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# THE CAUSES AND CONSEQUENCES OF MICROBIAL COMMUNITY STRUCTURE

Topic Editors
Diana R. Nemergut, Ashley Shade
and Cyrille Violle

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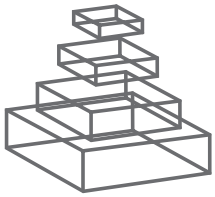
MICROBIOLOGY



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**ISSN** 1664-8714
**ISBN** 978-2-88919-361-5
**DOI** 10.3389/978-2-88919-361-5


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# THE CAUSES AND CONSEQUENCES OF MICROBIAL COMMUNITY STRUCTURE

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The causes and consequences of differences in microbial community structure, defined here as the relative proportions of rare and abundant organisms within a community, are poorly understood. Articles in "The Causes and Consequences of Microbial Community Structure", use empirical or modeling approaches as well as literature reviews to enrich our mechanistic understanding of the controls over the relationship between community structure and ecosystem processes. Specifically, authors address the role of trait distributions and trade-offs, species-species interactions, evolutionary dynamics, community assembly processes and physical controls in affecting 'who's there' and 'what they are doing.'

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frontiers in
MICROBIOLOGY

EDITORIAL
published: 26 September 2014
doi: 10.3389/fmicb.2014.00497

# When, where and how does microbial community composition matter?

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**Keywords: structure-function, biodiversity-ecosystem function, trait-based approaches, species-species interactions, ecological trade-offs, microbial community assembly, trait distributions**

Our planet is experiencing rates of environmental change unprecedented in modern times, and an understanding of how microbes both mediate and respond to these shifts is an important research challenge (De Vries and Shade, 2013). Because of the temporal and spatial scales over which microbes function as well as their extreme diversity, dynamics in microbial structure and processes are typically examined at the community level. However, the factors that drive patterns in microbial structure and function, and the links between them, remain widely debated (Prosser et al., 2007). In this issue, such patterns in microbial communities are further documented for soils, lakes, streams and ocean provinces (Arnosti et al., 2012; Jones et al., 2012; King et al., 2012; Larouche et al., 2012). Additionally, the importance of spatial and temporal dynamics (Armitage et al., 2012; Arnosti et al., 2012; Jones et al., 2012; Larouche et al., 2012) and interactions with macrobiota (King et al., 2012) in driving these patterns is demonstrated. Yet, a central but unanswered question is: "does knowing who is there help us to better understand what they are doing?" Indeed, as shown here by Salles et al. (2012), links between structure and function can often be weak, both at the level of the individual and at the level of the community. Several papers in this special issue, "The Causes and Consequences of Microbial Community Structure," use empirical or modeling approaches as well as literature reviews to enrich our mechanistic understanding of the controls over the relationship between community structure and ecosystem processes. Specifically, authors address the role of trait distributions and trade-offs, species-species interactions, evolutionary dynamics, community assembly processes and physical controls in affecting "who's there" and "what they are doing."

Trait-based approaches can provide mechanistic links between community structure and function, and are gaining popularity in microbial ecology (Krause et al., 2014). Importantly, the distribution of traits within a community may affect the relationship between structure and function (Webb et al., 2010). Thus, as highlighted in this issue by Comte et al. (2013), traits can be considered at both the individual and the community level, where trait distributions may have important implications for emergent properties (e.g., redundancy). Indeed, Shade et al. (2012) highlight a variety of traits that may govern the stability of individual organisms, populations and communities including plasticity, tolerance and dormancy. Folse and Allison (2012) used a multi-nutrient, multi-genotype model of enzyme activity, and showed that trait distributions could yield insight into the relationships between biodiversity and ecosystem function. They found that generalists dominated at low levels of community diversity when rates of enzyme production and enzyme diffusion were lowest. Matias et al. (2013) used a simple microcosm experiment and examined the response of assembled communities to fluctuations in salinity. Their results were somewhat different from Folse and Allison (2012), as they found that community diversity was positively related to productivity and that generalists were more productive and less variable over time. Their work also showed that there did not appear to be a fitness trade-off associated with generalization. Comte et al. (2013) took a novel approach to examine plasticity and redundancy in freshwater bacterioplankton communities, and described explicit metrics to track these traits within community transplant experiments. They showed that plasticity appeared to be an intrinsic community property while redundancy was affected by external environmental factors. Their work also revealed strong relationships between community plasticity and redundancy, with no evidence for trade-offs and a possible co-selection of these attributes.

As well, species-species interactions can affect the relationship between communities and processes. In the model presented by Folse and Allison (2012), the importance of both "coalitions" of complementary organisms and the abundance of "cheaters," or organisms that use a public good without contributing to its production, increased under high levels of enzyme production. They also found that the presence of cheaters could affect the relationship between biodiversity and function. Fox (2012) offered a cautionary tale in terms of our ability to interpret relationships

between abundance and "adaptedness" because of organismal interactions. He used a consumer-resource model to demonstrate that, at medium levels of niche overlap, outcomes of competition can be unpredictable, decoupling relationships between abundance and adaptation.

Evolutionary dynamics can also alter relationships between structure and function. In a Perspectives Article, Choudoir et al. (2012) advocate for population-level approaches to examining microbial community diversity, emphasizing that organisms with exactly the same 16S rRNA gene sequence can exhibit very different ecological dynamics. Indeed, Salles et al. (2012) examined the links between rates of denitrification and phylogenies and highlighted the potential importance of horizontal gene transfer (HGT) by showing that similarity in *nirK* genes, which are thought to be subject to HGT, is not related to $N_2O$ accumulation rates. Furthermore, for *nirS* and 16S rRNA genes, Salles et al. (2012) showed that there was more explanatory power between structure and function at finer scales of phylogenetic resolution for denitrification and metabolic profiles respectively. Pearce et al. (2012) used metagenomics to examine a soil microbial community from Mars Oasis, Antarctica, and showed that while genera-level diversity was limited, species-level diversity was high. They proposed that this suggests strong selection on the types of taxa that can inhabit this extreme environment combined with high rates of diversification within those lineages. Related, Knope et al. (2012) used a microcosm approach to examine the importance of evolutionary history for diversification in bacteria. They showed that prior exposure to an environmental challenge led to higher rates of diversification. These studies suggest that understanding the coupling of ecological and evolutionary processes is key for interpreting microbial community patterns of structure and function.

Community assembly processes may also alter the relationship between "who's there" and "what they do" (Nemergut et al., 2013). Knope et al. (2012) found that arriving in a community first led to a greater degree of diversification within bacteria, likely because of niche-preemption. Pholchan et al. (2013) used a variety of manipulations to alter microbial community assembly in sludge reactors and showed that relationships between biodiversity and ecosystem function in these systems were unpredictable. They hypothesized that the relative importance of stochastic vs. deterministic assembly processes could change the relationship between biodiversity and ecosystem function. In their comment on the Pholchan manuscript, Knelman and Nemergut (2014) provide a conceptual framework illustrating how assembly, biodiversity and function may be related. Together, these studies provide growing evidence for the importance of assembly processes in determining microbial community properties.

Physical dynamics may also be key in regulating the relationship between structure and function. Schimel and Schaeffer (2012) propose a conceptual framework that highlights a requirement that biological processes need to be rate limiting or fate determining in order for community structure to matter for ecosystem function. For example, they propose that structure is not likely to be relevant for organic matter breakdown in mineral soils, where diffusion is limited and organic particles may be occluded or sorbed to soil surfaces. Likewise, Folse and Allison (2012) demonstrate that rates of diffusion of enzymes can affect community diversity and the relative proportion of generalists to specialists. Their work also showed high rates of diffusion coupled to high rates of production can lead to community bottlenecks and increases in stochasticity. As well, King et al. (2012) found that physical dynamics may also affect biotic relationships. They found that associations between plants and microbial community composition were less pronounced at higher elevations, likely due to an increase in the influence of physical harshness on community composition.

Together, the studies in this special issue highlight the role of a variety of ecological, evolutionary and physical dynamics in microbial community structure and function (**Figure 1**). This body of work emphasizes the importance of emergent, aggregate community properties and the role of community dynamics in variations in the strength of the structure-function relationships. As Schimel wrote in 1995 "At a small enough scale, microbial community structure must be a dominant control on ecological processes, but as we move up in scale toward the ecosystem and integrate across many individual communities, the influence of microbial community structures decreases." Predicting when, where, how, and at what scale microbial communities may

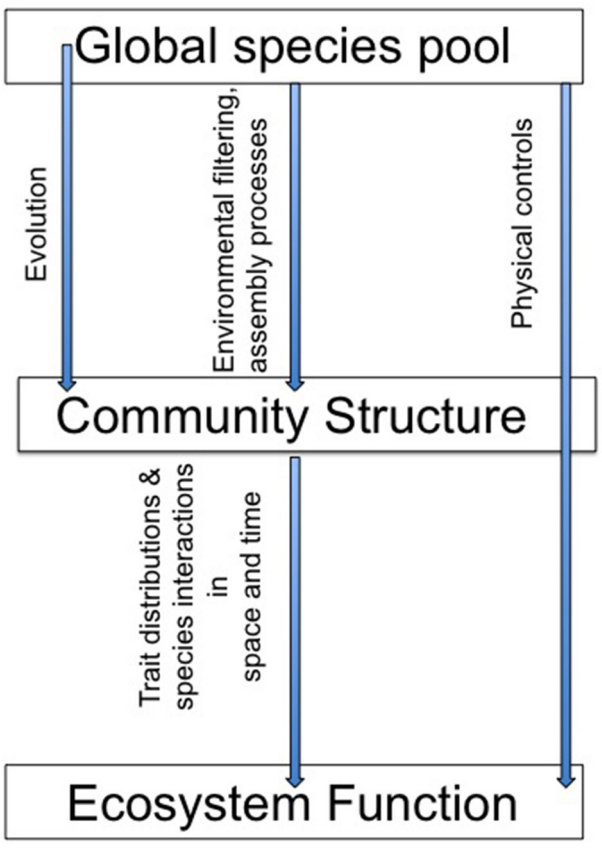


**FIGURE 1 | Does "who's there" matter for "what they do"?** The papers in this special issue use modeling, empirical approaches, and literature reviews to address a suite of controls over the relationship between community structure and ecosystem function.

respond to environmental changes remains a research priority and these papers present new insights into this challenge.

## ACKNOWLEDGMENTS

Cyrille Violle was supported by a Marie Curie International Outgoing Fellowship within the 7th European Community Framework Program (DiversiTraits project, no. 221060).

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

*Received: 06 August 2014; accepted: 03 September 2014; published online: 26 September 2014.*

*Citation: Nemergut DR, Shade A and Violle C (2014) When, where and how does microbial community composition matter? Front. Microbiol. **5**:497. doi: 10.3389/fmicb.2014.00497*

*This article was submitted to Terrestrial Microbiology, a section of the journal Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 10 August 2012
doi: 10.3389/fmicb.2012.00293

# Millimeter-scale patterns of phylogenetic and trait diversity in a salt marsh microbial mat

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Intertidal microbial mats are comprised of distinctly colored millimeter-thick layers whose communities organize in response to environmental gradients such as light availability, oxygen/sulfur concentrations, and redox potential. Here, slight changes in depth correspond to sharp niche boundaries. We explore the patterns of biodiversity along this depth gradient as it relates to functional groups of bacteria, as well as trait-encoding genes. We used molecular techniques to determine how the mat's layers differed from one another with respect to taxonomic, phylogenetic, and trait diversity, and used these metrics to assess potential drivers of community assembly. We used a range of null models to compute the degree of phylogenetic and functional dispersion for each layer. The SSU-rRNA reads were dominated by Cyanobacteria and Chromatiales, but contained a high taxonomic diversity. The composition of each mat core was significantly different for developmental stage, year, and layer. Phylogenetic richness and evenness positively covaried with depth, and trait richness tended to decrease with depth. We found evidence for significant phylogenetic clustering for all bacteria below the surface layer, supporting the role of habitat filtering in the assembly of mat layers. However, this signal disappeared when the phylogenetic dispersion of particular functional groups, such as oxygenic phototrophs, was measured. Overall, trait diversity measured by orthologous genes was also lower than would be expected by chance, except for genes related to photosynthesis in the topmost layer. Additionally, we show how the choice of taxa pools, null models, spatial scale, and phylogenies can impact our ability to test hypotheses pertaining to community assembly. Our results demonstrate that given the appropriate physiochemical conditions, strong phylogenetic, and trait variation, as well as habitat filtering, can occur at the millimeter-scale.

**Keywords: microbial mat, community assembly, biodiversity, phylogenetics, null models, metagenomics, salt marsh**

## INTRODUCTION

The mounting evidence that biodiversity *per se* positively affects the emergent functions of an ecosystem justifies further studies of the mechanisms by which taxa coexist (Loreau et al., 2001; Hooper et al., 2005). Much of this theory is built on data from eukaryotes, due mainly to our inability to survey bacterial, archaeal, and viral (henceforth microbial) assemblages in their natural environments (but see Bell et al., 2005). Beyond driving a number of critical biogeochemical functions, microbes encompass a tremendous pool of undescribed biodiversity on earth (Curtis and Sloan, 2004; Quince et al., 2008). Censuses of microbial diversity commonly encounter staggering levels of genetic and taxonomic information, and often lead to the discovery of novel biological functions (Cowan et al., 2005). In particular, the metagenomic and targeted-gene amplicon approaches to microbial ecology can be combined to visualize and statistically compare multiple dimensions of biodiversity within and between environmental samples (Tyson et al., 2004; Sogin et al., 2006).

Biodiversity within a microbial community can be defined in three fundamental ways. Taxonomic diversity, or the number of arbitrarily similar units and their abundance distributions, is the traditional metric by which communities are defined and compared. Trait diversity, or the breadth of phenotypic, rather than genotypic, differences among individuals also has a rich history of use in ecology. Certain traits are used to signify the role an organism plays in the context of biotic and abiotic interactions. These traits are termed "functional" in the sense that they influence the properties of the greater ecosystem. Because traits are a product of evolutionary dynamics within a population, a phylogenetic perspective on diversity is a useful bridge between taxonomic and trait diversity (Faith, 1992).

It is now recognized that communities are dynamic, arbitrarily bounded assemblages whose members are products of both

contingent, historical processes, and semi-deterministic assembly rules (Ricklefs, 2006). Historical processes that structure communities include the constraints on diversification imposed by biogeography (e.g., dispersal limitation) and evolution (e.g., adaptive radiation, Red Queen dynamics; MacArthur and Wilson, 1967; Vermeij, 1987; Losos et al., 1998; Gillespie, 2004). In contrast, assembly rules are generally defined as contemporary mechanisms which either permit or prohibit an individual or taxon from occupying a particular local habitat (Diamond, 1975; Weiher and Keddy, 1999). While phylogenetic approaches have been a cornerstone in evolutionary biogeography for some time, ecologists have only recently adopted a phylogenetic perspective to tease apart community assembly mechanisms (Webb et al., 2002; Cavender-Bares et al., 2004). Most commonly, the phylogenetic relatedness (or trait similarity) of taxa within a particular habitat is compared to the averaged relatedness of randomized communities whose taxa are sampled from a pool of potential colonizers (called a *regional pool*). If the observed phylogenetic relatedness of a community is significantly lower or higher than the mean (or median) of the randomized communities, then the community is said to be either phylogenetically clustered or overdispersed, respectively (Webb, 2000). If the assumption of phylogenetic niche conservatism is justified, then the differences between two organisms' traits should positively covary with phylogenetic distance (Peterson et al., 1999). Thus, if habitat filtering is a dominant community assembly mechanism, we expect to find phylogenetic and trait clustering in that habitat due to trait-driven niche conservatism. Alternatively, in a scenario involving character displacement, competition between sister taxa will result in divergent selection on their traits (and hence their realized niches). In this case, habitat filtering results in independence between the phylogenetic diversity and trait diversity of a community, and manifests as functional clustering with no pattern to the phylogenetic structure of the community.

The assumption of phylogenetic niche conservatism appears robust at very broad taxonomic levels (e.g., among all Angiosperms), but can break down within smaller clades (e.g., oak species; Cavender-Bares et al., 2006). In microbial communities, horizontal gene transfer (HGT) among distantly related taxa weakens such an assumption at all taxonomic levels. Conversely, certain mono- and polyphyletic clades do possess a suite of traits which make certain habitats much more favorable. For example, the Cyanobacteria require both light and oxygen to carry out photosynthesis and respiration, and thus should generally be found in oxic, photic habitats. Likewise, the microaerophilic and anaerobic non-oxygenic phototrophs require light of particular wavelengths, as well as reduced inorganic sulfur and hydrogen for photosynthesis. Many obligate anaerobes (e.g., order Clostridiales) can similarly be found in habitats satisfying certain abiotic conditions.

Metagenomic shotgun and targeted-gene amplicon sequencing are two complementary culture-independent approaches to assessing microbial biodiversity. The former offers a relatively unbiased view of the suite of genomic information in an environmental sample, provided adequate sequencing depth and assembly steps. Assembled sequence fragments can then be compared against reference databases to predict their structures and potential functions. The downsides to this approach include erroneous predictions of gene function and limitations assessing overall diversity due to inadequate sequencing depth. To overcome the second caveat, phylogenetically informative genes (e.g., SSU-rRNA) can be amplified by PCR and the resulting amplicon pool sequenced alongside or independent of a shotgun metagenome. Depending on the quality and length of shotgun contigs and amplicon sequences, both metagenomic and amplicon approaches can yield information on the taxonomic, phylogenetic, and functional aspects of microbial biodiversity (Burke et al., 2011). Consequently, these data can also be used to inform our understanding of the processes structuring microbial communities. For instance, many studies have found evidence for phylogenetic and functional clustering in microbial assemblages in marine (Barberán and Casamayor, 2010; Kembel et al., 2011; Bryant et al., 2012; Pontarp et al., 2012), freshwater (Horner-Devine and Bohannan, 2006; Newton et al., 2007; Amaral-Zettler et al., 2010; Barberán and Casamayor, 2010), and terrestrial habitats (Horner-Devine and Bohannan, 2006; Bryant et al., 2008; Wang et al., 2012). Although these results are often taken as evidence for habitat filtering, many are based on comparisons of samples collected at distances which are often orders-of-magnitude greater than the scale at which cells are known to interact (Long and Azam, 2001; Dechesne et al., 2006). Therefore, quantification of niche-based community assembly may be confounded by historical biogeographic and evolutionary processes, such as adaptive radiation, genetic drift, and serial founder effects (Ricklefs, 2006; Vamosi et al., 2009; Fine and Kembel, 2011). By measuring the phylogenetic and functional properties of adjacent habitats at a scale permissive of genetic admixture (so-called microhabitats), such results can be more reliably attributed to trait-driven differences in habitat specialization (Webb et al., 2008).

Microbial mats are one of the more conspicuous and well-studied microbial communities (Stal and Caumette, 1994; Seckbach and Oren, 2010). These mats typically form in habitats too extreme to support plant growth, such as hypersaline soils, geothermal springs, and tidal flats. Their laminated appearance is due to vertical segregation of particular guilds of bacteria and diatoms, which assemble in response to millimeter-scale gradients in both light intensity and redox potential (Jørgensen et al., 1979; Revsbech et al., 1983; van Gemerden, 1993). In temperate environments, the top layer is often dominated by oxygenic cyanobacteria and eukaryotic algae and takes on a green hue due to its chlorophyll *a* content. During daylight hours, the oxygen concentration of this layer is equal to or higher than atmospheric levels and decreases with depth to trace levels at 5 mm. Thus, this layer also supports a rich community of aerobic heterotrophs. The production of extracellular polysaccharides (EPS) in the upper layers, however, probably limits the efficacy of larger eukaryotic grazers in capturing prey (Awramik, 1984). Light becomes more diffuse past 3 mm depth, but can still drive non-oxygenic photosynthesis in groups such as purple sulfur bacteria and green sulfur bacteria, provided the appropriate reducing agents are available (Jørgensen and Des Marais, 1986; Pierson et al., 1990). At depths greater than 10 mm, light is absent at wavelengths $<1\,\mu$m and photosynthesis does not occur. Here, the microbial community primarily consists of anaerobic sulfate-reducers, although this form of respiration also

occurs in the mats' photic zones (Pierson et al., 1987; Risatti et al., 1994).

Despite the historical significance of microbial mats, such as their role in the ecology of early Earth (Des Marais, 2003), there have been few molecular surveys of such communities, and even fewer focusing on temperate salt marsh habitats (Ley et al., 2006; Buckley et al., 2008; Kunin et al., 2008; Bolhuis and Stal, 2011; Burow et al., 2012). Our aim was twofold: (1) to present the results of a shotgun metagenomic and targeted-gene amplicon survey of a particularly well-studied salt marsh microbial mat and (2) determine if patterns in the taxonomic, phylogenetic, and functional diversities of the microbial mat show evidence for non-random community assembly. The extreme biotic stratification and abiotic gradients evident in microbial mats led us to predict systematic differences in microscale biodiversity. For instance, because light, oxygen, and sulfur gradients in the mat favor particular metabolic strategies, and since many of these metabolic (particularly photosynthetic) strategies are phylogenetically conserved, taxa present within each layer should be more related to one another than expected by chance, or phylogenetically clustered, when measured over the entire bacterial domain. Under the assumption of phylogenetic niche conservatism, if trait-based habitat filtering is a dominant mechanism of community assembly, functional traits should also be clustered. Alternatively, if HGT is not a product of phylogenetic distance, a widespread prevalence of non-homologous recombination should decouple phylogenetic and trait diversity patterns, and manifest as clustered traits with random phylogenetic dispersion.

**FIGURE 1 | Greater Sippewissett salt marsh microbial mat showing typical lamination (photo credit: NDY).**

## MATERIALS AND METHODS

### STUDY SITE

The Great Sippewissett salt marsh is located west of Falmouth, MA, on Buzzard's Bay (N41°35′13.34″, W70°38′29.10″). The habitat is typical of New England salt marshes, with braided tidal creeks running around dense stands of *Spartina*. Microbial mats form in sandy intertidal sediments which lack colonization by plants, and are identifiable by the leathery, green/gold-colored top layer (Nicholson et al., 1987; Pierson et al., 1987; **Figure 1**).

We collected samples from the Great Sippewissett salt marsh on two occasions: June 23, 2010 and July 6, 2011. In 2010, two cores measuring 2 cm (diameter) × 5 cm were collected from two mat habitats. One of these cores was chosen as an example of an early successional mat. The early successional mat consisted of a wide light-green band of cyanobacteria descending from the surface to approximately 1 cm depth in unconsolidated sandy sediment. The early successional mat lacked a leathery surface layer, conspicuous bands attributable to anoxygenic phototrophs, and the cohesiveness which are all characteristic of climax mat communities in this system (Nicholson et al., 1987). The early successional mat was likely initiated at the end of winter and was developing in close proximity (1–3 m) to an area containing mature mats. Our second core from 2010 was taken from this climax mat system which consisted of a thick leathery surface layer and sharply defined dark green, pink, and brown cohesive layers (as described in Nicholson et al., 1987). Our assumption that these mat sections were of different ages is based on (1) their qualitative differences (slight green banding vs. multicolored layering, loose vs. stabilized sediment) and (2) their close proximity (similar biotic and abiotic characteristics). These definitions are consistent with descriptions of coastal mat development elsewhere (Stal et al., 1985; Mir et al., 1991; Stal and Caumette, 1994). Henceforth, we refer to the two cores from 2010 as "young mat" (YM-10) and "old mat," (OM-10). We collected an additional core from Great Sippewissett salt marsh in 2011. This core was representative of a climax microbial mat community and is referred to as OM-11. Intact mat sections (20 $cm^2$) were returned to the lab for processing. Cores were taken from the center of mat sections, the cores sectioned, and DNA extracted within 2–3 h of collection. The developed mats were sectioned with a sterile razor blade at the boundaries of their colored layers, and the young mat at corresponding depth.

Oxygen concentration and pH measurements were performed in 2011 using OX-50 and a pH-probes attached to a Microsensor Monometer (Unisense, Denmark). *In situ* depth profiling was conducted with a Unisense micromanipulator MM33. We also used abiotic data from previously published studies of the Sippewissett microbial mat including chlorophyll *a*, bacteriochlorophyll *a*, and sulfide (Pierson et al., 1987; Buckley et al., 2008). While these data are not perfectly matched to our samples, the descriptions and locations of the mats used in each study closely resemble our own.

### DNA EXTRACTION AND SEQUENCING

DNA was extracted from each sectioned layer using the MoBio PowerSoil kit according to the manufacturer's instructions. This extraction was prepared for sequencing at the Bay Paul Center (Marine Biological Laboratory, Woods Hole, MA, USA) and the procedure is detailed elsewhere (Huber et al., 2007 Supplementary Materials). Briefly, this involved PCR amplification of the SSU-rRNA v4 through v6 hypervariable regions using degenerate primer sets 518F (5′-CCAGCAGCYGCGGTAAN-3′) and 1064R (consensus: 5′-CGACRRCCATGCANCACCT-3′) for the 2010 cores and primers 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 907R (5′-CCGYCAATTCMTTTRAGTTT-3′) for the 2011 cores (Morrison and Sogin, Unpublished). The forward primers were synthesized to include (from 5′ to 3′) Roche A-adapters, a unique 8-base barcode specific to each sample, a 2-base linker sequence, and the forward primer. Reverse primers were synthesized with the Roche B-adapter linked to the primer via 2-base linker. Template concentration among all extractions was normalized to 15 ng/μl. PCR templates were amplified using 2× Phusion HF polymerase with 8% DMSO for 32–36 cycles, the first 10 cycles using a touchdown annealing temperature from 68 to 58°C followed by 12 cycles of three-step and 10–14 cycles of two step PCR. We used the PicoGreen assay to quantify PCR product and concentrate it to 100 μl. It was then gel-purified using the Montage Kit (Millipore) and shipped to Penn State Genomics Core Facility and sequenced on the Roche GS-FLX Titanium platform.

In addition to the SSU-rRNA amplicon libraries, we performed metagenomic sequencing on the layers of the old mat in 2010. All five samples were given unique multiplex identifier (MID) tags prior to pooling on the microtiter plate. Pyrosequencing was carried out as described above.

### SSU-rRNA PROCESSING

All analyses of the SSU-rRNA amplicons were conducted within the QIIME software package (Caporaso et al., 2010a). Raw 454 reads were quality filtered, dereplicated, and demultiplexed by removing Roche adapters, linkers, primers, and sample barcodes. Sequences shorter than 350 bp and longer than 550 bp were discarded. Next, to expedite chimera detection, we clustered identical reads using uclust (Edgar, 2010). One representative sequence of each cluster was aligned to the Greengenes core set with PyNAST 1.1 (DeSantis et al., 2006; Caporaso et al., 2010b). Chimeric sequences (7,804 total) were detected using the ChimeraSlayer algorithm and subsequently removed (Haas et al., 2011). To examine the effect of phylogenetic resolution on resulting diversity metrics, we used uclust to bin SSU-rRNA sequences into operational taxonomic units (OTUs) using a 97% similarity cutoff using a greedy algorithm. Singleton OTUs (those appearing in only one sample, or represented by only one sequence) were discarded. We assigned taxonomic identities to a sequence representing a particular OTU using the RDP naïve Bayesian classifier (Cole et al., 2009). These representative sequences were once again aligned to the Greengenes core set using PyNAST. Any sequences identified as belonging to archaea, eukarya, or chloroplasts were removed, as were singleton OTUs. The sequences were masked and used to build a phylogenetic tree with FastTree 2.1.4 using the GTR + CAT approximation for substitution rate heterogeneity (Stamatakis, 2006; Price et al., 2010). This software is optimized for phylogenetic inference from large datasets and makes use of nearest-neighbor interchange algorithms to significantly decrease computation time (Price et al., 2010).

### METAGENOME PROCESSING

Shotgun sequences from the 2010 old mat layers were quality filtered and demultiplexed using QIIME's split_libraries procedure. Sequences shorter than 75 bp were discarded. This filtering resulted in approximately 243.8 Mbp. These sequences were then submitted to MG-RAST for dereplication and screening against model organismal DNA (Meyer et al., 2008). Protein prediction was carried out in the MG-RAST pipeline using FragGeneScan (Rho et al., 2010). We used the categorization of the Kyoto Encyclopedia of Genes and Genomes (KEGG) for annotating our sequences (Kanehisa et al., 2012).

### DIVERSITY ESTIMATION

Rather than using a univariate index to characterize the taxonomic richness and evenness of each mat layer, we computed the series of effective numbers, ${}^{q}D^{\mathbf{Z}}(\mathbf{p})$, recently presented by Leinster and Cobbold (2012). This family of metrics is a simple extension of Hill numbers (Hill, 1973) that also account for the similarity between taxa, represented by a matrix $\boldsymbol{Z}_{ij}$. These numbers are generalizations of particular diversity indices, and can be plotted against the parameter $q$, the sensitivity to rare taxa. At $q = 0$, the number equals the taxonomic richness $S$, if a naïve similarity matrix (the identity) is used, otherwise, it is a measure of phylogenetic diversity (Faith, 1992; Chao et al., 2010). At $q = \infty$, the naïve solution is the reciprocal of the Berger–Parker index and gives the inverse proportional abundance ($p$) of the most dominant taxa. To measure the phylogenetic diversity of a community, we used the equation.

$$ {}^{q}D^{z}(\pi) = \left( \sum_{\substack{i,b:i\in I_b,\\ \pi_{(i,b)}>0}} \pi_{(i,b)} (Z\pi)_{(i,b)}^{q-1} \right)^{q/(1-q)} $$

where $\pi_{(i,b)}$ is the relative abundance of "historical species" $(i,b)$, where $b$ is a particular branch terminating in the subset of taxa present in the community: $i \in \{1, 2, 3, \ldots, S\}$, $I_b \subseteq \{1, 2, 3, \ldots, S\}$, and $\boldsymbol{Z}_{(i,b)(j,c)}$ is an asymmetric relatedness matrix (details in Leinster and Cobbold, 2012, Appendix). Additionally, we calculated ${}^{0}D^{\mathbf{Z}}(\mathbf{p})$ with $\boldsymbol{Z} = \boldsymbol{I}$, which is simply the taxonomic richness of the community. For each layer, these values were calculated over a range of $q$ (0–5) averaged over 100 subsamples rarefied to the smallest number of sequences in a layer (Old mat 2010, 10–15 mm, 1071 sequences).

Mean phylogenetic diversity (MPD) was estimated by calculating the average phylogenetic distance of all pairwise branch lengths. For examination of particular mono- and polyphyletic guilds within the overall phylogeny (e.g., Cyanobacteria, sulfur oxidizing bacteria), we trimmed the tree to only include particular clades and recalculated the distance matrix. These values were then averaged over 100 rarefactions normalized to the smallest sample.

### NULL MODEL ANALYSES

We estimated the standard effect size of each community's rarefied mean phylogenetic distance ($MPD_{SES}$) by comparing it to the rarefied values of 999 randomized communities generated using null models. Because the statistical power to detect niche-based community assembly varies with the choice of null model, we assessed the agreement of three different randomization routines: (1) the independent swap algorithm, which shuffles the taxa/sample table; (2) the phylogeny.pool algorithm, which draws random samples from the phylogenetic distance matrix to populate the taxa/sample table; and (3) the taxa.labels algorithm, which shuffles the labels of a phylogenetic distance matrix (Kembel and Hubbell, 2006; Kembel, 2009). In null model three, we calculated phylogenetic diversity both by weighting the relative number of sequences obtained for each OTU as either 0 or 1 (presence/absence) or as $p_{ij}$, the proportional relative abundance of the OTU in its respective sample. We acknowledge that measuring proportional abundance using sequence counts is not optimal, but it still provides a more realistic view of a community compared to presence/absence data. We assessed the robustness of our results by first comparing the results of null model three (taxa.labels) using three definitions of "taxa pools": (1) the entire set of OTUs detected in all samples during all years, (2) the set of OTUs detected in each year, separately, and (3) the OTUs only found in each particular core. We did this with the expectation that phylogenetic dispersion is sensitive to the spatiotemporal scale of the taxa pool (Kembel and Hubbell, 2006; Swenson et al., 2006; Lessard et al., 2012). The first, and least conservative species pool assumes that all OTUs detected in the study are equally likely to colonize all mat layers, independent of year. The last, and most conservative pool assumes that the only OTUs capable of colonizing all three mat cores are those that were detected within all three cores to begin with. Additionally, because weak bifurcation support values in our phylogeny might bias our estimates of standard effect size, we tested the robustness of our findings by collapsing bifurcating nodes into polytomies if the nodes' support values were below a certain threshold (50 and 80%). Samples were considered significantly overdispersed or clustered ($\alpha = 0.05$) if they fell above or below 95% of the randomized communities' values, respectively. We ran these tests for all OTUs detected in the sample, and for three functional guilds: (1) phylum Cyanobacteria (oxygenic phototrophs), (2) order Chromatiales (purple sulfur bacteria), and (3) orders Desulfobacterales, Desulfovibrionales, Syntrophobacteriales, and Clostridiales (anaerobic sulfate-reducers; Risatti et al., 1994).

Trait richness and dispersion of the OM-10 sample was measured using the function-level KEGG ortholog (KO) group, excluding hypothetical proteins at an *e*-value equal to or less than $10^{-5}$ with a minimum alignment length of 50 bp. To correct for sample size bias, we rarefied KO richness to the lowest number of KO genes detected in our sample (0–2 mm; 20,121). We followed the methods of Bryant et al. (2012) in re-sampling without replacement 1,000 simulated sets of KO genes for each community from the pool of KO genes detected throughout the mat core. We compared our observed KO richness to those of our simulated communities to assess whether or not layers contained more or fewer trait-encoding genes than would be expected by chance. We verified our findings by performing the same routine on non-supervised orthologous groups (NOGs), which are annotated algorithmically rather than by hand (Jensen et al., 2008), and clustered orthologous groups (COGs), which are manually curated (Tatusov et al., 1997). We also measured rarefied KO richness patterns for different groups of genes which may be ecologically important in structuring the microbial mat and compared these values to a randomized expectation. These gene categories included (1) total metabolism, (2) photosynthesis, (3) carbohydrate metabolism, (4) sulfur metabolism, (5) ABC transporters, and (6) oxidative phosphorylation. Significance was assessed using the rank test described for $MPD_{SES}$.

### STATISTICAL ANALYSES

To statistically compare phylogenetic richness between communities, we calculated the unweighted SSU-rRNA UniFrac distances between samples, rarefied to the sample with the lowest number of sequences, and clustered using Ward's minimum variance method (Lozupone and Knight, 2005). Statistical significance was assigned to clusters as determined by approximately unbiased (AU) *p*-values calculated from 10,000 multiscale bootstrap dendrograms (Shimodaira, 2004). We used the permutation-based analysis of variance ADONIS to test the null hypotheses of whether differences existed in community OTU composition between years, sites, and depth (Anderson, 2001). We used linear regression to test for a relationship between our Hill diversity measures and pH, depth, chl *a*, Bchl *a*, sulfide, and oxygen concentrations. Because our data appeared non-linear in exploratory analyses, we compared linear and power law (log–log-transformed) fits for the same data and selected the top model based on its coefficient of determination. We calculated regressions both with and without covariates for the age of the mat sample and the year in which it was collected. All quantitative analyses were carried out in R 2.14 (R Development Core Team, 2011) using the packages "vegan 2.0" (Oksanen et al., 2012), "picante 1.3" (Kembel et al., 2010), and custom scripts for rarefaction and diversity profiling (Bryant[1]; Cobbold[2]; Armitage[3]).

## RESULTS

Our measurements of variation in abiotic conditions with depth demonstrate clear gradients. Our oxygen profile showed the predicted spike in concentration between 2 and 3 mm, and decreased to trace levels past 5 mm (**Figure 2**). Likewise, pH declined with depth to 3 mm and remained constant to 6 mm (**Figure 2**).

After quality filtering, dereplication, and singleton removal, we were left with a total of 91,392 SSU-rRNA sequences representing 3,503 OTUs clustered at 97% sequence similarity. The top layers of each cluster were dominated by Cyanobacteria of the family Oscillatoriales, while the lower clusters were heavily dominated by Proteobacteria, including many OTUs classified to the family Chromatiales (**Figure 3**). UniFrac clustering revealed that samples were clustered by year and age (**Figure 4**). Two statistically significant clusters were identified: one containing all but

[1] http://openwetware.org/wiki/User:Jessica_A._Bryant
[2] http://www.maths.gla.ac.uk/∼cc/supplements/diversity.html
[3] http://github.com/darmitage

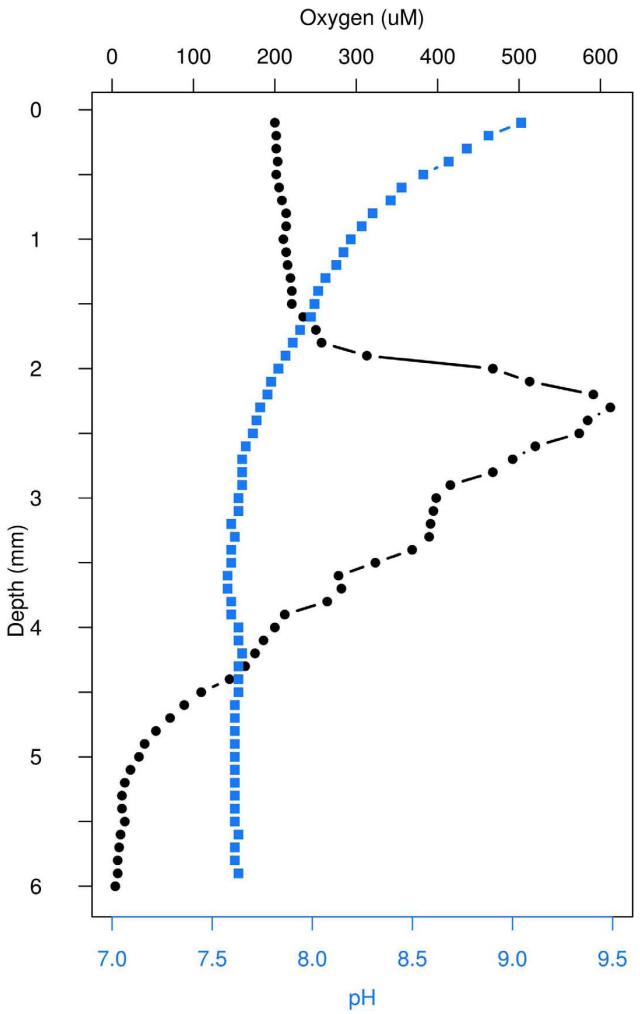


**FIGURE 2 | *In situ* microsensor depth profile for oxygen concentration and pH.**

the top layers of the old mats, and one containing the top layers of all mats and the young mat (AU test; $p < 0.001$; **Figure 4**). We rejected the null hypotheses that communities were compositionally identical ($\alpha = 0.05$) across years ($F_{1,13} = 4.20$, $p < 0.005$), ages ($F_{1,13} = 3.34$, $p < 0.005$), and depth ($F_{1,13} = 2.72$, $p < 0.005$). However, these three effects only explained 50.6% of the variance in our data.

Taxonomic richness, ${}^0D(\mathbf{p})$, and phylogenetic richness, ${}^0D^{\mathbf{Z}}(\pi)$, were both positively associated with depth on the log–log scale, and this result was significant ($p < 0.001$, $R^2 = 0.64$; $p < 0.0001$, $R^2 = 0.75$). Profiles of ${}^qD^{\mathbf{Z}}(\boldsymbol{\pi})$ show that the top layer of each mat had the lowest phylogenetic richness ($q = 0$) and evenness ($q > 0$). Overall phylogenetic richness was greatest in the young mat, with the exception of the top layer, which had the lowest phylogenetic and taxonomic richness of the three cores (**Figure 5**).

