycle that is known to be commonly active under anoxic conditions (Campbell and Cary, 2004; Nakagawa et al., 2005).

Labeling experiments as the one presented here are easily affected by the availability of unlabeled substrate. Dependent on the availability of unlabeled $CO_2$, determined incorporation rates are eventually influenced due to a dilution effect. The availability of $CO_2$ in soil pore space is strongly correlated with ongoing respiration processes, which are influenced by present organic substrates (Van Hees et al., 2005; Iqbal et al., 2009). Against this background our results appeared robust against the outlined dilution effect. The SOC content was highest in rice-rice soil, which presumably stimulated respiration. As a result $CO_2$ availability would have been rather high, potentially diluting added $^{14}C$-$CO_2$. Nevertheless, $^{14}C$ incorporation was the highest in these soils.

In line with previous work, fixed $^{14}C$ significantly decreased with soil depth in three cropping systems, indicating that a large proportion of microbially fixed $^{14}C$ was restricted to the surface soil (Wu et al., 2014). Nishimura et al. (2008) reported that land use change from paddy rice to upland crop not only affected soil properties within the surface soil layer, but also caused changes within the deeper soil layers. Therefore, the differences in soil properties across soil depths may affect the availability of substrate ($^{14}CO_2$) and electron donors to autotrophic bacteria, resulting in changes in $^{14}C$ fixation rates with soil depth (Jeffery et al., 2009; Kellermann et al., 2012; Wu et al., 2014). In addition, $^{14}CO_2$ reduced to methane in the flooded surface soil could be accessed by methane oxidizing bacteria, which might in part explain the large assimilation in the surface of rice-rice and rice-rapeseed soils as well. It cannot be ruled out that incorporated $^{14}CO_2$ was at least partially derived from $CO_2$ fixation by soil algae, which eventually released metabolites that could have been processed by heterotrophic organisms. Nevertheless, soil algae were presumably playing a minor role. From previous work it is known that their abundance is at least one order of magnitude lower compared to autotrophic bacteria (Yuan et al., 2012a). Future isotope based work could help to gain an insights regarding the active community fraction

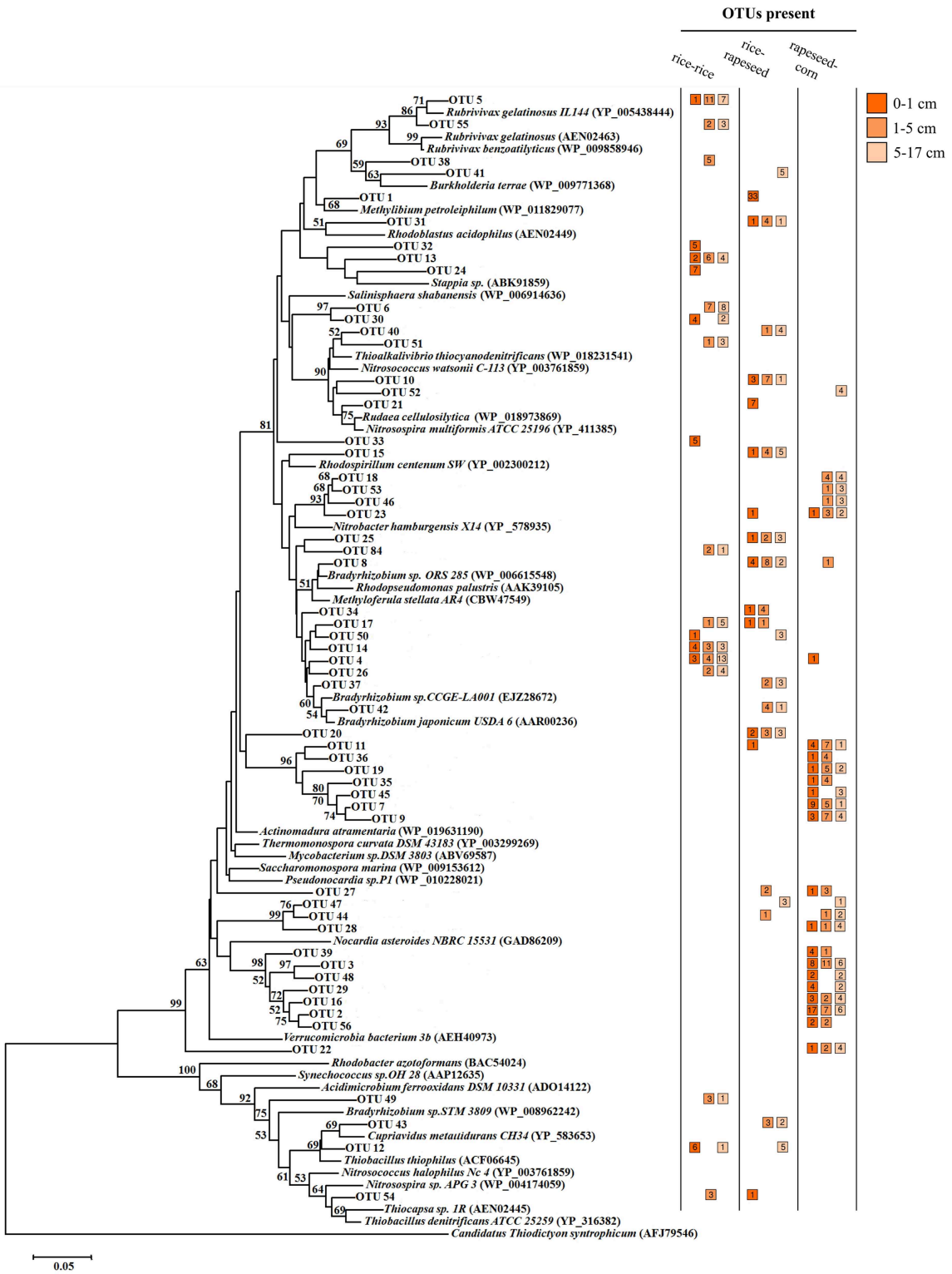


**FIGURE 4 | Neighbor-joining tree illustrating the phylogeny of the OTUs primarily responsible for the differences in *cbbL*-carrying bacterial communities.** The tree was constructed using 165 deduced amino acids of corresponding nucleic acid *cbbL* clone sequences and 36 reference sequences from public databases. The *cbbM* gene from *Candidatus* "Thiodictyon syntrophicum" strain Cad 16 (accession number AFJ79546) was used as outgroup. The number of sequences retrieved from different samples is displayed in the colored squares next to the OTUs. Bootstrap values above 50% are indicated at the branch nodes. The scale bar represents 0.05 substitutions per amino acid based on a p-distance matrix analysis.

consuming $CO_2$ among *cbbL*-carrying bacteria and algae as a whole, since our community structure analyses revealed only minor changes for *cbbL*–carrying bacterial communities within different depths.

The present study showed variations in $CO_2$ fixation by autotrophic bacteria in response to different cropping systems. Statistical analysis revealed higher $CO_2$ assimilation rates in rice-rice than rice-rapeseed and rapeseed-corn rotated soils. Observed differences in soil parameters caused by the applied cropping systems lead to changes in *cbbL* abundance, activity and bacterial community structure, and thus resulted in differences in $^{14}C$ incorporation rates in the three cropping systems. These results broaden our knowledge about the importance of autotrophic bacteria involved in the soil carbon sink. However, questions still remain, including the true extent of *cbbL* diversity. Here, next generation technologies such as high throughout sequencing appear represent promising follow up approaches, because the resolution of the analysis would substantially increase. Identifying active autotrophs involved in $CO_2$ fixation by studying community gene expression would provide a better understanding about organisms playing major roles under different soil management conditions and how active organisms eventually interact with each other.