Phylogenetic clustering increased linearly with depth when mat age was included as a covariate ($p < 0.05$, $R^2 = 0.65$; **Figure 6**). The old mat samples from 2010 to 2011 showed similar patterns in phylogenetic dispersion, with positive $\text{MPD}_{\text{SES}}$ values in the top layer and significantly clustered communities in the underlying layers. Additionally, we found evidence for an increase in phylogenetic clustering with mat age for depths >2 mm (**Figure 6**). The young mat was similarly patterned, but the significance of the clustering was less than that of the older mats. Our results did not change when phylogenetic bifurcations were collapsed into polytomies at 50 and 80% support values. Our choice of null model did not change most of our results, but the independent swap algorithm dampened the strength of statistical significance compared to the phylogeny-shuffling approaches (**Table A1** in Appendix). Compared to our first taxa pool (all OTUs in all years and samples), pools 2 (within-year), and 3 (within-core) altered magnitude of statistical significance for many of the samples (**Table A1** in Appendix). There were no differences in outcomes between relative abundance-weighted and censored randomizations (**Table A1** in Appendix). Chromatiales (purple sulfur bacteria) exhibited no significant phylogenetic dispersion in any layer. The same was true for Cyanobacteria, except for one significantly clustered community in the top layer of the young mat ($\text{MPD}_{\text{SES}} = -2.11$, rank = 15, $p < 0.05$). Sulfate reducing bacteria were phylogenetically clustered in the young mat, but only significantly so for the 2–5 mm (pink) layer ($\text{MPD}_{\text{SES}} = -3.79$, rank = 7, $p < 0.01$).

Trait richness, measured by rarefied KO and COG annotations, decreased with depth, and appeared to negatively covary with taxonomic and phylogenetic richness. Using the NOG gene annotation, functional richness appeared to slightly increase in the third and fourth layers, but could not be statistically evaluated with only four data points. Total trait richness was significantly lower than expected under a randomized sample for all layers and all ortholog databases, indicating fewer traits were present in each layer than expected by chance (**Figure 7**). Photosynthesis-related KO genes decreased with depth, and were significantly greater than expected by chance in the top layer, and reduced in the underlying layers (**Figure 8**). Richness of metabolic KO genes decreased with depth. Genes related to sulfur metabolism and carbohydrate metabolism remained relatively constant with depth, though the former category had very few KO hits. Genes encoding ABC transporters decreased in richness to the third (brown) layer, and then increased in the bottom layer. Genes coding for oxidative phosphorylation steadily declined with depth.

## DISCUSSION

### COMMUNITY COMPOSITION

Using a combination of bacterial SSU-rRNA amplicons and metagenomics, we quantified patterns in taxonomic, phylogenetic, and trait diversity within a salt marsh microbial mat. Our results, taken in concert with other studies, suggest cyanobacterial mats contain a very diverse community with large variation at the millimeter-scale (Ley et al., 2006; Villanueva et al., 2007; Kunin et al., 2008; Dillon et al., 2009). This variation appeared to be primarily driven by light limitation with depth. At the surface, light drives oxygenic photosynthesis in organisms possessing chlorophyll *a* and favors the oxidation of water to oxygen. As light becomes limited at depths greater than 2 mm, phototrophs possessing bacteriochlorophylls dominate, such as the purple bacteria (e.g., orders Rhodospirillales and Chromatiales) and the green

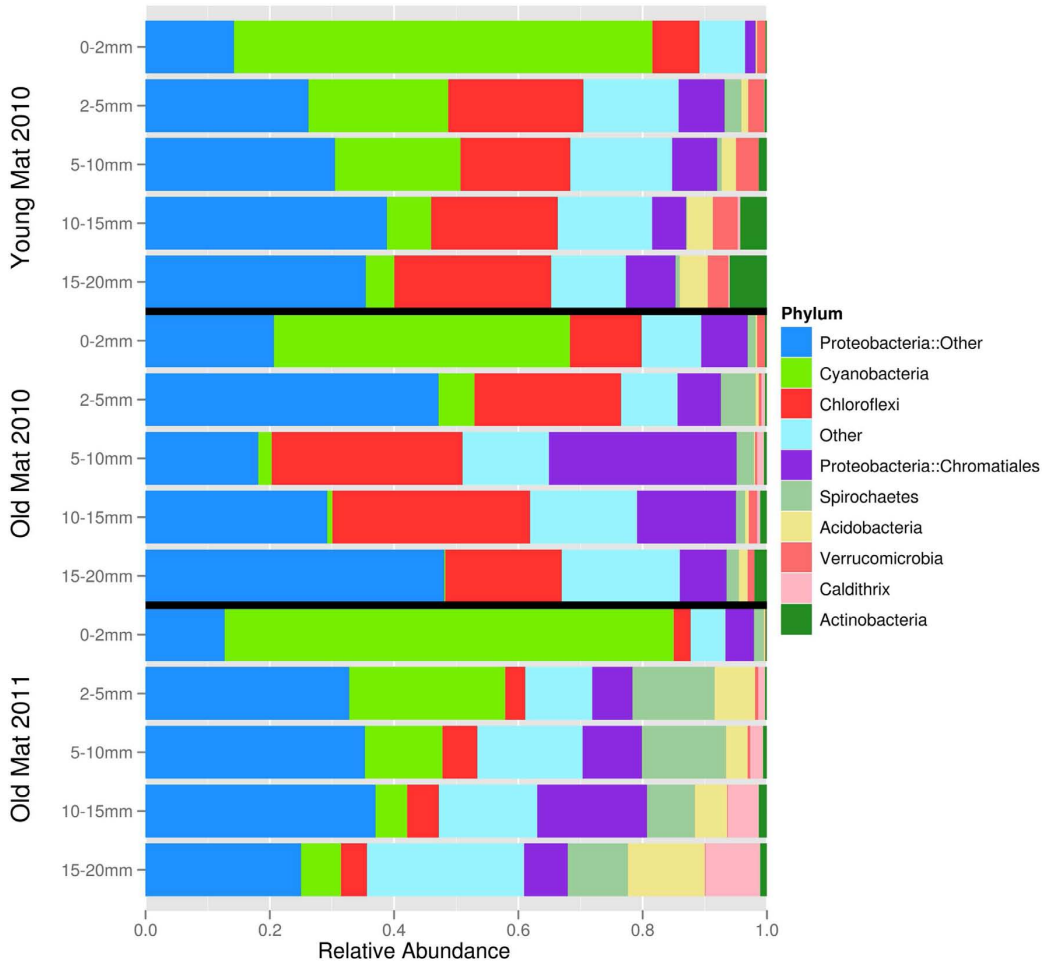


**FIGURE 3 | Phylum-level community composition for each mat layer and core sample.** 'Proteobacteria::Other' includes orders Syntrophobacterales, Rhodospirillales, Rhodobacterales, Desulfobacterales, Campylobacterales, Oceanospirillales, Myxococcales, Desulfovibrionales, Salinisphaerales, and Rhizobiales. Phyla in "Other" category (<1% relative abundance) include Bacteroidetes, Chlamydiae, Chlorobi, Firmicutes, Fusobacteria, Gemmatimonadetes, Lentosphaerae, Nitrospirae, Planctomycetes, Tenericutes, Thermi, and candidate divisions ABY1, BRC1, GN02, GN04, GN06, GN12, HYD24-12, KSB1, LCP89, MSBL6, MVP-15, NKB19, OP8, OP9, OP11, SAR406, SC4, TG3, TM6, TM7, WPS-2, WS1, WS3, ZB2.

sulfur bacteria (order Chlorobiales). These organisms use sulfide, hydrogen, and organics as reducing agents, scavenging it from the anaerobic reduction of sulfate carried out in the underlying layers. Our finding that all of the young mat's layers most closely resembled the top green layers of the developed mats is not surprising, considering that in the absence of thick EPS layers, light probably penetrates much deeper into the mat. While the young mat sample did not display laminae, it still contained numerous taxa identified as sulfate-reducers, as well as potential sulfur oxidizing bacteria. This result is in agreement with other studies of intertidal mats, which demonstrate patterns of succession initiated by *Oscillatoria* spp. and other oxygenic phototrophs (Stal et al., 1985; Bolhuis and Stal, 2011). These early colonizers contribute to the formation of the underlying layers by stabilizing the sediment and making available organic carbon to the heterotroph communities, whose activities in turn create anoxic conditions and permit dissimilatory sulfate reduction and photosynthetic oxidation of hydrogen sulfide as life history strategies (Herbert and Welsh, 1994).

## DIVERSITY PATTERNS

In all three mat cores, phylogenetic and taxonomic richness was lowest in the top layer. Similar patterns have been found in marine systems (Stevens and Ulloa, 2008; Kembel et al., 2011; but see Bryant et al., 2012), hypersaline lakes (Humayoun et al., 2003), subtidal sand (Böer et al., 2009), and hypersaline microbial mats (Dillon et al., 2009). Because the sharpest increase in diversity occurred during the transition between the first and second layers, we hypothesize that the top layer of the mat may be a habitat unfavorable to the majority of OTUs detected in our samples due to a combination of UV irradiation, temperature, and flood scouring (Calkins and Thordardottir, 1980; Garcia-Pichel and Castenholz, 1994; Ibelings and de Winder, 1994; Bossio and Scow, 1998). Because these factors fluctuate on a daily cycle, and because these communities undergo massive diel migrations, the daytime top layer of the mat is likely composed of stress-tolerant aerobes, while at night, microaerophiles and UV-sensitive taxa temporarily migrate to the top layer (Villanueva et al., 2007; Dillon et al., 2009).

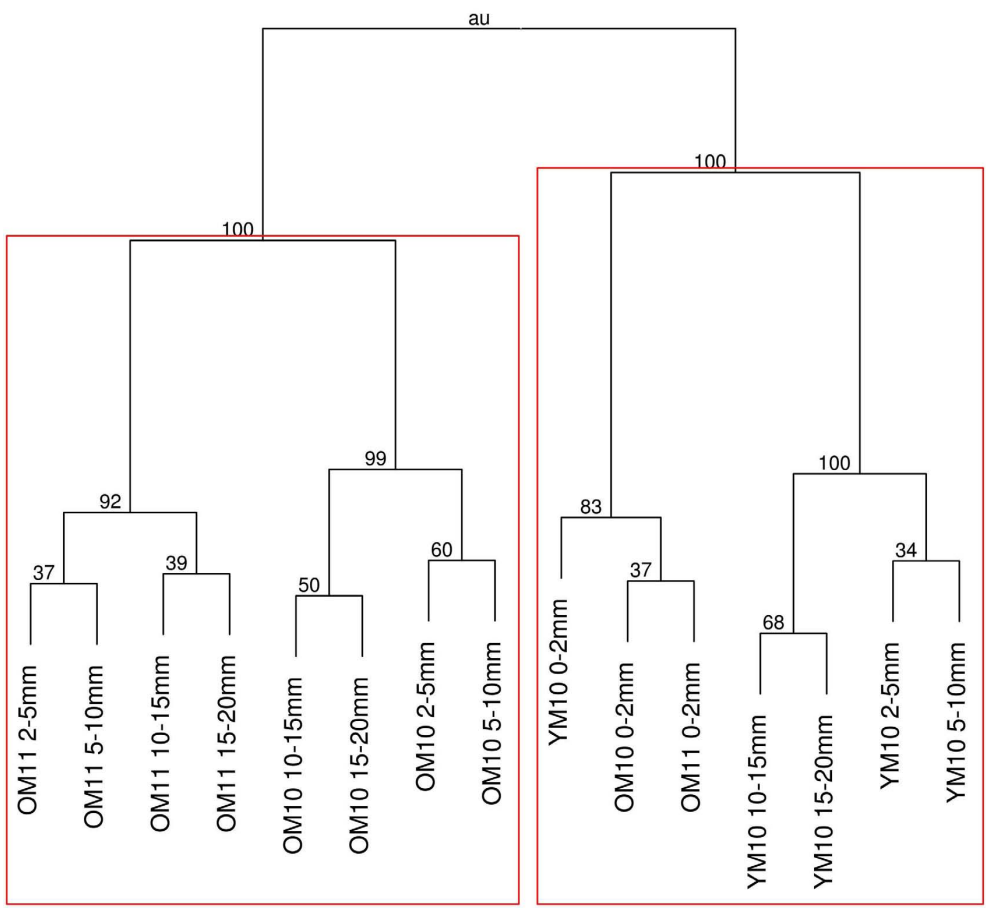


**FIGURE 4 | Dendrogram of UniFrac distances clustered using Ward's minimum variance method.** Node labels are AU *p*-values. Values greater than 95 indicate significant clusters. Rectangles indicate the deepest significant clusters within each clade. OM, old mat; YM, young mat; 10, 2010; 11, 2011.

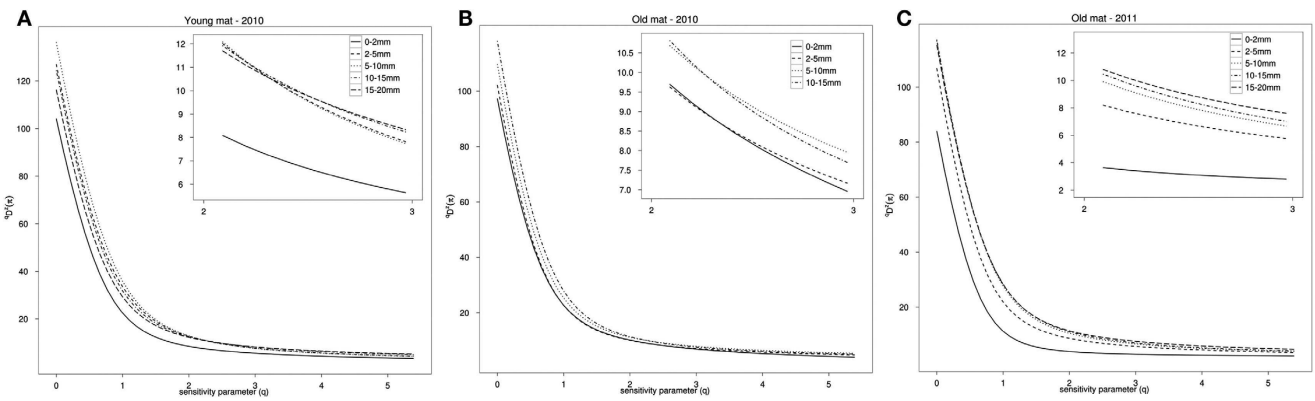


**FIGURE 5 | Rarefied phylogenetic diversity profile, ${}^{q}D^{Z}(\pi)$, for (A) the young mat sample, (B) the old mat 2010 sample, and (C) the old mat 2011 sample.** Increasing parameter $q$ decreases the metric's sensitivity to rare taxa and becomes a measure of evenness. Inset box shows values $2 \leq q \leq 3$.

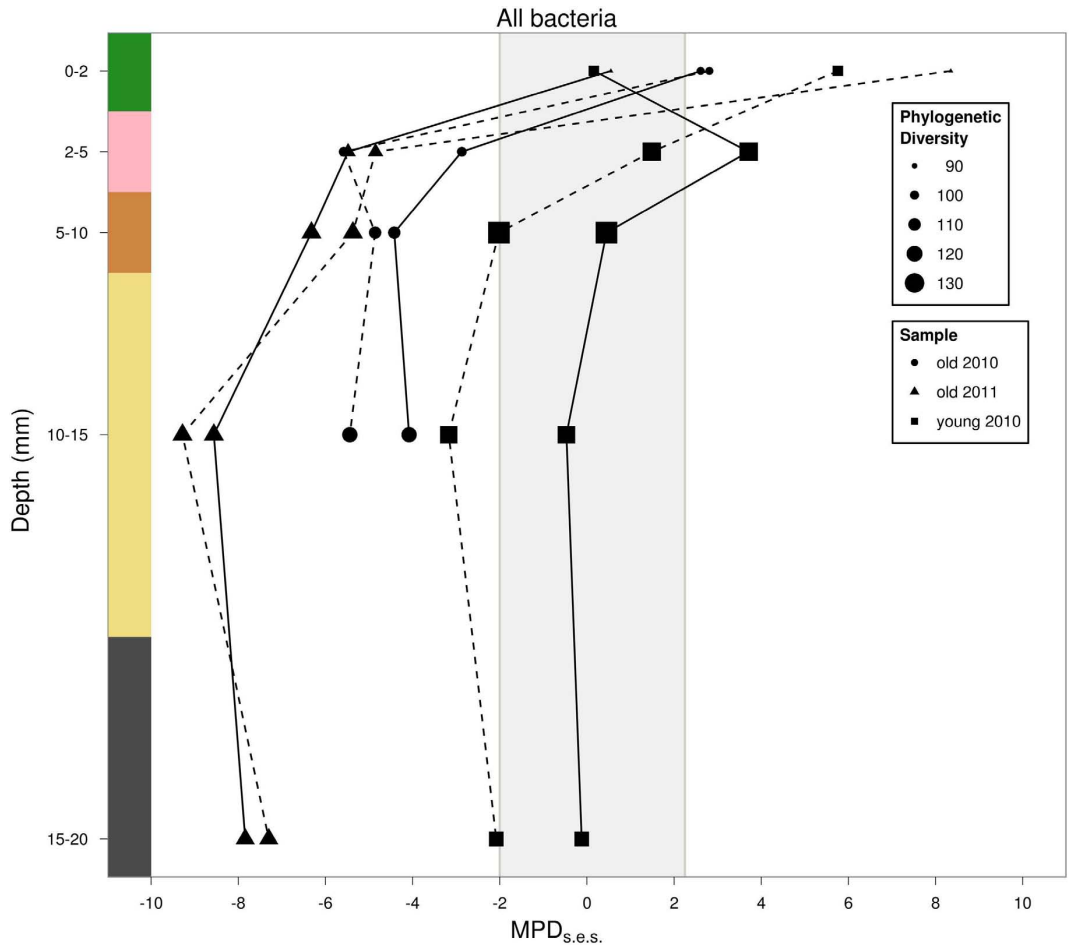


**FIGURE 6 | Estimated rarefied phylogenetic dispersion with depth.** Solid lines are results using taxa pool 1 (all years, all cores) as the regional pool, dashed lines are results using taxa pool 3 (individual cores) as the regional pool. Point size is scaled to phylogenetic diversity, ${}^{0}D^{\mathbf{z}}(\boldsymbol{\pi})$. Values falling below the shaded region indicate statistically significant phylogenetic clustering. Values above this region indicate phylogenetic overdispersion.

Niche theory predicts that areas of higher resource heterogeneity will support a greater diversity of taxa (MacArthur and Levins, 1967; but see Stevens and Carson, 2002). Thus, if a particular mat layer has a greater diversity of resources, we might expect an equally proportional diversity of genes relating to resource use. However, we did not find evidence of a decreasing diversity with depth of metabolic strategies measured by overall metabolic KO genes, nor genes encoding for carbohydrate metabolism, and ABC transporters, suggesting that an increase in resource heterogeneity does not explain the correlation between diversity and depth. Competition theory presents an alternative mechanism to explain the increase in diversity with mat depth. We speculate that the pool of nutrients available for assimilation (primarily *N* and *P*) may be limiting to heterotrophs in the oxic layer due to the large biomass of primary producers such as heterocystous Cyanobacteria and diatoms (Herbert and Welsh, 1994; Camacho and de Wit, 2003; Jonkers et al., 2003; Yannarell and Paerl, 2007). If competition for limiting nutrients, rather than competition for sources of C and energy, are resulting in competitive exclusion then we might expect competitive exclusion should not be as dominant a force deeper in the mat, allowing more taxa to coexist (Hutchinson, 1961). Indeed, we found indirect support for competition being greater in the top mat layer than in underlying layers based on phylogenetic dispersion measurements. At the domain level, the top layers of each core often showed phylogenetic overdispersion, meaning OTUs detected in this layer were less related than would be expected by chance. These results match those of Bryant et al. (2012), who found significant phylogenetic overdispersion only in the uppermost photic layers of their pelagic depth series, a region also dominated by primary production and aerobic metabolism. In soils, Horner-Devine and Bohannan (2006) found a negative relationship between total organic carbon and the phylogenetic relatedness of the community. If the assumption of phylogenetic niche conservatism holds for the domain bacteria, and if competitive interactions are greatest among sister clades, then phylogenetic overdispersion can be taken as evidence for limiting similarity within a set of interacting taxa (MacArthur and Levins, 1967; Webb et al., 2002). Currently, there is limited evidence for either assumption in microbial communities, and this remains a fertile area for research (Boyd et al., 2010; Mayfield and Levine, 2010; Violle et al., 2011).

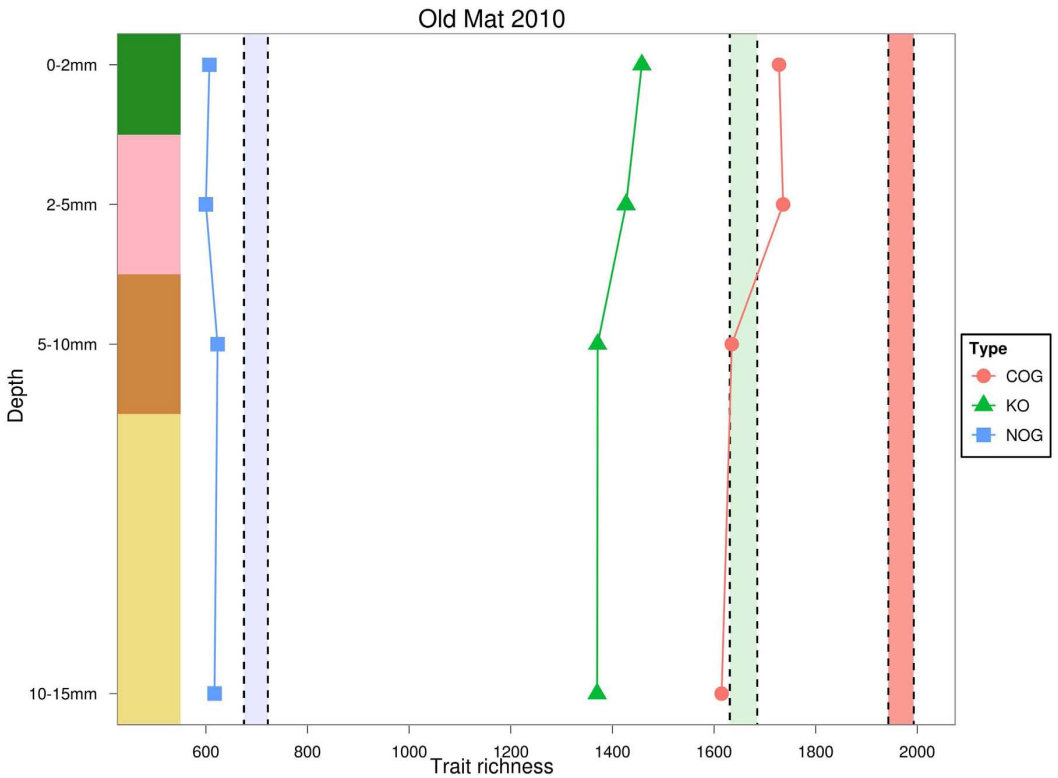


**FIGURE 7 | Estimated rarefied trait richness in the OM-10 core using three different orthologous gene annotations.** Values falling below or above their similarly colored shaded regions indicate significantly fewer ($\alpha = 0.05$) or greater trait richness than expected under 1,000 null model randomizations, respectively.

The qualitatively negative relationship between taxonomic/phylogenetic richness and trait richness measured via KO and COG pathways was unexpected. The KEGG and COG databases we used to annotate gene function in our samples were obtained from sequenced genomes, which are heavily biased toward model organisms and those from well-studied environments. Thus, the decrease in trait richness we observe may be due to annotation bias if deeper mat layers contain more organisms with poorly characterized genes and pathways. Our data do not support this claim, however, as we did not observe either a decrease in the proportion of unassigned reads nor an increased mean *e*-value with depth. Differences in OTU abundance between layers might also impact the observed pattern of trait richness. If the layer with the highest taxonomic richness is also the least even of the samples, the set of genes identified in that community will be biased toward the dominant taxa. Our diversity profiles clearly show that both diversity and evenness increase with depth, and so trait bias toward dominant OTUs cannot explain the decrease in trait richness that occurs with mat depth. Finally, our omission of archaeal reads from the amplicon library may explain the pattern, since Archaea are probably proportionally more abundant in the anaerobic layers. However, they do not exhibit as wide a physiological breadth as the domain Bacteria and are not expected to strongly bias measurements of trait diversity.

That our data show clear patterns of phylogenetic clustering with depth suggests that niche-based habitat filtering impacts community assembly as oxygen and light become limited. This claim is strengthened by our finding of significantly fewer KO genes in each layer than are expected by chance. When particular functional guilds of taxa were examined, however, this pattern dissolved, indicating that this clustering may only be apparent at very broad taxonomic levels, or in clades which we did not investigate. Other studies have also found an effect of taxonomic resolution on community clustering patterns (Horner-Devine and Bohannan, 2006; Pontarp et al., 2012). Habitat filtering in this community may be driven by the phylogenetic conservatism of phenotypes capable of anaerobic respiration. However, the metagenomic approach does not allow us to test this prediction directly due to both our inability to match taxon and gene, and our inability to match genotype with phenotype. Nonetheless, phylogenetic clustering patterns dominate the literature, and appear to covary with a number of factors, such as chlorophyll *a* content, organic carbon, nitrate (Horner-Devine and Bohannan, 2006), ocean depth (Kembel et al., 2011; Bryant et al., 2012), elevation (Bryant et al., 2008; Wang et al., 2012), and salinity (Barberán and Casamayor, 2010). More generally, the principle of habitat filtering has been the basis for enrichment culturing of microbes since the early days of microbiology, and it is not surprising that different habitats, characterized by different metabolic substrates and abiotic conditions, will selectively filter out all but a subset of taxa in the same way as a defined medium in a culture flask. One model of phylogenetic succession predicts that if the traits of early colonizers

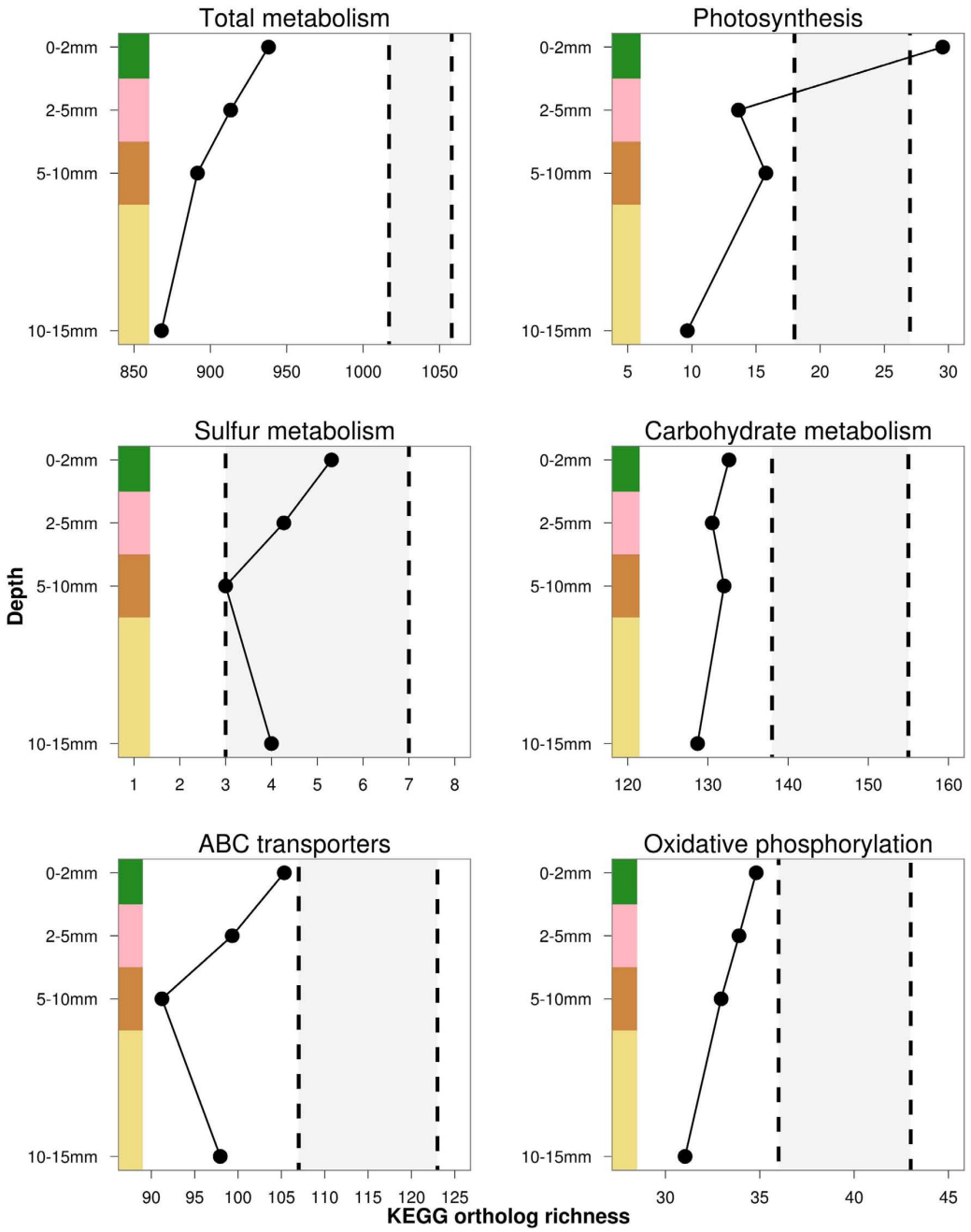


**FIGURE 8 | Estimated rarefied KEGG trait richness in the OM-10 core for a subset of functional gene categories.** Values falling below or above the shaded regions indicate significantly fewer ($\alpha = 0.05$) or greater trait richness than expected under 1,000 null model randomizations, respectively.

are phylogenetically conserved, then habitat filtering should give way to facilitative and competitive interactions as succession progresses, which should manifest as a switch from phylogenetically clustered to random/overdispersed communities with time (Verdú et al., 2009). In our samples, the increased phylogenetic clustering with mat age at depths below 2 mm, along with the compositional clustering among the young mat and the top layers of the old mats, suggests the increased importance of habitat filtering with succession, as poorly adapted initial colonizers are lost from underlying layers while taxa more suited to the changing abiotic conditions are recruited into the habitat.

## NULL MODEL CONSIDERATIONS

In many cases, our choice of null model affected the interpretation of our analyses. By decreasing the size of our regional taxa pools from the entire suite of OTUs in a sample to the set of OTUs in one particular mat layer, we were limiting the dispersal potential for taxa not shared among samples. Little is known about the

scale at which microbes disperse (Whitaker et al., 2003), but we found relatively similar results between the two most extreme taxa pools (**Table A1** in Appendix). It is likely that no two taxa have the same likelihood of occupying a habitat, and future studies should consider a spatially explicit metacommunity approach to identifying more realistic taxa pools (Lessard et al., 2012; Peres-Neto et al., 2012). Likewise, we found that the independent swap algorithm decreased the effect sizes of MPD. This null model has been identified as being the most conservative in detecting niche-based community assembly in simulations (Kembel, 2009). This randomization routine, compared with the two phylogeny-based approaches, did not identify overdispersion in our 0–2 mm layers. The statistical power to detect limiting similarity in communities requires testing, as do alternative null models which incorporate the effects of real ecological processes (Gotelli and Ulrich, 2012). By weighting our randomizations by a taxon's relative abundance, we were assuming that the probability of colonization in a mat layer was proportional to the taxon's abundance in the community, while presence/absence data give all taxa an equal probability (Kembel and Hubbell, 2006). In reality, the true dispersal probability for a taxon lies somewhere in between these two extremes, but has yet to be quantified so that an appropriate null model can be developed. In the absence of more sophisticated, semi-mechanistic null models, we advocate a pluralistic view of null modeling schemes focusing efforts on interpretation of a suite of models in light of their constraints and assumptions. Additionally, most, if not all, of the assumptions surrounding null models and community assembly (e.g., dispersal scale, niche conservatism) have yet to be assessed for the majority of organisms, microbial or otherwise, and we strongly urge ecologists to interpret their results in the light of these knowledge gaps.

## CONCLUSION

Microbial mats exhibit marked variation in diversity at scales much smaller than typically considered in ecological studies. Our observations of phylogenetic and trait clustering within the layers of a salt marsh microbial mat indicate habitat filtering as the main driver for community assembly. However, the effects of biotic interactions such as competition and syntrophy cannot be dismissed as alternative factors affecting community structure, especially in the mat's topmost Cyanobacteria-dominated layer. Nevertheless, until rigorous manipulative studies are carried out to test the assumptions of phylogenetic niche conservatism and dispersal limitation in bacteria, results from community phylogenetic approaches must be interpreted with caution. We suggest that these types of studies be carried out using microbial mat communities, which have historically proven tractable to both laboratory and field experimentation.

## ACKNOWLEDGMENTS

We thank C. Pepe-Ranney and L. Kelly for help with data processing. H. Morrison and the staff of the MBL Bay Paul center provided assistance with sequencing. This research was performed by participants in the MBL Microbial Diversity Course in Woods Hole, MA, and was supported in part by The Howard Hughes Medical Foundation, The Gordon and Betty Moore Foundation under grant No. (2493), the National Science Foundation under grant No. (DEB-0917499), the US Department of Energy under grant No. (DE-FG02-10ER13361), and the NASA Astrobiology Institute. David W. Armitage was supported by an NSF Graduate Research Fellowship. The authors thank the NSF Dimensions of Diversity distributed graduate seminar (DEB-1050680) and the Sousa Lab for valuable input.

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

*Received: 12 June 2012; paper pending published: 04 July 2012; accepted: 24 July 2012; published online: 10 August 2012.*
*Citation: Armitage DW, Gallagher KL, Youngblut ND, Buckley DH and Zinder SH (2012) Millimeter-scale patterns of phylogenetic and trait diversity in a salt marsh microbial mat. Front. Microbio. **3**:293. doi: 10.3389/fmicb.2012.00293*
*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

## APPENDIX

**Table A1 | Rarefied $MPD_{SES}$ values for all bacteria, Cyanobacteria, purple sulfur bacteria, and sulfate reducing bacteria.**

| Sample | Group | Taxa.labels | | | Ind. Swap | Phylo. Pool | RelAbund | |
|---|---|---|---|---|---|---|---|---|
| | | Depth | Pool 1 | Pool 2 | Pool 3 | Pool 1 | Pool 1 | Pool 1 |
| YM-2010 | All bacteria | 0–2 | 0.16 | 5.76* | −0.55 | −0.68 | 0.1 | −0.02 |
| | | 2–5 | 3.71* | 1.48 | 1.82 | 2.03 | 3.56* | 3.63* |
| | | 5–10 | 0.45 | −2.02* | −1.54 | 0.54 | 0.4 | 0.43 |
| | | 10–15 | −0.47 | −3.17* | −1.3 | −0.03 | −0.49 | −0.52 |
| | | 15–20 | −0.12 | −2.08* | −3.4* | 0.13 | −0.16 | −0.14 |
| | Cyanobacteria | 0–2 | −2.11* | −0.01 | −2.07 | | | |
| | | 2–5 | −1 | −0.45 | −0.63 | | | |
| | | 5–10 | −0.312 | −0.64 | 0.24 | | | |
| | | 10–15 | −0.86 | 0.79 | −0.53 | | | |
| | | 15–20 | −0.429 | 0.89 | 0.21 | | | |
| | Purple sulfur bacteria | 0–2 | −1.66 | −0.82 | −1.63 | | | |
| | | 2–5 | −1.13 | −0.17 | −0.4 | | | |
| | | 5–10 | 0.18 | −0.32 | 1.1 | | | |
| | | 10–15 | −0.96 | −1.22 | −0.53 | | | |
| | | 15–20 | −1.26 | −1.11 | −1.22 | | | |
| | Sulfate reducing bacteria | 0–2 | NA | NA | NA | | | |
| | | 2–5 | −2.71 | −1.25 | −0.32 | | | |
| | | 5–10 | −3.78 | −2.53 | −1.6 | | | |
| | | 10–15 | −1.88 | −0.54 | 0.27 | | | |
| | | 15–20 | −2.5 | −0.16 | −0.29 | | | |
| OM-2010 | All bacteria | 0–2 | 2.61* | 2.81* | 3.99* | 1.41 | 2.6* | 2.61* |
| | | 2–5 | −2.87* | −5.58* | −2.17 | −1.41 | −2.72* | −2.77* |
| | | 5–10 | −4.42* | −4.86* | −4.12* | −2.29* | −4.31* | −4.39* |
| | | 10–15 | −4.08* | −5.44* | −3.73* | −2.13* | −4.09* | −4.1* |
| | | 15–20 | NA | NA | NA | NA | NA | NA |
| | Cyanobacteria | 0–2 | −1.18 | −0.14 | −2.13 | | | |
| | | 2–5 | 0.74 | −1.18 | 0.54 | | | |
| | | 5–10 | 0.80 | 0.33 | 0.46 | | | |
| | | 10–15 | NA | NA | NA | | | |
| | | 15–20 | NA | NA | NA | | | |
| | Purple sulfur bacteria | 0–2 | −0.09 | 1.83 | −0.75 | | | |
| | | 2–5 | −0.95 | 2.1 | 0.19 | | | |
| | | 5–10 | −0.75 | 1.97 | −0.99 | | | |
| | | 10–15 | −0.20 | 1.13 | −0.19 | | | |
| | | 15–20 | −0.19 | 1.02 | 0.12 | | | |
| | Sulfate reducing bacteria | 0–2 | NA | NA | NA | | | |
| | | 2–5 | −1.66 | −0.76 | 0.04 | | | |
| | | 5–10 | 0.29 | 0.54 | 0.32 | | | |
| | | 10–15 | 0.69 | 0.82 | 0.94 | | | |
| | | 15–20 | NA | NA | NA | | | |
| OM-2011 | All bacteria | 0–2 | 0.55 | 8.34* | 6.49* | 0.33 | 0.56 | 0.52 |
| | | 2–5 | −5.48* | −4.86* | −1.69 | −2.61* | −5.62* | −5.5* |
| | | 5–10 | −6.32* | −5.36* | −2.03 | −2.98* | −6.46* | −6.35* |
| | | 10–15 | −8.56* | −9.28* | −7.24* | −4.04* | −8.65* | −8.61* |
| | | 15–20 | −7.84 | −7.29* | −5.14* | −3.84* | −7.92* | −7.92* |
| | Cyanobacteria | 0–2 | −1.211 | 1.22 | −1.5 | | | |
| | | 2–5 | −1.06 | −0.99 | −0.96 | | | |
| | | 5–10 | −0.156 | −0.49 | −0.65 | | | |
| | | 10–15 | −0.974 | −1.23 | −0.79 | | | |
| | | 15–20 | −1.266 | 1.03 | −0.46 | | | |

*(Continued)*

**Table A1 | Continued**

| Sample | Group | Taxa.labels | | | Ind. Swap | Phylo. Pool | RelAbund | |
|---|---|---|---|---|---|---|---|---|
| | | Depth | Pool 1 | Pool 2 | Pool 3 | Pool 1 | Pool 1 | Pool 1 |
| | Purple sulfur bacteria | 0–2 | −0.48 | 0.54 | 0.02 | | | |
| | | 2–5 | −0.39 | −0.69 | −1.09 | | | |
| | | 5–10 | −0.16 | −0.16 | −0.6 | | | |
| | | 10–15 | −0.02 | 0.91 | −0.1 | | | |
| | | 15–20 | −0.19 | −0.95 | −1.45 | | | |
| | Sulfate reducing bacteria | 0–2 | −0.08 | −0.51 | −1.02 | | | |
| | | 2–5 | 0.54 | −0.24 | 0.12 | | | |
| | | 5–10 | 0.70 | 0.61 | 0.56 | | | |
| | | 10–15 | 1.11 | −0.07 | 1.22 | | | |
| | | 15–20 | 0.94 | 0.27 | 0.97 | | | |

*Values are presented for three species pools, and four null models, including abundance-weighted randomizations (see text for details). *Indicates significant ($p < 0.05$) phylogenetic clustering (negative values) or overdispersion (positive values) based on rank test.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 13 December 2012
doi: 10.3389/fmicb.2012.00425

# Contrasting extracellular enzyme activities of particle-associated bacteria from distinct provinces of the North Atlantic Ocean

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Microbial communities play a key role in the marine carbon cycle, processing much of phytoplankton-derived organic matter. The composition of these communities varies by depth, season, and location in the ocean; the functional consequences of these compositional variations for the carbon cycle are only beginning to be explored. We measured the abilities of microbial communities in the large-particle fraction (retained by a 10-μm pore-size cartridge filter) to enzymatically hydrolyze high molecular weight substrates, and therefore initiate carbon remineralization in four distinct oceanic provinces: the boreal polar (BPLR), the Arctic oceanic (ARCT), the North Atlantic drift (NADR), and the North Atlantic subtropical (NAST) provinces. Since we expected the large-particle fraction to include phytoplankton cells, we measured the hydrolysis of polysaccharide substrates (laminarin, fucoidan, xylan, and chondroitin sulfate) expected to be associated with phytoplankton. Hydrolysis rates and patterns clustered into two groups, the BPLR/ARCT and the NADR/NAST. All four substrates were hydrolyzed by the BPLR/ARCT communities; hydrolysis rates of individual substrate varied by factors of ca. 1–4. In contrast, chondroitin was not hydrolyzed in the NADR/NAST, and hydrolytic activity was dominated by laminarinase. Fluorescence *in situ* hybridization of the large-particle fraction post-incubation showed a substantial contribution (15–26%) of CF319a-positive cells (*Bacteroidetes*) to total DAPI-stainable cells. Concurrent studies of microbial community composition and of fosmids from these same stations also demonstrated similarities between BPLR and ARCT stations, which were distinct from the NADR/NAST stations. Together, these data support a picture of compositionally as well as functionally distinct communities across these oceanic provinces.

**Keywords: extracellular enzymes, biogeography, particles-associated bacteria, hydrolysis, carbon cycling**

## INTRODUCTION

Heterotrophic microbial communities collectively process a large fraction of the organic matter biosynthesized in the ocean (Azam, 1998), remineralizing, repackaging, and respiring a variety of substrates, and thus playing a central role in the marine carbon cycle. These communities have been shown recently to exhibit distinct biogeographic patterns in the ocean, with community composition differing by location, season, water mass, and depth (e.g., Kan et al., 2006; Agogue et al., 2011; Gilbert et al., 2012; Hanson et al., 2012). The consequences of these compositional differences for microbial community function are only beginning to be explored, however. The distribution of functional genes among communities at different locations, depths, and times in the ocean demonstrates the potential for distinct functionalities among these communities (DeLong et al., 2006; Teeling et al., 2012), but the conditions under which and extent to which potential differences in function might be expressed are still largely unknown. Although transcriptomic investigations have yielded insight into broad categories of genes that are active in marine microbial communities (Poretsky et al., 2009; Shi et al., 2011), this approach has major limitations with respect to carbon cycling, due to the wide range of potential substrates in ocean waters and our very limited abilities to identify specific functional genes related to cycling of these substrates (e.g., Rebuffet et al., 2011).

Direct measurements of carbon processing by microbial communities demonstrate their abilities to take up specific low molecular weight substrates labeled with $^{14}C$ or $^{3}H$ (e.g., Rich et al., 1997; Vila-Costa et al., 2007; Alonso-Saez et al., 2008), but these studies yield only indirect information about the rate at which most natural marine organic matter is remineralized, since most marine organic matter is initially biosynthesized as macromolecules such as polysaccharides, proteins, and lipid complexes. Although marine macromolecules are present in much higher concentrations than low molecular weight organic matter, microbial access to these substrates requires hydrolysis by extracellular enzymes to produce substrates that can be transported across cell membranes for further processing (Arnosti, 2011). The activities of these enzymes thus initiate carbon cycling by heterotrophic

microbial communities, and determine the types of organic matter that can serve as substrates.

Efforts to measure activities of microbial extracellular enzymes also – paradoxically – usually rely on a small number of low molecular weight substrate proxies. These commercially available proxies consist of a monomer such as glucose or leucine linked to a fluorophore whose fluorescence increases greatly upon hydrolysis of the monomer-fluorophore bond (Hoppe, 1983). This experimental approach has been used to compare potential hydrolysis rates at a wide range of depths and locations in the water column (e.g., Huston and Deming, 2002; Baltar et al., 2009; Piontek et al., 2011), and results have frequently been extrapolated to the degradation of carbohydrates and proteins in general (Christian and Karl, 1995; Fukuda et al., 2000). These substrate proxies, however, do not adequately mimic the three-dimensional structure of organic macromolecules in solution, so the relationship between hydrolysis rates measured with these proxies and the hydrolysis rates of their putative macromolecular counterparts are highly uncertain (Warren, 1996).

In order to measure the activities of enzymes responsible for hydrolysis of high molecular weight organic matter, alternative methods have been developed. These methods are intended also to detect the activities of endo-acting enzymes that cleave macromolecules mid-chain, an essential step in microbial degradation of macromolecular organic matter (Weiner et al., 2008; McBride et al., 2009). These approaches require synthesis of specific labeled substrates: fluorescently labeled polysaccharides and phytoplankton extracts (Arnosti, 1995, 2003; Arnosti et al., 2005b) or peptides (Pantoja et al., 1997, 2009; Pantoja and Lee, 1999), and chromatographic separation of the hydrolysis products, and thus requires considerably more work pre- and post-experiment. The additional effort is compensated for by the fact that these substrates can be used to investigate differences in hydrolysis rates and patterns for substrates with closely related structures, yielding new insight into the specific enzymatic capabilities of heterotrophic microbial communities in the water column and sediments. Using this approach, major functional differences among heterotrophic microbial communities at different depths and locations in the ocean have been identified (Arnosti et al., 2005a; Arnosti, 2008; Steen et al., 2012), showing that specific complements of enzyme activities are found not just for individual organisms, but among entire microbial communities. Moreover, these patterns of differences in enzyme activities have been shown to extend along large spatial gradients in the ocean (Arnosti et al., 2011), perhaps reflecting large-scale changes in microbial community composition along latitudinal gradients (Pommier et al., 2007; Fuhrman et al., 2008).

Given previous evidence of substantial differences in microbial communities in the North Atlantic (Schattenhofer et al., 2009), we tested whether organisms from distinct oceanic provinces within near-surface waters of the North Atlantic differed in their abilities to hydrolyze high molecular weight substrates. We focused in particular on a fraction of seawater enriched in large particles, since particle-associated heterotrophic communities are believed to be well-equipped to metabolize high molecular weight substrates, with enzymes that are sufficiently active so as to provide hydrolysate to the surrounding water column community as well as to the attached community (Smith et al., 1992; Simon et al., 2002; Grossart, 2010). At the conclusion of our incubation experiments, we carried out fluorescence *in situ* hybridization (FISH) staining to compare communities from the same station that had been incubated with different substrates, as well as communities obtained from different locations that were incubated with the same substrate. Concurrently conducted investigations of microbial community composition (Gomez-Pereira et al., 2010; Schattenhofer et al., 2011) and *Bacteroidetes*-associated fosmids (Gomez-Pereira et al., 2012) from the same locations provided us with the opportunity to examine the link between microbial community composition and function, and thus provided a larger context within which to interpret our measurements of microbial community activity. Together, these data provide new insight into the functional capabilities as well as the composition of microbial communities in distinct oceanic provinces of near-surface waters of the North Atlantic.

## MATERIALS AND METHODS

### SAMPLE COLLECTION AND WATER MASS IDENTIFICATION

Water was collected at a depth of 20 m using Niskin bottles mounted on a rosette equipped with a CTD at four stations (S3, S6, S12, and S19) during the VISION cruise of the R/V *Maria S. Merian* (September/October 2006). The stations represented distinct oceanic provinces on a N–S gradient along the 30°W meridian. As discussed in detail in Gomez-Pereira et al. (2010), water mass provinces were defined via satellite-derived parameters including Advanced Very High Resolution Radiometer (AVHRR), sea surface temperature and Sea-viewing Wide Field-of-view Sensor (SeaWiFS) water leaving radiance, according to Oliver and Irwin (2008). By these definitions, S3 was in the boreal polar (BPLR), S6 was within the Arctic oceanic (ARCT) province, S12 was within the North Atlantic drift (NADR), and S19 was within the North Atlantic subtropical (NAST) province (Gomez-Pereira et al., 2010).

### SAMPLE PREPARATION AND MEASUREMENT OF EXTRACELLULAR ENZYMATIC HYDROLYSIS

At each station, ca. 100 l of seawater (combined contents of five Niskin bottles) were passed through a stainless steel cartridge filter (Wolf Technik, Weil der Stadt, Germany), resulting in approximately 1 l retentate with particles larger than 10 μm (**Table 1**). The microbial community of the retentate is operationally defined as the large-particle-associated fraction; this fraction was used for incubation experiments. We note that this operationally defined fraction necessarily contained some bacteria present in the surrounding seawater, since the particles in the retentate were not removed from surrounding water. Microscopy of the retentate revealed that protists (diatoms, dinoflagellates, ciliates, and many others) and ample debris of unknown origin were collected. Since large-particle-associated bacteria concentrated from the euphotic zone may preferentially be associated with phytoplankton cells, which are typically carbohydrate-rich (Parsons et al., 1961), we focused on measuring the potential of the bacteria retained within this fraction to hydrolyze high molecular weight substrates that would likely be associated

**Table 1 | Station location, sample volume, and physical and chemical characteristics of the water column at 20 m depth.**

| Station | Position | *In situ* temperature (°C) | Salinity (PSU)* | Water mass | 10 μm filter concentration | | Concentration factor | $PO_4^{3-}$ (μM)* | $NO_3^-$ (μM)* | $NO_2^-$ (μM)* | $NH_4^+$ (μM)* | Chl *a* ($\mu g\,l^{-1}$)* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Initial volume (l) | Final volume (ml) | | | | | | |
| S3 | 65°52.6′N 29°56.5′W | 0.6 | 33.0 | BPLR | 100 | 730 | 137 | 0.408 | 2.895 | 0.133 | 0.29 | 1.0 |
| S6 | 59°20.9′N 29°59.9′W | 10.9 | 35.0 | ARCT | 96 | 850 | 113 | 0.432 | 5.437 | 0.21 | 0.584 | 1.7 |
| S12 | 46°44.5′N 30°0.2′W | 18.3 | 35.9 | NADR | 93 | 750 | 124 | 0.062 | 0.315 | 0.051 | 0.707 | ~0.2 |
| S19 | 34°24.8′N 28°28.9′W | 24.1 | 36.6 | NAST | 95 | 500 | 186 | 0.011 | 0.022 | 0.015 | 0.169 | ~0.1 |

**Data from Gomez-Pereira et al. (2010).*

with phytoplankton. Such substrates would include carbohydrates exudates, carbohydrate-containing cellular components, and phytoplankton-derived transparent exopolymeric particles (TEP; Painter, 1983; Passow et al., 1994; Myklestad, 1995). Four different fluorescently labeled polysaccharides (laminarin, xylan, fucoidan, and chondroitin sulfate) were therefore used to measure the activity of extracellular enzymes, after the method of Arnosti (1995, 2003). The polysaccharides were purchased from Sigma or Fluka, labeled with fluoresceinamine (Sigma; Isomer II), and characterized as described in Arnosti (2003). Laminarin (β(1,3)-glucose), xylan (β(1,4) xylose), and fucoidan (sulfated fucose) are components of marine algae and phytoplankton (Parsons et al., 1961; Painter, 1983); fucoidan additionally has a chemical composition consistent with TEP (Zhou et al., 1998). Chondroitin sulfate is a marine-derived polysaccharide (a sulfated polymer of galactosamine and glucuronic acid (β-GlcA (1,3)-GalNAc (1,4)) that is rapidly hydrolyzed by heterotrophic microbial communities in seawater and sediments (Arnosti, 2008; Arnosti et al., 2009). Most of these polysaccharides are produced by marine phytoplankton and algae (Painter, 1983; Alderkamp et al., 2007). Moreover, activities of enzymes hydrolyzing all of these substrates have been measured in seawater and sediments (Arnosti et al., 2005a; Arnosti, 2008; Teske et al., 2011), and gene sequences corresponding to enzymes that would hydrolyze these substrate have also been identified in the genomes of recently sequenced marine bacteria (Glöckner et al., 2003; Bauer et al., 2006; Weiner et al., 2008).

At each station, four sets of substrate incubations were prepared: duplicate live incubations (15 ml each) and single killed control incubations (10 ml seawater + 3.5 ml formalin) for each substrate. Each substrate was added at a final concentration of 3.5 μM monomer-equivalent to the incubations. Incubations from S3 and S6 were incubated at 4°C in the dark in a temperature-controlled room; incubations from S12 and S19 were initially incubated at 18–23°C (in flowing seawater in the lab) and then were incubated at 20°C (temperature-controlled room) in the dark. Subsamples (ca. 2.5 ml) were removed from each incubation at 0, 5, and 15 days, filtered through a sterile 0.2-μm pore-size filter, the first 1 ml of filtrate was discarded, and the remaining 1.5 ml of the filtrate was stored frozen until analysis. In brief, substrate hydrolysis was determined from the changes in polysaccharide molecular weight with time, as measured using gel permeation chromatography and fluorescence detection, described in detail in Arnosti (2003). Gel columns consisted of a 20 cm × 1 cm column of Sephadex G-50 gel, connected in series to a 18.5 cm × 1 cm column of Sephadex G-75 gel. Mobile phase (phosphate buffer, pH 8.0) was pumped at 1 ml $min^{-1}$ by a Shimadzu LC-10AT pump; the column outflow passed through a Hitachi L-7480 fluorescence detector, set to excitation and emission wavelengths of 490 and 530 nm, respectively. Hydrolysis rates were calculated from differences in substrate molecular weight at the different time points, as described in detail in Arnosti (2003).

### FLUORESCENCE *IN SITU* HYBRIDIZATION AND CELL COUNTS

At the end of the 15-day incubation, the remaining incubation water (ca. 7.5 ml) from one of the two live replicates was fixed with particle-free formaldehyde, filtered through polycarbonate 0.2-μm pore-sized filters, and the filters were stored frozen (−20°C) until analysis. *In situ* identification with the standard catalyzed reporter deposition (CARD) FISH protocol (Pernthaler et al., 2002) and cell counting relative to total cell counts (DAPI) was carried out using the general bacterial probe mix EUB338 I–III (Daims et al., 1999) as well as the group-specific probes CF319a (Manz et al., 1996) and PLA46 (Neef et al., 1998; for a recent update of probe specificity see Amann and Fuchs, 2008) targeting *Bacteroidetes* and *Planctomycetales*, respectively. For two samples, insufficient volume was obtained to make accurate FISH counts, so relative abundances were estimated.

## RESULTS

### ENZYMATIC HYDROLYSIS RATES AND PATTERNS

The four stations sampled represent different North Atlantic provinces, as characterized by distinctive temperature and salinity signatures, as well as nutrient and chlorophyll concentrations

(**Table 1**; see Gomez-Pereira et al., 2010 for further details). They also showed distinct patterns and rates of extracellular enzymatic activities. **Figure 1** shows the maximum hydrolysis rate of each substrate at each station, which was measured after either 5 or 15 days incubation. Maximal rates were observed after 5 days of incubation for fucoidan from all stations, for laminarin from all stations except S6, and for xylan from S12 and S19. The maximal hydrolysis rates of xylan from S3 and S6, of laminarin from S6, and of chondroitin at S3 and S6 were observed after 15 days of incubation. Data from both time points (5 and 15 days) are plotted in **Figure 1** because in cases where substrates are hydrolyzed rapidly (e.g., maximum values at 5 days), later time points (e.g., 15 days) typically show a lower calculated hydrolysis rate, reflecting the fact that hydrolysis was nearly complete at the earlier time.

The differences in timescales over which hydrolysis rates reach a maximum rate reflect the activity and distribution of extracellular enzymes within a given community. Since hydrolysis of fluorescently labeled polysaccharides is detected as a change in the molecular weight distribution of the entire pool of added substrate (see Arnosti, 1995, 2003 for more details), extended incubations (timescales of days) are typically required to measure activity in pelagic samples. An enzyme activity that is low or is uncommon among a community is first observable after a more extended period of incubation, while enzyme activities that are intrinsically rapid or are widespread among a given community may be observed at an earlier time point. Since these timescales of measurement (days) allow sufficient time for community growth as well as enzyme expression, enzyme activities measured using this technique represent the potential of a community to react to substrate input – e.g., by induction of genes and/or by growth of a numerically small or slow-growing members of the community – rather than reflecting only the activity of enzymes already present at the time substrate is added to the sample. We note also that in this incubation, the time points were quite widely spaced; maximum hydrolysis rates might have been reached prior to 5 or 15 days, so the rates reported here could be underestimated. We also note that these measurements necessarily represent the potential of the microbial community present in the sample at the time of collection. Samples collected at different seasons could show different patterns, depending on larger-scale factors that could drive seasonal variation in microbial community composition.

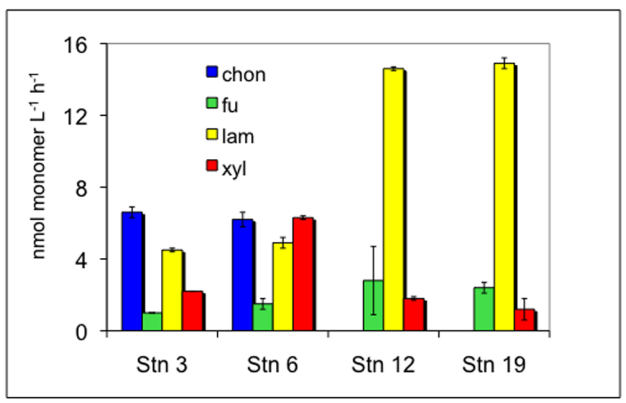


**FIGURE 1 | Enzymatic hydrolysis rates of four polysaccharides in 10 μm retentate from each station.** Chon, chondroitin sulfate; fu, fucoidan; lam, laminarin; xyl, xylan. Bars show standard deviation of two replicate incubations. Hydrolysis rates are maximum rates that were measured after 5 days incubation for all substrates except chondroitin, laminarin at S6, and xylan at S3 and S6, which were from 15 days incubation (see text).

### HYDROLYSIS PATTERNS BY STATION

As a pair, S3 and S6 resembled each other in hydrolysis rates and patterns. At these stations, all four substrates were hydrolyzed by the large-particle-associated microbial fraction. Hydrolysis rates of chondroitin were quite high; the hydrolysis rates of the other substrates were for the most part within a factor of ca. 1–4 of the chondroitin hydrolysis rate. At S12 and S19, in contrast, hydrolytic activity was highest for laminarin, which was very rapidly hydrolyzed (14.6 and 14.9 nmol $l^{-1}$ $h^{-1}$, respectively). Hydrolysis rates of fucoidan and xylan at S12 and S19 were 5–20 times lower than that of laminarin, and there was no indication of chondroitin hydrolysis in the large-particle-associated fraction from these stations.

### MICROBIAL COMMUNITY COMPOSITION

The composition of the microbial communities at the conclusion of the incubation was investigated using CARD-FISH. The general bacterial probe mixture EUB338 I–III hybridized to 75–99% of all DAPI-stained cells. Of these bacteria, a substantial proportion was assigned to *Bacteroidetes* with probe CF319a: on average, 26, 22, 15, and 17% for S3, S6, S12, and S19, respectively (**Table 2**). Counts with the probe PLA46 specific for *Planctomycetes* were low or below the detection limit at all stations (data not shown), serving also as a negative control for unspecific staining and autofluorescence. For any given station, there was no consistent relationship between hydrolysis rate of a specific substrate and fraction of cells detected by CF319a.

## DISCUSSION

The enzymatic complement of heterotrophic bacteria is variable even among closely related organisms (Gao et al., 2003;

**Table 2 | CF319a hybridized cells, as % DAPI-stained cells, after 15-day incubation with substrate.**

| Substrate | Station | | | |
|---|---|---|---|---|
| | S3 | S6 | S12 | S19 |
| Lam | 23.6 | 13.5 | 19.4 | 10–20* |
| Xyl | 28.3 | 22.7 | 13.9 | 5–10* |
| Fu | 17.3 | 13.9 | 10.2 | 19.3 |
| Chon | 34.3 | 36.7 | 15.8 | 14.7 |
| *Average* | *25.9* | *21.7* | *14.8* | *17.0* |
| Bulk water** | 19.7 | 17.5 | 30.6 | 5.4 |

*Lam, laminarin; xyl, xylan; fu, fucoidan; chon, chondroitin sulfate.*
**Estimated abundance: volume too low to count precisely (see text). These values not included in the average.*
***Bulk water: CF319a-stained cells (as % of DAPI-stained cells) from a depth of 20 m at each station; data from Gomez-Pereira et al. (2010).*

Gomez-Pereira et al., 2012), so the nature and type of substrate that can be accessed by a given organism is quite specific, as has been demonstrated by microbiological and genomic investigations of a variety of prokaryotes (e.g., Martinez et al., 1996; Ensor et al., 1999; Glöckner et al., 2003; Bauer et al., 2006; Weiner et al., 2008). Some bacteria are able to use low molecular weight hydrolysis products although they cannot effectively hydrolyze the initial substrates (Cotta, 1992), demonstrating the necessity for close interactions among specific bacteria within a community. The extent to which substrate preference patterns extend from specific organisms to entire microbial communities, however, is only beginning to be explored in marine environments. Previous studies of polysaccharide hydrolysis by pelagic microbial communities demonstrate site-specific differences in extracellular enzymatic hydrolysis rates and patterns (Arnosti et al., 2005a; Steen et al., 2008) as well as large-scale latitudinal gradients in the spectrum of polysaccharide hydrolases activities in surface ocean waters (Arnosti et al., 2011). The observation that enzymatic hydrolysis patterns measured here clustered into two groups, consisting of the northern BPLR/ARCT stations S3 and S6, and the more temperate NADR/NAST stations S12 and S19 (**Figure 1**) suggests that there are biogeographic patterns in enzymatic capabilities also among the large-particle-associated fraction of microbial communities.

These functionally distinct communities also differed in composition, as shown by FISH staining of whole water (unsorted) as well as flow cytometry-sorted samples collected concurrently with the present study. Although members of the SAR11 were the single largest constituents of the unsorted population, contributing 25–32% of the total stained cells at a depth of 20 m at all four stations, the contribution of other prokaryotic cells was quite variable by station (Schattenhofer et al., 2011; **Figure 2**). CF319a-stainable cells (*Bacteroidetes*) contributed a large fraction of the identifiable cells, Syn405 (*Synechococcus*) counts were significant only in S3 and S6, while Pro405 (*Prochlorococcus*) counts were significant only at S12 and S19 (**Figure 2**; Schattenhofer et al., 2011). The fundamental factors shaping these differences in microbial community composition remain to be determined (Hanson et al., 2012); as noted by Schattenhofer et al. (2011), specific groups of bacterioplankton were statistically correlated with physical, chemical, and biological parameters, in agreement with investigations of other microbial communities at other locations (Kan et al., 2006; Gilbert et al., 2012). In any case, the general trend of a north to south decrease in nutrient and chlorophyll *a* concentrations as well as picoeukaryotic abundance and prokaryotic counts at S3, S6, S12, and S19 tracks the changes in prokaryotic community composition and extracellular enzyme activities measured at the four stations (**Table 1**; **Figure 2**).

Compositional distinctions among the microbial communities at these four stations were supported also by more detailed analysis of bacteroidetal communities, carried out on the filtrate that had passed through the 10 μm cartridge. A focus on members of the class Flavobacteria of the phylum *Bacteroidetes* is especially relevant due to their proven abilities to degrade organic macromolecules in marine waters (Kirchman, 2002; Bauer et al., 2006), as well as their abundance at these stations and in our incubations (**Table 2**). Analysis of 16S rRNA gene clone libraries and direct cell counts via CARD-FISH demonstrated that the flavobacterial composition of S3 and S6 had considerable overlap, sharing 25–40% of flavobacterial phylotypes, while S3 and S18 were mostly unrelated, sharing just 2–5% of flavobacterial phylotypes (Gomez-Pereira et al., 2010). Flavobacteria are a major target of FISH probe CF319a, which comprised a substantial proportion (5–31%) of cells in bulk seawater (Gomez-Pereira et al., 2010) as well as 15–26% of the post-incubation large-particle-associated fraction from these stations (**Table 2**). Since the complement of glycosyl hydrolase genes in fully sequenced members of the *Bacteroidetes* differs substantially (Gomez-Pereira et al., 2012), these differences in flavobacterial community composition indicate the potential for functional differences.

Evidence of biogeographic patterns in polysaccharide hydrolase activities (**Figures 1 and 2**) is also supported by the results of a metagenomic investigation based on analysis of *Bacteroidetes*-associated fosmids obtained from S3 and S18, the same samples Gomez-Pereira et al. (2010) used for clone libraries and CARD-FISH staining. Fifteen glycosyl hydrolase-associated genes were identified in the fosmids, some of which included signal sequences that predicted enzyme export to the outer membrane (Gomez-Pereira et al., 2012), where they could hydrolyze extracellular polysaccharides. At S3, genes corresponding to sulfatases (used to remove sulfate groups from polysaccharides) were also identified; most of these enzymes were likewise predicted to be exported from the cytoplasm (Gomez-Pereira et al., 2012), and thus to play a role in extracellular hydrolysis. Sulfatase genes were also identified at S18, in lower numbers than at S3. Overall, the relative content of glycosyl hydrolases and sulfatases at S3 was higher than at S18; differences in gene abundance were correlated with the abundance of the organisms from which the fosmid sequences were derived (Gomez-Pereira et al., 2012). The current study thus provides evidence of functional differences that are implied by the genomic and community population data from samples collected concurrently from these same stations (Gomez-Pereira et al., 2010, 2012; Schattenhofer et al., 2011).

Although differences in patterns of enzymatic hydrolysis (**Figures 1 and 2**) suggest fundamental differences in function of the large-particle-associated fraction of the community, information about relative gene abundance at different stations (Gomez-Pereira et al., 2012) cannot be linked directly to specific rates of hydrolysis. The extent and conditions under which a given gene is expressed in the environment are unknown; moreover, the kinetic characteristics of the enzymes themselves as well as the quantity of enzymes produced contribute to observed hydrolysis rates. Furthermore, the hydrolysis rates measured in this study may be affected by the fraction of large particles retained by the 10 μm cartridge filter, as well as by the extent of particle colonization by bacteria at each station. The DNA extractions of Gomez-Pereira et al. (2010, 2012) were carried out on the fraction of water that passed through the 10 μm cartridge, i.e., on the filtrate, rather than the retentate, and thus specifically excluded the large-particle-associated fraction used to measure enzyme activities in the current study. The retentate fraction, however, was isolated within a background of retained seawater (i.e., particles were not isolated from their surrounding solution), and therefore also contained some cells that were present in the last fraction

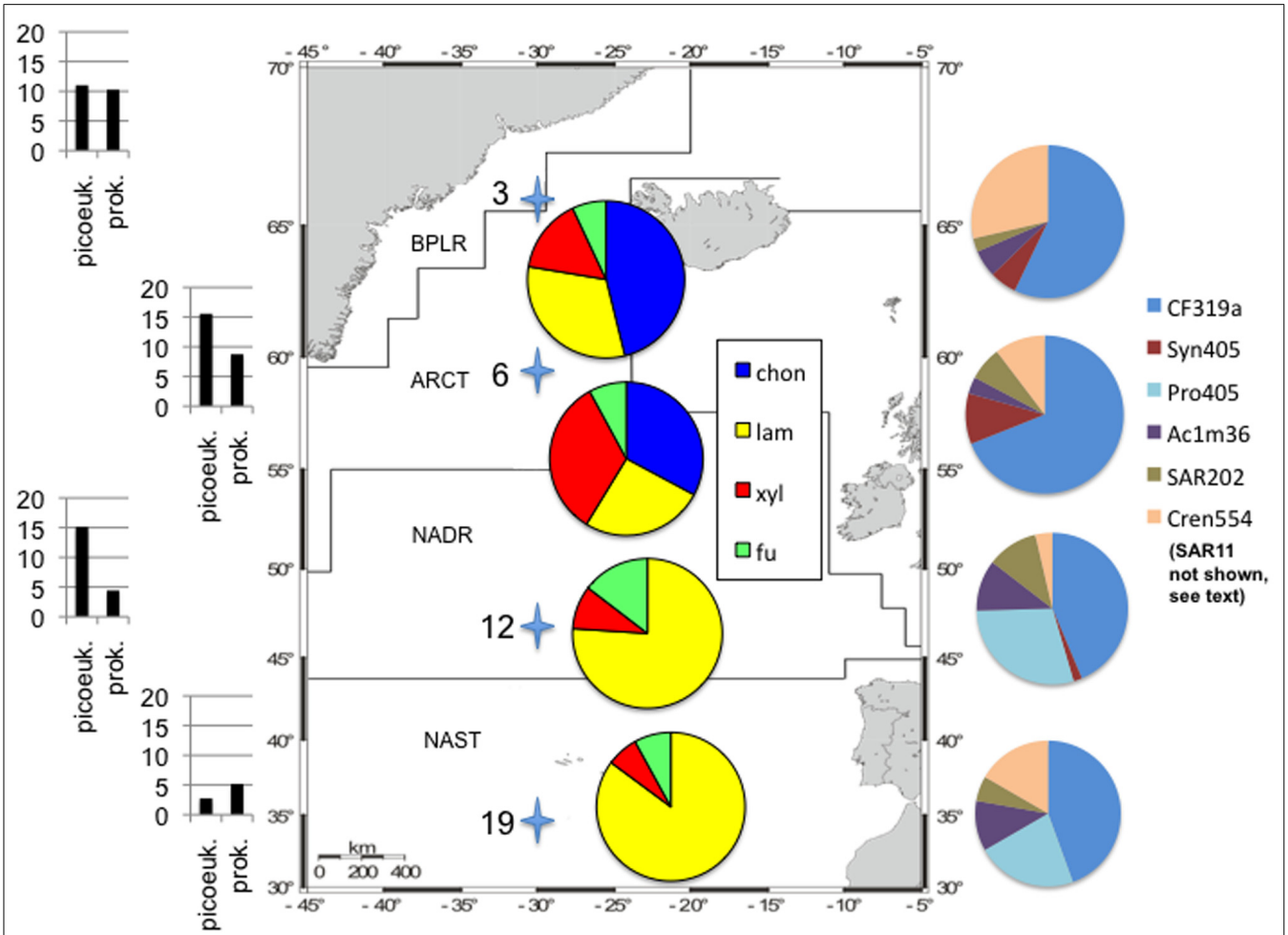


**FIGURE 2 | Study area with oceanic provinces, sampling stations, enzymatic hydrolysis patterns, cell counts, and community composition information.** Station locations shown as stars (station numbers on left); map modified from Gomez-Pereira et al. (2010). Cell counts on left side of figure: total prokaryotic cell counts as "prok," picoeukaryotes counted via flow cytometry as "picoeuk." Left hand scale: for picoeukaryotes, $\times 10^3$ cells ml$^{-1}$, for prokaryotes, $\times 10^5$ cell ml$^{-1}$. Data from Schattenhofer et al. (2011) and Gomez-Pereira et al. (2010). Pie charts superimposed on map: relative contribution of each enzyme activity to total activity measured at a station; substrate names as in **Figure 1**. Pie charts on right hand side: relative contribution of FISH-stained cells to total cell counts from water samples that were not subject to flow cytometry ("unsorted cells"); data from Schattenhofer et al. (2011) SI **Table 2**. Note that SAR11 cells contributed 25–32% of total DAPI-stained cells at each station, and are not plotted here for clarity. Pie charts as shown represent 17.5, 29, 27.5, and 18% of the total DAPI-stainable cells at S3, S6, S9, and S19, respectively. CF319a, *Bacteroidetes*; SYN405, *Synechococcus*; PRO405, *Prochlorococcus*; AC1M36, marine clade I *Actinobacteria*; SAR202, SAR 202 clade; CREN554, *Crenarchaeota* marine group I.

to pass into the cartridge filter. Substrate hydrolysis rates showed no systematic relationship with concentration factor (**Table 1**), also indicating that the differences observed among stations were not an outcome of sample manipulation. Furthermore, Schattenhofer et al. (2011) did not prefilter their samples, and the population they studied (**Figure 2**) would thus also include the fraction defined here as large-particle-associated bacteria. In any case, any differences between stations in bacterial cell numbers of the large-particle-associated fraction would be reflected primarily in differences in the rates of hydrolysis, and not in the patterns of substrates hydrolyzed. Different patterns of substrate hydrolysis point instead at functionally different communities.

The patterns evident at these stations additionally demonstrate that enzymatic hydrolysis rates in pelagic waters are not a simple function of environmental temperature. Although *in situ* as well as incubation temperatures at S12 and S19 were substantially warmer than at S3 and S6, only laminarin hydrolysis rates appear to track temperature. Hydrolysis rates of fucoidan, chondroitin, and xylan, in contrast, were comparable to or higher at the colder stations (S3/S6) than at the warmer stations (S12/S19). A correlation of laminarin hydrolysis with temperature, and little temperature correlation for other polysaccharides, is in fact a pattern consistent with enzyme activities in surface ocean waters on latitudinal gradients (Arnosti et al., 2011).

The lack of measurable chondroitin hydrolysis at S12 and S19 is particularly interesting in light of the observation that it is one of the activities frequently measured in surface ocean waters (Arnosti et al., 2011). At these stations, hydrolytic activity may be

associated with the free living or small-particle-associated microbial fraction that would not have been retained by a 10-μm filter. This activity may also simply be missing in this biogeographic province, since evidence to date suggests that enzymatic capabilities are non-uniformly distributed in the surface ocean (Arnosti et al., 2005a, 2011; Steen et al., 2008), and polysaccharide hydrolase gene distribution varied among these stations (Gomez-Pereira et al., 2012). Conversely, fucoidan hydrolysis at all four stations is also notable, since it is an activity that is relatively infrequently measured in surface ocean waters (Arnosti et al., 2011), and perhaps is primarily associated with large-particle-associated bacteria. Both chondroitin and fucoidan are sulfated polysaccharides, and their rates and extent of utilization may also be related to the activities of sulfatase enzymes whose genes were identified in the fosmids from these stations (Gomez-Pereira et al., 2012). The pattern of more rapid chondroitin hydrolysis upon extended incubation (i.e., 15 days) is consistent with previous observations of chondroitin hydrolysis in marine waters and sediments, as well as experiments indicating that chondroitin hydrolysis is induced in marine bacteria (Arnosti, 2004).

Our CARD-FISH identifications focused on the potential enrichment of two bacterial phyla that had been linked to degradation of (sulfated) polysaccharide, *Planctomycetales* (Glöckner et al., 2003; Woebken et al., 2007) and *Bacteroidetes* (Kirchman, 2002; Bauer et al., 2006). Whereas we could not detect significant numbers of *Planctomycetales* with probe PLA46, there was consistently a strong contribution of CF319a-positive bacteroidetal cells in the large-particle-associated fraction post-incubation (**Table 2**). This result is consistent with the observation that members of the phylum *Bacteroidetes* – and in particular those of the class *Flavobacteria* – are frequently associated with phytoplankton blooms (Gomez-Pereira et al., 2012; Teeling et al., 2012). FISH staining showed somewhat higher average counts of CF319a-stainable cells at S3/S6 compared to S12/S19, a result likely due to initial differences in phytoplankton (**Figure 2**; Gomez-Pereira et al., 2010) as well as to microbial growth in response to substrate addition. Specific differences in functional potential of these organisms, however, became evident only through direct measurement of extracellular enzyme activities, using an experimental approach that provides information about structural specificities of these enzymes. This investigation is the first to specifically focus on the hydrolytic capabilities of large-particle-associated bacteria to hydrolyze these substrates; it is also the first investigation to combine FISH staining directly with these measurements. These communities evidently exhibit distinct patterns in enzyme activities, as has been observed for unfiltered surface and subsurface waters (Arnosti et al., 2005a, 2011; Steen et al., 2008, 2012). The extent to which patterns of enzyme activities may differ for large particles vs. whole water should be a focal point of further work, as should the extent to which such patterns may change through annual cycles of phytoplankton and bacterial succession (Allison et al., 2012; Gilbert et al., 2012). Such investigations will help us define more precisely at a functional level the contributions of specific microbial communities to carbon processing in the ocean.

## ACKNOWLEDGMENTS

We thank the captain, crew, and scientific party of R/V *Maria S. Merian* for a successful cruise, and Antje Wichels and Gunnar Gerdts help in sample collection. We are particularly grateful to Jörg Wulf and Birgit Rattunde for their meticulous efforts to obtain FISH data from minimal sample volume. This project was supported by the Max Planck Society. Additional support to Carol Arnosti was provided by the U.S. National Science Foundation (OCE-0323975 and OCE-0848703), as well as by the Alfred Wegener Institute for Polar and Marine Research, and the Hanse-Wissenschaftskolleg (Delmenhorst). Additional support to Uta Passow was provided by the U.S. National Science Foundation (OCE-0926711 and OCE-1041038).

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

*Received: 29 July 2012; accepted: 27 November 2012; published online: 13 December 2012.*

*Citation: Arnosti C, Fuchs BM, Amann R and Passow U (2012) Contrasting extracellular enzyme activities of particle-associated bacteria from distinct provinces of the North Atlantic Ocean. Front. Microbio.* ***3****:425. doi: 10.3389/fmicb.2012.00425*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 11 September 2012
doi: 10.3389/fmicb.2012.00336

# Grappling with Proteus: population-level approaches to understanding microbial diversity

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The emerging fields of microbial population genetics and genomics provide an avenue to study the ecological rules that govern how communities form, function, and evolve. Our struggle to understand the causes and consequences of microbial diversity stems from our inability to define ecologically and evolutionarily meaningful units of diversity. The 16S rRNA-based tools that have been so useful in charting microbial diversity may lack sufficient sensitivity to answer many questions about the ecology and evolution of microbes. Examining genetic diversity with increased resolution is vital to understanding the forces shaping community structure. Population genetic analyses enabled by whole genome sequencing, multilocus sequence analyses, or single-nucleotide polymorphism analyses permit the testing of hypotheses pertaining to the geographic distribution, migration, and habitat preference of specific microbial lineages. Furthermore, these approaches can reveal patterns of gene exchange within and between populations and communities. Tools from microbial population genetics and population genomics can be used to increase the resolution with which we measure microbial diversity, enabling a focus on the scale of genetic diversity at which ecological processes impact evolutionary events. This tighter focus promises to improve our understanding of the causes and consequences of microbial community structure.