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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.2015.00379/abstract

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REVIEW
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# Stream microbial diversity in response to environmental changes: review and synthesis of existing research

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The importance of microbial activity to ecosystem function in aquatic ecosystems is well established, but microbial diversity has been less frequently addressed. This review and synthesis of 100s of published studies on stream microbial diversity shows that factors known to drive ecosystem processes, such as nutrient availability, hydrology, metal contamination, contrasting land-use and temperature, also cause heterogeneity in bacterial diversity. Temporal heterogeneity in stream bacterial diversity was frequently observed, reflecting the dynamic nature of both stream ecosystems and microbial community composition. However, within-stream spatial differences in stream bacterial diversity were more commonly observed, driven specifically by different organic matter (OM) compartments. Bacterial phyla showed similar patterns in relative abundance with regard to compartment type across different streams. For example, surface water contained the highest relative abundance of Actinobacteria, while epilithon contained the highest relative abundance of Cyanobacteria and Bacteroidetes. This suggests that contrasting physical and/or nutritional habitats characterized by different stream OM compartment types may select for certain bacterial lineages. When comparing the prevalence of physicochemical effects on stream bacterial diversity, effects of changing metal concentrations were most, while effects of differences in nutrient concentrations were least frequently observed. This may indicate that although changing nutrient concentrations do tend to affect microbial diversity, other environmental factors are more likely to alter stream microbial diversity and function. The common observation of connections between ecosystem process drivers and microbial diversity suggests that microbial taxonomic turnover could mediate ecosystem-scale responses to changing environmental conditions, including both microbial habitat distribution and physicochemical factors.

**Keywords: ecosystem structure and function, lotic ecosystems, microbial diversity, rivers, streams**

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## Introduction

Aquatic microbial diversity is well understood to be a key component of aquatic ecosystem functioning (Nold and Zwart, 1998; Cotner and Biddanda, 2002; Gessner et al., 2010), and major

advances toward linking microbial diversity with ecosystem function have been made in aquatic systems (Horner-Devine et al., 2003; Smith, 2007; Singer et al., 2010; Comte et al., 2013). While the responsiveness of microbial diversity to environmental variability is an ongoing topic of inquiry (Finlay, 2002; Allison and Martiny, 2008; Langenheder et al., 2012; Shade et al., 2012), it is clear that global environmental change poses many potential threats to the structure and function of all aquatic ecosystems (Malmqvist and Rundle, 2002; Dudgeon et al., 2006), and that changing environmental factors at the watershed scale directly impact the biological function of lotic ecosystems (Likens et al., 1970; Hynes, 1975; Mulholland et al., 2008; Palmer and Febria, 2012). However, a recent survey of microbial diversity studies in aquatic habitats showed that microbial diversity in lotic environments is less commonly studied than in marine and lake ecosystems, and that impacted systems are less commonly studied than unimpacted systems (Zinger et al., 2012). Given the importance of microbial processes to lotic ecosystem function, and the microbial genetic diversity that contains the information supporting those functions as well as the potential for resilience under environmental changes, it is critical to study stream microbial diversity in the context of shifting environmental drivers.

Streams and rivers are hotspots of microbially mediated carbon (C) and nutrient processing within landscapes (Hynes, 1975; Fisher et al., 1998; McClain et al., 2003). Microbial activity drives organic matter (OM) decomposition, whole-stream respiration and C flow to higher trophic levels (Lindeman, 1942; Meyer, 1994; Hall and Meyer, 1998; Hieber and Gessner, 2002; Tank et al., 2010). Thus, microbial processes are at the center of the conceptual model of OM processing and food web structure through the river continuum (Vannote et al., 1980; Minshall et al., 1985). Also, microbial nitrification, denitrification, and heterotrophic nitrogen (N) uptake in small streams affects downstream water quality (Peterson et al., 2001; Mulholland et al., 2008; Valett et al., 2008). There is a strong history and impact of studying microbial processes within and among stream and river ecosystems, yet molecular methods, which enable the study of microbial diversity within the context of ecosystem function, have not been widely utilized in lotic ecosystems (Findlay, 2010).

Studies on bacterial and fungal diversity in lotic ecosystems have been historically more associated with the research themes of microbial transport and leaf litter decomposition, respectively. Stream fungal diversity research has traditionally been rooted in an ecological context, investigating the patterns and mechanisms of aquatic hypomycete succession that occur on leaf biofilms concomitant with the progression of leaf decomposition (Suberkropp and Klug, 1976; Suberkropp et al., 1976; Webster and Descals, 1981; Bärlocher, 1982; Gessner et al., 1993). Early stream bacterial diversity research consisted of culture-for-diversity assessments of bacterial loads in the water column, resulting in predominantly *Pseudomonas* sp. isolates with variation in diversity correlated most strongly with water temperature, storm events and sunshine (Bell et al., 1982a,b). These studies relied upon microscopy for identification of fungal conidia following sporulation or substrate utilization profiling of bacterial isolates. Bacterial diversity was particularly difficult to define, beyond differentiating gram-negative from gram-positive cells (Geesey et al., 1977) or conducting plate counts on selective media (Milner and Goulder, 1984), before the availability of molecular tools.

The application of molecular techniques to measure stream microbial diversity produced new insights. Genetic markers within each "*Pseudomonas*" sp. isolate differed among isolates derived from the same stream, and between stream- and soil-derived isolates (McArthur et al., 1992); somewhat analogously, the distribution of selected loci differed among *Tetrachaetum elegans* monosporic isolates from different leaf species within the same stream (Charcosset and Gardes, 1999). Also, the genetic composition of the entire microbiota found on fine particulate OM was more similar than expected given differences in water chemistry among the streams sampled (Sinsabaugh et al., 1992). Following the establishment of the ribosomal rRNA gene as a conserved marker of taxonomic lineage (Pace, 1997), studies on water column biota began to resolve longitudinal patterns in microbial diversity (Crump et al., 1999; Crump and Baross, 2000), and efforts to integrate molecular and traditional tools in studies of fungal diversity were mounted (Nikolcheva et al., 2003; Nikolcheva and Bärlocher, 2005). In the 21st century, studies of molecular microbial diversity in lotic ecosystems are increasing to an extent that a review and synthesis of progress on the topic is needed.

Because microbial processes in stream and river ecosystems are variable in space and time in response to differences in nutrient availability (Dodds et al., 2000), temperature (Boyero et al., 2011), OM quality or quantity (Gessner and Chauvet, 1994), hydrological factors (Valett et al., 1997), or land use (Mulholland et al., 2008), a reasonable initial prediction is that microbial diversity might also respond to changes in these environmental variables. In fact, many recent studies of molecular microbial diversity in streams have been initiated based on this rationale. Among these case studies, there are many examples of variation in microbial diversity in response to environmental variability, many examples of microbial diversity showing no response to the predicted driver, and many examples of microbial diversity changing with one environmental factor, but not another, in the same study. This leads to a large degree of uncertainty in the extent to which lotic microbiota are sensitive or resistant to environmental perturbation (Allison and Martiny, 2008; Shade et al., 2012). If microbial functions are phylogentically conserved to any extent, there should be some predictability to the changes in microbial diversity along environmental gradients in space and time (Phillippot et al., 2010). If functional redundancy among microbial taxa or physiological flexibility due to functional diversity within microbial taxa is high, microbial diversity will be more static in the face of environmental fluctuation. One literature review cannot tease apart these complex mechanisms; however, it can attempt to identify which environmental factors are demonstrated to be more or less likely to affect microbial diversity. Any pattern can inform hypotheses regarding the sensitivity or resilience of diverse aquatic microbiota to the multiple categories of current environmental threats to aquatic ecosystems (Malmqvist and Rundle, 2002).