**Keywords: microbial population, ecology, biogeography, evolution, microbial diversity**

## INTRODUCTION

According to Greek mythology, Proteus knew of all things past, present, and future, but only shared this knowledge if captured. When pursued he would change shape, and the closer his pursuer the more quickly he changed, his knowledge just beyond grasp. Our efforts to define the causes and consequences of microbial community structure are like grappling with Proteus. A rigorous framework for understanding microbial community structure remains beyond our grasp because our concepts of what constitutes a community, a species, or a population are ill-developed and fungible. We argue that our comprehension of microbial communities is severely hampered by our inability to recognize ecologically and evolutionarily meaningful units of diversity. We believe observations from microbial population genetics and genomics promise to change the manner in which we define microbial diversity. Population genetics and population genomics both focus on the evolutionary dynamics of populations. While these approaches employ different analytical techniques, conceptually they represent points along a continuum, the differences being the amount and type of genetic information used to make evolutionary inference. Population-based approaches represent a powerful new paradigm for exploring the fundamental units of community structure.

The ribosomal RNA (rRNA) paradigm has been invaluable in charting the diversity of the microbial world by providing both a phylogenetic framework for understanding microbial diversity and tools for characterizing microbial communities without the biases imposed by cultivation. The remarkable conservation of rRNA gene sequences makes them excellent for determining phylogenetic relationships between diverse microbial assemblages. However, as a result of their high conservation these molecules are insensitive to evolutionary changes that occur in response to ecological dynamics. As the field of microbial ecology matures, we increasingly seek to understand the ecological rules that govern how communities assemble, function, and evolve. The rRNA-based tools that have fueled the growth and development of our field may be poorly suited to answering many of the ecological questions we now face.

The most common unit of diversity employed in analyses of microbial community structure is the operational taxonomic unit (OTU) based on the 16S rRNA gene. An OTU is generally defined as a group of sequences that differ by less than 3% of nucleotide positions in the 16S rRNA gene ($OTU_{0.03}$; Hughes et al., 2001). This cutoff is based on current criteria for defining microbial species (Wayne et al., 1987). In other fields of biology, a "species" is considered a distinct and coherent evolutionary unit in terms of ancestry, range, or ecological function (Coyne and Orr, 2004). The current microbial species concept, however, is primarily based on the needs of taxonomic analysis. This taxonomic species concept was established before the use of molecular sequence data and before phylogenetic analysis of microorganisms was possible. Sequence data was applied to the microbial species framework in a *post hoc* manner as a way of supporting the pre-existing species definition. Hence, the foundation that underpins the $OTU_{0.03}$ does not have a firm ecological basis and we should consider carefully the range of ecological and evolutionary hypotheses

that can be addressed effectively on the basis of this unit of diversity.

The nascent field of microbial biogeography provides a compelling example of the limitations that the current microbial species concept imposes on our understanding of microbial diversity. As demonstrated by *Escherichia coli* and *Salmonella enterica*, 2.8% divergence of 16S rRNA between species can take approximately 63–120 million years (Ochman et al., 1999). Hence, strains within an $OTU_{0.03}$ may have shared a most recent common ancestor during the early Cretaceous, a period when many of the Earth's continents were still joined. Consider for a moment the consequences if this unit of diversity were employed in the study of plants and animals. For example, all species in the tortoise family have diverged in the last 50 million years, and thus, if studied using units of diversity that have the same sensitivity as the $OTU_{0.03}$, would represent a single globally distributed taxonomic unit. Were Darwin to have used this definition of diversity, he would have observed only one type of tortoise in the Galapagos, and we would all be the poorer for it. It should not surprise us that this unit of diversity is poorly suited to resolve current patterns of microbial biogeography.

The use of rRNA-based OTUs obscures the recent evolutionary history of microbial lineages. We know that organisms with exactly the same 16S rRNA gene sequence can share as few as 38% of the genes in their genomes (Welch et al., 2002; Tettelin et al., 2005; Hall et al., 2010), and that organisms with the same 16S rRNA gene sequence can have different ecological characteristics (Jaspers and Overmann, 2004). Units of diversity defined by rRNA genes are valuable in terms of discovering and characterizing new lineages, charting the scope of microbial diversity, and resolving evolutionary relationships at temporal scales ranging approximately from $10^7$ to $10^9$ years. However this unit of diversity is not well suited to addressing ecological and evolutionary processes, such as dispersal and speciation, which operate at timescales of less than $10^6$ years.

A focus on genetic diversity at a smaller phylogenetic scale than the $OTU_{0.03}$ is vital to understanding the forces that govern community structure. Rather than attempting to define the appropriate units for studying microbial diversity *a priori*, we should currently be making observations of the patterns of genetic diversity that exist in nature. This can be achieved by focusing on groups of closely related strains and adopting a flexible and methods-free concept of microbial populations: a group of organisms characterized by a genetic, spatial, temporal, or ecological boundary. Populations of isolates can be studied through multilocus analyses which provide greater phylogenetic resolution than 16S rRNA sequence analyses and which allow for estimation of recombination rates. Genomic analyses, including analyses of single-nucleotide polymorphisms (SNPs) or whole genome sequences, provide even greater phylogenetic resolution and the ability to explore patterns of gene exchange and signatures of selection. Population genetics and genomics now provide a solid foundation to study evolutionary dynamics at the sale of ecological interactions and a framework for addressing specific questions such as: (1) How are microbial lineages distributed spatially, and what are the roles of migration and local adaptation in defining the genetic and functional characteristics of communities? (2) How do patterns of gene flow vary with respect to the genetic and geographic distance between strains? (3) How do population dynamics influence community dynamics and ecological processes? The following is a brief overview of how our understanding of microbial ecology may be enhanced by taking a population genomics approach.

## IMPLEMENTING POPULATION-LEVEL APPROACHES

The forces governing microbial biogeography can be best evaluated at fine scales of genetic diversity (Pearson et al., 2009; Vogler et al., 2009), and *Bacillus anthracis* provides a case study to demonstrate this point (Kenefic et al., 2009). The potential use of *Bacillus anthracis* in terror attacks created a need to distinguish naturally occurring strains from those used as biological weapons and to understand the genetic diversity within this species. Multiple *Bacillus anthracis* genome sequences were used to identify canonical SNPs that resolve branching points in the phylogeny of the species, and these SNPs were subsequently used to explore the origins of *Bacillus anthracis* in North America (Kenefic et al., 2009). The introduction of anthrax to North America was hypothesized to have occurred along the US Gulf Coast by infected European cattle during the colonial period of American history. However, analysis of SNPs in 285 geographically diverse isolates from North America indicates a Eurasian ancestor originating from the north, likely entering the continent along the Bering land bridge and introduced by ungulate migrations during the last ice age (Kenefic et al., 2009). Through this approach, we see that discernible patterns of microbial biogeography were established as a result of dispersal at a temporal scale of thousands of years. These patterns could only be observed through genome-level analyses.

In another example, Pearson et al. (2009) reconstructed the evolutionary history of *Burkholderia pseudomallei* and *Burkholderia mallei* using >14,000 orthologous SNPs from 33 whole genome sequences of *Burkholderia pseudomallei* and *Burkholderia mallei* sampled across Australia, Southeast Asia, and the rest of the world. This phylogeny was supplemented with data from multilocus sequence analysis (MLSA) of >1,700 global *Burkholderia* isolates. Population structure of these isolates supports the existence of two geographically distinct *Burkholderia pseudomallei* subpopulations, originating in Australia and Southeast Asia and separated by the Wallace Line, a geographical pattern well-documented in macroorganisms. The deeply branching Australian *Burkholderia pseudomallei* group was determined to be the most genetically diverse lineage, thus, representative of the ancestral gene pool. Molecular clock estimates suggest these *Burkholderia pseudomallei* populations diverged between 16,000 and 225,000 years ago (Pearson et al., 2009). The biogeographical patterns observed in *Burkholderia* underlie patterns of genome differentiation and ultimately govern the origins of diversity within the genus. These genetic boundaries would be overlooked if sequence analyses were restricted to rRNA genes.

*Helicobacter pylori* inhabits the stomachs of over half the world's human population and provides another useful case study. MLSA of 769 *H. pylori* isolates representing 51 distinct human populations identified six extant subpopulations of *H. pylori*. These ancestral populations correlate well with geographical regions at a global scale. Extant strains of *H. pylori* demonstrate distinct

patterns of ancestral admixture influenced by the geographic origin and mixing of hosts (Linz et al., 2007). There is an inverse relationship between genetic diversity and geographic distance from East Africa in both *H. pylori* and its human host, and thus *H. pylori* dispersal patterns are believed to mirror human migration patterns from East Africa approximately 58,000 years ago (Linz et al., 2007).

These studies demonstrate that patterns of microbial biogeography, veiled in analysis of 16S rRNA genes, become evident through more sensitive genetic analyses. In the case of *Bacillus anthracis*, we learn that European strains did not routinely colonize and persist in North America, despite multiple introductions over hundreds of years during the time of European settlement (Kenefic et al., 2009). This raises interesting new questions about the factors that govern the competitive fitness of strains introduced to new habitats. Issues of dispersal and colonization are critical for understanding constraints on community structure. An advantage of population genetics and genomics is that they provide a route for investigating microbial biogeography and also provide data that can be used to explore the ecological adaptations that impact colonization success and ultimately the environmental distribution of species.

Analyses of strain collections spanning discrete sites make it possible to determine how ecological traits map onto the evolutionary history of a lineage (Connor et al., 2010; Becraft et al., 2011; Preheim et al., 2011). For example, using an approach to map habitat traits onto microbial phylogeny, ecological populations within coastal *Vibrio* isolates can be predicted based on seasonal occurrence and particulate size fractionation (Hunt et al., 2008; Preheim et al., 2011). Populations adapted to a free-living lifestyle can be distinguished from those adapted to living on the surface of organic matter particulates, or on the surface of phytoplankton (Preheim et al., 2011). This approach has also been used to identify ecological populations in *Bacillus* (Connor et al., 2010) and *Synechococcus* (Becraft et al., 2011). Both solar exposure and soil texture are important predictors of ecological populations among the *Bacillus subtilis–Bacillus licheniformis* clade (Connor et al., 2010), while ecological populations of *Synechococcus* correspond with gradients of temperature and depth in microbial mats (Becraft et al., 2011). Recognizing the existence of meaningful ecological units is the first step to understanding both the ecological factors that govern the spatial and temporal dynamics of microbial communities and the evolutionary dynamics that govern the origins and maintenance of microbial diversity.

An advantage of using population genomics over single or multilocus methods is the ability to evaluate the impact of horizontal gene transfers (HGTs) on microbial evolution and ecology. HGT can blur the lines of ancestry between lineages, shuffling adaptive genes, and HGT may prevent the development of genetically and ecologically cohesive populations (Fraser et al., 2009; Shapiro et al., 2012). Genomic studies provide evidence that patterns of gene exchange may be controlled by propinquity, with local adaptation facilitated by sampling genes from the environment. For example, environmental co-localization governs exchange of integron cassettes in *Vibrio* species regardless of species boundaries (Boucher et al., 2011), while, interspecies exchange of core genes is not observed (Boucher et al., 2011). Likewise, Caro-Quintero et al. (2011) demonstrated that *Shewanella baltica* isolates in the Baltic Sea exchange genes more frequently with isolates found at similar depths than with isolates at different depths. Lastly, gene flow in the thermoacidophilic crenarchaeon *Sulfolobus islandicus* is influenced by geographical isolation, driving patterns of speciation (Whitaker et al., 2003; Reno et al., 2009). These observations suggest that evolutionary processes may vary between microbial lineages and even between core and auxiliary genes (Riley and Lizotte-Waniewski, 2009; Lefebure et al., 2010). In the case of *Shewanella baltica*, as much as 20% of the genome was inherited from co-localized strains (Caro-Quintero et al., 2011), suggesting that ecological interactions can have strong impacts on genome dynamics.

At a gross level, it is clear that the composition of a microbial community has strong impacts on environmental biogeochemistry, though the biotic and abiotic mechanisms that link community structure and function remain poorly described. If we want to understand community structure–function relationships at a fundamental level, we need to start with measurements of diversity that capture adaptive differences within and between lineages. We need to understand how the genomic diversity within a lineage impacts ecological function and is distributed in the environment, how evolutionary and ecological forces regulate gene exchange, and how patterns of gene exchange within and between lineages impact community function. These inquiries can be enabled by studying populations as the fundamental units from which communities are constructed.

## WHERE DO WE GO FROM HERE?

Microbial population genetics and genomics are opening avenues for understanding the ecological and evolutionary mechanisms governing microbial diversity. However, a focus on the dynamics of populations and species still faces several formidable obstacles. Foremost is the current lack of coherent and pragmatic definitions for populations and species. Without such criteria it is difficult to objectively compare results between studies. As our understanding of microbial populations improves, it will be important to develop objective criteria for defining lineages that will be relevant and applicable across a wide range of microorganisms.

Another challenge to the application of microbial population genomics is a lack of information about how to properly sample the genetic diversity of microbial populations, both in terms of spatial scale and numbers of strains. Rigorous population studies require robust genetic sampling across appropriate spatial, temporal, or habitat scales in order to achieve the ultimate goal of accurately depicting patterns of biodiversity existing in nature. Sufficient individuals must be sampled to represent the breadth of genetic diversity across an organism's geographic range and to capture genetically informative loci that reflect its evolutionary history. What spatial scales best capture the genomic diversity of microbial populations? Over what spatial and temporal scales does a microbial cell sample genetic material from its environment? What scales are most suitable for inferring adaptive traits from an organism's habitat distribution? How do we define a microhabitat, or an ecological niche? What environmental parameters should be measured as part of the sampling design? All of these questions are fundamental and must be addressed as we move forward.

An obvious limitation to population genomic approaches is the need for strains to be cultivated in isolation. While most microorganisms have yet to be cultivated, much progress can still be made with organisms that we can cultivate now. Model systems can be developed and used to explore the evolutionary and ecological mechanisms that regulate microbial diversity. Once revealed, these mechanisms can be used to make predictions relevant to organisms more recalcitrant to cultivation. In addition, a solution to the cultivation problem is available through application of single-cell genomics. Single-cell methods of genome analysis can be used to perform genome sequencing or multilocus analysis on individual microbial cells without the need for cultivation (Stepanauskas and Sieracki, 2007; Swan et al., 2011; Tadmor et al., 2011; Martinez-Garcia et al., 2012). Metagenomics offers another avenue through which theory developed through microbial population genetics and genomics may be applied to more complex communities without the need for cultivation (Allen and Banfield, 2005; Simmons et al., 2008; Dick et al., 2009; Morowitz et al., 2011; Denef and Banfield, 2012; Narasingarao et al., 2012).

The ongoing exponential decline in sequencing costs has made population genomics a reality, but the computational tools and theory for understanding these data still lag. While a wide variety of computational population genetics tools exist, many are based on theory developed for eukaryotic organisms. There are fundamental differences between macroorganisms and microorganisms that may impact assumptions implicit in population genetic models. In addition, not all models can equally capture the range of ecological and evolutionary dynamics that operate in the microbial world. For example, the algorithm eBURST determines founding genotypes of clonal complexes from MLSA and has been used to describe evolutionary patterns in many microbial lineages (Feil et al., 2004). However, in populations with high allelic diversity or recombination rates, like *H. pylori*, eBURST may not be appropriate (Turner et al., 2007). The program STRUCTURE uses multilocus data to infer population structure and gene exchange (Pritchard et al., 2000) and has been applied to a number of bacteria including *Moraxella catarrhalis* (Wirth et al., 2007), *H. pylori* (Falush et al., 2003), and *Streptomyces* (Doroghazi and Buckley, 2010). However, it remains challenging to estimate accurately the true number of ancestral populations contributing to a collection of strains. The program LDhat (McVean et al., 2002) is based on coalescent theory and estimates recombination rates from population genetic data. However, LDhat assumes an unstructured population in equilibrium and will misestimate recombination when these assumptions are not met. An alternative to this approach is the program ClonalFrame (Didelot and Falush, 2007), which maps recombination events onto a phylogenetic pattern of clonal ancestry. However, ClonalFrame can have difficulty modeling populations with very high rates of recombination. While each of these tools may be applicable to certain microbial lineages, they can give incorrect results if applied to lineages that violate model assumptions. There is a continuing need to develop computational strategies that focus on microbial population genomics and to test the assumptions that underlie these analyses.

Ultimately, population-level approaches promise to shed light on the forces that govern microbial diversification and evolution. By providing evidence to interpret adaptive traits and identify selective habitats, these approaches will inform our understanding of the competitive interactions within and between lineages, laying the framework for an understanding of community-level interactions. These approaches should also provide insight on the genetic and ecological forces that govern gene exchange. We should focus now on making empirical observations to inform our understanding of the vertical and horizontal components of ancestry and whether and how microbial lineages form ecologically and genetically cohesive units. A better grasp of the relevant units with which to measure microbial diversity is essential for progress in microbial ecology. Insights from microbial population studies promise to improve our understanding of microbial diversity, providing access to knowledge about the causes and consequences of microbial community structure.

## ACKNOWLEDGMENT

This material is based upon work supported by the National Science Foundation under Grant No. DEB-1050475.

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

*Received: 31 May 2012; accepted: 29 August 2012; published online: 11 September 2012.*

*Citation: Choudoir MJ, Campbell AN and Buckley DH (2012) Grappling with Proteus: population-level approaches to understanding microbial diversity. Front. Microbio. **3**:336. doi: 10.3389/fmicb.2012.00336*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 09 May 2013
doi: 10.3389/fmicb.2013.00112

# Links between metabolic plasticity and functional redundancy in freshwater bacterioplankton communities

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Metabolic plasticity and functional redundancy are fundamental properties of microbial communities, which shape their response to environmental forcing, and also mediate the relationship between community composition and function. Yet, the actual quantification of these emergent community properties has been elusive, and we thus do not know how they vary across bacterial communities, and their relationship to environmental gradients and to each other. Here we present an experimental framework that allows us to simultaneously quantify metabolic plasticity and functional redundancy in freshwater bacterioplankton communities, and to explore connections that may exists between them. We define metabolic plasticity as the rate of change in single-cell properties (cell wall integrity, cell size, single-cell activity) relative to changes in community composition. Likewise, we define functional redundancy as the rate of change in carbon substrate uptake capacities relative to changes in community composition. We assessed these two key community attributes in transplant experiments where bacterioplankton from various aquatic habitats within the same watershed were transplanted from their original water to waters from other systems that differ in their main resources. Our results show that metabolic plasticity is an intrinsic property of bacterial communities, whereas the expression of functional redundancy appears to be more dependent on environmental factors. Furthermore, there was an overall strong positive relationship between the level of functional redundancy and of metabolic plasticity, suggesting no trade-offs between these community attributes but rather a possible co-selection. The apparent continuum in the expression of both functional redundancy and plasticity among bacterial communities and the link between them, in turn suggest that the link between community diversity and function may also vary along a continuum, from being very tight, to being weak, or absent.

**Keywords: freshwater bacterioplankton, metabolic plasticity, traits selection, functional redundancy, community composition, environmental forcing**

## INTRODUCTION

There is now ample evidence that the overall metabolic performance of aquatic bacterial communities is mainly driven by environmental factors in a manner that is roughly predictable (Ducklow, 2008; Lennon and Cottingham, 2008; Comte and del Giorgio, 2009), and yet there is much unexplained variation in the response of microbes to environmental changes (e.g., Comte and del Giorgio, 2009). The extent to which the composition and diversity of bacterial communities play a role in shaping their overall performance and their responses to environmental forcing has been a focus of research in aquatic microbial ecology (Langenheder et al., 2005, 2006, 2010; Findlay and Sinsabaugh, 2006; Bertilsson et al., 2007; Gamfeld and Hillebrand, 2008; Boucher and Debroas, 2009; Lindström et al., 2010; Comte and del Giorgio, 2010, 2011; Peter et al., 2011; Reed and Martiny, 2013). Results from both field studies (Shade et al., 2007; Jones et al., 2009; Nelson, 2009) and laboratory experiments (Judd et al., 2006; Kritzberg et al., 2006; Newton and McMahon, 2011) have shown, for example, that shifts in the nature or the source of organic matter can induce changes both in community composition and in various aspects of community metabolism, yet it is still uncertain to what extent the actual metabolic response may be mediated by these changes in community structure, or whether the two simply covary.

One of the reasons it has been so difficult to establish clear links between bacterial community composition (BCC) and the functional and metabolic responses of the community to environmental forcing is that this link is not direct, but rather may be mediated by aggregate properties of these communities. One such aggregate property is the degree of metabolic plasticity that exists at the community level (also termed community "resistance" by Allison and Martiny, 2008), which reflects the capacity of a community to accommodate environmental changes by adjusting the overall performance of existing dominant phylotypes.

Another key emergent community property that may mediate community composition and overall response is functional

redundancy, which implies that different phylotypes can perform similar functional role in the community. Therefore, this emergent community property may explain the reported lack of connection between community composition and key aspects of bacterial community performance and function, as well as the fact that stable ecosystem function is generally maintained under very different configurations of community composition (e.g., Fernàndez et al., 1999; Langenheder et al., 2005). Although there is debate on whether functional redundancy is high (Wohl et al., 2004) or moderate (Peter et al., 2011) in microbial communities, the reality is that this community property has seldom been quantified, and we do not know how functional redundancy varies among microbial communities.

These community-level properties are key for two reasons: They influence the response of bacterial communities to environmental forcing, and on the other hand, they modulate the relationship that may exist between community composition and community function and performance. Lack of correlation between features of bacterial function and of composition has traditionally been interpreted as evidence of a high degree of functional redundancy (e.g., Rosenfeld, 2002; Allison and Martiny, 2008). In contrast, significant relationships between these variables are usually interpreted as diversity and composition having a significant influence on community performance (and therefore on ecosystem functioning), suggesting in turn that functional redundancy may be more constrained.

Likewise, there is ample evidence that individual aquatic bacterial taxa may be extremely flexible in terms of the breadth of physiological and morphological adjustments to their environment (Hahn et al., 2003; Jaspers and Overmann, 2004; Meyer et al., 2004; Buchan et al., 2005; Hahn, 2006; Walker et al., 2006; Schimel et al., 2007; Allison and Martiny, 2008; Comte and del Giorgio, 2011). However, metabolic plasticity at the community level, aka community resistance (Allison and Martiny, 2008), like functional redundancy, is a difficult concept to quantify, and we still do not know its magnitude and variability among communities.

These emergent properties, i.e., plasticity and redundancy, result from the sum of the physiologic and life history traits of the ensemble of individual players within the community, but the scaling of these individual traits to the community level is still not well understood (Bohannan et al., 2002; Suding et al., 2008; Hillebrand and Matthiessen, 2009; Shade et al., 2012). As with species or ecotype traits, we can question whether there are trade-offs associated to these two community-level traits, or whether they are in fact co-selected by the environmental and the biological forcing factors that shape the structure of these communities. The rules of co-selection and trade-offs associated to traits within a single-organism, however, may not apply to community-level features that emerge from the combination of individual traits (e.g., Suding et al., 2008; Hillebrand and Matthiessen, 2009). For example, we can hypothesize that communities that are intrinsically more plastic may be less functionally redundant, because there may be trade-offs at the individual level between the breadth of physiologic tolerance and flexibility, and the breadth of resource utilization. Moreover these trades-off can vary along environmental gradients as previously shown for other microbial traits (Jessup and Bohannan, 2008). These questions have not been addressed for natural aquatic bacterial communities.

There are thus major conceptual and technical challenges associated to these two community properties, and although they have been extensively discussed and invoked in contemporary microbial ecology, they have seldom, if ever, been actually quantified and compared. We do not know how they vary among communities, how they are regulated, if they are ecosystem-specific, and more importantly, we do not know what the relationships and trade-offs are between these two key properties of microbial communities. In this paper we explicitly address these issues in freshwater bacterioplankton communities.

## CONCEPTUAL FRAMEWORK

Bacterial plasticity is linked to the average breadth of morphological and physiological characteristics of cells affecting their individual performances such as growth, cell division, and respiration. For example, there is clear evidence that bacteria can respond to shifts in environmental conditions by modifying their size, physiology and activity, which may ultimately lead to dormancy (Schimel et al., 2007; Lennon and Jones, 2011; Evans and Hofmann, 2012) or tolerance to environmental perturbations (Meyer et al., 2004). In this context, high community plasticity is associated to shifts in single-cell properties with little or no change in community composition, and therefore to communities that can accommodate environmental change with physiological and morphological adjustments of the dominant phylotypes. In our framework, community plasticity can be quantified as changes in these single-cell characteristics (SCC), evidenced as shifts in a host of individual properties of cells measured by flow cytometry (cell wall integrity, cell size, single-cell DNA content, and activity), relative to changes in BCC. In practice, this can be quantified as the slope of the regression model of SCC as a function of BCC under circumstances where both SCC and BCC are varying, for example, in time within a given site, in space along natural environmental gradients, or under experimental manipulations of environmental factors. The change in SCC and in BCC under these circumstances can be quantified in terms of dissimilarity between successive states of the same community, and high values of slopes between the dissimilarities observed in SCC relative to those in BCC would indicate a high degree of plasticity for that particular community, and vice versa.

Redundancy, on the other hand, is linked to the level of overlap in functional capacities (FCs) among the dominant phylotypes, such that different taxa provide similar functions to the community (Burke et al., 2011). In our experimental framework, functional redundancy is defined as the magnitude of change in FCs relative to the magnitude of change in community composition, again under circumstances where both FC and BCC are varying (as for SCC above); we use the organic substrate uptake profiles of the community as a measure of FC. In this framework, small changes, and therefore a high level of similarity in FC together with stronger changes in BCC, for example along an environmental gradient, would indicate a high level functional redundancy within the community.

We have applied the above metrics to assess the extent of metabolic plasticity and functional redundancy among local

communities within same regional metacommunity, and how these two emerging properties relate to each other, in experiments where we manipulated the environment in order to allow these communities to express their intrinsic levels of metabolic plasticity and functional redundancy. Rather than exposing these bacterial communities to artificial conditions, we chose to use reciprocal transplant experiments, which have been commonly used to test for effects of both environment and composition (and their interactions) on the functioning of microbial communities in multiple environments (e.g., Gasol et al., 2002; Kirchman et al., 2004; Langenheder et al., 2005; Reed and Martiny, 2007, 2013; Strickland et al., 2009; Bell, 2010; Shade et al., 2010), and wherein local freshwater bacterial communities were grown in their own original water, and also in water originating from other habitat types that exist within the same watershed (**Figure 1**). We transplanted lake bacterial communities into river water and vice versa, and lake bacterial communities into marsh waters and vice versa, and these environments differed greatly in terms of chemistry and organic matter (**Table 1**). These transplant experiments allowed us to derive two alternative but complementary estimates of both plasticity and redundancy. On the one hand, we calculated an "absolute" measure of functional redundancy and plasticity in both controls and transplanted communities, and used these to test whether redundancy and plasticity are intrinsic features of these communities. On the other, we estimated a more integrative measure of both bacterial communities properties (i.e., "relative plasticity and redundancy"), where plasticity and redundancy measured in transplanted communities were calculated relative to control communities. We derived these metrics as follows:

(1) "Absolute" plasticity and redundancy (**Figure 2**): For each individual treatment we estimated the rate of change in SCC, FC, and BCC over time, and then combined these to derive plasticity (as the slope of SCC vs BCC) and redundancy (as the slope of the FC vs BCC). We did this for the community incubated in its original water, and the same community incubated in a different source of water, such that we had two separate estimates

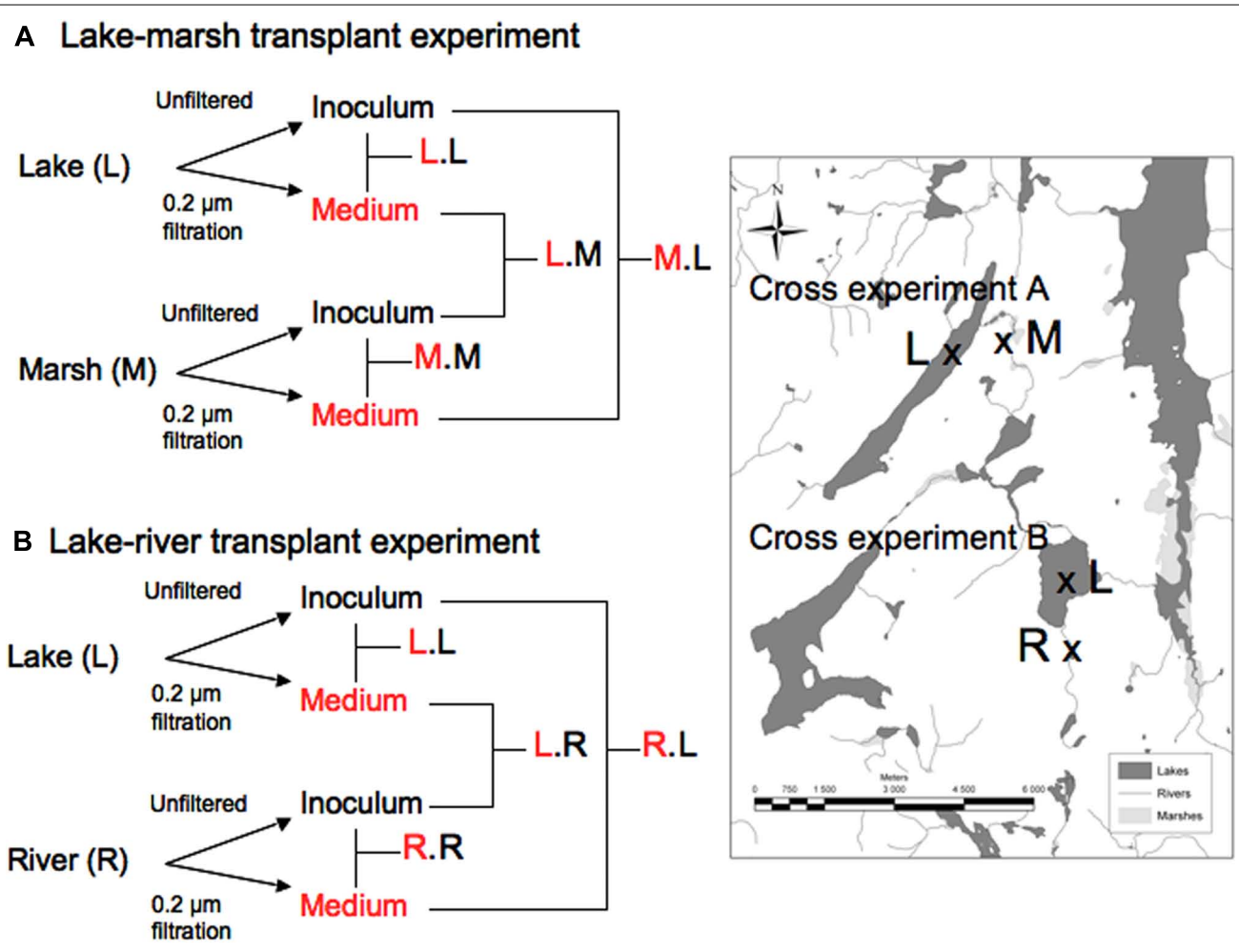


**FIGURE 1 | Experimental design of the study – Two-way factorial design with two series of experiments: Lake Bowker-Marsh (A) and Lake Fraser-River (B) Each experiment was replicated two times (June and July 2006).** Samples were taken at days 0, 2, 3 and 5. L, M, R correspond to lake, marsh and river respectively. The treatment assignment presents the source of medium first (in red) and the source of the inoculum.

**Table 1 | Characteristics of the aquatic ecosystems sampled**

| Site | DOC (mg $L^{-1}$) | TP ($\mu$g $L^{-1}$) | TN (mg $L^{-1}$) | Temp (°C) | Cond (mS $cm^{-1}$) | BP ($\mu$g C $L^{-1}$ $h^{-1}$) | BA ($10^6$ $mL^{-1}$) |
|---|---|---|---|---|---|---|---|
| Lake Bowker | 2.1 ± 0.05 | 2 ± 1.04 | 0.14 ± 0.02 | 22.1 ± 0.8 | 0.056 ± 0.01 | 0.21 ± 0.07 | 1.5 ± 0.1 |
| Marsh | 5.1 ± 0.46 | 7.1 ± 1.5 | 0.26 ± 0.02 | 21.8 ± 1.3 | 0.057 ± 0.01 | 1.15 ± 0.05 | 4.1 ± 1.9 |
| River | 10.2 ± 1.7 | 21.6 ± 1.2 | 0.45 ± 0.02 | 16.8 ± 3 | 0.046 ± 0.02 | 2 ± 0.63 | 3.7 ± 1.3 |
| Lake Fraser | 6 ± 0.38 | 6 ± 1.9 | 0.22 ± 0.03 | 19.3 ± 1.8 | 0.057 ± 0.01 | 1.41 ± 0.4 | 4.2 ± 1.5 |

*Values represent in situ mean (±standard deviation) concentrations of dissolved organic carbon (DOC), total phosphorus (TP) and total nitrogen (TN), average water temperature (Temp) and conductivity (Cond), mean rates of bacterial production in terms of $^3$H-leucine uptake (BP) and bacterial abundance (BA).*

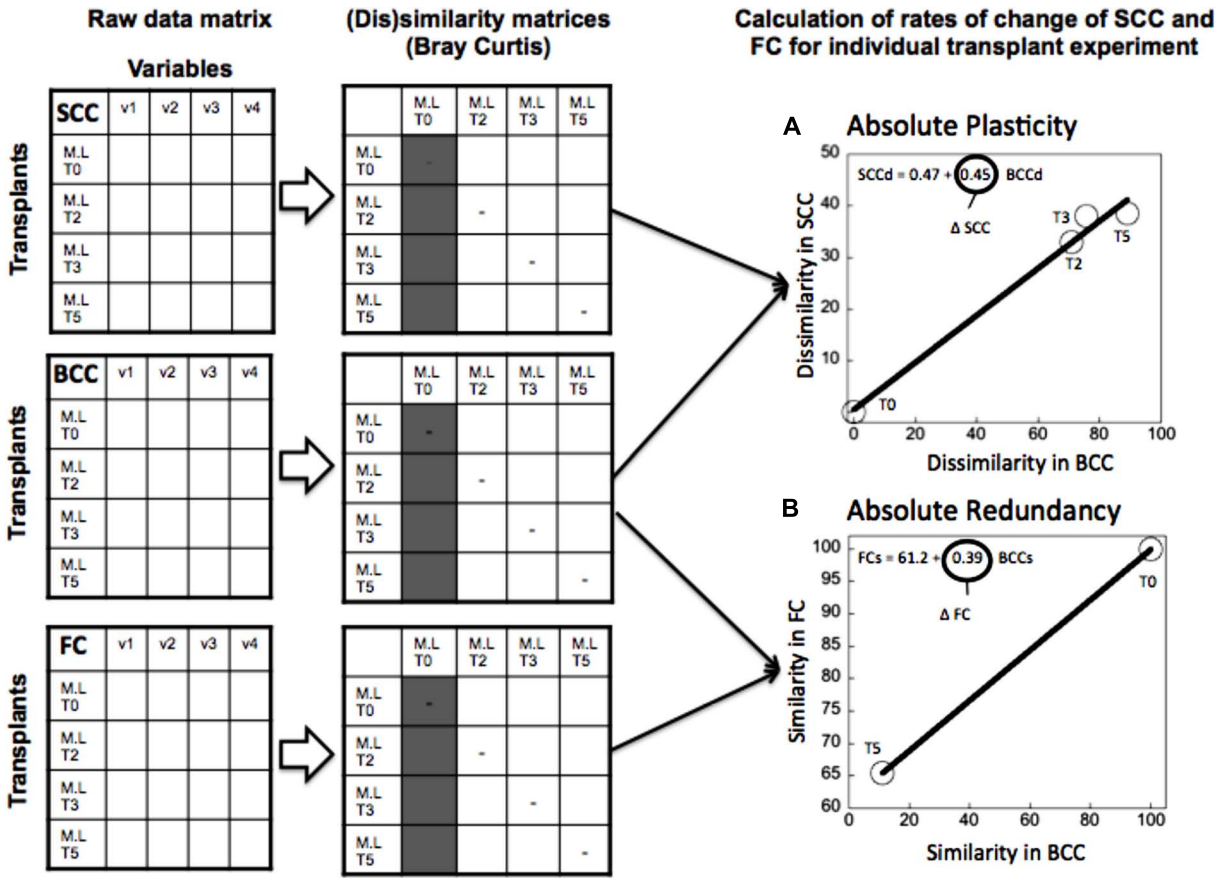


**FIGURE 2 | Example of calculation of the "absolute" plasticity (A) and "absolute" functional redundancy (B) for the transplant experiment (June 2006), where lake bacteria were inoculated in marsh water.** Functional redundancy (Δ FC) was calculated as the slope of the relationship between the rate of change in similarity (Bray Curtis) of functional capacities at the different time points of the experiment relative to the community at time 0 of the experiment (marked in gray in the similarity matrix), and the corresponding changes in similarity in community composition (BCCs). Metabolic plasticity (Δ SCC) was estimated as the slope of the relationship between the dissimilarity in single-cell characteristics (SCCd) relative to the community at time 0, and the corresponding changes in the relative dissimilarity in BCC (BCCd) over the same period. Each circle represents the average similarity in FC (or dissimilarity in SCC) per unit similarity (or dissimilarity) in BCC at each time point of the experiment. Dissimilarity and similarity indices were estimated using Bray Curtis metric. The line represents the least square regression fit and the slope of the regression model refers to either Δ FC or Δ SCC.

of plasticity and redundancy for any given community. This allowed us to test whether there was a correlation between plasticity and redundancy expressed under different environmental scenarios, and therefore, whether these properties are intrinsic to the community or whether they are driven by environmental forcing.