With this goal in mind, I gathered published papers on microbial diversity in streams and rivers for a focused review. After collecting papers, I recorded the findings of each in a categorical manner: I noted the significance or lack thereof for each comparison within each study, differentiated among the effects of defined environmental drivers and contrasting levels of spatiotemporal variation, tallied the frequency of studies using various methodologies, and harvested coarse taxonomic data from relevant studies. The result is a synthesis that is a step beyond a traditional review, but not as quantitatively rigorous as a true meta-analysis. There was a great deal of evidence for sensitivity of lotic microbial communities to environmental variation, and somewhat surprising implications regarding the relative influence of physical versus geochemical factors on microbial diversity.

## Methods

To collect as many published, peer-reviewed studies on stream microbial diversity as possible, I searched the Web of Science database using the parameters TS = ("stream" OR "river" OR "lotic") AND TS = (microb* OR bacteria* OR fung*) AND TS = "diversity" for all full years available (through 2013). I screened search results and kept all papers that (i) reported microbial taxonomic diversity including data collected using molecular, microscopy, and "culture for diversity" methods and (ii) evaluated the effect of some environmental variable on microbial diversity. I accepted papers that reported diversity as richness, as a diversity index incorporating some estimate of relative abundance of taxa, or as the relative abundance of measured taxa (community structure). These search parameters may not catch some relevant studies, however, the resulting synthesis of 294 papers is to my knowledge the most comprehensive to date.

These studies were categorized based on (i) the microbial group of interest (fungi, bacteria, protozoa, archaea, stramenopiles); (ii) the methodology used (microscopy, denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), sequencing of genes from clone libraries, sequencing of genes from next-generation technology libraries, fluorescent *in situ* hybridization (FISH), other molecular methods and other non-molecular methods; and (iii) the environmental driver(s) evaluated by the study (temporal variation, among-stream variation, longitudinal variation, differences in nutrient concentrations, differences in OM quantity or quality, OM compartment type, hydrologic variation, differences in metals concentrations, differences in surrounding land-use, and differences in temperature, **Table 1**). All studies were also categorized by the OM compartment sampled coarse particular organic matter (CPOM, leaves or wood), water column, epilithon (biofilm attached to any hard surface), streambed sediment, hyporheic biofilm or water, and other [including, e.g., foam, fine benthic organic matter (FBOM)], and by the scale of investigation (within-stream comparison, among-stream comparison, or among-region comparison). Many studies included multiple experiments or comparisons, or comparisons that could be classified under multiple categories (e.g., a study evaluating the effect of a wastewater treatment plant on bacterioplankton diversity was categorized as a longitudinal comparison and as a comparison of nutrient concentration effects).

It is not valid to compare the values of derived diversity metrics or the abundance of microorganisms based on data collected with different methodologies and taxonomic resolutions, so a fully quantitative metaanalysis, using a response index, was not possible. However, it is valid to accept significant results of a study as informative, no matter the data type. For example, a meta-analysis of heterogeneity in soil microbial communities showed greater-than-random spatial similarity no matter the

**TABLE 1 | Categories of environmental variation evaluated for effects on stream microbial diversity.**

| Category | Definition/Variables included |
|---|---|
| **Spatiotemporal variation** | |
| Temporal variation ($n = 101$) | Samples collected at multiple time points |
| Among-stream variation ($n = 82$) | Samples collected at different streams |
| Longitudinal variation ($n = 70$) | Samples collected at different sites from up-to-downstream |
| Compartment type ($n = 38$) | At one site within a stream, samples collected from different OM/surface types (including rocks, coarse particular organic matter (CPOM), benthic surface sediment, subsurface sediment, or no surface i.e., water column) |
| **Variation in defined environmental drivers** | |
| Nutrient concentrations ($n = 56$) | Variation in surface water nutrient concentrations; nutrient = any form of N or P, or C:N, C:P, or N:P stoichiometry |
| Organic matter (OM) quality/quantity ($n = 52$) | Variation in surface water DOC concentrations, particulate OM stock, or substrate quality (e.g., different species of leaf litter) |
| Hydrological variation ($n = 32$) | Variation in stream flow, hydrological regime, or before/after a defined flooding or drying event |
| Metals effects ($n = 31$) | Variation in soluble metals concentrations (e.g., Al, Cd, Cu, Fe, Mn, Pb, Zn), or generalized acid mine drainage effects |
| Land-use ($n = 28$) | Variation in riparian or watershed land-use (e.g., agricultural, urban, undeveloped) |
| Temperature ($n = 23$) | Variation in water temperature |

*Studies were classified into categories of spatiotemporal variation or variation in environmental drivers based on the sampling strategy employed in each study, with number (n) of studies noted for each category.*

technique used to measure diversity, but the magnitude of heterogeneity detected was greater if a lower-resolution taxonomic definition was utilized (Horner-Devine et al., 2007). Therefore, for this study, a qualitative comparative approach was used: Each comparison of environmental variability on stream microbial diversity was categorized as "significant" or "non-significant," based on the criteria used in the publication, and the distribution of significant results was compared among all studies. With this approach, a semi-quantitative evaluation of the most and least commonly observed effects of environmental drivers on stream microbial diversity was possible.

Some studies in the database reported the 16S rRNA gene sequence abundance of microbial taxa. While individual datasets had varied sequencing depths, particularly the clone library versus next-generation sequencing studies, the most abundant microbial populations should be the most commonly sequenced using any method. In the interest of extracting as much information as possible from the database, I harvested relative abundances of dominant phyla and subphyla in bacterial 16S rRNA gene libraries from the 29 studies that reported relative abundance in addition to diversity/heterogeneity summary metrics. This data extraction included neither studies that reported sequence information for only a subset of sequences gathered (thus skewing relative abundances higher), nor FISH data, which, while based on the 16S rRNA gene sequence, tend to not quantify bacterial phyla such as Acidobacteria, Cyanobacteria, or Verrucomicrobia. While differences in extraction protocol, sequencing primers, sequencing depth, and other technical particulars introduce potentially large sources of non-environmental variation to this analysis, signals that rise above the noise must be particularly strong. I tested the hypothesis that OM compartment type affected bacterial community composition across studies with a one-way analysis of variance (ANOVA) and Bonferonni *post hoc* comparisons using R Commander (Fox, 2005; R Core Development Team, 2010).

## Results

A total of 294 papers published between 1976 and 2013 were included in the analysis; these papers contained 520 comparisons of the effects of spatiotemporal heterogeneity or defined environmental drivers on microbial community composition in streams (Appendix). The majority of papers examined bacterial communities (56%), many examined fungal communities (36%), and the remainder examined archaeal, protozoan, or stramenopile communities (**Figure 1**). Many fungal studies utilized a definition of taxonomic identity based on conidial morphology, making microscopy the most common methodology in the database (26% of studies), followed by the "community fingerprint" techniques of DGGE and T-RFLP (20 and 11% of studies, respectively) and sequencing of ribosomal genes from clone libraries (10%; **Figure 1**). Only 4% of studies utilized next-generation sequencing, all published in 2012 or 2013; this reflects a more rapid increase in papers using molecular methods in the past decade (**Figure 2**). In total, almost half of the

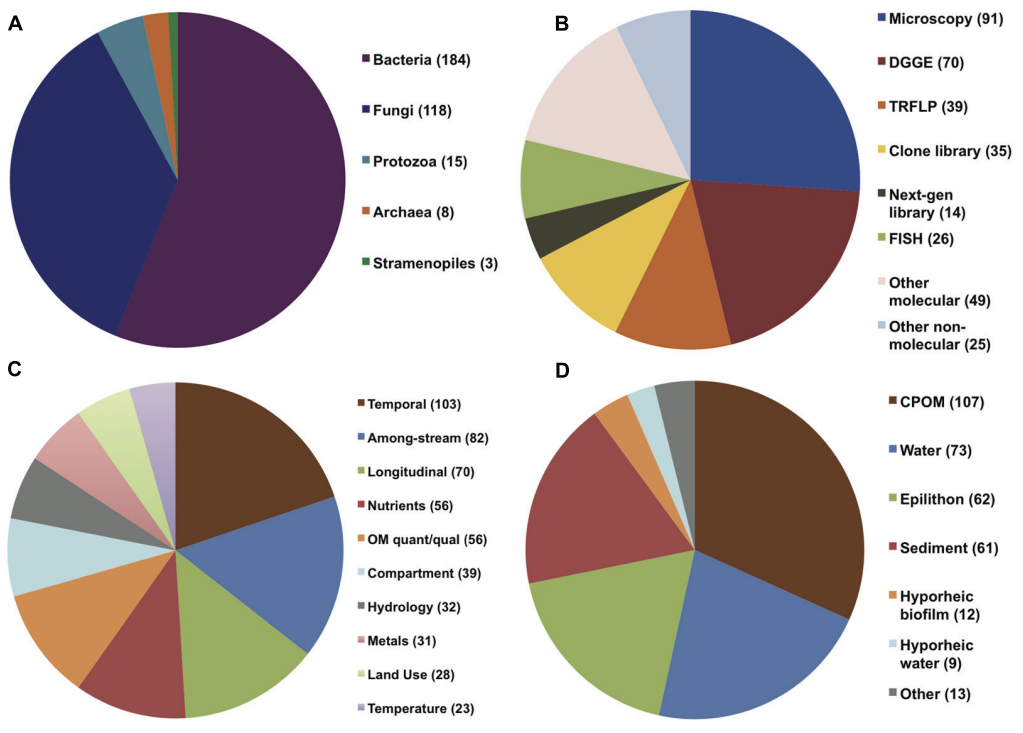


**FIGURE 1 | Pie charts showing distribution of (A) organism of study, (B) methodology used in study, (C) environmental drivers, and (D) compartment of study among all papers (294) included in the analysis.**

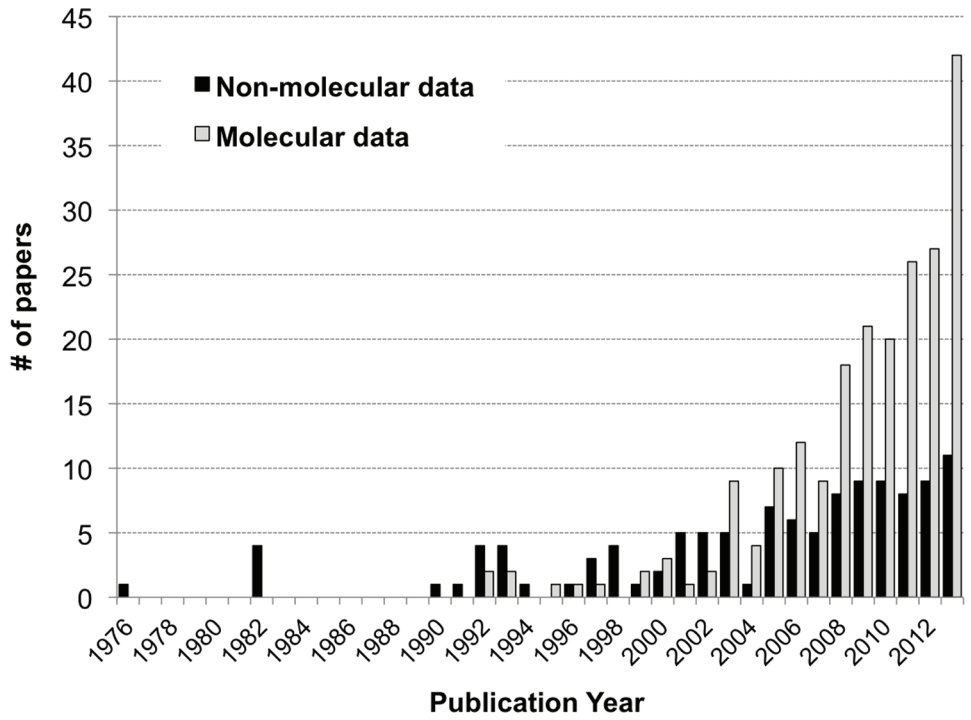


**FIGURE 2 | Histogram of the number of papers reporting stream microbial diversity from 1992 to 2013: papers using molecular methods as gray bars, non-molecular methods as black bars.**

comparisons measured spatiotemporal variation in microbial community composition (temporal, 20%; among-stream, 16%; longitudinal, 14%), and the most commonly evaluated environmental drivers were nutrient concentration (11%) and OM quality or quantity (11%; **Figure 1**). Finally, among compartment types studied, CPOM was best represented (32%, primarily fungal communities), followed by the water column (22%, primarily bacterioplankton), epilithic biofilms (18%), and benthic sediment (18%; **Figure 1**).

The overall distribution of published significant versus non-significant effects of environmental variation on stream microbial community composition was 88.5% versus 11.5%. The most common source of spatio-temporal heterogeneity was within-stream heterogeneity among different OM compartments (97% significance among published effects), followed by within-stream temporal, among-stream, and within-stream longitudinal heterogeneity (93, 87, and 83% significant effects, respectively; **Figure 3**).

Because a good number of studies reported the dominant bacterial phyla and subphyla in 16S rRNA gene sequence libraries from defined sample types (either clone libraries, 21 studies; or next-generation sequencing, eight studies), it was possible to evaluate which taxonomic groups varied in relative abundance among compartments (**Figure 4**). The results of ANOVA *post hoc* comparisons showed significant among-compartment differentiation in a number of phyla, including the Acidobacteria, Actinobacteria, Bacteroidetes, Cyanobacteria, and other Bacteria (**Table 2**). Among different stream compartments, water column samples contained the highest relative abundance of Actinobacteria, epilithon samples contained the highest relative abundance of Bacteroidetes and Cyanobacteria, FBOM samples contained the highest relative abundance of Acidobacteria, and sediment samples contained the second highest relative abundance of Acidobacteria and the highest relative abundance of other Bacterial sequences.

The category of defined environmental drivers with the most commonly significant effect was metals (100%), followed by temperature, OM quantity or quality and hydrology, land use and nutrient concentrations (91, 88, 88, 86 and 79% significant effects, respectively; **Figure 5**). The studies evaluating metals effects did not report widely comparable taxonomic data (these included a mix of fungal microscopic, fungal sequencing, bacterial sequencing, and non-sequencing data), so it was not possible to perform an evaluation of metals effects on microbial community composition.

This meta-analysis of environmental variation in stream microbial diversity was dominated by within-stream studies (358 comparisons, 88.5% significant); to contrast with other study scales, the 73 among-stream comparisons showed a total distribution of 86% significant effects, with 100% significance within all categories of variation except land use and nutrient concentrations (88 and 73% significant effects, respectively), and the 11 among-region comparisons showed 100% significance within all categories of variation (**Figure 6**).