(2) "Relative" plasticity and redundancy (**Figure 3**): In the second approach, we followed the change in SCC, FC, and BCC not between time points within a given treatment, but rather between the control and the transplanted samples of the same community. This approach yielded a single estimate of plasticity (also as change in SCC relative to change in BCC),

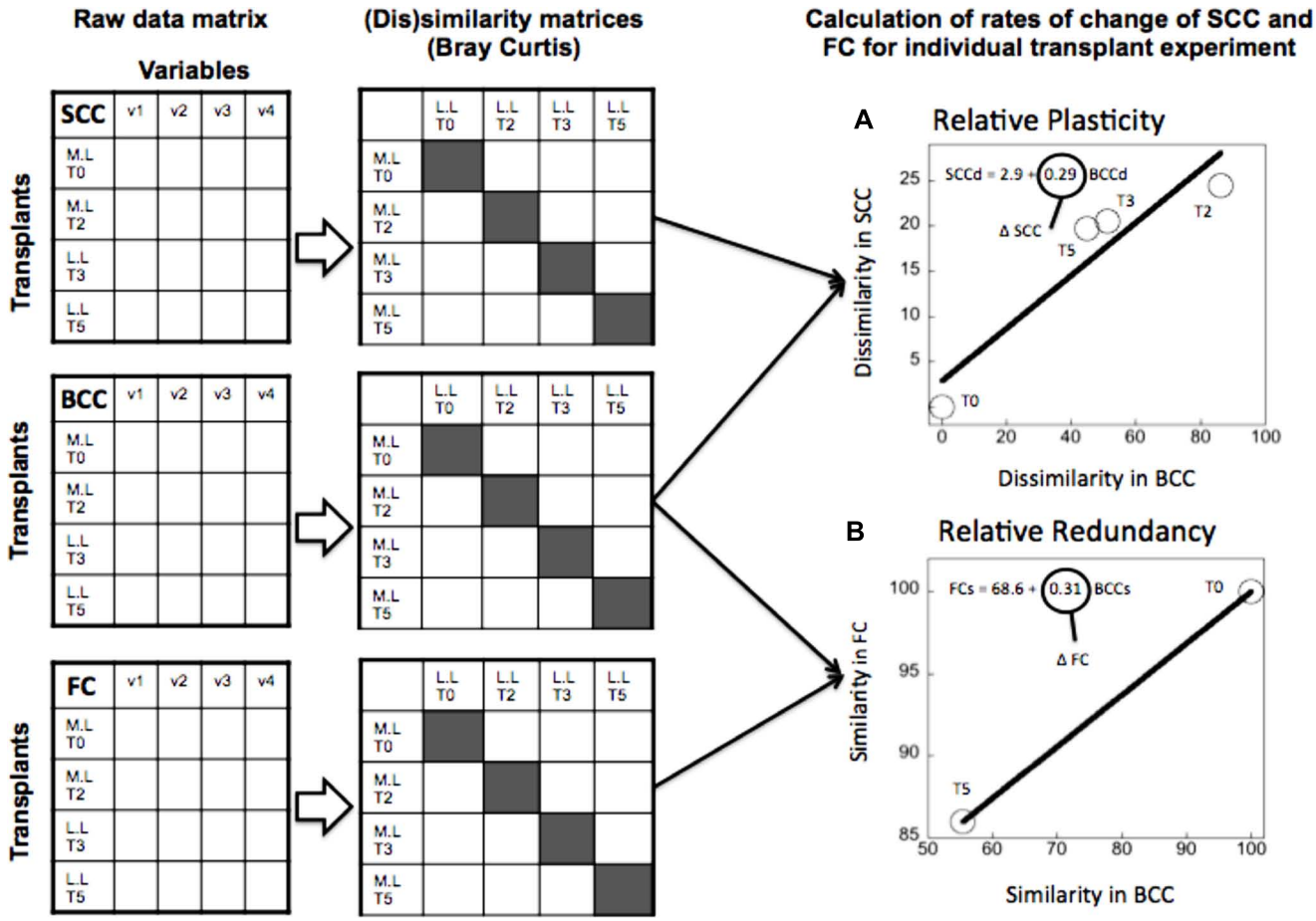


**FIGURE 3 | Example of calculation of the extent of "relative" plasticity (A) and "relative" functional redundancy (B) for the transplant experiment (June 2006) where lake bacteria were inoculated in marsh water.** Functional redundancy (Δ FC) was calculated as the rate of change in community functional capacities similarity (FCs) relative to similarity in community composition (BCCs) between control and transplanted communities at each time point of the experiment (highlighted in gray in the similarity matrix). Metabolic plasticity (Δ SCC) was estimated from the rates of changes in dissimilarity in single-cell characteristics (SCCd) relative to BCC dissimilarity (BCCd) over time. Each circle represents the average similarity in FC (or dissimilarity in SCC) per level of similarity (or dissimilarity) in BCC at each time point of the experiment. Dissimilarity and similarity indices were estimated using Bray Curtis metric. The line represents the least square regression fit and the slope of the regression model refers to either Δ FC or Δ SCC.

and redundancy (as change in FC relative to change in BCC) per community, which is based on the breadth of response in SCC, FC, and BCC when the community is taken outside its native environment. This alternative estimate provides a more integrative quantification of plasticity and redundancy that takes into account the background variability that these communities express in their native environment, and which therefore highlights their response to environmental forcing.

## MATERIALS AND METHODS

### SAMPLING AND EXPERIMENTAL SET-UP

The experiments involved two lakes, a headwater river and a freshwater marsh, all located within the same temperate watershed in southeastern Québec (45.508N, 73.588W). These systems are part of the same hydrological network but differ in their limnological characteristics (**Table 1**).

The experimental design was the following: we carried out two series of transplants: The first series (Experiment A), consisted of (i) inoculating the upstream oligotrophic lake water (lake Bowker) with bacteria originating from the downstream marsh (L.M indicating that lake water received marsh bacteria), (ii) inoculating marsh water with lake bacteria (i.e., M.L), (iii) inoculating each medium with its own microbial assemblages (L.L and M.M) (**Figure 1**, top panel). The second series (Experiment B), consisted of (i) inoculating lake water (Lake Fraser) with the inflowing river bacteria (L.R); (ii) inoculating the upstream river water with bacteria from the receiving lake Fraser (R.L); (iii) inoculating each medium with its own microbial assemblages (L.L and R.R; **Figure 1**, bottom panel). Controls with only medium but no inoculum were also prepared for each experiment. We carried out these two experiment series twice, in June and July of 2006, so that there are a total 8 transplant experiments with their respective controls.

The transplant experiments were carried out using dialysis bags (Spectrum labs, MWCO: 12–14 kDa). The bags were cut to accommodate a volume of 600 ml, and were thoroughly washed, rinsed and soaked in Nanopure water before use. Water for media was prepared by sequentially filtering the sample through 3 μm precombusted glasfiber filters (A/D filter, Pall Corporation), and 0.2 μm filter Capsule (Acropak 1000 supor capsule membrane, Pall Corporation) to remove most of the ambient organisms; this medium was then inoculated with unfiltered water (1% vol/vol), and the bags were sealed with clamps. We prepared a total of six bags for each treatment, which were incubated submerged in a tank filled with 40 L of the corresponding unfiltered medium water, kept in the dark at 20°C. The tank water was renewed at day 3 to maintain this water as close to ambient as possible. We took samples at time zero, and then removed duplicate bags at time = 2, 3, and 5 days for further analyses.

### BIOLOGICAL VARIABLES

Bacterial communities metabolism was assessed as rates of incorporation of $^3$H-leucine following protocol described in Kirchman (1993). We used the profile of carbon substrate utilization, measured from BIOLOG Ecoplates as a proxy for community FC. For logistical purpose, measures were taken at the beginning and end of each incubation only. The plates were inoculated with the water samples and incubated in the dark at room temperature, and the absorbance was recorded in a microplate reader (Tecan GENios) every day for 5 days. We used the time at which the average well color development (AWCD) was closest to the reference absorbance of 0.5, as the end point (Garland et al., 2001). We measured several SCC using flow cytometry (FACScalibur, Becton Dickinson) at each sampling time points (time = 0, 2, 3 and 5 days). Total bacterial abundance, and the abundance of high and low DNA populations (HNA and LNA respectively) was determined using SYTO 13. Respiring and dead cells were enumerated using CTC and Live/Dead kit respectively, in addition to cells with compromised (DiBAC4) and intact (DiOC6 (3)) membranes. In all cases, we used the average fluorescence values for each of the assays (from the different fluorochromes) as well as the average side scatter as measures of single-cell properties. BCC was determined by denaturing gradient gel electrophoresis (DGGE). DNA was extracted using CTAB buffer and chloroform/isoamyl alcohol. PCR reactions were performed using GC clamp-358 F and 907 rM primers (HPLC purified, Sigma Genosys). DGGE gels were build on 100 ng of DNA and ran for 16 h at 100V and 60°C on 40–65% acrylamide gels and analyzed using Quantity one software (Biorad). All procedures described above are detailed in Comte and del Giorgio (2009).

### CONSTRUCTION OF RAW DATA AND DISSIMILARITY MATRICES

We constructed raw data matrices for each of the three components considered in the study (FC, SCC, and BCC), where rows represent the different treatments at the different time points during the experiment, and columns correspond to averages (from the duplicate bags) of the variables measured for each component (SCC, FC, and BCC) (see conceptual **Figures 2** and **3**). In the case of the BCC matrix, each column corresponds to the relative contribution of each band to the overall fluorescence of the sample. The matrix of SCC consisted of the average fluorescence and side-scatter estimates obtained from the cytometric analyses. In the case of the FC matrix, each column represents the absorbance values for each of the 31 substrates that are included in the Ecoplates. For each raw matrix, data were $\log_{10}$-transformed, (except for the BCC data, which were arcsine transformed), normalized, and standardized. We then generated a dissimilarity matrix (for the estimation of plasticity based on SCC and BCC), or a similarity matrix (for the estimation of redundancy based on FC and BCC) in both cases based on the Bray Curtis metric (Primer 5.2 software). We chose the Bray Curtis metric because it is bound between 0 and 100, which allowed us to compare the above three components: For example, a Bray Curtis dissimilarity value of 0 means that two communities have the same composition (or FC or SCC), and 100 means the two communities do not share any phylotypes or any SCC or FC features.

### CALCULATION OF BACTERIAL PLASTICITY AND FUNCTIONAL REDUNDANCY

As described previously, we produced two alternative estimates of plasticity: (1) We regressed the dissimilarity in SCC and in BCC in time for each individual treatment, and used the resulting slope as a measure of "absolute" plasticity (**Figure 2A**). (2) We calculated the dissimilarity in SCC between the transplanted and the control community at each time point, and we regressed this against its counterpart in BCC, and used the slope as a measure of "relative" plasticity (**Figure 3A**). We proceeded likewise to estimate "absolute" and "relative" redundancy (**Figures 2B** and **3B**), except that we used similarity rather than dissimilarity so as to obtain a more intuitive positive relationship between FC and BCC, such that higher slopes imply higher redundancy and vice versa. The relationship between plasticity and functional redundancy was investigated using least square regression model (Jmp 7.0).

## RESULTS

### HETEROGENEITY IN ECOSYSTEMS CHARACTERISTICS

The aquatic ecosystems sampled differed greatly in terms of their limnological characteristics (e.g., conductivity, nutrients, and dissolved organic carbon concentration). These differences in physicochemical variables coincide with differences in the total abundance and biomass production rates of bacteria as assessed by the uptake of $^3$H-leucine (**Table 1**).

### VARIABILITY IN PLASTICITY AND REDUNDANCY BETWEEN AND WITHIN COMMUNITIES

We found a relatively large range in "absolute" plasticity, both among different communities, and also within a given community, between the control and the transplanted treatments, the latter generally having higher values. There was a significant positive relationship between the "absolute" plasticity in the controls and in the transplanted treatments for any given community ($r^2 = 0.62$, $p = 0.034$, **Figure 4A**). Bacterial communities that had high "absolute" plasticity when grown in their native environment, also tended to express high plasticity when transplanted into a different environment, and vice versa. There was one clear outlier that corresponds to experiment B conducted in June, in

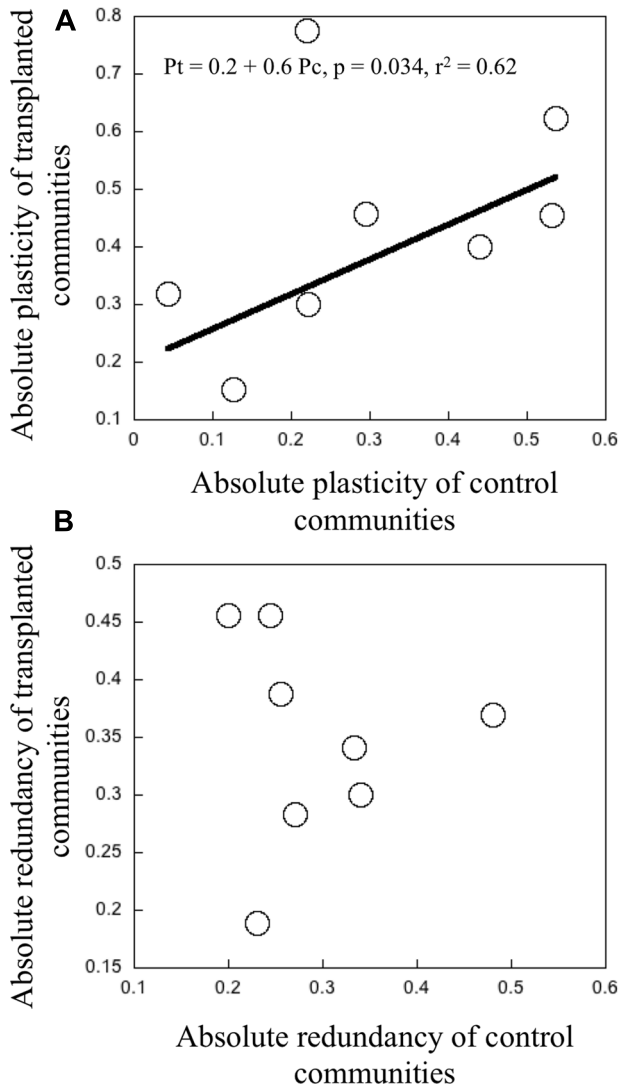


**FIGURE 4 | (A)** Relationship between the "absolute" plasticity in transplanted communities as a function of "absolute" plasticity in control communities. **(B)** The relationship between the "absolute" redundancy measures in transplanted and control communities. Each point represents the average magnitude of plasticity and redundancy per treatment of transplant experiments in June and July 2006. The black line represents the fit from a least regression model with a significant threshold at $p < 0.05$.

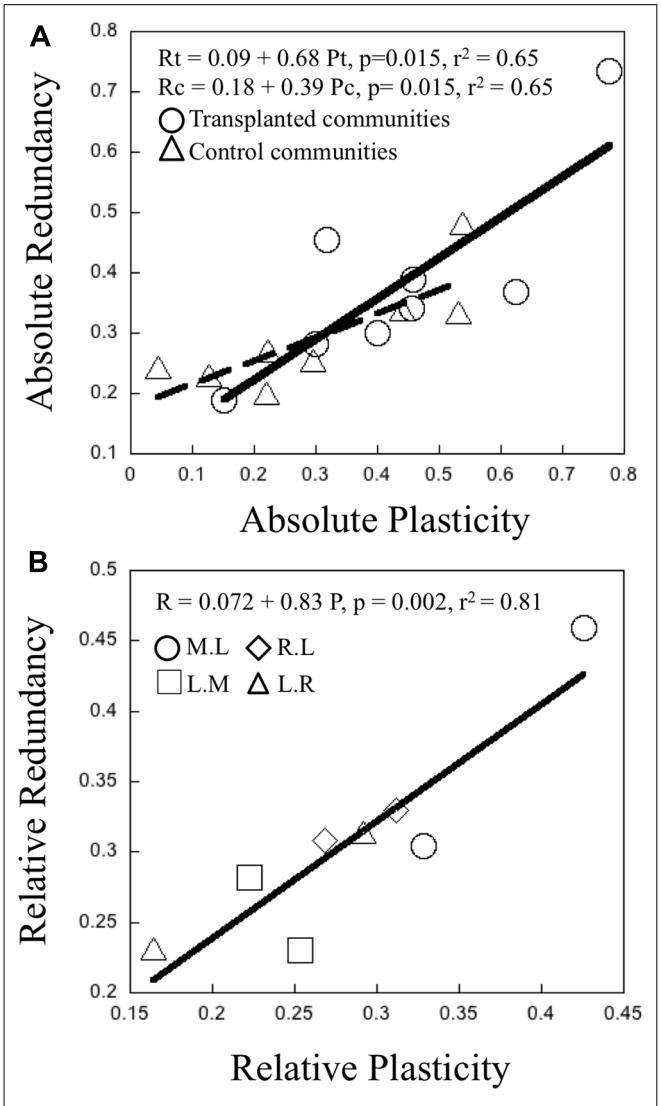


**FIGURE 5 | (A)** Relationship between the magnitude of "absolute" community functional redundancy and "absolute" bacterial metabolic plasticity. Each point represents the average plasticity and redundancy per treatment of transplant experiments in June and July 2006. Open circles and triangles refer to transplanted and control communities respectively. The black full and dashed lines represent the trend generated from a least regression model ($p < 0.050$ for transplanted and control communities respectively. **(B)** Relationship between "relative" community functional redundancy and "relative" bacterial metabolic plasticity. Each point represents the average magnitude of plasticity and redundancy per treatment of transplant experiments in June and July 2006. Open circles, squares, diamonds, and triangles refer to M.L, L.M, R.L, and L.R treatments respectively. The black line represents the trend generated from a least regression model ($p < 0.05$).

which bacterial assemblage from lake Fraser were inoculated in the water from the inflowing river. However, there is no explanation for it. "Absolute" redundancy also showed a wide range, both among and within communities, but as opposed to plasticity, there was no relationship at all between the "absolute" redundancy in the controls and in the transplanted communities (**Figure 4B**).

### RELATIONSHIP BETWEEN COMMUNITY PLASTICITY AND FUNCTIONAL REDUNDANCY

There was a significant positive relationship between the "absolute" plasticity and "absolute" redundancy, both for the control and transplant communities (**Figure 5A**). The latter had overall higher values for both variables, as was to be expected. There was an even stronger positive relationship between "relative" plasticity and "relative" redundancy (**Figure 5B**). Overall, communities characterized by high intrinsic metabolic plasticity (absolute and relative), also tended to express higher functional redundancy and vice versa. There was a tendency for lake communities to

have higher overall "absolute" and also "relative" plasticity and redundancy, and for rivers to have the lowest, but these differences were not statistically significant.

## DISCUSSION

The relationship between biodiversity and ecosystem functioning has generated much interest and debate in recent years. There are two main lines of thought in this regard, the first increasing the number of species (increasing richness) should result in increased ecosystem functioning and/or stability (e.g., Bell et al., 2005; Horner-Devine et al., 2006), and the second is that patterns in microbial function should correlate with the presence or absence of specific species or phylotypes (e.g., Peter et al., 2011). In both conceptual frameworks, functional redundancy and metabolic plasticity are relegated to serving as null hypotheses (Naeem, 1998; Loreau, 2004). These two community traits play a key role in influencing the response of bacterial communities to environmental forcing and in modulating the relationship between community composition and function. Although they have often been evoked and discussed, they remain however difficult to quantify in natural communities, and their actual patterns within and across bacterial communities, and more importantly their relationship to each other have seldom been explored.

In previous studies (Comte and del Giorgio, 2011), we had hypothesized that metabolic plasticity plays no role in the actual outcome in terms of metabolism, but does play a role in terms of the pathways of response to environmental forcing, because this property determines whether the community response is mediated by changes in composition or not. We also hypothesized that both metabolic plasticity and functional redundancy are intrinsic, emerging properties of bacterial communities, which do not depend on the environmental conditions but rather is determined by community composition. In this paper we explicitly addressed these hypotheses, and in addition, we have explored the potential connections that might exist between these two fundamental community properties.

In order to address these questions, we first had to develop a conceptual framework and an experimental approach, which would allow us to quantify these properties and empirically test their patterns. There are no doubt drawbacks in our approach. For example, substrate uptake profiles are only one aspect of bacterial function, and these results thus cannot be directly extrapolated to other aspects of function. Likewise, we used single-cell properties to derive a metric of metabolic plasticity, but there are many other dimensions to bacterial metabolism that are not considered here. Nevertheless, we feel that the approach does provide quantitative metrics for both metabolic plasticity and functional redundancy that can be compared among samples and between each other.

The first major question we addressed is how redundancy and plasticity vary within and among bacterial communities. In particular, we explored whether these properties are driven by environmental factors, or whether they can be considered intrinsic features of freshwater bacterial communities, in the sense that a given community will consistently show a high or a low degree of redundancy and plasticity under different environmental scenarios. Our results using our "absolute" metrics suggest that plasticity is indeed an intrinsic feature of these communities, because there was a strong relationship between the level of plasticity expressed by any given community under two very different environmental scenarios, with communities that express a high level of plasticity in one environmental scenario tend to also express high plasticity when exposed to a different environment, and vice versa. If the expression of community plasticity were entirely environmentally-driven, we would not necessarily expect such positive relationship, and that was in fact the case for functional redundancy. We also found significant variability in functional redundancy, but the lack of relation between control and transplant treatments would suggest that the expression of this property might be more strongly controlled by environmental factors, such as the nature of the DOC pool or other environmental drivers. If this is the case, it would suggest that all bacterial communities may have a high potential for functional redundancy, but that the expression of this property is directly related to environmental forcing or other factors.

Although there was a consistent pattern of plasticity within a given community, it is worth pointing out that the expression of plasticity for a given community somewhat differed between the two environmental scenarios, the magnitude being lower in the controls relative to the transplants as would be expected, suggesting that although this appears to be an intrinsic community trait, there is nevertheless an environmental component in the expression of this trait. The expression of functional redundancy, on the other hand, appears to be more strongly driven by environmental factors, and there was no consistent pattern in the expression of redundancy under the two environmental scenarios. These findings in turn suggest that the distribution of functional redundancy may be neutral, with each community presenting the same capacity to express a similar level of redundancy under environmental forcing, with the level of functional redundancy expressed being random or influenced by external forces, which nevertheless result in comparable patterns in C substrates utilization among the different communities.

Since there is indication that metabolic plasticity appears to be intrinsic emergent community properties, likely defined by the collective genetic composition, this would suggest that microbial communities might differ in terms of their extent of functional redundancy and plasticity, with some communities being more plastic than others. In this regard, there was some indication that there may be ecosystem-specific patterns in plasticity, with lake communities presenting higher extent of both plasticity estimates (and redundancy), although the limited samples size precludes any statistical strength. These results would suggest, nevertheless, that environmental differences between the different ecosystems may select taxa that are either more functionally specialized or present a wider niche breadth, as has been previously suggested (Kirchman, 2002; Hahn, 2006; Newton et al., 2011).

On the other, our results further suggest that the metabolic plasticity as a community "trait" should be bound by the genetic composition of the dominant taxa of the community, and not by extrinsic environmental factors. This would in turn mean that the individual traits are co-selected in all the dominant taxa,

but the basis for this co-selection are not known. Individual traits are defined as morphological, physiological or phenological characteristics of an organism affecting its individual performance (see Hillebrand and Matthiessen, 2009). This definition can be extended to encompass traits at the community level, and which refer to aggregate, community-level physiological or functional characteristics that influence community performance. These community-level traits influence the performance of the entire community, and in particular, the responses of these communities to environmental or biological changes, and influence the ability of these communities to cope with disturbance or stress. There is still debate as to how the individual traits scale up to determine community-level features. In the simplest scenario, these emergent community properties reflect the abundance- or biomass-weighted mean of individual traits (Diaz et al., 2007). However, there are more complex scenarios, for example, if traits are context-specific and the link between functional traits and contribution to community performance changes with changes in either the environment or in biodiversity (Fox and Harpole, 2008). Our results may offer examples of both scenarios of scaling: Metabolic plasticity appears to be an intrinsic property of the community, which is probably defined by the sum of the relative contributions of the dominant players, whereas the expression of functional redundancy appears to be more influenced by factors extrinsic to the community.

Metabolic plasticity, however, could be shaped by quite different pathways. We still do not know, for example, how metabolic plasticity is distributed among the different taxa that form the community. The null hypothesis is that the dominant taxa all have roughly the same degree of plasticity, such that the sum of the traits of these players yields a community that is also on average more plastic. The alternative hypothesis would be that bacteria within a given community vary widely in terms of their metabolic plasticity, and that the community level integrates this diversity but without reflecting the properties of any specific taxa. These two scenarios are similar in terms of the final outcome, but very different in terms of their underlying regulation and ecological significance, for whereas the first would require the co-selection of taxa that share a similar level of plasticity, the second would involve the coexistence of taxa that differ greatly in this particular trait, with the overall community property shaped by the differential expression of the trait, perhaps driven by shifts in the relative contribution of the different taxa. The fact that metabolic plasticity appears to be an intrinsic property of these bacterial communities would suggest that the former scenario is most likely.

The second major question that we explored here is whether metabolic plasticity and functional redundancy are linked across these communities, and if they are, in what manner. We found that these two community properties are strongly positively related, regardless of whether we used our "relative" or "absolute" metrics. The relationship reported in **Figure 5** therefore suggests few or no trade-offs between plasticity and redundancy, but rather co-selection of taxa that are simultaneously more metabolically plastic and which can also express a broader range of function, the latter resulting in increased functional redundancy at the community level. Trade-offs and co-selection of traits have been traditionally investigated at the strain or species level, generally focusing on functional traits (Violle et al., 2007), for example, growth capacity, tolerance to pollutants or conditions, or intrinsic characteristics such as cell size, all potentially affecting its individual performance. Trade-offs between bacterial growth and plasticity have been reported in the literature, such that bacteria that can withstand a broader range of conditions and resources tend to have low intrinsic growth rates (Hall et al., 2010).

There is a priori no reason to think that bacterial taxa that are intrinsically more metabolically plastic should also be more generalist in terms of FC. There are in fact examples in the literature of trade-offs between metabolic or growth-related traits and resource acquisition and processing capabilities in bacteria (Hall et al., 2010). For metabolic plasticity to covary positively with functional redundancy requires that when dominant taxa are intrinsically more plastic, that they would also have a broader niche breadth in terms of substrate uptake capacities. Whether these two traits tend to be co-selected, or one actually results in the other, has yet to be explored.

In summary, our experimental design together with our conceptual approach allowed us to explore how functional redundancy and metabolic plasticity vary in terms of their magnitude among communities, and also within a given community as a function of environmental forcing. We have shown that metabolic plasticity is an intrinsic emerging property of freshwater bacterial communities, whereas the expression of functional redundancy appears to be more strongly determined by environmental forcing. There thus appears to be a co-selection of taxa that share a certain degree of metabolic plasticity, although the underlying mechanisms are not known yet. In particular our results suggest that both redundancy and plasticity are key properties that do not necessarily shape the response of bacterial communities to the environmental forcing, but rather modulate the role that community composition and diversity play in this response. A major consequence of this tight link between redundancy and plasticity, and the fact that there is a continuum in their expression among bacterial communities, is that the apparent link between community diversity and function may also vary along a continuum, from being very tight, to being weak or even completely absent, which may in part explain the apparently conflicting results that abound in the literature. This in turn suggests that we may have to reassess not only our interpretation of current data, but also our future strategies to more effectively actually explore the role that community composition plays in the functioning of aquatic bacterial communities.

## ACKNOWLEDGMENTS

We thank P. Tremblay-Dauphinais, A. Guindon, M. Bergevin, A. Parkes, and C. Beauchemin for assistance in the field and lab. We are grateful to Ashley Shade and three anonymous reviewers for their valuable comments on previous versions of the manuscript. This work was supported by grants from the National Science and Engineering Research Council of Canada to Paul A. del Giorgio.

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

*Received: 02 July 2012; accepted: 18 April 2013; published online: 09 May 2013.*

*Citation: Comte J, Fauteux L and del Giorgio PA (2013) Links between metabolic plasticity and functional redundancy in freshwater bacterioplankton communities. Front. Microbiol. **4**:112. doi: 10.3389/fmicb.2013.00112*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 27 September 2012
doi: 10.3389/fmicb.2012.00338

# Cooperation, competition, and coalitions in enzyme-producing microbes: social evolution and nutrient depolymerization rates

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Extracellular enzymes represent a public good for microbial communities, as they break down complex molecules into simple molecules that microbes can take up. These communities are vulnerable to cheating by microbes that do not produce enzymes, but benefit from those produced by others. However, extracellular enzymes are ubiquitous and play an important role in the depolymerization of nutrients. We developed a multi-genotype, multi-nutrient model of a community of exoenzyme-producing microbes, in order to investigate the relationship between diversity, social interactions, and nutrient depolymerization. We focused on coalitions between complementary types of microbes and their implications for spatial pattern formation and nutrient depolymerization. The model included polymers containing carbon, nitrogen, or phosphorus, and eight genotypes of bacteria, which produced different subsets of the three enzymes responsible for hydrolyzing these polymers. We allowed social dynamics to emerge from a mechanistic model of enzyme production, action, and diffusion. We found that diversity was maximized at high rates of either diffusion or enzyme production (but not both). Conditions favoring cheating also favored the emergence of coalitions. We characterized the spatial patterns formed by different interactions, showing that same-type cooperation leads to aggregation, but between-type cooperation leads to an interwoven, filamentous pattern. Contrary to expectations based on niche complementarity, we found that nutrient depolymerization declined with increasing diversity due to a negative competitive effect of coalitions on generalist producers, leading to less overall enzyme production. This decline in depolymerization was stronger for non-limiting nutrients in the system. This study shows that social interactions among microbes foraging for complementary resources can influence microbial diversity, microbial spatial distributions, and rates of nutrient depolymerization.

**Keywords: nutrient depolymerization, cooperation, spatial model, density-dependence, extracellular enzymes, facilitation, microbe, decomposition**

## INTRODUCTION

Microbial public goods are products that are secreted outside the cell, and therefore benefit not only the individuals producing them, but also neighboring cells (Velicer, 2003; West et al., 2006, 2007). They include substances crucial to intercellular interactions, such as quorum sensing molecules, biofilm polymers, siderophores, exoenzymes, and many other cell products (West et al., 2006, 2007). Public goods are ubiquitous in microbial ecosystems. However, evolutionary theory predicts that producers of public goods are vulnerable to cheating by individuals that receive the benefits without paying the cost of production. Exoenzyme production by bacteria and fungi is particularly important for ecosystem function because these enzymes catalyze the rate-limiting step in the depolymerization of carbon and nutrients from organic polymers in the environment (Schimel and Bennet, 2004). Thus the quantity and types of enzymes produced by microbes influence the rate at which these nutrients flow through the ecosystem. However, despite their importance to nutrient cycling, these enzymes have received little attention from a social evolution perspective relative to other public goods. At the same time, models of enzyme-mediated decomposition by microbes (for example Schimel and Weintraub, 2003) do not account for social interactions.

Allison (2005) applied a public goods framework to exoenzyme production by microbes and demonstrated that the presence of cheaters reduces nutrient depolymerization, and that the cost of cheating increases with the diffusion rate of the enzyme and the rate of constitutive enzyme production. This study considered an environment with one limiting nutrient and two types of microbes, producers, and cheaters. However, real microbial communities are highly diverse, with thousands of taxa (Roesch et al., 2007), and they depend on multiple nutrients. The present study extends the model of Allison (2005) to investigate the social dynamics of enzyme production in a multi-genotype community in a multi-nutrient environment.

We used this model to examine how diversity and social interactions modulate each other's effects on nutrient depolymerization.

Our first objective was to determine the conditions for the maintenance and loss of diversity. Second, in the context of social evolution, we determine the conditions under which coalitions can form and compete successfully. We define a coalition to be a mutualistic interaction between two or more complementary types that each produce enzymes lacking in the other. Since interactions due to diffusion occur locally, this would lead to greater growth and survival when complementary types are close together. We predict that complementary types should therefore be spatially associated and take such spatial correlation to be evidence for coalitions. Finally, in the context of ecosystem function, our third objective was to examine how diversity and coalitions affect rates of nutrient depolymerization. If social interactions limit the production of enzymes, these effects could cascade upwards, limiting the depolymerization of nutrients, and therefore the overall rate of flow through the ecosystem.

## MATERIALS AND METHODS

### MODEL OVERVIEW

The model is an individual-based, stochastic simulation coded in C++, built on a previous model by Allison (2005). It consists of a $100 \times 100$ lattice grid, where each grid box represents $1\,\mu m^3$. In each box of the grid, the model tracks the substrate, enzyme, and product concentrations for each of the three nutrients, as well as the resident microbe if one is present. Each microbe has a genotype and a pool of nutrients internal to itself. At each time step of the model, the following processes occur in each grid box in this order: substrate input, substrate decay, product decay, product diffusion, enzyme decay, enzyme diffusion, product formation, nutrient uptake and enzyme production, microbial metabolism, death, and reproduction. Microbes optimize their nutrient uptake and enzyme production in order to balance their internal nutrients in their stoichiometric ratios.

The original study (Allison, 2005) analyzed the social dynamics of producers and cheaters in the case where only carbon was limiting. In the current model, carbon (C), nitrogen (N), and phosphorus (P) were all present only as substrates that must be hydrolyzed by enzymes in order to be available. We used a genetically explicit model with three loci, each of which coded for an enzyme that breaks down C, N, or P. At each locus, there was one allele for enzyme production and one for no enzyme production (i.e., cheating). In naming a genotype, we represent the former with the capital letter of the respective nutrient and the latter with the lowercase letter. Thus the genotype *CNp* represents a microbe that produces C- and N-enzymes, but not P-enzymes. There are eight genotypes in total: *cnp*, *Cnp*, *cNp*, *cnP*, *CNp*, *CnP*, *cNP*, and *CNP*. We also include mutation, which allows for the reintroduction of new types after they have been lost.

### INITIALIZATION

At the start of the model, the concentrations of substrate, enzyme, and product are all initialized to 0 over the entire grid. Each grid box may contain zero or one microbe, but no more than one microbe may occupy a single grid box. Thus the maximum density is 1 microbe/$1\,\mu m^3$, so the population is limited by space. Microbes are introduced randomly with a total frequency of 0.02, and each is assigned a random genotype with equal probability. The C biomass of each microbe is initialized to 150 fg C (Button et al., 1998), and the other nutrients within the microbe are initialized to maintain the stoichiometric ratios of C:N = 6 and C:P = 60.

### ITERATION

Although conceptually the following processes occur simultaneously across all grid boxes, the program must compute them sequentially. The order of the grid boxes and of the nutrients is randomized so as to avoid bias, which would arise if the same order were used each time.

### INPUTS

Substrate is added to each grid box at each time step, at rates of 0.1 fg C/min/$\mu m^2$., 0.01 fg N/min/$\mu m^2$, and 0.001 fg P/min/$\mu m^2$. No product or enzyme is directly added to the grid.

### DECAY AND DIFFUSION

The substrate, product, and enzymes were removed from the grid at a constant rate of 0.01/min. Substrate does not diffuse. Product diffusion rates are set to 0.5 $\mu m^2$/min, meaning that the concentrations in two adjacent grid boxes will equilibrate in 1 min of model time. When diffusion occurs at a box, a random neighbor box is chosen, and an amount proportional to the difference in the concentrations of the two boxes is moved from the box with the higher concentration to that with the lower concentration. These values are based on diffusion and loss rates reported by Vetter et al. (1998).

### PRODUCT FORMATION

Product is formed by the action of enzymes on substrate, following Michaelis–Menten kinetics:

$$\Delta\,[\text{Product}] = \left[\text{Enzyme}\right] * V_{\max} * \frac{[\text{Substrate}]}{K_m + [\text{Substrate}]} * \Delta t$$

where $V_{\max} = 10$ fg/fg/min is the maximum rate of product formation at substrate saturation, and $K_m = 0.001$ fg/$\mu m^3$ is the half-saturation constant. These values fall within the range reported in the literature for hydrolytic enzymes (Schomburg and Schomburg, 2001). This quantity is also deducted from the substrate pool.

### PRODUCT UPTAKE

Microbes only take up nutrients when they are in demand relative to the microbe's stoichiometric ratios of C:N = 6 and C:P = 60. For example, if the microbe's internal ratio is C:N > 6, this would mean that N was in demand, and the microbe would take up N in order to maintain its stoichiometric ratio. Although microbes are actually more flexible in their nutrient uptake, this constraint reflects a microbial tendency to maintain stoichiometric ratios within limits (Sterner and Elser, 2002).

The rate of product uptake is proportional to the surface area of the microbe and also follows the Michaelis–Menten kinetics:

$$\Delta\,[\text{Nutrient}] = \text{Enz Per Area} * \text{Area To Mass} * \text{Biomass}^{2/3} * V_{\max} * \frac{[\text{Product}]}{K_m\,[\text{Product}]} * \Delta t$$

where Enz Per Area = 0.1 is the density of uptake enzymes on the exterior of the microbe, Area To Mass = 0.0428 is the ratio of surface area to volume of the microbe, $V_{max} = 10$ fg/fg/min is the maximum rate of uptake at product saturation, and $K_m = 0.001$ fg/$\mu$m$^3$ is the half-saturation constant. These values fall within the ranges reported by Button et al. (1998).

#### ENZYME PRODUCTION

In each time step, if a microbe has the gene to produce an enzyme, it must produce the enzyme at a minimum constitutive rate, which by default is set to $10^{-7}$ fg/fg/min, times its biomass.

Facultative enzyme production occurs only if the nutrient is still in demand following nutrient uptake, in which case a maximum of 1% of uptake from the current time step is allocated to enzyme production. This value is within the range of 0.7–2.1% reported for α-glucosidase production by yeasts (Giuseppin et al., 1993) and slightly higher than the 0.3–0.9% reported for protease production by *Bacillus clausii* (Christiansen and Nielsen, 2002). Production of N and P-enzymes is initially calculated in units of N or P mass, respectively. This quantity is then converted to C mass using the stoichiometric ratios of C:N = 3.5 and C:P = 200. The first value is based on the stoichiometry of proteins, and the second on the assumption that small amounts of P could be lost during enzyme secretion, especially if protein phosphorylation is involved. When enzymes are produced, a quantity of C equal to 10% of enzyme C mass is respired due to the metabolic costs of enzyme production.

If producing enzyme at the maximum level will cause another nutrient to become limiting, the microbe will produce less enzyme. For example, the ratio of C:N for microbes is 6, nearly twice that of enzymes. Suppose initially the microbe's C:N ratio is less than 6, indicating that C is limiting, and the microbe takes up a quantity of C equal to *uptake*. Then its maximum enzyme production will be 0.01 × *uptake*. However, producing this quantity may reduce the microbe's N pool to the point where its C:N ratio is now greater than 6, making N limiting. In this case, the microbe will produce a quantity equal to

$$\text{Enz Prod} = \frac{\text{C to N}_{\text{mic}} * \text{N} - \text{C}}{\text{C to N}_{\text{mic}}/\text{C to N}_{\text{enz}} - 1 - \text{Resp}_{\text{enz}}}$$

Here, C to $N_{mic}$ is the microbial C:N ratio of 6, C to $N_{enz}$ is the enzyme C:N ratio of 3.5, $Resp_{enz}$ is the rate of respiration due to enzyme production, 0.1, and N and C are the current pools of N and C in the microbe. This quantity of enzyme production equalizes the microbe's C:N ratio at its target level of C to $N_{mic} = 6$. Analogous calculations are applied to the other nutrient combinations.

#### MICROBIAL PROCESSES

In addition to product uptake and enzyme production, microbes also undergo metabolism, reproduction, and mortality. Microbes respire C at a constant basal metabolic rate (BMR), of 0.00015 fg/fg/min to account for cellular maintenance. This rate is 10 times higher than the range reported by Price and Sowers (2004) because we assume that actively growing microbes require more energy for maintenance metabolism. Thus the BMR also includes growth metabolism. Microbes also lose N and P in proportion to the amount of C lost by a factor of 0.1 divided by the C:nutrient ratio.

When a microbe reaches a critical mass of 300 fg, it divides, producing an exact copy of itself (unless mutation occurs). Mutations, which occur independently at each locus and change the allele to its complement, occur at a rate of $10^{-5}$/locus/division. One copy remains in the current grid box, and the other moves into a neighboring grid box. If that box is occupied, then one of the microbes dies randomly with probability 0.5.

Microbial mortality occurs randomly at a fixed rate of $3 \times 10^{-5}$/min and also occurs if a microbe's biomass falls below 30 fg C. This minimum mass is based on the low end of bacterial sizes reported by Button et al. (1998). When a microbe dies, its biomass and nutrients are added back to the grid, half as substrate and half as product.

For more details on the parameterization of the model, see Allison (2005).

#### MODEL RUNS

We varied the enzyme diffusion rate (EDiff) over the values $10^{-4}$, $10^{-3}$, and $10^{-2}$, and the constitutive enzyme production rate (EConstit) over the values $10^{-7}$, $10^{-6}$, $10^{-5}$, and $10^{-4}$. In these non-mixed runs, direct interactions are only between neighboring grid boxes, defined as the eight grid boxes surrounding a focal box. The model was also run in a well-mixed mode, in which the interacting box is drawn randomly from the entire grid, so that each box is equally close to every other box, removing spatial effects. For the well-mixed scenarios, the EDiff was always set to 0.5. These scenarios were run both with and without mutation enabled. We also ran the model with only two types, *CNP* (generalist producers) and *cnp* (cheaters). These runs did not include mutation.

We ran the model for 35,000 h for the full genotype set and 8,000 h for the two-typeset and for the full set in the case with EConstit = $10^{-4}$. For the 35,000 h runs, we ran five replicates both with and without mutation. For the scenario with EConstit = $10^{-5}$ and E Diff = $10^{-2}$, we ran six additional replicates.

#### MODEL OUTPUTS

The outputs of the model are population density by genotype, diversity, and depolymerization of C, N, and P. Densities were computed as the number of microbes over the area of the grid, which is 10,000 $\mu$m$^2$. Diversity was calculated as$1 - \Sigma p_i^2$, where $p_i$ is the proportion of genotype $i$ in the community. This quantity is bounded by 0 and 1, and higher values represent greater diversity. Nutrient depolymerization is the amount of substrate that is converted into product per hour, whether or not this product is taken up and used by microbes. Diversity and nutrient depolymerization were averaged over the last 10,000 model hours of all replicate runs.

#### SPATIAL ANALYSIS

To test for spatial associations between microbial types, we used spatial statistics on independent samples of the grid outputs. Grid outputs that are close in time cannot be considered independent because of temporal autocorrelations. Therefore, we calculated the time required for these autocorrelations to disappear and used

these intervals as a basis for sampling the grid. The number of time steps required for two time slices to be considered independent is $N_t = (1+r_t)/(1-r_t)$, where $r_t$ is the correlation between time slices, computed as the proportion of grid boxes that retained the same value at times $t$ and $t+1$. This value tended to increase over the course of a run, so one cannot assume that it is constant. We took the first time slice at $t = 1$, and then additional slices moving in steps of $N_t$, where $t$ is in units of 1,000 h, and $N_t$ is computed dynamically at each time slice and rounded up to the next integer.

At each independent time point, we analyzed the spatial associations of the microbial types using a multi-way "join-count" analysis. This analysis "counts" the number of "joined" (neighboring) cells of a pair of focal types and compares this number to the expected count assuming a purely random spatial distribution, yielding a $z$-statistic for each pair of types (Cliff and Ord, 1973, 1981). We used the "joincount.multi" and "cell2nb" functions in the "spdep" package in R (Bivand, 2012) to perform the calculations.

The $z$-statistics from the join-count analysis were squared and then summed over all time slices for all replicates of a given scenario to give a $\chi^2$ statistic. We also computed a total $\chi^2$ statistic summed over all scenarios. These statistics were then compared to a $\chi^2$-distribution with degrees of freedom equal to the number of independent time slices analyzed for that scenario (or over all scenarios) in which the pair was present. As there are 45 pairs (including empty boxes), we applied the Bonferroni correction to get a cutoff of $0.05/45 = 0.0011$. For each pair, we computed the average $z$-statistic over all cases for which there was a significant positive association, in order to get a score for the strength of the association.

The default implementation of the "cell2nb" function allows one to analyze a grid only in the case where each cell is considered "joined" to only its nearest neighbors. However, this spatial scale was too small to yield significant positive correlations between different types. We modified this function to join all cells in a neighborhood with a radius of any length. We ran the analysis with neighborhood radius lengths of 1, 2, 3, 4, and 5 grid boxes. As the relative values of the $z$-scores did change depending on the radius length, we averaged the $z$-scores for each pair over all radius lengths. Since smaller neighborhoods are contained within larger neighborhoods, this method implicitly weights closer neighbors more than farther neighbors by $1/x$, where $x$ is the neighborhood radius. We used these average $z$-scores to rank the pairs by strength of association.

To quantify the degree of complementarity between pairs of types, we calculated a complementation score as the number of enzyme loci with different alleles. For example, the pair (*CNp*, *Cnp*) has a score of 1, because it has the same alleles at the C and P loci and different alleles at the N locus. Across all pairs of types, we tested the correlation between this complementation score and the strength of the spatial association based on $z$-scores from the join-count analyses.

## RESULTS

In both the two-type and eight-type models, under low diffusion and constitutive production, producers dominated the community. As constitutive enzyme production and enzyme diffusion increased, cheaters made up a larger proportion of the community.

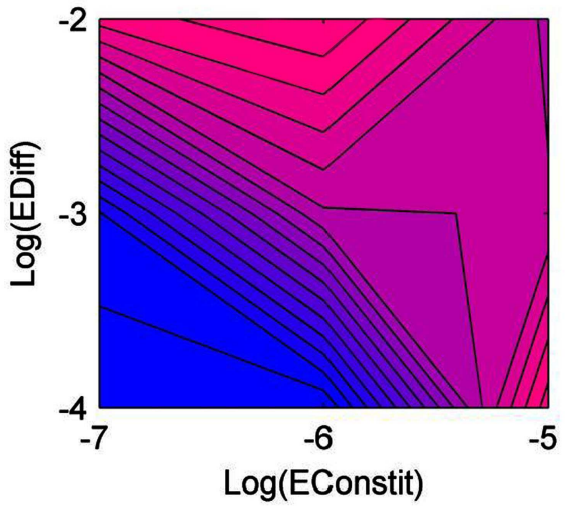


**FIGURE 1 | Diversity of the community as a function of diffusion rate (EDiff) and constitutive enzyme production rate (EConstit), averaged over all replicate runs.** Blue represents low diversity, and pink high diversity.

When EConstit was increased to $10^{-4}$, the high cost of production caused the community to go extinct. Community extinction also occurred in the well-mixed scenarios. In the scenario with high constitutive production ($\text{EConstit} = 10^{-5}$) and diffusion ($\text{EDiff} = 10^{-2}$), the two-type model resulted in extinction, but in the eight-type model, the community survived in 64% of replicates.

### DIVERSITY AND COMMUNITY COMPOSITION

Diversity was lowest when constitutive production and diffusion were both low (**Figure 1**). Generalist producers (*CNP*) dominated, driving all other types to extinction (**Figure 2A**). Under intermediate constitutive production and diffusion, although generalist producers (*CNP*) were still most common, they only made up approximately half of the community, with the remainder including the three other C-producers, *CNp*, *CnP*, and *Cnp* (**Figure 2B**). Increasing constitutive production while holding diffusion constant caused a shift in the community composition, but had little effect on diversity (**Figure 1**). Cheaters (*cnp*) made up half of the community, and types *CNP*, *CNp*, and *cnP* were also moderately successful (**Figure 2C**). The highest diversity was found under intermediate constitutive production and high diffusion (**Figure 1**). All types other than *cNP* and *cNp* were able to persist, and at relatively even frequencies, but with *CNP* at the highest frequency (**Figure 2D**). Diversity also peaked under high constitutive production and low diffusion, showing that high diversity could be maintained either by high diffusion or high constitutive production, but not both (**Figure 1**). The mixture of types at this scenario included more of types *cnp* and *Cnp*, because high constitutive production favors cheating more than high diffusion (**Figure 2E**).

Diversity was intermediate under high constitutive production and diffusion, which favor cheaters and coalitions (**Figure 1**). Competition from cheaters drove the community to very low densities, with most types going extinct. However, as densities

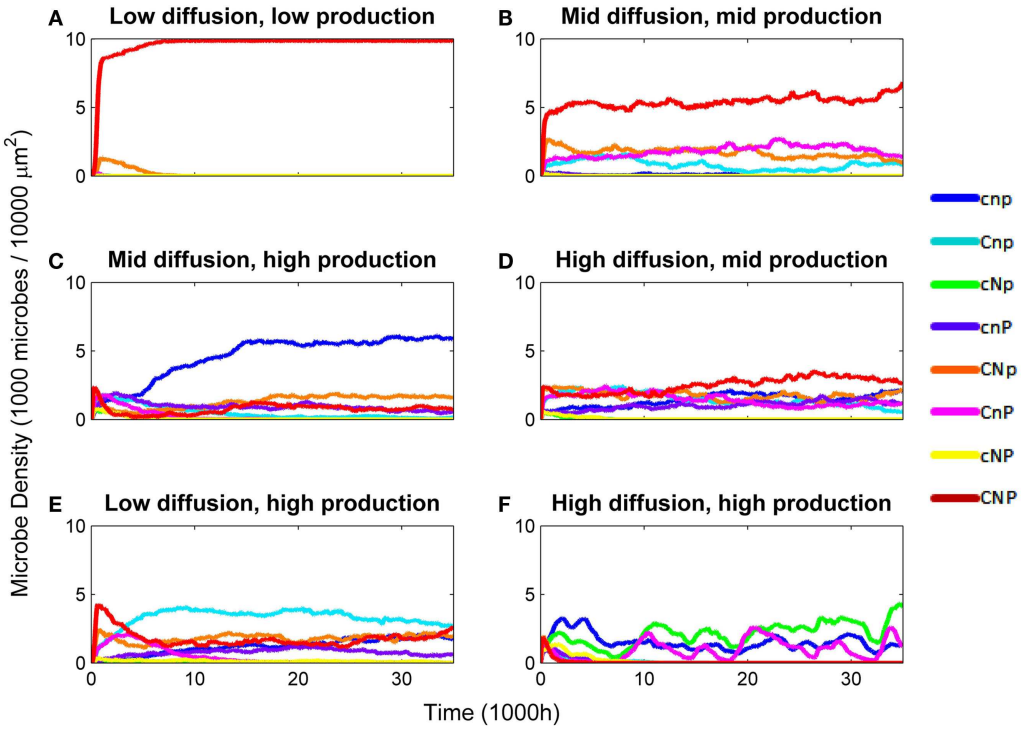


**FIGURE 2 | Density of microbes by genotype over various combinations of diffusion rate (EDiff) and constitutive enzyme production rate (EConstit).** Densities are in 1,000 microbes/10,000 $\mu m^2$. **(A)** EDiff $= 10^{-4}$, EConstit $= 10^{-7}$. **(B)** EDiff $= 10^{-3}$, EConstit $= 10^{-6}$. **(C)** EDiff $= 10^{-3}$, EConstit $= 10^{-5}$. **(D)** EDiff $= 10^{-2}$, EConstit $= 10^{-6}$. **(E)** EDiff $= 10^{-4}$, EConstit $= 10^{-5}$. **(F)** EDiff $= 10^{-2}$, EConstit $= 10^{-5}$.

declined, competition from cheaters was relaxed, and the surviving types were able to rebound in some cases. The specific types that survived this bottleneck were determined randomly, but in order to survive, the community must include a set of types that produce all three nutrients, for example, types *CnP* and *cNp* (**Figure 2F**). Cheaters (*cnp*) were nearly always successful in this scenario, due to the high diffusion and constitutive production. Density-dependent competition from cheaters caused the total density to cycle. Mutation has the ability to reintroduce types lost during the bottleneck, so diversity tended to be higher under mutation in this scenario.

The variability of the outcomes also increased with diffusion and constitutive production, and the last scenario was much more variable than the others, due to the bottleneck. **Figures 1** and **4** show results averaged over all replicate runs, and **Figures 2** and **3** show representative individual runs.

## SPATIAL ASSOCIATIONS AND COALITIONS

The strongest spatial associations were between microbes of the same type (**Table 1**). All of these associations were statistically significant at all spatial scales. C-enzyme producers were more strongly autocorrelated, as indicated by a significant correlation between C production and rank ($r = -0.87$, $p = 0.048$). However, there was no association between N- or P-enzyme production and the degree of autocorrelation.

Associations involving five pairs of different types were significant when summed across all scenarios at the 5-μm radius scale (**Table 2**). Fewer were significant at shorter distances, with none significant at the 1-μm radius scale. The correlation between average *z*-score and complementation score was significant ($r = 0.71$, $p = 0.035$), indicating that complementary types were more strongly associated.

Although associations between complementary types could be statistically detected when averaged across all scenarios, coalitions only played a major role in scenarios with high constitutive production. Under intermediate diffusion, the coalition between *CNp* and *cnP* was moderately successful, despite the high density of cheaters (**Figure 2C**). Colonies of these two types were strongly associated, as were types *CNP* and *cnp* (**Figure 3A**). The scenario with high diffusion and constitutive production (EDiff $= 10^{-2}$, EConstit $= 10^{-5}$) displayed uniquely variable outcomes due to a bottleneck caused by density-dependent competition from cheaters. These variable outcomes highlight how mutualistic, competitive, and parasitic interactions shaped the spatial patterns of the community.

When only types *CNP* and *cnp* survived, the producers formed dense aggregations due to their facilitative interactions, whereas cheaters were more diffuse due to their competitive relationship with each other, and clung to the edges of producer colonies due to their parasitic relationship with them (**Figure 3B**; Movie S1 in Supplementary Material). Densities of both types cycled due to negative density-dependent fitness of the producer and delayed tracking of producer density by the cheater.

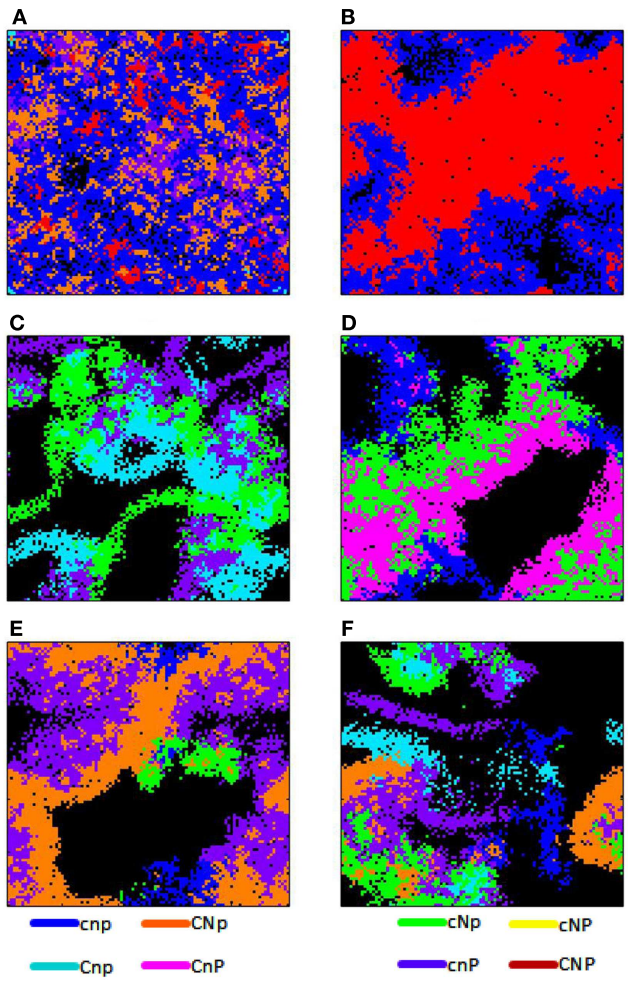


**FIGURE 3 | Spatial grid at a time slice for various scenarios.** In all figures, constitutive enzyme production is high (EConstit = $10^{-5}$). **(A)** Enzyme diffusion is intermediate (EDiff = $10^{-3}$), $t = 34{,}000$ h. **(B–D)** Show different replicates with high enzyme diffusion (EDiff = $10^{-2}$). **(B)** Types CNP (red) and (cnp) blue, $t = 20{,}000$ h. **(C)** Types *CnP* (pink), *cNp* (green), and *cnp* (blue), $t = 21{,}000$ h. **(D)** Types Cnp (light blue), cNp (green), and cnP (purple), $t = 25{,}000$ h. **(E)** Types CNp (orange), cNp (green), cnP (purple), and cnp (blue), $t = 27{,}000$ h. **(F)** Types CNp (orange), Cnp (light blue), cNp (green), cnP (purple), and cnp (blue), $t = 27{,}000$ h.

**Table 1 | Ranking of spatial autocorrelation between pairs of the same genotype with average *z*-score.**

| Types | Score |
|---|---|
| CNp | 8.86* |
| CNP | 8.29* |
| Cnp | 6.98* |
| CnP | 6.86* |
| cnP | 6.64* |
| cnp | 6.48* |
| cNp | 6.44* |
| cNP | 6.21* |

**Denotes statistical significance with Bonferroni correction at all scales.*

**Table 2 | Ranking of spatial association between pairs of different genotypes, with average *z*-score and complementation score, defined as the number of loci at which they have different alleles.**

| Types | Score | Comp. |
|---|---|---|
| cnP,CNp | 0.62* | 3 |
| CnP,cNp | 0.40* | 3 |
| cNp,Cnp | 0.34* | 2 |
| CNP,cnp | 0.26* | 3 |
| cNP,Cnp | 0.23* | 3 |
| CnP,CNp | 0.20 | 2 |
| cNP,CNp | 0.20 | 2 |
| CNp,cnp | 0.17 | 2 |
| cNP,CnP | 0.14 | 2 |
| cnP,Cnp | 0.10 | 2 |
| CNP,cNp | 0.09 | 2 |
| CNp,cNp | 0.08 | 1 |
| cNP,cNp | 0.08 | 1 |
| CNp,Cnp | 0.07 | 1 |
| CNP,cNP | 0.07 | 1 |
| Cnp,cnp | 0.06 | 1 |
| CnP,cnP | 0.06 | 1 |
| cNP,cnP | 0.05 | 1 |
| CnP,cnp | 0.05 | 2 |
| CNP,CnP | 0.05 | 1 |
| cNp,cnp | 0.05 | 1 |
| CNP,cnP | 0.05 | 2 |
| cnP,cNp | 0.04 | 2 |
| cNP,cnp | 0.03 | 2 |
| CnP,Cnp | 0.02 | 1 |
| cnP,cnp | 0.02 | 1 |
| CNP,Cnp | 0.02 | 2 |
| CNP,CNp | 0.02 | 1 |

**Denotes statistical significance with Bonferroni correction at the 5 μm scale.*

When types *Cnp*, *cNp*, and *cnP* survived, they formed a three-way coalition (**Figure 3C**; Movie S2 in Supplementary Material). In this case, the relationship is opposite to that described above, in that microbes of the same type inhibit each other's growth due to competition for the nutrients for which they cannot produce enzymes, but facilitate the growth of the other types by providing their complementary enzymes. This led to coalitional colonies of interwoven filamentous shapes.

When types *CnP* and *cNp* survived, they formed a successful coalition (**Figures 2F** and **3D**; Movie S3 in Supplementary Material). The relationship between these types includes both mutualistic and parasitic aspects. Both produce the complementary enzymes required by the other type, causing them to facilitate each other's growth. However, there is an inherent asymmetry, because one produces only one enzyme and the other two. The net impact of *cNp* on the fitness of *CnP* was positive at low density, but negative at high density, when competition for C became overwhelming, leading to cycling. Types *CNp* and *cnP* have a similar relationship and formed similar spatial patterns (**Figure 3E**; Movie S4 in Supplementary material).

The outcome with the highest diversity included five types, *CNp*, *Cnp*, *cNp*, *cnP*, and *cnp*, and displayed patterns of both mutualistic and parasitic interactions (**Figure 3F**; Movie S5 in Supplementary Material). Type *CNp* had a higher growth rate and was more independent relative to other types, and so formed larger colonies, whereas the single-enzyme producers formed thinner, more filamentous colonies because of competitive interactions with their own type. Density-dependent effects also led to cycling of *CNp* in this scenario.

### NUTRIENT DEPOLYMERIZATION

Overall, holding constitutive production constant, nutrient depolymerization was highest for low diffusion (**Figure 4**). Under low diffusion, depolymerization was highest under intermediate constitutive production, and under intermediate to high diffusion, depolymerization was highest under low constitutive production (**Figure 4**). A negative correlation was found between diversity and nutrient depolymerization of −0.45, −0.29, and −0.38 for C, N, and P, respectively. The *p*-values for these correlations were 0.020, 0.051, 0.030. This indicates weak significance for C and P, although if a Bonferroni correction with $n = 3$ is applied, none are significant.

In comparison with the two-type model, the eight-type model showed reduced depolymerization rates, especially for N and P. This effect was strongest under high constitutive production and low diffusion. In the two-type model, producers were able to dominate cheaters in this scenario. However, in the eight-type model, a diverse mix of types persisted, including a relatively high frequency of *Cnp* (**Figure 2E**), accounting for the low depolymerization of N and P. This effect was reversed under high constitutive production and diffusion, because in this case, the two-type model went extinct, whereas the eight-type model was able to survive the bottleneck caused by the initial crash in some replicates. Therefore, its average nutrient depolymerization rates were much higher than the two-type model.

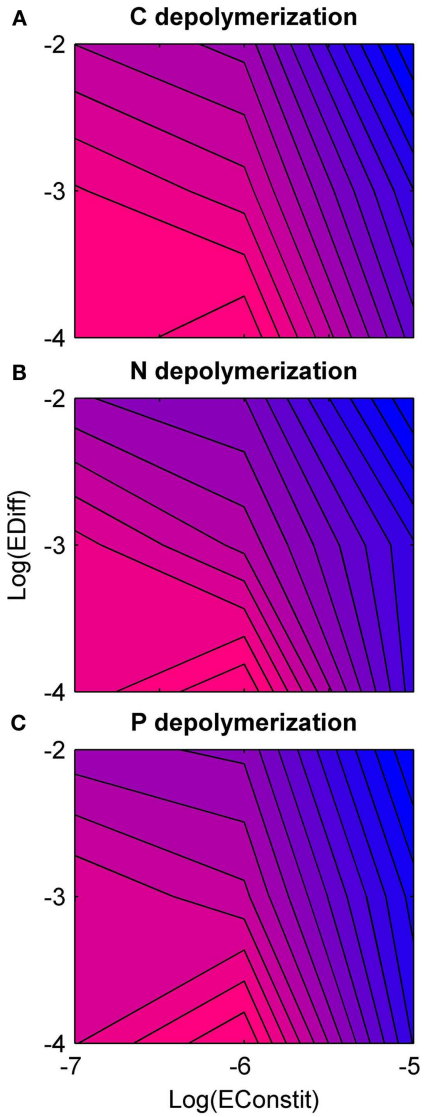


**FIGURE 4 | Rate of nutrient depolymerization as a function of diffusion rate (EDiff) and constitutive enzyme production rate (EConstit) for each of the three nutrients, averaged over all replicate runs.** Blue represents low rates of depolymerization, and pink high rates. **(A)** Carbon depolymerization. **(B)** Nitrogen depolymerization. **(C)** Phosphorous depolymerization. Across all three nutrients, the depolymerization rate is highest for intermediate production and low diffusion, and lowest for high production and high diffusion. The depolymerization rate decreases with the diffusion rate over the domain studied.

## DISCUSSION

We have modeled the enzyme-mediated depolymerization of nutrients by microbes as a public goods game in a diverse community of microbes. Our model differs from previous models in several important ways. While many models specify fitness payoffs of an evolutionary game exogenously (Durrett and Levin, 1994; Kerr et al., 2002; Hauert and Doebeli, 2004; Gore et al., 2009; Wakano et al., 2009), this model mechanistically models the production and action of enzymes by the microbes, and allows the game dynamics to emerge from them. Furthermore, rather than arbitrarily specifying the efficiency of resource capture (as do Gore et al., 2009) or the size of the interaction neighborhood (as do Wakano et al., 2009), we simply allow enzymes to diffuse through the environment. By increasing the diffusion rate, we reduce the efficiency of resource capture for enzyme producers, thus reducing the benefits of enzyme production for producers and increasing the amount of product available for cheaters. For this reason, faster diffusion benefits cheaters at the expense of producers (Allison, 2005).

This model builds on the previous one by Allison (2005) by the addition of multiple types of microbes, which may or may not produce enzymes for the three types of nutrients in the model. Thus there are two (not completely orthogonal) axes on which types vary, the number of enzymes they produce (from 0 to 3) and the types of enzymes they produce (C, N, or P). Most models of public goods games include only two types of agents, producers and cheaters. Some models of allelopathy include three types of agents, which can lead to rock-paper-scissors dynamics that allow for the maintenance of diversity (Durrett and Levin, 1997; Kerr et al., 2002). However, real microbial communities are highly diverse, as are the enzymes produced by them and the substrates they hydrolyze. While this model does not seek to model the full diversity of a real system, increasing the number of types from

two to eight allows us to gain some heuristic insight into the relationship between the diversity of enzyme production strategies and three important biological properties: diversity, social interactions, and nutrient depolymerization.

## DIVERSITY

Diversity was highest under intermediate conditions, specifically relatively fast diffusion (but not well-mixed), and intermediate constitutive production. Under these conditions, most types were able to coexist at relatively even frequencies. The intermediate level of constitutive production maintained a balance between producers and cheaters, and the fast diffusion allowed for the sharing of enzymes between complementary types. Diffusion rate is a function of both the size of the enzymes and the physical environment. The diffusion rates considered in this study are much slower than published diffusion coefficients in liquid media (Vetter et al., 1998; He and Niemeyer, 2003). They are intended to model diffusion in complex soils or sediment matrices, in which diffusion is slowed due to complex pore structures (Moldrup et al., 2001). Therefore the model predicts that environments allowing for an intermediate rate of diffusion should have higher diversity than either well-mixed environments or ones where diffusion is highly constrained.

Consistent with our model predictions, empirical studies show that differences in diffusion rates can influence microbial diversity and community composition. In a soil microcosm study, Carson et al. (2010) found that bacterial diversity increased at lower water potential, indicative of reduced soil pore connectivity and diffusion rates. Bacterial diversity and evenness also increased systematically as water content declined across a gradient of 29 soils sampled in the field (Zhou et al., 2002). Competition experiments under controlled conditions further suggest that rates of diffusion influence coexistence among bacteria. In soil microcosms, coexistence between *Ralstonia* and *Sphingomonas* bacteria increased with decreasing soil water potential (Treves et al., 2003). Another laboratory experiment found that two competing *Pseudomonas putida* strains coexisted on diffusion-limited agar plates but not in liquid culture (Dechesne et al., 2008). Thus there is good empirical support for the prediction that increasingly well-mixed conditions reduce the diversity of bacterial communities. However, our model also predicts that very low rates of diffusion could reduce diversity by selecting against cheaters that depend on diffusion to access enzyme reaction products. Since most prior studies have focused on relatively high diffusion environments, additional experiments should be conducted to test this prediction.

## SOCIAL INTERACTIONS

In the domain of social interactions, our model reveals an alternative strategy to generalist production and cheating: the formation of coalitions between complementary types. Our model assumes that if a microbe has the potential to make an enzyme, it must do so at constitutive levels or more, making the generalist producer strategy (*CNP*) inherently costly. Under low constitutive production, these costs are small, generalist producers dominate, and neither coalitions nor cheaters are observed. However, as constitutive production increases, enzyme costs reduce the competitive ability of generalist producers. While this level of public goods production favors the evolution of cheaters, it also favors coalitions that reduce the costs of enzyme production by allowing microbe types to obtain resources through the activity of complementary types. The same conditions that favor cheaters also favor coalitions because mechanistically, coalitions can be thought of as mutual "cheating," since both types take advantage of the enzymes produced by the other. Interactions between complementary types can be mutualistic, if they facilitate each other's growth, or parasitic, if one benefits at the expense of the other. We found that complementary types were spatially associated regardless of whether their interaction was mutualistic or parasitic, but the spatial association was stronger for mutualistic interactions. Therefore, if different microbe types are spatially associated, they should produce complementary enzymes, and their interaction is probably mutualistic, but need not be.

Coalitions were most important under high constitutive production and diffusion. Under these conditions, the two-type community was not stable, and crashed due to cheating. However, the eight-type community was able to survive the bottleneck of the initial crash and rebound in some replicates, albeit with reduced diversity. In some cases, the community even entered a cyclic state of repeated crashes and rebounds. This behavior is due to the density-dependence of microbe fitness, since producers have the advantage at low density, but cheaters the advantage at high density. More abstract models of evolutionary games have also shown that the addition of an advantage to cooperators at low densities allows for coexistence in a public goods game (Durrett and Levin, 1994; Wakano et al., 2009). These theoretical predictions are also supported by experimental evidence from the yeast *Saccharomyces cerevisiae*, which produces an extracellular enzyme that hydrolyzes the disaccharide sucrose into glucose. By varying the density of cells, Greig and Travisano (2004) showed that cooperators have higher fitness at low densities, but that cheaters have higher fitness at high densities. Since cheaters rely on the enzymes produced by cooperators, they are not able to survive at low densities, but enzyme producers are self-sufficient, and so are able to survive even at low density. However, at high densities, the enzyme is plentiful, and the cheaters are able to outcompete the cooperators because they do not pay the cost of enzyme production.

In addition, the high diffusion and constitutive productions scenario revealed differences in the emergent spatial patterns of different microbial associations. Fully cooperative interactions led to a dense, highly aggregated pattern with high autocorrelation, as observed for *CNP–CNP* interactions. In contrast, fully competitive interactions led to a dispersed pattern with lower autocorrelation, as observed for *cnp–cnp*. Interactions between complementary types include aspects of both cooperation and competition, and therefore produced more complex spatial patterns.

## NUTRIENT DEPOLYMERIZATION

Social interactions could complicate the relationship between microbial diversity and ecosystem function (Nielsen et al., 2011). Nutrient depolymerization was highest for low diffusion and low to intermediate constitutive production. In the absence of competition from cheating, depolymerization increased with increasing diffusion and constitutive production (Allison, 2005), due to the

fact that high constitutive production directly increases the quantity of enzymes produced, and high diffusion reduces enzyme saturation by spreading enzymes from areas of high concentration to areas of low concentration. However, these conditions also favored cheaters and coalitions of intermediate types in competition with generalist producers, and when these effects were accounted for, the net effects of high constitutive production and diffusion were reversed, reducing nutrient depolymerization.

By comparing nutrient depolymerization rates between the two-type and eight-type models, we found that nutrient depolymerization for all nutrients tended to be lower in the more diverse model (except under high constitutive production and diffusion where the two-type community went extinct). This effect may seem counterintuitive, because it is often assumed that increasing diversity will increase the rate of resource use due to niche complementarity between types (Tilman, 1999; Tilman et al., 2001). However, when social interactions are also considered, the effect of diversity may also be reversed, reducing nutrient depolymerization.

The reduction in depolymerization was strongest for P, intermediate for N, and weakest for C. This pattern was due to C-limitation of microbial growth in the model imposed by a higher stoichiometric demand for C relative to the substrate supply. Therefore C-only producers could acquire the most valuable resource while paying less cost of enzyme production than generalist producers. Competition from C-only producers reduced the density of generalist producers, and consequently N- and P-depolymerization were reduced relative to C-depolymerization. Therefore a prediction of our model is that N- and P-depolymerization are reduced more by competition than C-depolymerization, a prediction that could not be made by a simpler two-type model. Furthermore, this prediction is general and not restricted to C-limitation. Cheating is constrained for the most limiting nutrient, so the less limiting a nutrient is biologically, the more its depolymerization will be reduced by social interactions.

In this study, we showed that increasing diversity from two to eight types reduced nutrient depolymerization rates due to social interactions. However, real microbial communities contain thousands of taxa, although at this time it is unknown how much functional diversity is represented by this taxonomic diversity. Therefore, an important challenge for future work is to understand how social interactions influence nutrient dynamics at the high levels of microbial diversity observed in real ecosystems. Understanding these scaling rules could improve our ability to predict carbon and nutrient cycling processes driven by complex microbial communities.

## ACKNOWLEDGMENTS

This research was supported by grants from the NSF Advancing Theory in Biology program and the Office of Science (BER), US Department of Energy.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Terrestrial_Microbiology/10.3389/fmicb.2012.00338/abstract

**Movie S1 | Cycling of generalist producers and cheaters.** Only three types survive the initial bottleneck: generalist producers (red), cheaters (blue), and P-only producers (purple). When densities are low, generalist producers are capable of very high growth rates because they catalyze their own growth, and so form large aggregations. However, their high cost makes them poor competitors at high densities, and they are outcompeted by cheaters. Once density drops due to the cheater load, producers rebound, leading to cyclical behavior (http://dx.doi.org/10.6084/m9.figshare.92321).

**Movie S2 | Three-way coalition of single-enzyme producers.** Only three types survive the initial bottleneck: C-only (light blue), N-only (green), and P-only (purple). These types compete with their own type for the resources of enzymes they cannot produce, but catalyze each other's growth by providing their complementary enzymes. This leads to a spatial pattern in which the shapes of colonies maximize their perimeter, and different types are highly interwoven. Mutations can be seen when small colonies of other colors appear, but none of these are able to invade, indicating that this community is stable to invasion. This is the same replicate as **Figure 3D** (http://dx.doi.org/10.6084/m9.figshare.92322).

**Movie S3 | Asymmetric coalition between a two-enzyme producer and a one-enzyme producer.** Only three types survive the initial bottleneck: *CnP* (pink), *cNp* (green), and *cnp* (blue). *CnP* and *cNp* catalyze each other's growth at low density, but *cNp* becomes parasitic on *CnP* at high densities, leading to complex spatial patterns and cycling (http://dx.doi.org/10.6084/m9.figshare.92323).

**Movie S4 | Four types survive the initial bottleneck: CNp (orange), cNp (green), cnP (purple), and cnp (blue).** *CNp* and *cnP* complement each other, forming a mutualistic coalition at low densities, but at high densities, competition from the other types causes collapse of the *CNp* population, nearly leading to extinction. Once densities are low enough, *CNp* rebounds quickly, leading to cycling (http://dx.doi.org/10.6084/m9.figshare.92325).

**Movie S5 | High diversity. In this scenario, five type survive the bottleneck: CNp (orange), Cnp (light blue), cNp (green), cnP (purple), and cnp (blue).** This scenario includes the mutualistic interactions between single-enzyme producers as in Movie S2, the asymmetric coalition between *CNp* and *cnP* in Movie S4, and competition from cheaters, leading to a complex set of interactions. *CNp* is able to grow much faster than other types because it is more independent and catalyzes its own growth. This community is stable to invasion by other types due to mutation and represents the highest diversity outcome of this scenario (http://dx.doi.org/10.6084/m9.figshare.92324).

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

*Received: 01 June 2012; accepted: 01 September 2012; published online: 27 September 2012.*

*Citation: Folse HJ III and Allison SD (2012) Cooperation, competition, and coalitions in enzyme-producing microbes: social evolution and nutrient depolymerization rates. Front. Microbio.* ***3****:338. doi: 10.3389/fmicb.2012.00338*

*This article was submitted to Frontiers in Terrestrial Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

PERSPECTIVE ARTICLE
published: 02 August 2012
doi: 10.3389/fmicb.2012.00268

# When should we expect microbial phenotypic traits to predict microbial abundances?

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Species' phenotypic traits may predict their relative abundances. Intuitively, this is because locally abundant species have traits making them well-adapted to local abiotic and biotic conditions, while locally rare species are not as well-adapted. But this intuition may not be valid. If competing species vary in how well-adapted they are to local conditions, why doesn't the best-adapted species simply exclude the others entirely? But conversely, if species exhibit niche differences that allow them to coexist, then by definition there is no single best adapted species. Rather, demographic rates depend on species' relative abundances, so that phenotypic traits conferring high adaptedness do not necessarily confer high abundance. I illustrate these points using a simple theoretical model incorporating adjustable levels of "adaptedness" and "niche differences." Even very small niche differences can weaken or even reverse the expected correlation between adaptive traits and abundance. Conversely, adaptive traits confer high abundance when niche differences are very strong. Future work should be directed toward understanding the link between phenotypic traits and frequency-dependence of demographic rates.

**Keywords: trait-abundance correlations, coexistence, competitive exclusion, local adaptation, frequency dependence**

## SPECIES' TRAITS AS PREDICTORS OF THEIR ABUNDANCES

The underlying causal processes that determine organismal distribution and abundance presumably reflect phenotypic traits rather than taxonomic identity. It is the phenotypic traits of an organism, not its taxonomic identity, that actually determine its rates of reproduction, mortality, and movement (these rates will of course also depend on current and past biotic and abiotic environmental conditions). For this reason, ecologists working on both microorganisms and macroorganisms have called for a turn away from a focus on taxonomic identity and toward a focus on traits (e.g., Lavorel and Garnier, 2002; McGill et al., 2006; Violle et al., 2007; Green et al., 2008). A focus on traits might be particularly useful in microbial ecology. Microbial "species" are difficult to define, but microbial "functional traits"—phenotypic traits that directly or indirectly determine reproduction and mortality—are increasingly easy to measure (e.g., Fierer et al., 2007; Green et al., 2008; Gudelj et al., 2010; Edwards et al., 2012; Lennon et al., 2012).

Many studies using traits as predictors of distribution and abundance explicitly or implicitly assume that there is a straightforward and direct causal connection between the measured traits and abundance. Studies of spatial variation in species composition often assume that local environmental conditions "filter out" organisms with inappropriate traits (e.g., Keddy, 1992; Lavorel and Garnier, 2002; Cavender-Bares et al., 2004). On this view, sites are occupied only by organisms with traits sufficiently well-adapted to the local environmental conditions. In microbial systems, extremophiles provide a clear example: a 70°C hot spring will only harbor microbes with traits allowing them to survive and reproduce at 70°C (e.g., traits such as possession of proteins that retain a functional conformation at 70°C). Many less-extreme examples exist (e.g., Fierer et al., 2007; Schwaderer et al., 2011). For instance, relative abundance of freshwater phytoplankton varies with light availability: species with physiological traits conferring high fitness in low-light environments are most abundant in low-light lakes, while species with physiological traits conferring high fitness in high-light environments are most abundant in high-light lakes (Schwaderer et al., 2011). This view of the environment as filtering out (or at least making rare) species with the "wrong" traits resonates with Baas Becking's famous remark concerning microbial distributions: "everything is everywhere, but the environment selects." On this view, what evolutionary biologists term "local adaptation" is ubiquitous: the organisms found at any given site will tend to be those that are fittest at that site, and taxa will tend to occur at the sites where they are fittest (Kawecki and Ebert, 2004; Hereford, 2009). Similarly, studies of relative abundance within sites often assume that abundance is at least roughly proportional to how well-adapted taxa are to local conditions (e.g., Pärtel et al., 2001; Poullin and Mouillot, 2004; Harpole and Tilman, 2006; Shipley et al., 2006, 2011; Fierer et al., 2007; Partensky and Garczarek, 2010). For instance, Pärtel et al. (2001) attributed the high relative abundance of "core" alvar grassland plant species, as compared to non-core species, to the possession by core species of traits like low stature, conferring "adaptation to low-fertility conditions." As a microbial example, the cyanobacterium *Prochlorococcus* is relatively abundant compared to other microbes in oligotrophic tropical and subtropical ocean sites. This has been attributed to its relatively small size and reduced genome, as these adaptive traits reduce nutrient requirements, thereby improving relative fitness in nutrient-poor environments (Partensky and Garczarek, 2010).

However, there are both empirical and conceptual reasons to question whether variation in species composition and relative abundance generally should map so neatly onto trait variation. On the empirical side, studies that directly test for local adaptation by reciprocally transplanting organisms among sites typically do find local adaptation—but it is often quite weak (Hereford, 2009). This is true in microbes as well as in macroorganisms (Belotte et al., 2003). Local adaptation is trivially obvious (and so is rarely tested) when different sites have strongly contrasting environments (e.g., a 70°C hot spring vs. a 20°C pond). But when differences among localities are less extreme, local adaptation can become not just non-obvious, but non-existent. One important reason for this, though not the only one, is dispersal among sites, which decouples local abundance from local demography (Kisdi, 2002; Kawecki and Ebert, 2004; Paul et al., 2011).