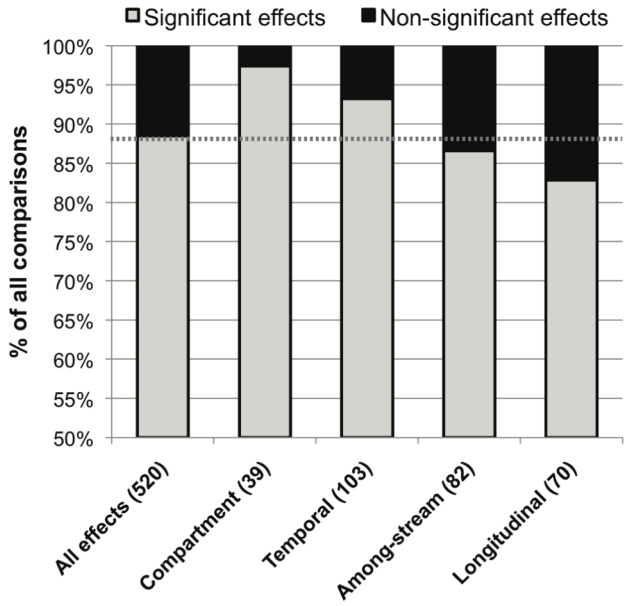


**FIGURE 3 | Distribution of significant (in gray) and non-significant (in black) reported effects of categories of spatiotemporal variation on stream microbial diversity, with number of included studies noted for each category along the x-axis, and the percentage of significant effects for all comparisons combined noted as a dashed line for reference.**

## Discussion

The results of this data synthesis reflected a high incidence of microbial diversity responses to environmental variation in stream and river ecosystems. Among spatiotemporal factors, within-stream compartment differences and temporal differences were most common, while longitundinal differences were least common. Among defined drivers, "metals" effects were ubiquitous and land-use and nutrients effects were least common. Overall, lotic microbial communities are quite sensitive to environmental changes, but their functional redundancy may be greater in relation to certain environmental variables than others.

The relative distribution of significant and non-significant effects of different categories of environmental drivers on lotic microbial diversity provides an informative synthesis of the current state of the literature. However, the high number of significant comparisons – 88.5% – raises some concern of a literature bias favoring the publication of significant results. This is an established concern in the interpretation of metaanalyses, and can result from a lack of enthusiasm by authors or reviewers to publish a study with "no effect" (Rosenthal, 1979; Hedges, 1992). This phenomenon may occur to some extent, which is unfortunate since non-significant results are important information within the context of the broader state of knowledge on a subject, as this synthesis demonstrates. On the other hand, investigators form an hypothesis based on a justified rationale; in this case, the expectation that an environmental factor known to affect microbial processes might also affect microbial diversity (Allison and Martiny, 2008). The high prevalence of significant variation in lotic microbial diversity in response to all evaluated drivers is most likely a consequence of widespread microbial sensitivity to environmental change (Shade et al., 2012).

### Spatiotemporal Heterogeneity

Microbes may be particularly sensitive to changes in their environment due to their small size and rapid growth rates. This high microbial turnover potential is reflected as a higher prevalence of temporal variation (93%) than longitudinal (83%) or among-stream (86%) variation in lotic microbiota (**Figures 3** and **6**). This temporal variation includes seasonal changes (Feris et al., 2003b; Olapade and Leff, 2004; Crump and Hobbie, 2005; Hullar et al., 2006), successional turnover on the order of days to weeks (Gessner et al., 1993; Lyautey et al., 2005; Wey et al., 2012; Wymore et al., 2013), and shifts with transient or less predictable durations directly associated with hydrological fluctuations (Battin et al., 2001; Rees et al., 2006; Chiaramonte et al., 2013; Fazi et al., 2013). These temporal changes can be correlated with multiple defined environmental drivers: Most notably, seasonal changes in water temperature, hydrology, OM quality and nutrient availability can be relatively predictable, and seasonal synchrony among years and streams in lotic bacterioplankton, epilithon, and sediment microbial diversity has been documented (Sutton and Findlay, 2003; Hullar et al., 2006; Crump et al., 2009). This is intriguing since it suggests that microbial community composition could be predictable based on environmental factors, as has been shown in lakes, estuaries, and oceans (Fuhrman et al., 2006; Ladau et al., 2013). However, predictability could be particularly challenging in stream and river ecosystems due to their variable hydrology and associated potential for cell dispersal via microbial transport (Bell et al., 1982a; Crump et al., 2012). Compared to other aquatic ecosystems, soils, and several other broad habitat types, stream ecosystems have the highest measured indices of temporal variability in microbial community composition (Portillo et al., 2012; Shade et al., 2013). Stream and river ecosystems, and their macrofauna, are characterized by temporal variability (Poff and Ward, 1989), and a high level of temporal turnover in microbial community composition may also be characteristic of lotic systems.

An even stronger and perhaps more surprising result was the widespread effect of compartment type on microbial diversity (**Figure 3**). Differences in stream microbiota between surface water, rock surfaces, leaves and wood, and streambed sediment – within the same stream – were more common than any other spatiotemporal effect. Only one study found similar microbial diversity between compartment types; this study took place in a contaminated stream where the high metals concentrations were hypothesized to limit fungal diversity (Sridhar et al., 2008). Bacterial community composition was remarkably consistent within compartment types in samples collected from a wide range of sites (**Figure 4**). The high relative abundance of Cyanobacteria and Bacteroidetes in epilithic biofilm makes sense, as autotrophic microbes such as Cyanobacteria are more competitive on an inorganic substrate, and Bacteroidetes are known to be characteristic of well-developed stream biofilms (Besemer et al.,