However, I will focus on a conceptual problem with species' traits as predictors of their abundances, relevant even when local demography completely determines local abundances. Consider the expectation that the species best adapted to local environmental conditions will tend to be the most locally abundant. This expectation raises a question: why doesn't the best-adapted species simply exclude all the others? The intuitive answer is that it's generally unrealistic to expect competition to lead to exclusion of all but the best-adapted species. Rather, interspecific niche differences will prevent weaker competitors from being excluded. But if that's the case, why expect species' traits to predict their abundances at all, even roughly? Insofar as there are niche differences—species with different traits "make their living" in different ways and so compete less strongly with heterospecifics than conspecifics—then there is no single best-adapted species. Rather, species' demographic rates will depend on their relative abundances (frequencies), with rare species having an advantage over common ones because common species necessarily experience mostly intraspecific competition while rare species necessarily experience mostly interspecific competition (Chesson, 2000). Laboratory experiments with microbes provide many of the most rigorous demonstrations of niche differences leading to stable coexistence (Grover, 1997; Rainey and Travisano, 1998; Le Gac et al., 2012). But to my knowledge the consequences of niche differences for trait-abundance correlations have not been much explored in either micro- or macroorganisms (but see Harpole and Suding, 2007).

Here I use a simple, classic theoretical model to ask how niche differences affect trait-abundance correlations. Under what circumstances can species with traits conferring high adaptedness coexist with, but also maintain higher abundance than, species with traits conferring lower adaptedness? I focus on trait-abundance relationships within a single site for the sake of simplicity, but the results also have implications for variation in species' relative and absolute abundances across sites. This simple model is not a realistic description of any particular natural system, microbial or otherwise, but it is not intended to be. Rather, the model is intended to sharpen intuition and aid hypothesis development. It is widely assumed that locally abundant species should have traits making them well-adapted to local environmental conditions (e.g., Pärtel et al., 2001; Poullin and Mouillot, 2004; Harpole and Tilman, 2006; Shipley et al., 2006, 2011; Harpole and Suding, 2007; Violle et al., 2007; Partensky and Garczarek, 2010). If this intuitively appealing hypothesis is valid, then it ought to hold in the context of a deliberately simplified theoretical model lacking complicating factors like dispersal. And if the hypothesis is not valid, the model can help identify the reasons why not, thereby aiding development of better hypotheses.

## A SIMPLE MODEL OF TRAIT-ABUNDANCE CORRELATIONS

The model is that of MacArthur (1970). The model considers two or more consumer species competing for two or more nutritionally substitutable limiting resources such as different sugars. Consumer $j$ consumes resource $i$ at a constant per-capita rate $c_{ij}$, converts a consumed unit of resource $i$ into $b_{ij}$ new consumer individuals, and dies at constant per-capita rate $m_j$. Resource $i$ grows logistically with intrinsic rate of increase $r_i$ and carrying capacity $K_i$. Logistic growth is more appropriate for living, self-reproducing resources than non-living resources like carbon compounds, but other forms of resource growth, such as chemostat-type resource supply, would not alter the results. Assuming a well-mixed system in which reproduction and mortality occur continuously, consumer and resource dynamics are given by

$$\frac{dR_i}{dt} = r_i R_i \left(1 - \frac{R_i}{K_i}\right) - \sum_j c_{ij} R_i C_j$$
$$\frac{dC_j}{dt} = \sum_i c_{ij} b_{ij} R_i C_j - m_j C_j \quad . \qquad (1a,b)$$

The behavior of this model is well-studied, particularly for certain analytically tractable special cases (reviewed in Chesson, 1990). This facilitates interpretation of the results presented below. To use the model to study trait-abundance correlations, I considered model parameters as consumer "traits." As noted above, we expect species' phenotypic traits to predict their abundances because those traits affect species' demographic rates. In the context of this model, the $c_{ij}$, $b_{ij}$, and $m_j$ parameters are features of the biology of consumer $j$ which determine its demographic rates as a function of resource levels. These parameters, therefore, can be considered as consumer traits.

In general, it is not possible to solve analytically for equilibrium consumer abundances as a function of the model parameters, so I relied on numerical simulations. In order to aid interpretation, I considered a special case in which there is a clear-cut separation between traits governing how well-adapted different consumer species are to their shared environment, and traits governing the amount of niche differentiation among different species. I varied "adaptedness" by allowing per-capita mortality rates to vary among consumer species. The lower the $m_j$ value, the better consumer species $j$ is adapted to the habitat. I vary niche overlap by varying the $c_{ij}$ values, while constraining all consumer species $j$ to have the same sum of their $c_{ij}$ values (i.e., $\sum_i c_{ij} = T$ for all $j$). Consumers thus varied in the relative rates at which they consumed different resources, but no consumer was intrinsically better than any other at consuming resources in general. I further

assumed that all $b_{ij} = b$, so no consumer was better than any other at converting consumed resources into new consumers, and no resource was more valuable or nutritious than any other. I assumed that all $r_i = r$ and all $K_i = K$, so all resources grew in an identical fashion, independent of consumers. Thus, $m_j$ values completely dictated the intrinsic "adaptedness" of consumers to the shared environment.

Classical analyses of this model measure consumer niche differentiation by making very strong assumptions about the distributions of the $c_{ij}$ values (Roughgarden, 1989). These strong assumptions are made for mathematical convenience and aren't necessary here. Instead, I follow Chesson (1990) and measure niche differentiation in a more generally applicable way: as "linear independence" of the $c_{ij}$ values. Imagine plotting the $c_{ij}$ values of one consumer against those of another. If the points lie on a straight line through the origin, then the two consumers have completely non-independent diets: the $c_{ij}$ values of one consumer are identical to, or differ by only a constant of proportionality from, the $c_{ij}$ values of the other consumer. Such consumers exhibit no niche differentiation and cannot coexist at equilibrium (Chesson, 1990). Conversely, if the two consumers do not consume any of the same resources, then their $c_{ij}$ values are linearly independent. They exhibit maximally large niche differentiation, do not compete at all, and so are guaranteed to coexist as long as each would be capable of persisting on its own. I used equation (12) from Chesson (1990) to measure linear independence between each pair of consumers, and used the mean linear independence of all possible pairs as a measure of the average or overall strength of niche differentiation for the community as a whole. Mean linear independence equals 0 when all consumers have identical diets, and 1 when no consumer shares a resource with any other consumer.

I simulated communities of 10 consumers and 10 resources, with $m_j$ values chosen randomly from a uniform distribution and $c_{ij}$ values chosen randomly under various constraints so as to explore the full range of possible values of mean linear independence. I ran each simulation to equilibrium, and calculated the Spearman rank correlation between consumer equilibrium abundances and $m_j$ values. I calculated mortality-abundance correlations for all 10 species, and for only persisting species (those with non-zero equilibrium abundances). There were no resource extinctions in most simulations. My choices of initial consumer and resource species richness, parameter values, and initial conditions were arbitrary, but the results are robust to these arbitrary choices.

## MODEL RESULTS

When species had very similar diets, only those few species with the lowest per-capita mortality rates persisted. This was due to competitive exclusion; for the parameter values I used, each species would persist if it were growing on its own. When niche differences were weak, the only important difference among species was their mortality rates. In this case, only species with the lowest mortality rates, and thus the highest adaptedness, can persist (Chesson, 1990; Grover, 1997). In the limit when all species have identical diets, the single species with the lowest mortality rate competitively excludes all others. Competitive exclusion of all but the few species with the lowest mortality rates leads to a strongly negative mortality-abundance correlation across all species (**Figure 1A**; points in extreme lower right of panel). However, considering only the few persisting

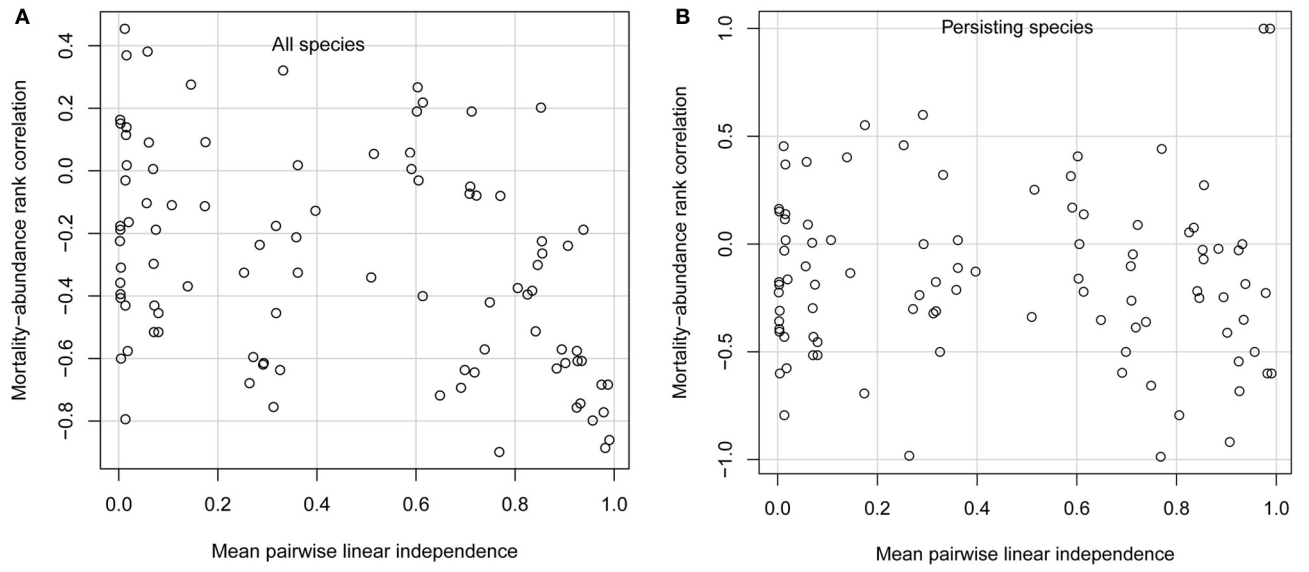


**FIGURE 1 | Simulation model results.** Spearman rank correlation between consumer per-capita mortality rate and consumer equilibrium abundance, for **(A)** all 10 consumer species, and **(B)** only persisting consumer species, as a function of mean linear independence of consumer diets. Each open circle gives results from one simulation. Note that, in the limiting case when every species consumes a different resource, so that species' diets are completely independent of one another, the rank correlation would be −1 (not shown).

species, the mortality-abundance correlation can take on any value (**Figure 1B**).

Species with low mortality rates also attain higher abundances when niche differences are as large as possible, meaning that every species consumes a different resource so that interspecific competition is absent. That is, when species have such large niche differences that they don't interact at all, their abundances are determined solely by their adaptedness to the habitat. In this limiting case, the rank correlation between adaptedness and abundance is −1 (not shown in **Figure 1**). Thus, both extremely small and extremely large niche differences create conditions favoring high abundance of species with traits conferring high adaptedness.

But in between these extremes, a wide range of trait-abundance correlations can arise. Whether niche differences are small (but not so small as to lead to exclusion of most species), large (but not so large as to prevent any interspecific competition), or intermediate, mortality-abundance correlations can range from strongly negative to moderately positive (**Figures 1A,B**). Positive mortality-abundance correlations indicate that less well-adapted species with higher per-capita mortality rates attain higher abundance. For non-extreme levels of niche differentiation, mortality-abundance correlations are highly variable when considering all species, or only persisting species. This means that species with low per-capita mortality rates are almost as likely to be competitively excluded as species with high per-capita mortality rates. On average, the typical mortality-abundance correlation is moderately negative, but the variability around this average is much the most striking feature of the results.

This variability in the strength and direction of the trait-abundance correlation arises because, when there is any diet overlap at all, species compete. Further, they don't just compete with others sharing the same resources, but interact indirectly with every species in the community (e.g., species 1 and 3 interact indirectly if both share resources with species 2). This means that a species' equilibrium abundance depends not just on its own traits, but depends in a complex way on the traits of every species in the community. Even quite small diet overlap can decouple equilibrium abundance from adaptedness, because even quite small diet overlap causes all species to interact with one another.

The same results hold if the trait governing adaptedness is not mortality rate, but some other trait such as total per-capita feeding rate or efficiency at converting consumed resources into new consumers (results not shown). Traits conferring high adaptedness to local conditions reliably confer high abundance only when niche differences are either maximally large or nonexistent. Non-extreme levels of niche differentiation, even if quite small, frequently weaken and even reverse expected trait-abundance correlations.

## DISCUSSION

In many respects, the deliberately simplified scenario considered here is a best-case scenario for trait-abundance correlations. The ecology of the system (Equation 1) is known, and is very simple. For instance, there are no environmental fluctuations or perturbations that might cause species' abundances to temporarily deviate from those expected based on their trait values. The system lacks many factors, such as dispersal, known to weaken or alter trait-abundance correlations. A single trait governs adaptedness to the local environment. Trait values and species' abundances, including those of species which are absent due to competitive exclusion, are known without error. Despite all this, trait values often fail to explain species' abundances, or do so only when competitively excluded species are included in the analysis.

Unfortunately, these results don't suggest any "rules of thumb" for when non-extreme levels of niche differentiation will or will not prevent trait-abundance correlations. When levels of niche differentiation are non-extreme, as they usually are, species' realized abundances, and thus trait-abundance correlations, will be sensitive to idiosyncratic details (here, the precise $c_{ij}$ values).

These results caution against inferring adaptiveness of traits from species' abundances. In general, the most abundant species at a given site will not necessarily possess traits making them better-adapted to local conditions than rare or absent species.

These theoretical results accord with empirical data. Studies of trait-abundance correlations in both micro- and macroorganisms often identify traits that explain a statistically significant but biologically modest fraction of interspecific variation in abundance (e.g., Harpole and Tilman, 2006; Fierer et al., 2007; Schwaderer et al., 2011). Investigators often attribute unexplained variation in abundance to unmeasured traits, dispersal, or other confounding factors. In contrast, the results shown here suggest that biologically modest trait-abundance correlations are likely to be the rule rather than the exception, and so demand no special explanation.

These results have implications for variation in species' absolute and relative abundances across sites. For instance, the results show that extinct species are not always those most poorly adapted to local conditions. This implies that the local environment cannot be viewed as simply "filtering out" the worst-adapted species. Variation in species composition across sites, therefore, cannot necessarily be ascribed to different local environments "filtering out" different species.

An interesting direction for future research would be to try to link measurements of species' traits not just to adaptedness of different species, but also to the strength of niche differentiation. I further suggest that, in empirical studies, niche differentiation is best quantified by manipulating relative abundances and directly measuring the strength of the resulting negative frequency dependence of species' demographic rates. Such direct, trait-independent measurements of the strength of niche differentiation can then form the basis of follow-up studies to identify their phenotypic basis. Quantifying niche differentiation in this way is standard in microbial laboratory experiments (e.g., Rainey and Travisano, 1998; Brockhurst et al., 2006; Zhang et al., 2009; Le Gac et al., 2012), increasingly common in studies of macroorganisms (Harpole and Suding, 2007), and requires no *a priori* assumptions about how species' traits relate to the strength of negative frequency dependence. This approach has already had success with both macro- and microorganisms. For instance, Angert et al. (2009) showed how an interspecific trade-off between traits conferring high growth capacity and traits conferring tolerance of low resource levels combines with

fluctuations in rainfall to generate negative frequency dependence and promote coexistence of desert annuals. In microbes, Brockhurst et al. (2006) and Zhang et al. (2009) demonstrated surprisingly strong negative frequency dependence among strains of *P. fluorescens* lacking any obvious trait differences, illustrating the value of testing for niche differentiation directly.

## ACKNOWLEDGMENTS

This work was supported by an NSERC Discovery Grant to the author. The author thanks Graham Bell for first pointing out to him that frequency dependent selection should undermine trait-abundance correlations. Comments from two referees improved the ms.

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

*Received: 31 May 2012; accepted: 09 July 2012; published online: 02 August 2012.*
*Citation: Fox JW (2012) When should we expect microbial phenotypic traits to predict microbial abundances? Front. Microbio. **3**:268. doi: 10.3389/fmicb.2012.00268*
*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 31 August 2012
doi: 10.3389/fmicb.2012.00318

# Spatial and temporal scales of aquatic bacterial beta diversity

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Understanding characteristic variation in aquatic bacterial community composition (BCC) across space and time can inform us about processes driving community assembly and the ability of bacterial communities to respond to perturbations. In this study, we synthesize BCC data from north temperate lakes to evaluate our current understanding of how BCC varies across multiple scales in time and space. A hierarchy of average similarity emerged with the highest similarity found among samples collected within the same lake, especially within the same basin, followed by similarity among samples collected through time within the same lake, and finally similarity among samples collected from different lakes. Using decay of similarity across time and space, we identified equivalent temporal (1 day) and spatial (10 m) scales of BCC variation. Finally, we identify an intriguing pattern of contrasting patterns of intra- and inter-annual BCC variation in two lakes. We argue our synthesis of spatio-temporal variation of aquatic BCC informs expectations for the response of aquatic bacterial communities to perturbation and environmental change. However, further long-term temporal observations will be needed to develop a general understanding of inter-annual BCC variation and our ability to use aquatic BCC as a sensitive metric of environmental change.

**Keywords: lake, beta diversity, spatial, temporal**

## INTRODUCTION

A central goal of ecology is to understand the patterns and processes of biodiversity. In particular, community ecologists seek to describe species richness at local scales (alpha diversity), differences in diversity across space and time (beta diversity), and diversity within a region (gamma diversity). Following the emergence of molecular techniques to classify bacteria, the field of freshwater microbial ecology has generated a number of studies describing the extant alpha diversity in these systems (Zwart et al., 2002; Newton et al., 2011). Like many ecologists (Soininen, 2010; Anderson et al., 2011), researchers studying microbial diversity are now transitioning to focus more intently on beta diversity (i.e., species turnover) in an effort to identify both extrinsic and intrinsic factors that explain differences in taxon composition among communities separated in space or time.

Distinct and consistent taxon distribution patterns along gradients in space and time are emerging (Schauer et al., 2005; Wu et al., 2006; Newton et al., 2007, 2011; Jones et al., 2009; Jezberova et al., 2010; Simek et al., 2010; Eiler et al., 2012). For example, repeatable compositional responses to predation (Pernthaler, 2005), interactions with phytoplankton (Pinhassi et al., 2004; Kent et al., 2007) and chemical or resource gradients (Schauer et al., 2005; Wu and Hahn, 2006a; Newton et al., 2007; Jones et al., 2009) have been observed. Many of the initial efforts to quantify aquatic microbial beta diversity have focused on traditional biogeographical concepts, including taxa-area relationships (Reche et al., 2005; Lindstrom et al., 2007; Logue et al., 2011) and the niche vs. neutral debate (Langenheder and Ragnarsson, 2007; Jones and McMahon, 2009; Lindstrom et al., 2010). Despite numerous studies addressing these biogeographical concepts, we, as aquatic microbial ecologists, lack a basic understanding of the characteristic scales of variation in aquatic bacterial community composition (BCC) (Lindstrom and Langenheder, 2012); this is especially true for variation in time. Arguably, this is a key gap in our basic understanding of aquatic bacterial diversity that hinders our ability to develop theories about how microbial mediated function and the stability of those functions are maintained across space and time.

Freshwater microbial ecologists are not alone in their plight to understand the characteristic temporal and spatial scales of microbial community beta diversity. Recent work in the marine environment and among human body sites has revealed systematic variation in bacterial community similarity with distance and time (Fuhrman et al., 2006; Caporaso et al., 2011; Gilbert et al., 2012). Human microbiome research has identified bacteria that are endemic to a particular body site and temporal decay of community similarity with time at a given body site (Caporaso et al., 2011). Work in the marine environment has also shown distance decay relationships and cyclic, seasonal patterns in the similarity of bacterial communities at a given site (Fuhrman et al., 2006; Gilbert et al., 2012). Despite widespread patterns in spatial and temporal decay of bacterial community similarity

across ecosystems, we lack any mechanistic understanding of the underlying processes driving these microbial biogeographic and temporal patterns (Hanson et al., 2012). We argue that an understanding of the temporal and spatial scales over which these patterns occur will be indicative of underlying process.

In this study, we use a collection of unpublished and previously published datasets from north temperate lakes to explore patterns of variation in freshwater BCC across multiple temporal and spatial scales. We employed a "distance-decay approach" (Soininen et al., 2007) to examine species turnover among samples. Our questions included: (1) how variable are PCR-based measures of community composition and does this limit our ability to quantify microbial beta diversity? (2) How does within-lake compositional variation compare to across-lake compositional variation? and (3) At multiple scales, how does spatial compositional variation compare to temporal compositional variation?

## MATERIALS AND METHODS

### STUDY SITES AND SAMPLE COLLECTION

Lake Mendota (ME) and Crystal Bog (CB) Lake (both in Wisconsin, USA; **Table 1**) were each sampled at 32 sites to evaluate the BCC within the lakes at a relatively high spatial resolution, on June 18, 2007. Sampling of both lakes was conducted on the same day by two separate research teams. In the smaller CB, approximately 5 min was spent at each site and all 32 sites were sampled in approximately 4 h. Sampling of the larger ME occurred over 6 h, with approximately 5 min spent at each site. ME and CB samples were filtered in the lab after being stored on ice in a dark cooler during the field sampling effort. Hold times in the field prior to filtering varied between 0.5 and 5.5 h. Sampling locations were determined based on a rectangular, uniform grid that was fit over a map of the lake in such a way that 32 grid cells (15.5 m by 11.5 m in CB and 1000 m by 1300 m in ME) encompassed the entirety of the lake surface. For each grid cell, a latitude and longitude was randomly selected to determine the sampling point. At each location, three integrated samples of the top 1 m of the water column (representing the epilimnion) were collected, transferred to 1-L bottles, and stored on ice. A 200-mL subsample was vacuum filtered onto a 0.2-μm filter (Pall Life Sciences) and stored at −80°C until DNA was extracted.

In addition to our highly resolved spatial survey, 62 total integrated-epilimnion samples were collected across three open-water seasons (2003, 2005, and 2007) from the center of CB, as previously described (Kent et al., 2004). Exact dates of sample collection can be found in Supplementary Material.

**Table 1 | Characteristics of Lake Mendota (ME) and Crystal Bog (CB) Lake.**

| Feature | Mendota | Crystal Bog |
|---|---|---|
| Location | 46.01°N, 89.61°W | 43.10°N, 89.41°W |
| Surface area (ha) | 3938 | 0.5 |
| Mean depth (m) | 12.8 | 1.7 |
| Max depth (m) | 25.3 | 2.5 |
| Trophic status | eutrophic | dystrophic |
| Shoreline development | high | low |
| Total phosphorus ($\mu g\ L^{-1}$) | 109.5 | 18.2 |
| pH | 8.4 | 5.2 |
| DOC ($mg\ L^{-1}$) | 5 | 9.8 |
| Conductivity ($\mu S\ m^{-1}$) | 412 | 11 |
| Wind speed ($ms^{-1}$) | 4.7 | 1.0 |

*We present long-term averages from routine sampling by the NTL-LTER (1981–2010 for CB and 1995–2010 for ME). Mean wind speed data are from high-resolution data collected 2005–2011 for both lakes.*

### DNA EXTRACTION AND AUTOMATED RIBOSOMAL INTERGENIC SPACER ANALYSIS (ARISA)

DNA was extracted from each of the filters using the FastDNA kit using the manufacturer's protocol (QBiogene) and stored at −80°C until needed. The DNA was quantified using Picogreen (Molecular Probes) and a Molecular Devices Spectramax fluorometric-capable plate reader. Samples were diluted with sterile water in order to add 5–10 ng of template DNA for each ARISA PCR reaction. Samples were then amplified (Eppendorf Mastercycler) using the 1406F fluorescently labeled primer (5′-TGYACACACCGCCCGT-3′) and the 23SR primer (5′-GGGTTBCCCCATTCRG-3′; bacterium specific, 23S rRNA gene) according to the following conditions: 2 min at 94°C, followed by 30 cycles of 94°C for 35 s, 55°C for 45 s, and 72°C for 2 min, then finishing with 72°C for 2 min. After amplification, the samples were mixed with a formamide buffer and a 100–2000-bp custom internal size standard (Bioventures) before denaturing capillary electrophoresis was carried out on an ABI 3730 genetic analyzer (PE Biosystems). For pictoral examples of ARISA profiles see Fisher and Triplett (Fisher and Triplett, 1999). Electropherograms were analyzed using custom fragment analysis utilities developed in the R Statistics Environment (Jones and McMahon, 2009). Briefly, community profiles were de-noised, individual peaks were binned into operational taxonomic units (OTUs), and the presence or absence of each OTU in a profile was based upon peak presence or absence in that profile. In general, ARISA can resolve OTUs with a single bp difference in length when fragments are 300–1000 bp in length. As lengths increase from 1000 to 1500 bp (the maximum length considered) resolution decreases to 3–5 bp due to smearing or stretching of bands migrating through the sequencer capillary. The width of OTU bins is adjusted as a function of length to accommodate this change in resolution. Further details of the ARISA method can be found elsewhere (Fisher and Triplett, 1999; Brown et al., 2005; Jones et al., 2007; Jones and McMahon, 2009). This resulted in sample-by-OTU-presence-absence matrices for each sample set that was analyzed statistically, as described below.

### METHODOLOGICAL VARIATION

In order to explore variation in BCC across time and space, we must understand the repeatability of our methods. To this end, we quantified the error in all steps of ARISA by sequentially replicating DNA extraction, ARISA PCR, and capillary gel electrophoresis for a single sample from ME, WI collected on July

17th, 2007. We filtered 250 ml of ME water collected from the top 1 m of the water column, onto each of four filters. We then extracted DNA from these filters with the methods described above. DNA from each of the four extractions was used as template in four replicate ARISA PCR reactions. Finally, we ran four replicate fragment analysis capillaries from each PCR reaction (yielding a total of 64 ARISA profiles from a single lake sample).

### PREVIOUSLY PUBLISHED NORTH TEMPERATE LAKES MICROBIAL OBSERVATORY (NTL-MO) DATASETS

The collection of studies conducted by the North Temperate Lakes Microbial Observatory (NTL-MO) over the past decade represent a set of consistently collected and treated (both molecularly and analytically) data spanning broad ranges in space and time. All samples were derived from the integrated epilimnion water column. ARISA data from three years (2000, 2001, and 2005) of a multi-year temporal survey (36 samples from a single location in ME) (Shade et al., 2007), a purely spatial survey (90 samples from 13 Wisconsin lakes) (Yannarell and Triplett, 2004), and a combined spatial/seasonal survey (90 samples from 30 Wisconsin lakes sampled in June, July, and October) (Yannarell and Triplett, 2005) were accessed from the NTL-MO database (http://microbes.limnology.wisc.edu/) and used to supplement the previously unpublished data described above. Methods for collection, molecular analyses, and electropherogram interpretation can be found in the original publications. All samples were collected during the open-water season. **Table 2** contains the total number of samples used from each study and the spatio-temporal extent of the datasets. A complete list of samples can be found in Supplementary Material.

### STATISTICAL APPROACHES

To assess the extent of variation introduced by our data collection methods, we compared the 64 ARISA profiles collected from a single sample from ME using minimum and mean similarities across replicates and analysis of similarity (ANOSIM) from the *vegan* package in the R Statistics Environment (R Development Core Team, 2010).

To assess beta diversity (i.e., species turnover), we used multivariate similarities (Sørensen's Index; $\frac{2C}{A+B}$, where $C$ is the number of species shared between the two samples and $A$ and $B$ are the richness of each sample; Legendre and Legendre, 1998) amongst a set of samples as our response variable. Using this standardized metric of compositional variability allowed for comparison across studies despite slight differences in OTU bin definitions.

The Sørensen's similarity matrices from our single day, high spatial-resolution sampling, were used to create Principle Coordinate Analysis (PCoA) ordinations. Mean centroids of triplicate samples taken at each of the 32 sites were used for display. Simultaneous display of compositional similarity and geographic location of samples is challenging. To depict this information, we assigned gradients of color to the first two axes of our PCoA ordinations and therefore the color of a location on the lake map is indicative of the composition of the community at that location. Geographic locations with similar colors on the map had similar BCC. The spatial interpolation and plotting of the compositional data were conducted using the *spatstat* package (Baddeley and Turner, 2005) in the R Statistics Environment (R Development Core Team, 2010).

In an attempt to compare rates of change in BCC through time and space, we fit distance-decay relationships for all of our data. Before estimating the distance-decay relationship, both the predictors (time or geographic distance) and Sørensen's similarity values were log transformed after adding a small value (1 day/meter or 0.001 for Sørensen's indices) to avoid log of zero issues, as is traditionally done when estimating the decay of community similarity over space or time (Soininen et al., 2007). When considering decay in community similarity over time, documented differences in inter-annual variation in CB (each year is distinct from previous; Kent et al., 2004) and ME (repeated annual phenology has been observed; Shade et al., 2007) forced us to use continuous time and julian day-based time, respectively, for the two time series. We followed the approach of Soininen and colleagues (2007) to calculate the predicted compositional similarity between samples separated by one meter or one day and the distance or time between samples required to halve the compositional similarity. We also used $T$-tests to identify significant differences in mean Sørensen's similarity between groups of samples.

When using pair-wise similarity scores for both the distance-decay model fitting and $T$-tests issues of non-independence are encountered. To avoid these non-independence issues, we used a randomization technique for assessing the significance of our distance-decay relationship (Green et al., 2004; Horner-Devine et al., 2004). For our $T$-tests we used two different approaches depending on the nature of the data. If we were comparing similarities within a single pair-wise similarity matrix we used a

**Table 2 | Studies and number of samples included in the comparison of spatial and temporal scales of aquatic bacterial community compositional similarity.**

| Study | Site | Basin | <1 week | <1 month | <1 year | >1 year | Cross-lake |
|---|---|---|---|---|---|---|---|
| Shade et al., 2007 | | | | ■ | ■ | ■ | |
| Yannarell and Triplett, 2004 | ■ | ■ | | | | | ■ |
| Yannarell and Triplett, 2005 | | | | | ■ | | ■ |
| This study | ■ | ■ | ■ | ■ | ■ | ■ | ■ |
| Total number of samples | 282 | 333 | 114 | 114 | 204 | 114 | 372 |

*Black cells indicate the spatial and temporal scales considered in the study.*

sub-sampling-based, pair-wise-adjusted *T*-test (paT) approach as described by Danforth and Freeman-Gallant (1996). When comparing similarities from multiple studies and therefore from different pair-wise similarity matrices we used the Pooled Mean Diversities test outlined by Gilbert, Rossini, and Shankarappa (GRStest; Gilbert et al., 2005). Briefly, the test estimates the mean pair-wise difference for two groups of samples as the empirical mean, but uses a variance estimate that takes into account non-independence of pair-wise comparisons through the use of U-statistic theory. All statistical analyses were conducted using custom created functions in the R Statistics Environment (R Development Core Team, 2010).

## RESULTS

### QUANTIFICATION OF METHODOLOGICAL VARIABILITY

The nested replication of the ARISA procedure allowed us to quantify and partition methodologically induced variability. The minimum Sørensen's similarity across the entire set of 64 analyses from a single sample was 0.92, and the mean was 0.95. The minimum and mean Sørensen's similarity of profiles generated from a single DNA extraction was 0.94 and 0.97, respectively. ARISA profiles significantly clustered by extraction, and differences among replicate PCR reactions carried out on a single DNA extraction were also detected, but as indicated by overall mean similarity across all profiles, these differences were small (**Table 3**). The minimum and mean similarity across capillary runs was 0.98, and no difference was detected between replicate capillary runs conducted on a single PCR reaction (**Table 3**). Overall, methodological variation was small relative to differences observed across space and time.

### WITHIN-LAKE vs. ACROSS-LAKE SPATIAL HETEROGENEITY

In both ME and CB we observed substantial horizontal variation in BCC on our single date of collection (**Figure 1**). In both lakes, triplicate samples collected within a 1-m$^2$ site were significantly more similar than cross-site (within-lake) comparisons (paT, CB: $df = 74$, $t = 2.57$, $p < 0.05$; ME: $df = 75$, $t = 8.61$, $p < 0.01$). Site-to-site differences were greater in ME, as demonstrated by comparison of *t*-values for the above paT (2.57 and 8.61 for CB and ME, respectively), but the magnitude of greatest differences in each lake were similar (**Figure 2**).

### SPATIAL vs. TEMPORAL COMPOSITIONAL VARIABILITY

In both ME and CB, spatial variation at a single time point was significantly smaller than variation through time (GRStest—CB: $T = 17.8$, $p < 0.001$; ME: $T = 11.3$, $p < 0.001$), but average spatial similarity across lakes was smaller than similarity within a lake through time (**Figures 3A,B**). However, the ranges of similarities observed through time and across lakes were approximately the same. When more lakes were considered, a hierarchy of average similarity emerged with the highest similarity found among samples collected within the same lake, especially within the same basin, followed by similarity among samples collected through time within the same lake, and finally similarity among samples collected from different lakes (**Figure 3C**).

A comparison between variation in time and space can be made using the decay of compositional similarity with distance and time. We observed significant log-log relationships between Sørensen's similarity decay over distance ($R^2 = 0.25$, $p < 0.001$) and time ($R^2 = 0.39$, $p < 0.001$) (**Table 4**). Comparison of estimated similarity of two communities separated by a single meter or day (0.90 vs. 0.84) and the halving distance of community similarity across distance or time (∼4000 m vs. ∼350 days) suggests that one day of temporal separation is approximately equivalent to 10 m of physical separation in terms of community change or turnover.

### A CONTRAST IN INTER-ANNUAL COMMUNITY COMPOSITIONAL PATTERNS

Inter-annual patterns of change in CB were distinct from those in ME (**Figure 4**). Although intra-annual variation was similar in magnitude across the two lakes (GRStest: $T = -1.16$, $p > 0.1$), CB had much greater inter-annual variation (GRStest: $T = -18.7$, $p < 0.001$). In fact, CB inter-annual similarity was significantly smaller than intra-annual similarity (paT: $df = 64$, $t = 9.18$, $p < 0.01$). This was not the case for ME (paT: $df = 42$, $t = -0.12$, $p > 0.1$). Effectively, ME BCC varied as much within a single year as it varied across any given year, suggesting a repeated annual phenology as previously documented by Shade and coauthors (Shade et al., 2007). Alternatively, CB BCC across years was more variable, suggesting directional change from year to year with smaller scale variation within a given year (**Figure 4**).

## DISCUSSION

The goal of our study was to calibrate expectations for beta diversity of aquatic lake bacterial communities across multiple spatial and temporal scales. The majority of spatial surveys have focused on cross-lake comparisons, and those that do consider within-lake variations generally do not consider it in a continuous manner (but see Yannarell and Triplett, 2004). Temporal studies are rarer than spatial lake surveys, but time appears to be an equally important axis of variation in aquatic microbial communities. Therefore, we sought to compare spatial and temporal beta diversity in order to evaluate their relative importance in structuring microbial communities.

**Table 3 | Minimum and mean Sørensen's similarity of replicate profiles at three major steps of the ARISA procedure.**

| Portion of method | Minimum similarity | Mean similarity | ANOSIM R | *p*-value |
|---|---|---|---|---|
| Water filtering and DNA extraction | 0.92 | 0.95 | 0.88 | 0.001 |
| PCR on a single DNA extraction | 0.94 | 0.97 | 0.81 | 0.001 |
| Capillary run on a single PCR reaction | 0.98 | 0.98 | −0.17 | 1.0 |

*In addition, statistics for analysis of similarity (ANOSIM) comparisons run across replicates at each step of the ARISA procedure.*

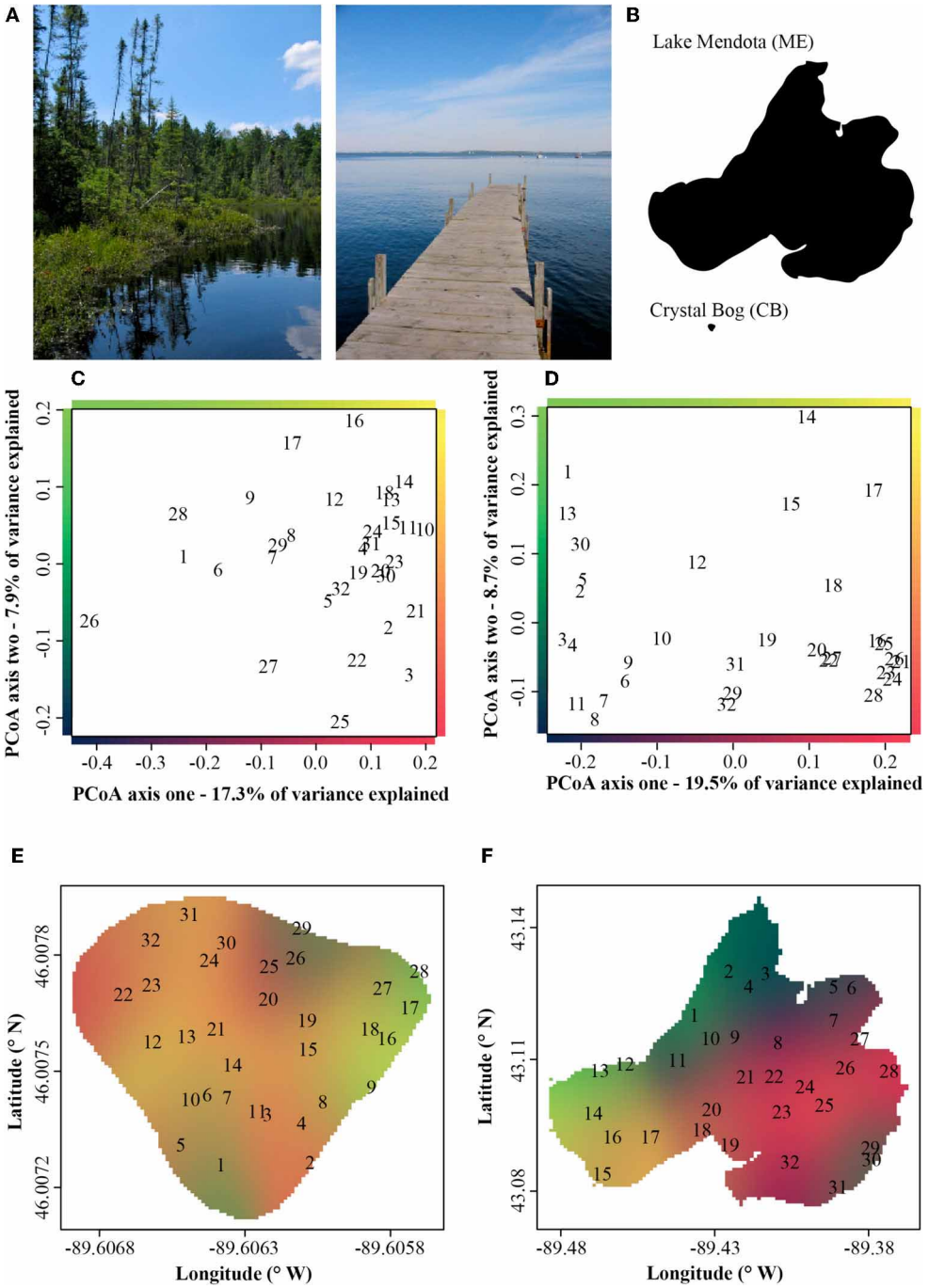


**FIGURE 1 | (A)** Pictorial and **(B)** areal contrast in the Lake Mendota (ME) and Crystal Bog (CB) environments. Principle coordinate analysis (PCoA) ordinations of bacterial community composition at 32 sites in Crystal Bog **(C)** and Lake Mendota **(D)**, collected on a single day. Points indicated as sample site numbers represent the mean of triplicate ordinated samples. The axes of the ordinations are color-coded and these colors are used to indicate bacterial community composition on the lake maps in panels **E** (Crystal Bog) and **F** (Lake Mendota).