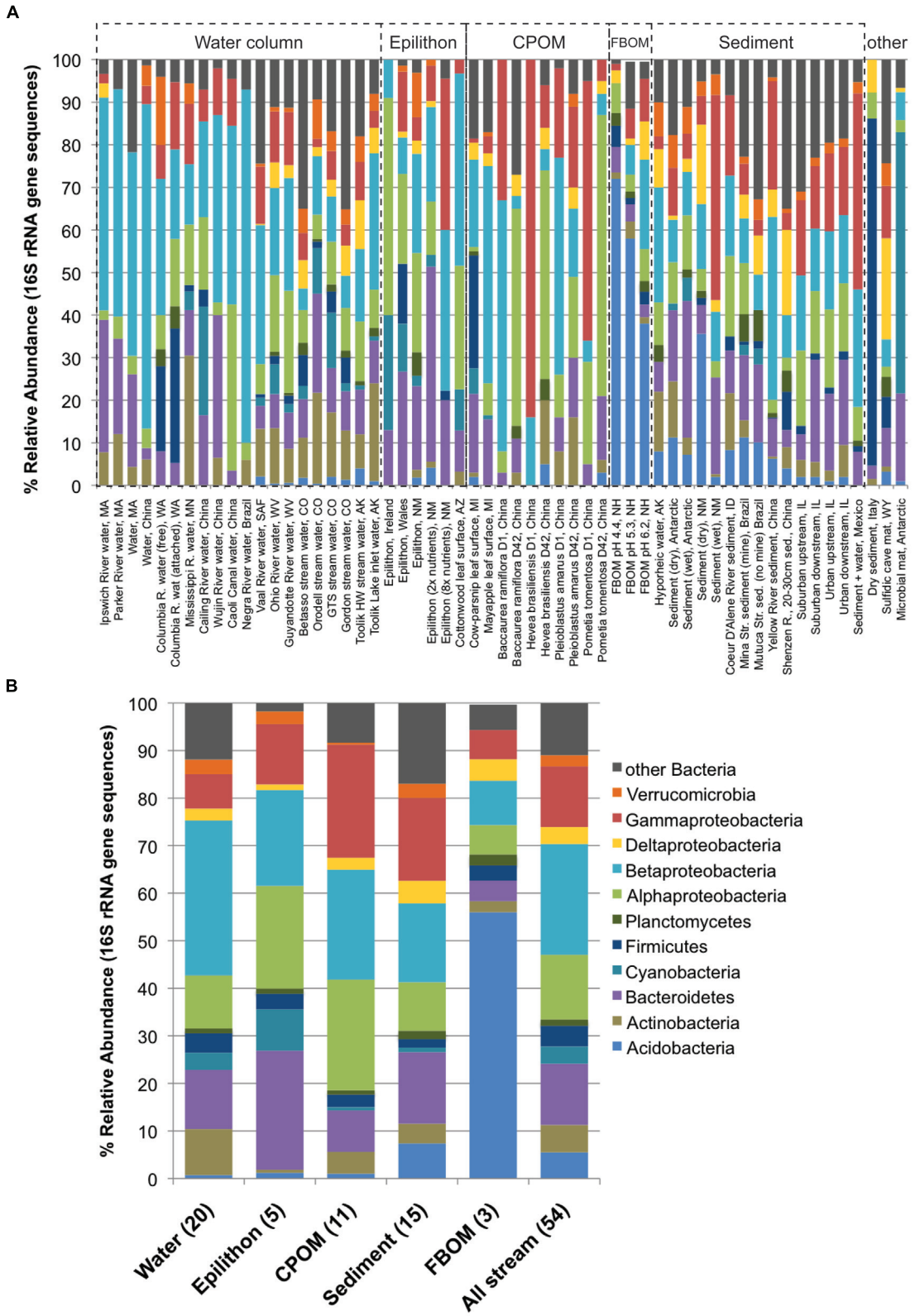


**FIGURE 4 | Relative abundance of major bacterial phyla and subphyla (based on 16S rRNA gene sequence libraries, available from 29 papers) for all available defined compartments: (A) all samples with habitats and locations noted along the *x*-axis and compartment type bracketed by dashed lines, noted above each group; (B) average community composition for each compartment.**

**TABLE 2 | Relative abundance [%, (1 SE)] of bacterial phyla and subphyla (rows) in stream compartments (columns) including analysis of variance (ANOVA) results for among-compartment comparisons: omnibus results in first column, and significant multiple comparisons groups (Bonferonni *post hoc* test, $\alpha = 0.05$) in lower case superscripts (a-d).**

| | ANOVA results (*F, P*) | Water column | Epilithon | CPOM | Fine benthic organic matter (FBOM) | Sediment |
|---|---|---|---|---|---|---|
| Acidobacteria | 60.9, <0.0001 | 0.69[a] (0.24) | 1.20[ab] (0.82) | 0.91[ab] (0.51) | 56.0[d] (9.9) | 7.40[c] (2.2) |
| Actinobacteria | 3.15, 0.022 | 9.70[b] (1.8) | 0.65[a] (0.40) | 4.48[ab] (1.8) | 2.33[ab] (1.3) | 4.81[ab] (0.83) |
| Bacteroidetes | 4.02, 0.007 | 12.5[a] (9.3) | 25.0[b] (13) | 8.79[a] (6.4) | 4.33[a] (1.5) | 14.5[ab] (8.1) |
| Cyanobacteria | 2.46, 0.058 | 3.57[ab] (1.4) | 8.69[b] (5.6) | 3.25[ab] (0.98) | 0.00[a] (0) | 0.88[ab] (0.44) |
| Firmicutes | 0.319, 0.864 | 4.09 (1.8) | 3.27 (2.7) | 2.41 (2.4) | 3.17 (1.0) | 1.68 (0.58) |
| Planctomycetes | 0.852, 0.499 | 1.03 (0.34) | 1.10 (1.1) | 0.82 (0.50) | 2.33 (0.44) | 1.91 (0.65) |
| Alphaproteobacteria | 2.74, 0.039 | 11.1 (2.0) | 21.6 (8.4) | 23.8 (6.8) | 6.17 (1.1) | 10.2 (1.6) |
| Betaproteobacteria | 2.26, 0.076 | 32.6 (2.7) | 20.2 (5.4) | 25.2 (6.6) | 9.33 (6.2) | 17.3 (2.4) |
| Deltaproteobacteria | 1.27, 0.296 | 2.50 (0.51) | 1.17 (0.57) | 2.27 (0.69) | 4.50 (2.3) | 5.04 (1.7) |
| Gammaproteobacteria | 1.96, 0.115 | 7.26 (1.1) | 12.7 (6.2) | 21.9 (8.3) | 6.17 (2.5) | 16.5 (3.6) |
| Verrucomicrobia | 2.10, 0.095 | 3.07 (0.92) | 2.64 (1.97) | 0.36 (0.28) | 0.00 (0) | 3.30 (0.64) |
| Other bacteria | 3.03, 0.026 | 11.9[ab] (2.3) | 1.78[a] (0.87) | 7.59[ab] (2.8) | 5.33[ab] (3.0) | 16.5[b] (2.8) |

2009). Stream and river sediments might contain more pore-scale heterogeneity and anaerobic microsites (Kemp and Dodds, 2001) that promote a greater abundance of narrower phyla or unknown taxa. It is less clear why Actinobacteria might be characteristic of surface waters or Acidobacteria might be characteristic of FBOM. This result does suggest, however, that characteristics of the within-stream physicochemical environment could select for specific groups of microorganisms, and that some physiological characteristics allowing successful colonization of different microbial habitats may be conserved at the phylum level (Phillippot et al., 2010).

The major physicochemical differences among lotic "microbial habitat" compartments are primarily OM type, which could select for microbes best suited to different categories of C processing, and surface type, which could select for microbes best suited to attachment and growth under certain conditions. The CPOM habitat consists of primarily complex polymeric OM, lignin, cellulose and lignocellulose, which can select for microbes that produce extracellular enzymes with oxidative and hydrolytic capabilities, such as fungi. Fine particulate OM habitat contains fragmented and processed OM with more surface area for bacterial colonization (Sinsabaugh et al., 1992). The sediment habitat may be the most heterogeneous in terms of OM type, containing a mixture of buried OM of all sizes and ages, and in terms of physical structure, with inorganic particles of widely varied texture and the potential to set up steep diversity and productivity gradients under saturated conditions (Ellis et al., 1998; Findlay et al., 2003). The epilithic habitat provides no OM at early stages in biofilm development, though mature biofilms contain primary producers that exude C compounds, and entrain particulate OM. Water column habitat supports both suspended and particle-associated bacterioplankton (Crump et al., 1999) and contains a combination of algal and exogenous C. In addition to the contrasting OM substrates characterizing these habitats, they present surfaces with different texture and area for microbial colonization: surface roughness and water flow conditions affect the trajectory and diversity of cells inhabiting biofilms and the exchange of cells and particles between biofilms and the surface water (Battin et al., 2003; Arnon et al., 2010; Singer et al., 2010). This mosaic of selective habitats within a stream (Pringle et al., 1988), combined with the strong mass effects of flowing waters (Crump et al., 2007, 2012), make lotic ecosystems prime test grounds for questions about microbial community assembly within a metacommunity context (Logue et al., 2011).

## Defined Drivers Effects

Specific drivers of environmental change had varied frequencies of significant influence on stream and river microbial diversity (**Figure 5**), with the exception of the always-significant effect of metals. As noted earlier, the only study that reported no significant differentiation among OM compartments was undertaken in metal-contaminated streams (**Figure 3**; Sridhar et al., 2008). This ubiquitous result reflects the acute toxic effects that metals can have on cells with no tolerance adaptations (Genter and Lehman, 2000). Also, large changes in pH and concentrations of electron donors and acceptors, as found in acid mine drainage

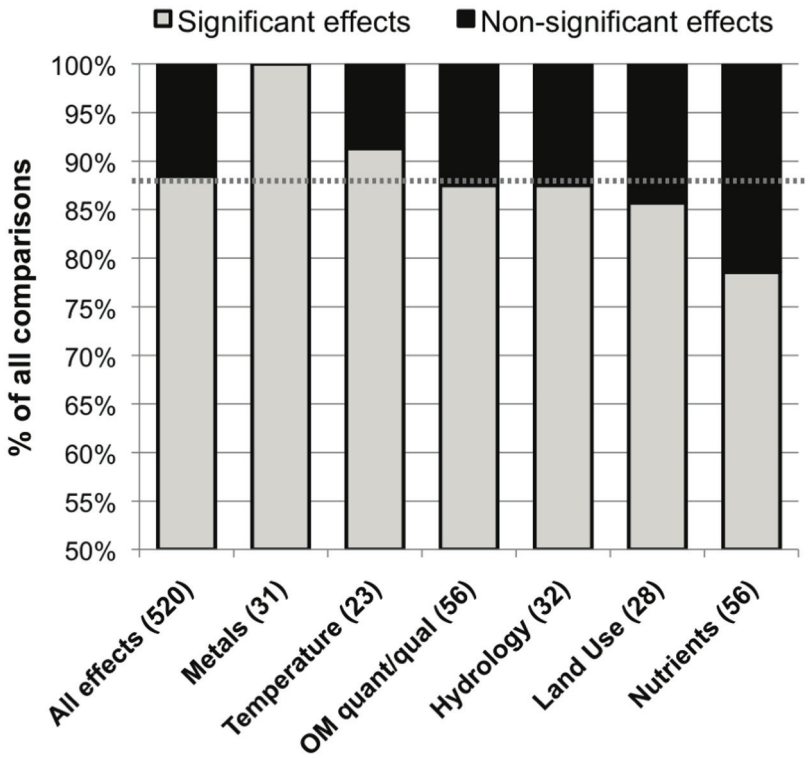


**FIGURE 5 | Distribution of significant (in gray) and non-significant (in black) reported effects of categories of variation in defined environmental drivers on stream microbial diversity, with number of included studies noted for each category along the x-axis, and the percentage of significant effects for all comparisons combined noted as a dashed line for reference.**

streams, creates conditions favorable for very specific microbial metabolic functions and taxonomic groups (Niyogi et al., 2002; Kim et al., 2009; Yu et al., 2010). In soils, pH can vary widely and is correlated with broad differences in microbial community composition and function (Sinsabaugh et al., 2008; Lauber et al., 2009; Rousk et al., 2010); however, in lotic ecosystems the isolated effect of pH on microbial diversity has been studied less frequently and has varied effects on microbial diversity (Fierer et al., 2007; Simon et al., 2009). Field studies within the "metals" category were associated with current or historic mining or industrial activities in the watershed (Feris et al., 2003a; Ancion et al., 2010); however, none were associated with an effect of pH in isolation from metals contamination (Baudoin et al., 2008). This reflects the common covariance of multiple environmental stressors, such as metals and pH, associated with watershed mining activities (Palmer et al., 2010) and urbanization (Grimm et al., 2008), and highlights the dramatic impact that these factors may have in delimiting microbial niches.

In contrast, significant land-use and nutrients effects on lotic microbial diversity were least often observed (**Figures 5** and **6**). This may point to a relatively high level of functional diversity and redundancy regarding nutrient kinetics within natural microbial communities: cells may have strategies to operate under a range of nutrient concentrations (Findlay and Sinsabaugh, 2003), and taxa with contrasting nutrient affinities may often coexist (Martens-Habbena et al., 2009), possibly due to dormancy or low but persistent viability during suboptimal conditions (Jones and Lennon, 2010; Shade et al., 2014). In complement, other environmental drivers may have a stronger effect on limiting the competitive advantage of some microbial taxa than nutrient availability. Metals concentrations (Ancion et al., 2013), temperature (Sliva and Williams, 2005; Zhang et al., 2012), OM quantity or quality (Zoppini et al., 2010; Marano et al., 2011), hydrological factors (Sliva and Williams, 2005; Zoppini et al., 2010), or other site-specific factors (Oliveira and Goulder, 2006; Comte and del Giorgio, 2009; Perez et al., 2012; Washington et al., 2013) could cause stricter physiological limitations on cell success than nutrient availability. This functional redundancy and relative insensitivity of stream and river microbiota to changing nutrient concentrations helps to explain situations in which nutrient concentrations are better predictors of microbial function than microbial diversity (Baxter et al., 2012).

On the other hand, it is important to remember that, while least common, nutrient effects on lotic microbial diversity were significant in 79% of studies. These effects included a number of experiments that applied nutrient enrichment in the absence of other environmental variation (Sridhar and Bärlocher, 2000; Olapade and Leff, 2005; Artigas et al., 2008; Van Horn et al., 2011). Many environmental nutrient gradients include covariates, however. Wastewater treatment effluent, which can cause shifts in river temperature, salinity and bacterial load, as well as increased nutrient concentration, was often influential on

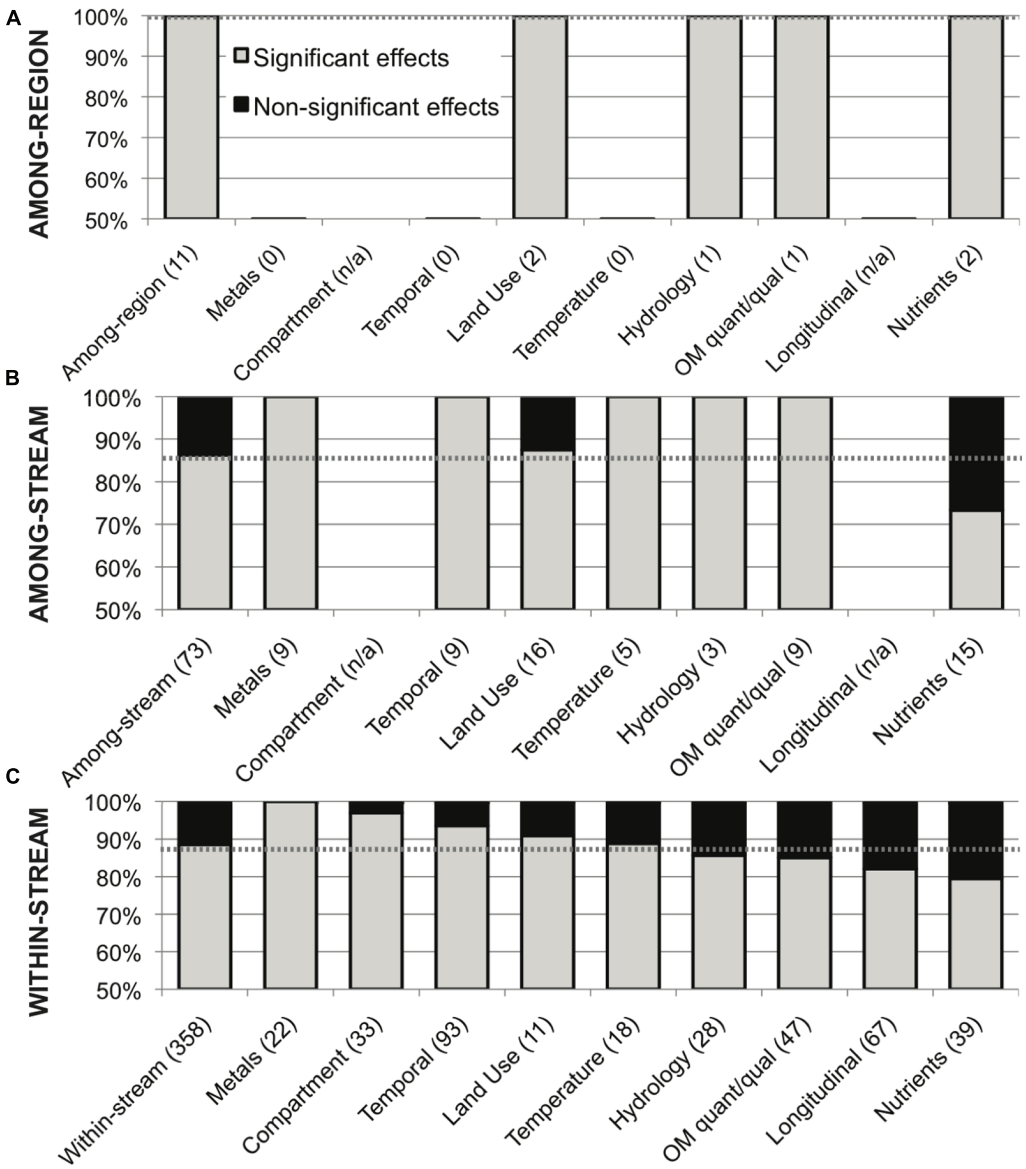


**FIGURE 6 | Distribution of significant (in gray) and non-significant (in black) reported effects of categories of variation in all environmental drivers on stream microbial diversity, for (A) among-region comparisons, (B) among-stream comparisons and (C) within-stream comparisons; with number of included studies noted for each category along the x-axis, and the percentage of significant effects for all comparisons combined noted as a dashed line for reference, for each scale.**

microbial diversity (Wakelin et al., 2008; Angel et al., 2010; Drury et al., 2013; Sonthiphand et al., 2013). Nutrient impacts on stream microbiota from land-use change are often accompanied by differences in riparian vegetation and cover (thus OM quality), temperature, geomorphology and hydrological connectivity, and organic pollutants (Findlay and Sinsabaugh, 2006; Dorigo et al., 2009; Villeneuve et al., 2011). Thus, it is critical to consider both the individual and interactive impacts of changes in nutrient concentrations and other environmental alterations on microbial diversity, functional redundancy, and integrated function. Future studies on lotic microbial nutrient sensitivity should strive to utilize experimental data, in addition to observational data, to make the largest strides in understanding the functional redundancy and hierarchical environmental controls on stream microbial structure and function (Goodman et al., 2015).

## Lotic Microbial Diversity is Critical and Understudied

While the importance of microbial diversity to ecosystem function, and the threats that environmental changes pose to diversity and function, are key areas of research in all aquatic ecosystems, lotic microbial diversity has generally received less attention than marine and lentic microbial diversity (Zinger et al., 2012).

Comparing and contrasting key points in the current state of knowledge in both lotic and better-studied aquatic systems highlights areas where complementary research can further advance understanding of diversity-function relationships in aquatic systems generally.

While most work on aquatic microbial diversity has focused on planktonic communities (Zwart et al., 2002; Hahn, 2006; Smith, 2007; Newton et al., 2011; Ladau et al., 2013), in lotic ecosystems the diversity of surface-attached biofilms in benthic habitats is better studied (**Figure 1D**). The dominant controls on bacterioplankton diversity, such as light and algal abundance, predation, temperature and salinity (Fuhrman et al., 2006; Kent et al., 2007; Lefort and Gasol, 2013), may differ from dominant controls on benthic microbial diversity, due to the contrasting energy sources and physical conditions of pelagic versus benthic habitats (Covich et al., 2004). A predominant contrast between these habitats is the relative importance of algal vs. terrestrial-derived OM substrates, and the coarse differences between water column, epilithon, CPOM, FBOM, and sediment associated bacterial community composition in lotic systems (**Figure 4**; **Table 2**) may be related in part to autochthonous versus allochtonous OM source, as noted earlier. While it is possible that the structure and function of microbiota on allochthonous substrates responds differently to environmental changes than those associated with autochthonous substrates (Dodds, 2006), like primary production, decomposition and microbial mineralization of terrestrial OM in streams responds positively to changes in nutrient concentrations and temperature (Boyero et al., 2011; Rosemond et al., 2015). The topic of microbial metabolism of algal versus terrestrial OM is increasingly relevant in all aquatic systems (Del Giorgio et al., 1997; Pace et al., 2004), and hypotheses based on microbial diversity and function in heterogeneous lotic habitats can inform understanding of carbon cycling across changing, and connected, aquatic landscapes (Battin et al., 2008).

In addition to the organic substrate heterogeneity presented by the benthos-dominated lotic ecosystem, the level of interaction between benthic and pelagic habitats affects aquatic diversity and function (Covich et al., 2004). Bacterioplankton community composition can be temporally predictable, in concert with temporal variation in environmental parameters such as light and temperature (Fuhrman et al., 2006; Kan et al., 2006; Kent et al., 2007; Crump et al., 2009; Eiler et al., 2012). Benthic surface- and sediment-attached microbiota are exposed to variability in physical factors, such as shear stress and surface roughness, as well as changes in light and water quality, and biofilm formation is a predominant characteristic of lotic microbiota. Thus, the large heterogeneity in surfaces available for colonization within a stream reach presents a variety of contrasting selective environments (Battin et al., 2007). Also, bacterioplankon diversity is impacted by water residence time, (Crump et al., 2004; Lindström and Bergström, 2004), and the flowing water of lotic systems clearly acts to transport microbial cells relatively quickly from up- to downstream habitats (Crump et al., 2007; Riemann et al., 2008). Thus, both dispersal and environmental filtering are strong forces in microbial biofilm community assembly in stream and river ecosystems, making lotic systems highly appropriate for understanding metacommunity dynamics (Logue et al., 2011; Besemer et al., 2012, 2013).

Surface-attached and bed sediment-entrained cells are not always accounted for in broad views of microbial life in aquatic ecosystems (Whitman et al., 1998), yet their activity drives biogeochemical processes at reach, watershed and continental scales (Likens et al., 1970; Mulholland et al., 2008; Rosemond et al., 2015). Further research on lotic microbial diversity, including themes such as community assembly, OM source and metabolism, and functional diversity and redundancy under multi-factor environmental variability, is critical to understanding and managing aquatic ecosystem functions in a changing world. Yet lotic microbial diversity is still understudied: Many studies to date are observational, few are experimental; most take place within one stream, few employ cross-site comparisons that would facilitate identification of universal drivers. Given the importance of stream microbes to global biogeochemical cycles, the rapidly increasing accessibility of molecular tools and data, and the relevance of stream microbiota to larger ecological questions, there is still a lot to be learned about lotic microbial diversity.

## Author Contribution

LZ collected and synthesized the data and wrote the manuscript.

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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.2015.00454/abstract

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