As BCC is compared across smaller and smaller spatial and temporal scales, we may approach the limits of our methods to discriminate assemblages. To identify the limitations of our methods we conducted a nested evaluation of our selected method to quantify microbial beta diversity (ARISA; **Table 3**). The ARISA method proved to be extremely repeatable. Our results suggest that we can detect compositional differences, as quantified by Sørensen's Index, of approximately 0.05 or greater. The nested-nature of our method evaluation indicated that DNA extraction generates the greatest variation, followed by PCR, while

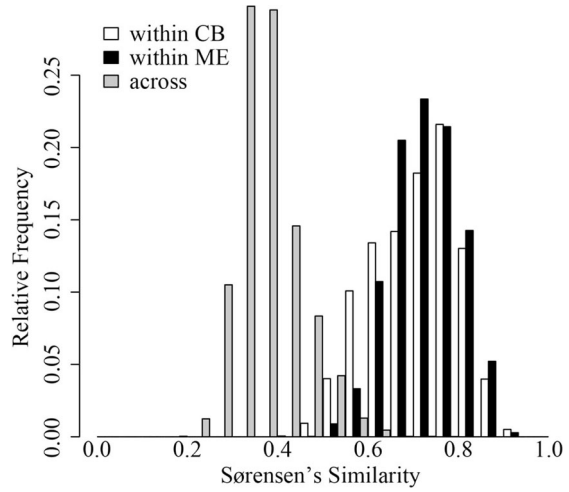


**FIGURE 2 | Distribution of Sørensen's similarity indices within 32 sites in Crystal Bog (within CB; open bars), within 32 sites in Lake Mendota (within ME; black bars), and between sites in Crystal Bog and Lake Mendota (across lakes; gray bars).** Similarities were calculated as pair-wise comparisons of all samples within a set (e.g., 512 pairwise combinations for the 32 sites in CB).

the capillary gel electrophoresis and analysis imparts nearly no methodological variability. Similar evaluation should be conducted for newer molecular methods being used to characterize microbial beta diversity (e.g., tag pyrosequencing; Prosser, 2010).

It should be noted that ARISA, as well as any other fingerprinting technique or even low coverage tag pyrosequencing, are biased toward the detection of abundant community members. As a result, our analyses neglect the contribution of likely numerous rare members of aquatic bacterial communities (Pedros-Alio, 2006). It is difficult to predict how the inclusion of the rarer members of aquatic bacterial communities would impact the patterns we observe here. On one hand, if these rare members are extremely endemic spatial differences may be magnified, while temporal changes may be muted. On the other hand, if the rarer bacterial community members represent a relatively homogeneous, seed bank (Jones and Lennon, 2010; Caporaso et al., 2012) across the landscape, spatial and temporal differences may be reduced. It does, however, seem that the use of a consistent sampling depth in all systems generates a comparable rate of community similarity change with time or distance where increasing the sampling depth only impacts the intercept of this relationship (Horner-Devine et al., 2004).

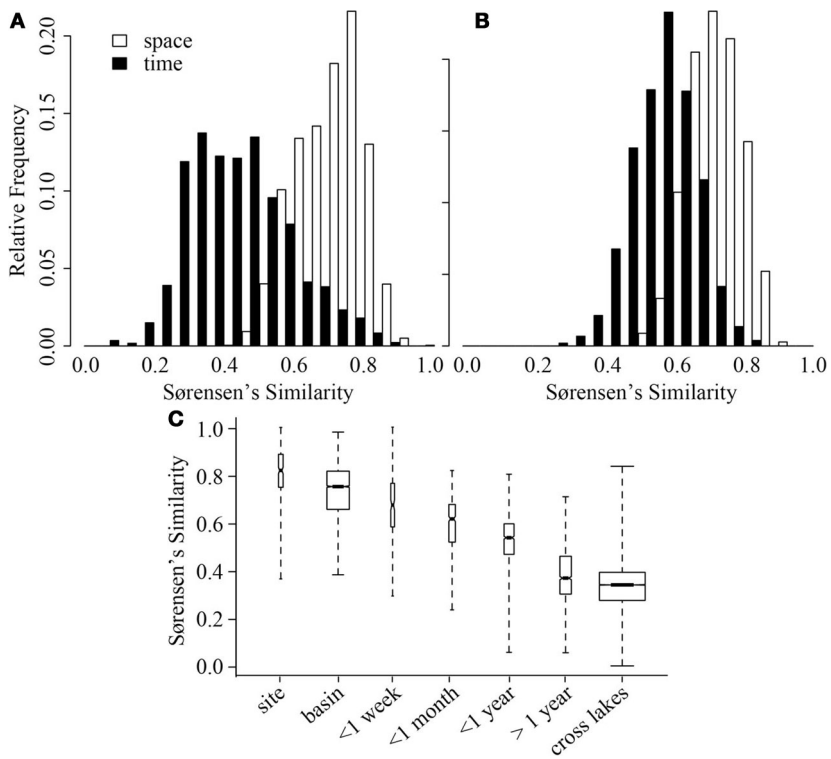


**FIGURE 3 | Distribution of Sørensen's similarity indices for samples from Crystal Bog (A) and Lake Mendota (B) distributed spatially within the lake (open bars) and through time (black bars).** Comparisons across space were conducted using the 32 samples collected on a single day from each lake. Comparisons across time were conducted using 68 samples from CB collected in 2003, 2005, and 2007 (previously unpublished), and 46 samples from ME collected in 2000, 2001, and 2005 (Shade et al., 2007). **(C)** Distribution of pairwise Sørensen's similarities calculated for multiple scales of time and space. Bold lines delineate medians, box heights represent the interquartile range, and whiskers mark the extremes of the data. Width of a box is proportional to the number of similarities in the category. Data from: Yannarell and Triplett (2004, 2005); Shade et al. (2007), and the current study, see **Table 2**.

Spatial surveys have been a popular approach to characterize freshwater bacterial diversity and structuring features of aquatic BCC. Broad spatial surveys have identified stark contrasts in occurrence patterns of some freshwater bacterial taxa (Lindstrom et al., 2005; Yannarell and Triplett, 2005; Wu et al., 2006; Newton et al., 2007; Simek et al., 2010). Phylogenetically narrow freshwater lineages appear to respond strongly to environmental variables, including pH (Newton et al., 2007; Simek et al., 2010), carbon substrate characteristics (Jones et al., 2009; Salcher et al., 2011), temperature (Wu and Hahn, 2006b), and salinity (Wu et al., 2006). Indeed, environmental characteristics seem to be key in determining BCC of aquatic ecosystems (Lindstrom et al., 2005; Yannarell and Triplett, 2005; Berdjeb et al., 2011) and dispersal limitation or biogeography seems to be less important in spatial structuring of aquatic bacterial communities (Crump et al., 2007; Van der Gucht et al., 2007; Jones and McMahon, 2009; Nelson et al., 2009). However, robust biogeographic patterns, such as distance-decay of community similarity and taxa-area relationships, have been observed in aquatic microbes (Soininen et al., 2011) and other microbial systems (Green et al., 2004; Horner-Devine et al., 2004; Martiny et al., 2006) indicating some relationship between geographic distance and likelihood of dispersal in microbial communities.

Although freshwater bacterial biogeographic patterns have been considered previously, only a few prior studies have considered intra-lake differences (Yannarell and Triplett, 2004; De Wever et al., 2005). Some might argue that lakes are horizontally well-mixed and one should expect to see very little intra-lake heterogeneity in the x-y dimensions. Under this premise, we would expect a flat distance-decay slope until spatial scales included comparisons of communities across lake boundaries. The results of our highly resolved intra-lake spatial survey suggest that within-lake x-y spatial heterogeneity in BCC does indeed exist (**Figure 1**). In addition, we did not observe any habitat-specific patterns of community composition. For example, there was not a consistent contrast in composition between littoral and pelagic sites. Instead, we observed a consistent decay in community similarity from spatial scales of meters to hundreds of kilometers (**Figure 3C**). In fact, the halving distance of community similarity is surprisingly similar to that observed by Soininen et al. (2011), who only evaluated cross-lake community similarity and geographic distances [4000 m in our study vs. 2965 m in Soininen et al. (2011)]. As a result of similar slopes, the initial similarities observed in our study at a distance of 1 m (0.9) were much higher than the 0.5 observed by Soininen and colleagues (2011).

Surprisingly, we observed comparable levels of beta diversity in the two lakes sampled in our intense spatial survey (**Figure 2**). Based upon previous work (Yannarell and Triplett, 2004), we expected a greater level of beta diversity in the larger lake. Our results indicate that even very small lakes (∼1 ha) can have significant horizontal spatial beta diversity (**Figure 2B**). The presence of horizontal heterogeneity in community composition may indicate the rates of biological and ecological interactions driving

**Table 4 | Summary statistics for distance- and temporal-decay relationships fit to our bacterial community similarity data from north temperate lakes.**

| | Slope[a] | Intercept[b] | Similarity[c] when separated by 1 meter or 1 day | Halving distance (m) or time (day) of similarity[c] |
|---|---|---|---|---|
| Distance decay | −0.084 | −0.046 | 0.90 | 4000 |
| Temporal decay | −0.118 | −0.076 | 0.84 | 350 |

[a] *Slope of the distance-decay linear relationship fit to log transformed Sørensen's similarity values.*

[b] *Intercept of the distance-decay linear relationship fit to log transformed Sørensen's similarity values.*

[c] *Predicted similarity, halving-distance, and halving-time were predicted using the methods of Soininen and colleagues (2007).*

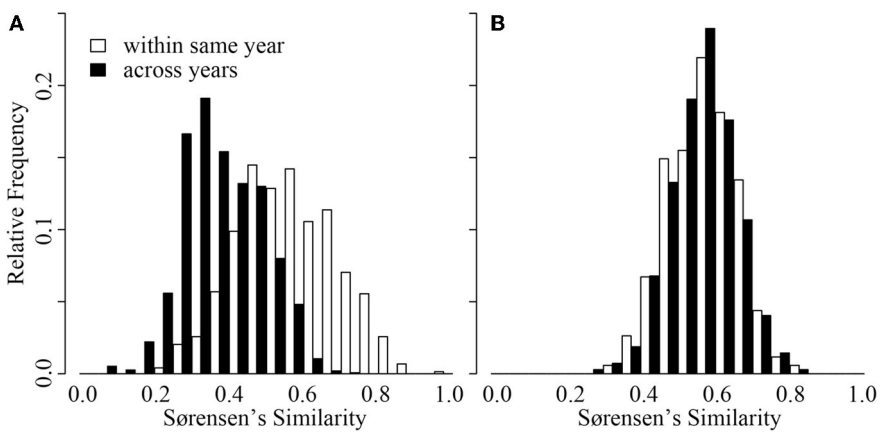


**FIGURE 4 | Distribution of Sørensen's similarity indices for samples collected within the same year (open bars) and samples collected across years (black bars) from Crystal Bog (A) and Lake Mendota (B).**

Comparisons were conducted using 68 samples from CB collected in 2003, 2005, and 2007 (previously unpublished), and 46 samples from ME collected in 2000, 2001, and 2005 (Shade et al., 2007).

bacterial community assembly are occurring more rapidly than rates of water movement and turbulence in lakes; this heterogeneity may include neutral dynamics occurring in temporarily separated parcels of water.

Although long-term or well-resolved temporal surveys targeting aquatic bacterial communities are fairly rare, some progress has been made toward understanding key factors influencing BCC in freshwater habitats through time (Kent et al., 2004; Allgaier and Grossart, 2006; Wu and Hahn, 2006a; Shade et al., 2007; Crump et al., 2009). Successional or phenological patterns have also been linked to changes in the physical and chemical environment (Kritzberg et al., 2006; Wu and Hahn, 2006a; Shade et al., 2007; Nelson, 2009) as well as changes in other components of the microbial loop (Muylaert et al., 2002; Kent et al., 2004; Wu and Hahn, 2006a). Temporal analogs of the approaches used to investigate spatial beta diversity have been developed, including the species-time relationship (Preston, 1960; Adler and Lauenroth, 2003; White, 2004) and temporal decay of compositional similarity over time (Collins et al., 2000; Korhonen et al., 2010). Using these tools we can compare the strength of temporal and spatial effects on freshwater bacterial beta diversity.

Our results suggest that spatial variation and temporal variation are quite comparable (**Figure 3**; **Table 4**). For example, the average community similarity across multiple temporal scales falls between average intra- and inter-lake community similarity values and there is a large amount of overlap in the distribution of these values (**Figure 3**). Using the temporal and spatial similarity decay relationships, we were able to calibrate temporal and spatial beta diversity to each other. Communities separated by a single day or meter are comparably similar, and we expect compositional similarity to halve across approximately one year or 4000 m (**Table 4**; Soininen, 2010). The equivalence of a day and a few meters in their impact on bacterial community similarity suggests similar ecological processes driving community assembly occur over these scales in space and time (Soininen, 2010). Soininen (2010) highlighted intrinsic factors, such as body size and dispersal rate, and extrinsic factors, such as ecosystem size and isolation, as likely drivers of bacterial turnover in both space and time. We agree with this theoretical assessment and suggest the results of our study support this assertion. Perhaps the change in environmental characteristics that occur over a day is equivalent to aquatic spatial heterogeneity occurring on the scale of meters. Alternatively, aquatic bacteria generation times (approximately on the order of days) may closely correspond to aquatic bacterial dispersal distances or rates. As has been highlighted recently, a shift in focus to the processes underlying current microbial biogeographic and temporal observation is now required (Hanson et al., 2012), and our results may indicate at what spatial and temporal scales to begin investigation of underlying processes, such as competitive exclusion, dispersal, and neutral drift in community composition.

An additional intriguing temporal observation from our study was the contrast in inter-annual patterns between CB and ME (**Figure 4**). Despite comparable spatial beta diversity, our analysis (**Figure 4**) and previous work (Kent et al., 2004) suggest that repeated seasonal patterns in BCC do not occur in CB, while extremely repeatable phenological patterns occur in ME each year (**Figure 4**; Shade et al., 2007). This represents empirical support for hypothetical patterns describing temporal decay of community similarity in seasonal and non-seasonal communities presented by Korhonen et al. (2010). However, we are uncertain what could drive this contrast in dynamics. ME and CB differ in a number of characteristics, including trophic status, lake size, pH, and surrounding land use, making it difficult to identify what system features drive this divergence. We emphasize the need for larger datasets of intra- vs. inter-annual variation in BCC in multiple lakes representing various gradients (e.g., size, trophic status) to develop more robust expectations.

## ACKNOWLEDGMENTS

We thank the staff at the University of Wisconsin Madison Center for Limnology and Trout Lake Station for supporting the fieldwork described herein. We also thank the original authors of the previously published work used in our meta-analyses for sharing their data. This work was supported by grants to Katherine D. McMahon from the National Science Foundation [Awards CBET-0644949 (CAREER) and MCB-0702653 (Microbial Observatories Program)], and Wisconsin SeaGrant (R/BT-24). This material is based upon NTL LTER work supported by the National Science Foundation under Cooperative Agreement DEB-0822700.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/AquaticMicrobiology/10.3389/fmicb.2012.00318/abstract

**Table S1 | Collection details for all samples included in this study.** All samples were integrated samples extending from the lake surface to the indicated depth. NA indicates the information was unavailable.

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

*Received: 28 May 2012; accepted: 14 August 2012; published online: 31 August 2012.*

*Citation: Jones SE, Cadkin TA, Newton RJ and McMahon KD (2012) Spatial and temporal scales of aquatic bacterial beta diversity. Front. Microbio.* ***3****:318. doi: 10.3389/fmicb.2012.00318*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

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MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 11 October 2012
doi: 10.3389/fmicb.2012.00347

# Co-occurrence patterns of plants and soil bacteria in the high-alpine subnival zone track environmental harshness

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Plants and soil microorganisms interact to play a central role in ecosystem functioning. To determine the potential importance of biotic interactions in shaping the distributions of these organisms in a high-alpine subnival landscape, we examine co-occurrence patterns between plant species and bulk soil bacteria abundances. In this context, a co-occurrence relationship reflects a combination of several assembly processes: that both parties can disperse to the site, that they can survive the abiotic environmental conditions, and that interactions between the biota either facilitate survival or allow for coexistence. Across the entire landscape, 31% of the bacterial sequences in this dataset were significantly correlated to the abundance distribution of one or more plant species. These sequences fell into 14 clades, 6 of which are related to bacteria that are known to form symbioses with plants in other systems. Abundant plant species were more likely to have significant as well as stronger correlations with bacteria and these patterns were more prevalent in lower altitude sites. Conversely, correlations between plant species abundances and bacterial relative abundances were less frequent in sites near the snowline. Thus, plant-bacteria associations became more common as environmental conditions became less harsh and plants became more abundant. This pattern in co-occurrence strength and frequency across the subnival landscape suggests that plant-bacteria interactions are important for the success of life, both below- and above-ground, in an extreme environment.

**Keywords: community assembly, co-occurrence networks, facilitation, mutualism, Niwot Ridge, plant-microbe interactions, soil microbial communities, symbiosis**

## INTRODUCTION

Interactions between plants and microorganisms play a central role in ecosystem functioning (Bonfante and Anca, 2009; Hayat et al., 2010). However, it has been difficult to characterize plant-microbial relationships for all but the most closely interacting species (Kremer, 2006; van der Heijden et al., 2008; Hayat et al., 2010). For example, while mutualistic and parasitic symbiotes inhabiting root tissue have been well studied (mycorrhizae: Bianciotto et al., 1996; Bonfante and Anca, 2009; diseases: Compant et al., 2005; Kremer, 2006; N-fixation: Hayat et al., 2010; heat tolerance: Márquez et al., 2007; plant growth hormone production by bacteria: Hayat et al., 2010), it appears that these interactions just scratch the surface of the probable range of symbioses (Li and Kremer, 2006; Rudgers et al., 2012). Similarly, the indirect associations involved with decomposition and nutrient cycling between microorganisms and plants, despite being well studied from a biogeochemical perspective, have remained difficult to characterize with respect to the soil microbial species involved (van der Heijden et al., 2008). In addition to the difficulty imposed by frequent findings of new plant-bacteria interactions, predicting the frequency and types of known plant-soil microbe associations is often difficult even when species lists and environmental conditions are known (Bever et al., 2010). The success of targeted studies using cultures (Kremer, 2006; Hayat et al., 2010) and stable isotope probing (Haichar et al., 2008) to identify plant-soil interactions suggests that larger scale surveys using high-throughput methods may allow for the detection of broad patterns for plant-soil microbe interactions from bulk soil. Here, for the first time, we use culture-independent methods to test hypotheses regarding the drivers of co-occurrence relationships between plants and soil bacteria in bulk soil samples and at the landscape scale.

The co-occurrence of species that share resource requirements is governed by competitive (Diamond, 1975) and facilitative (Bruno et al., 2003) interactions between species as well as independent environmental sorting in the case of strong environmental filters (Weiher and Keddy, 1995; Diaz et al., 1998; Ackerly, 2003). As such, co-occurrence patterns represent an important tool for inferring potential interactions involving microorganisms, particularly bacteria, which are most commonly identified indirectly via isolation of organisms or sequencing their DNA from soil extracts (Ruan et al., 2006; Horner-Devine et al., 2007; Fuhrman and Steele, 2008; Freilich et al., 2010; Barberán et al., 2012). Because the methods enabling the analysis of high-throughput, sequencing based, broad-scale surveys of bacteria are very new (Caporaso et al., 2011; Gonzalez et al., 2011), the frequency and strength of plant-bacteria co-occurrence relationships at a landscape scale are poorly understood. Thus, high-throughput sequencing of bulk soil samples represents a potentially powerful resource for the detection

of plant-bacteria interactions such as saprotrophic bacteria that specialize on senesced material from a particular plant species, bacteria sharing similar environmental preferences, and symbiotic rhizosphere bacteria which are often found to be a well represented subset of bulk soil samples (Mahaffee and Kloepper, 1997; Graff and Conrad, 2006; Berg and Smalla, 2009).

We examine plant-bacterial co-occurrence relationships along an abiotic harshness gradient in a high-alpine subnival ecosystem because the resulting gradient in plant abundance represents increasing availability of reduced carbon for soil microorganisms (Ley et al., 2004) and the oligotrophic soils mean plant symbioses with soil microorganisms are particularly important (Chapin et al., 1994; Tscherko et al., 2005). The harsh environmental conditions of the subnival zone result from high altitudes with their concurrent high solar radiation, low humidity, large daily temperature fluctuations that often cross the freezing point, large snowpack volumes or high wind exposure, and low soil nutrients (Ley et al., 2004; Freeman et al., 2009; King et al., 2010). Thus, subnival organisms must persist in a state of strong environmental stress which prohibits continuous plant cover, but is not extreme enough that there is year-round snow or ice cover. Previous studies in slightly less harsh, alpine ecosystems have shown that the majority of plants are able to form mutualistic associations with fungi, N-fixing bacteria, and growth promoting bacteria (Mullen et al., 1998; Cazares et al., 2005; Sheng et al., 2010), suggesting that mutualistic associations are important for the survival of plants and bacteria in these systems (Hobbie et al., 2005; Wang and Qiu, 2006; Schmidt et al., 2008a; Sheng et al., 2010; Zinger et al., 2011). However, in subnival glacial fore-fronts these same well-colonized plant species often lack root mutualistic symbionts and this lack of symbionts is thought to be due to dispersal limitation of the microorganisms in the newly exposed substrate (Cazares et al., 2005). Pathogens of alpine plants, on the other hand, are rarely reported (Olofsson et al., 2011); it may be that pathogens are rare or it could be that few studies explicitly focus on this group. Thus, positive plant-bacterial interactions are of high potential importance in subnival landscapes.

We draw from the body of theory regarding community assembly (Diamond, 1975; Chapin et al., 1994; Chase and Leibold, 2003; Reynolds et al., 2003) to develop simple yet fundamental hypotheses about the drivers of the frequency and distribution of plant-bacteria co-occurrence relationships. A basic tenet of community assembly theory is that abiotic and biotic "filters" select species from the regional species pool to assemble a local community most suited to the prevailing conditions in a process known as ecological sorting (Weiher and Keddy, 1995; Diaz et al., 1998; Ackerly, 2003). In the context of plant-bacteria co-occurrence in the alpine subnival environment, we expect the presence of biotic filters that restrict one group to occur only when its symbiont/facilitator is present as well as a common set of abiotic and dispersal filters that may constrain both plants and microbes directly. Biotic filters may be reflected by bacteria with plant growth promoting/inhibiting abilities or plants that promote the growth of mutualistic soil bacteria. If certain bacteria and plants have similar environmental preferences, they may co-occur across space independently without directly interacting with each other. For soil bacteria that are, in fact, interacting with plant species, negative correlations can arise from inhibitory and competitive interaction whereas positive correlations can arise from facilitative, mutualistic, or parasitic interactions.

Based on this community assembly framework, we explicitly test three hypotheses about the drivers of the frequency and distribution of plant-bacteria co-occurrence relationships. First, analogous to the suitable habitat area-diversity relationship from island biogeography (MacArthur and Wilson, 1967; Martiny et al., 2006), we expect bacteria to more often co-occur with plant species' that provide larger and more consistent nutrient sources across the landscape. Assuming that a plant species contribution to nutrient pools is proportional to its abundance (Grime, 1998; Vile et al., 2006), we thus predict that abundant plants will have more bacterial clades associated with them than less abundant plants.

Second, we expect that bacterial clades related to isolates with plant growth promoting/inhibiting abilities will show stronger species-specific associations with plants than will bacteria related to isolates with plant-independent metabolic strategies. Thus, the hypothetical functional capacity for associating with a plant species represents a biotic filter (e.g., Chase and Leibold, 2003) for the occurrence of bacteria in the subnival landscape. Importantly, evaluation of this driver is also testing the utility of a correlation approach using culture-independent environmental sequencing of the 16S to identify clades that have a specific function.

Lastly, we predict that facilitation should be more common in portions of the landscape furthest from intact tundra due to increasing environmental harshness. Importantly, theory predicts that facilitative and mutualistic plant-microbe interactions are more common in harsher, low density, low nutrient availability, successional environments (Reynolds et al., 2003) because they promote the growth of both bacteria and plants. Such facilitative interactions are thought to be able to overcome environmental filters that would otherwise exclude either symbiont (Choler et al., 2001). Although it is likely endemic extremophiles are adapted to the harsh environment of the subnival zone, our previous work has shown that the ancestry of bacteria does not change significantly across the landscape (King et al., 2010). Therefore, we expect that the lower temperatures, greater UV radiation, and less soil nutrients with increasing remoteness (Körner, 2007) represent real increases in stress to bacteria in this landscape.

## MATERIALS AND METHODS

### STUDY SITE

The dataset used in this analysis comes from a previously published spatially explicit study of the Niwot Ridge LTER's south-facing subnival slope (eastern face of the Continental Divide in Colorado, USA; **Figure 1**; King et al., 2010). This slope is covered with snow from October to June and the deepest snow fields do not melt fully until August. Precipitation averages 930 mm/year with 80% falling as snow (Nemergut et al., 2005). The study area extends over 2 $km^2$ and is predominately covered by granite blocks greater than 1m in diameter. Interspersed are patches of soil up to 20 m in diameter with plant cover ranging from 0% in late melting snowbanks up to 100% in a few exposed areas receiving water from upslope

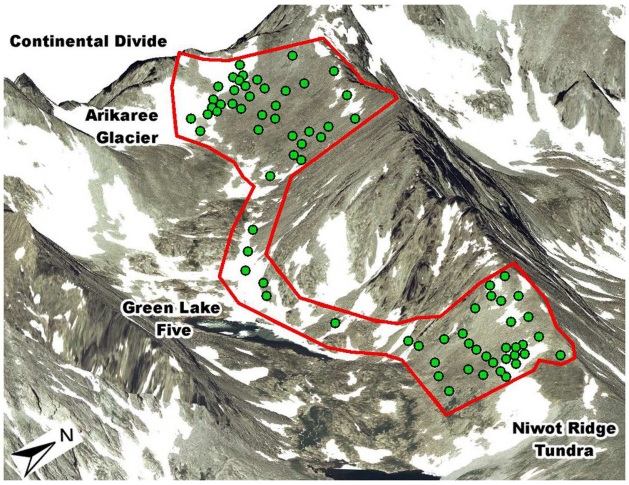


**FIGURE 1 | Our 76 sample sites in the subnvial zone of the Green Lakes Valley within the Niwot Ridge LTER study site on eastern face of the Continental Divide in Colorado, USA (red outline represents the south-facing extent of exposed soils).** From the north-east corner to the north-west corner of the study area is 2 km. These sites represent a subset of the sites in our previous study (shown in Figure 1 of King et al., 2010).

snowmelt. Plants in this environment are 10–20 cm in height and have approximately the same biomass per individual.

## PLANT SPECIES AND SOIL SAMPLING

As previously reported (King et al., 2010), sampling locations were chosen based on a pilot study (King et al., 2008) in order to provide optimal spacing for the calculation of spatial autocorrelation models across the landscape. Sampling was partitioned into a grid of 160 samples across the landscape, spaced every 50 m, with three targeted 30 m × 30 m areas sampled at every 5 m (Figure 1 in King et al., 2010). In September of 2007, at each sampling location a pen was tossed into the air and at the spot where it landed all vascular plants within a 3 $m^2$ circle were identified and a soil sample was collected. Plant abundance measures were based on stem counts. Because alpine species are constrained to a low stature growth form, stem counts are an appropriate estimate of species biomass within a plot (Grytnes, 2000). Species identification was based on Webber and Whittmann (2001).

Soils samples were collected by homogenizing a ∼50 $cm^2$ patch of soil *in situ* to a depth of 4 cm and then filling a 50 mL sterile conical tube with soil. Because of the high degree of spatial autocorrelation in bacterial community diversity in this system, an individual sample is likely to be highly representative of the surrounding 25 $m^2$ of soil (King et al., 2010). Samples were transported to the lab within 5 h of collection, held at 4°C for up to a week while subsampling for soil biogeochemistry was conducted (results presented in King et al., 2010), and afterward frozen at −20°C until DNA could be extracted (up to 9 months). 85 of the original 160 samples were randomly selected for bacterial community analysis. From those 85 samples, we include in the current study only plots that had at least two plants per $m^2$ (76 samples, **Figure 1**).

## BACTERIAL COMMUNITY ASSESSMENT

Bacterial community composition was also measured as described in King et al. (2010). Briefly, for each of the 76 soil samples, DNA was extracted from 1 g of soil using a Mobio PowerSoil DNA Isolation Kit (Mobio Laboratories) and PCR was used to amplify the V1–V2 hypervariable region of the bacterial 16S SSU ribosome gene using the 27F and 338R primers and protocol from Fierer et al. (2008). Sequencing was performed on the Roche 454 platform using FLX chemistry. Raw sequence data was processed using the methods of Hamady et al. (2010), resulting in 6151 representative OTUs at 3% similarity with 10,000 total reads and an average of 200 reads per sample. For downstream analysis, sequence reads of each OTU in each sample were converted to relative abundance by dividing by the total number of sequences in an individual sample.

## SELECTION OF CLADES USING CORRELATION STRENGTH WITH PLANT SPECIES ABUNDANCES

Because most methods of assigning taxonomic IDs to individual sequences are based on the extent of knowledge contained in a reference database (e.g., DeSantis et al., 2006; Wang et al., 2007) and because classical taxonomies can be polyphyletic, we used a phylogenetic tree to assign sequences to clades for downstream analysis (the phylogenetic tree was the same as used in King et al. (2010)). This approach is essentially a phylogenetic comparative method (Harvey and Pagel, 1991) with association with plants as the trait of interest. Initially we considered as candidate clades all nodes on the tree that had greater than 1% of the sequences (therefore, clades are independent of phylogenetic resolution and a particular clade might correspond to any gradation between phylum and species). Each candidate clade's relative abundance per sample was then regressed against the abundances of the 13 most prevalent plant species (all plant species averaging greater than one individual per sampling location; the total plant species pool for this landscape is 26). Regressions were performed in R using the Akaike Information Criterion (Akaike, 1974) to select the plant species with the most explanatory power via the step and lm functions (R Development Core Team, 2011). A strictly linear approach was chosen to maintain a low number of parameters and residuals from both the models, and pairwise regressions of clades against individual plant species were found to be normally distributed.

The initial list of clades was then filtered so that clades were mutually exclusive; this filtering was achieved via sorting the clades by $r^2$, removing all clades that had a lower $r^2$ but shared sequences with a clade that had the highest $r^2$, and then reiterating with the next highest remaining $r^2$ until reaching a clade without a significant correlation or an $r^2$ less than 0.15. The remaining 22 clades were tested for significance with an ANOVA with the AIC selected variables as explanatory variables and significance cutoff adjusted for multiple comparisons using the Bonferroni correction for $n = 22$ comparisons ($p < 0.002$; Bonferroni, 1936). The final result was 14 bacterial clades. Taxonomic IDs were then assigned as the finest resolution at which at least 50% of the sequences shared the same classification in their closest match in NCBI GenBank.

## PLANT-BACTERIA CO-OCCURRENCE ORDINATION

To visualize the correlations between plant species and the bacterial clades identified by the above regressions we used a NMDS

approach. First, we constructed a correlation matrix with all possible pairwise comparisons between both bacteria clades and plant species. We then used a common approach (Cox and Cox, 1994) to transform each Pearson correlation value (ranging from 1 to −1) by taking the absolute value and subtracting it from one such that both positive and negative 100% correlations became a distance of 0 and a 0% correlation became a distance of one. The resulting distance matrix was used as an input to the metaMDS function in the vegan package for R (Oksanen et al., 2010). While co-occurrence networks in microbiology have most commonly been arranged so that the nodes with the greatest numbers of edges are in the center of the plot (Barberán et al., 2012), NMDS is very commonly used in plant ecology to represent strength (rather than number) of interactions (McCune et al., 2002). We chose NMDS so that the strength of interaction between each clade or plant species in the dataset would be represented by proximity. In addition, we used NMDS to ordinate our plant-bacteria co-occurrence relationships rather than the more common metric MDS approach (Cox and Cox, 1994) because NMDS gives greater weight to the strongest correlations (Cox and Cox, 1994) and relatively little weight to weaker correlations (MDS techniques would have used the raw correlation value and the large number of non-significant correlations with values of 0.20 or less would have introduced extra noise into the ordination). After the species and clades were ordinated, we visually overlaid the significant interactions according to our models.

### STATISTICAL ANALYSIS

To quantitatively estimate the average strength of association for a particular plant species or bacterial clade, we averaged the individual correlation $r$ values (absolute value) that were significant according to our AIC supervised linear models. ($p < 0.05$, two-tailed $t$-test for each regression coefficient not equal to 0). Bonferroni correction of the $p$-value for multiple comparisons was done for each model's coefficient significance tests (clade relative abundance models ranged from one to eight AIC selected plant species, Supplementary Materials).

To address the hypothesis that more abundant plants are better predictors of the abundance of associated bacterial clades, the number of significantly correlated clades per plant species was regressed against plant species abundance and we used a $t$-test to determine if the correlation was significantly greater than zero. The mean correlation strength of clades associated with each plant species was also regressed against plant species abundance and we used a $t$-test to determine if the correlation was significantly greater than zero. These tests were performed both including and excluding plant species with no significantly correlated bacterial clades.

To address the hypothesis that average association strength would be greatest for bacterial clades closely related to bacterial species that are known to form symbioses with plants, we performed a $t$-test comparing mean correlation strengths of each model's significant factors for clades related to plant symbiosis capable versus plant-independent bacteria [i.e., Avg Corr Str ($r$) column of **Table 1**].

To address the hypothesis that environmental harshness affects association strength, we reweighted all already identified sets of bacterial clade relative abundances by a harshness index (a proxy for the subnival landscape's general gradient of increasing exposure, temperature variability, and time before complete snowmelt with increasing distance away from intact tundra). The environmental harshness index for each sample was calculated as distance from intact tundra + distance from valley floor. In addition to distance from tundra and altitude, the degree of shelteredness from wind is also expected to play a role in structuring species co-occurrence in this subnival landscape (Litaor et al., 2008). However, although both high and low snow accumulation areas vary in the nature of the primary stressor (late snowmelt versus high wind exposure), snowfields and windswept areas occur throughout our study area (Erickson et al., 2005). Due to this variability, our harshness metric is not correlated with snow depth (Correlation test, $p = 0.2$, $r^2 = 0.02$) in the Niwot subnival zone, suggesting that harshness overlays a matrix of sheltered and unsheltered areas which occur throughout the landscape. Thus, our proposed harshness metric is similar in nature to altitudinal gradient analysis in that it is strongly correlated with gradients in temperature/solar insolation that overlay landscape heterogeneity in shelteredness (Körner, 2007).

We examined the effect of multiplying abundances by the harshness index (up-weighting harshness). We also examined the effect of multiplying abundances by the harshness subtracted from the max harshness value (down-weighting harshness). For up- and down-weighted datasets we recalculated linear model fits for each of the previously identified bacterial clades using AIC selection of significantly predictive plant species. The effect size of reweighting was then calculated by averaging the individual correlation $r$ values (absolute value) between plant species and bacterial clades that were significant first for the upweighted and then for the down-weighted datasets. To test if a significant effect of up- or down-weighting was observed for a specific clade, we used an ANOVA with the AIC selected variables as explanatory variables and significance cutoff adjusted for multiple comparisons using the Bonferroni correction for $n = 14$ comparisons ($p < 0.004$; Bonferroni, 1936). Again, the clade average correlation strengths reflect only significant correlations after Bonferroni correction for each model's set of AIC selected variables.

## RESULTS

### GENERAL FINDINGS

The NMDS ordinated co-occurrence network contained 14 bacterial clades, representing 31% of the total sequences in our study, and 10 of the 13 plant species (**Figure 2**). Significant correlations were not dependent on the diversity of bacterial OTUs, total branch length within the clade, or relative abundance of clades (**Figure 3**). The percentage of variance in clade relative abundance significantly explained by plant species abundance ranged from 40% to our minimum cutoff of 15% (**Table 1**). Negative correlations, at 3, were less common than positive correlations, at 14 (**Figure 2**; **Table 1**). Bacterial clades had one or two significantly correlated plant species; only one had exclusively negative significant correlations with plant species (Desulfovibrionales), and two had multiple positive associations (Clostridiales and Rhodospirillales). Our models predicting bacterial clade relative abundance based on plant species abundance with 13 potential explanatory plant species (Datasheet S1 in Supplementary Material) only slightly under-perform compared to previously published environmental

**Table 1 | A summary of known metabolic capabilities, OTU richness, number of significant plant-associations, and model fits for plant-associated subnival zone bacterial clades.**

| Clade | Plant symbiosis | Symbiosis location | Free-living metabolism | Reference | Average abundance (SD) | OTU richness | Positive interactions | Negative interactions | Model $r^2$ | Avg corr str ($r$) | Harsh environ up $r^2$ | Corr number harsh env up | Avg corr harsh up ($r$) | Harsh env down $r^2$ | Corr number harsh env down | Avg corr harsh down (r) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Acidimicrobiaceae | Unknown | | S-oxidation, Fe oxidation, N-fixation chemotrophy | Buckley et al. (2007), Norris et al. (2011) | 1.11 (1.1) | 58 | 1 | 0 | 0.26 | 0.41 | 0.29* | 2 | 0.37 | 0.34* | 2 | 0.27 |
| Acidobacteria_Gp1 | Unknown | | Unknown | Lee et al. (2006) | 2.76 (2.2) | 84 | 1 | 1 | 0.34 | 0.29 | 0.21* | 2 | 0.26 | 0.32* | 1 | 0.51 |
| Acidobacteria_Gp3 | Unknown | | Unknown | Lee et al. (2006) | 1.18 (1.4) | 63 | 1 | 0 | 0.26 | 0.3 | 0.27* | 1 | 0.28 | 0.3* | 2 | 0.32 |
| Acidobacteria_Gp4 | Unknown | | Unknown | Lee et al. (2006) | 1.22 (1.5) | 67 | 1 | 0 | 0.32 | 0.51 | 0.13 | 0 | | 0.42* | 1 | 0.63 |
| Acidobacteria_Gp7 | Unknown | | Unknown | Lee et al. (2006) | 2.77 (2.7) | 67 | 0 | 1 | 0.24 | 0.31 | 0.08 | 0 | | 0.16* | 1 | 0.14 |
| Burkholderiales | N-fixation, P-mobilization pathogenic | Intra/ extracellular roots stems | Heterotrophy endosymbiont | Rodrigues-Diaz et al. (2008), Compant et al. (2008) | 1.47 (1.7) | 54 | 1 | 0 | 0.4 | 0.52 | 0 | 0 | | 0.41* | 2 | 0.32 |
| Clostridiales | Growth promoting pathogenic | Extracellular | Heterotrophy, N-fixation | Rodrigues-Diaz et al. (2008) | 1.32 (1.8) | 3 | 2 | 0 | 0.38 | 0.46 | 0.07 | 0 | | 0.69* | 3 | 0.55 |
| Deltaproteobacteria | Unknown | | Heterotrophy, S-reduction, Fe-reduction | Brenner et al. (2005) | 5.01 (2.7) | 503 | 1 | 0 | 0.24 | 0.26 | 0.27* | 2 | 0.26 | 0.3* | 2 | 0.24 |
| Desulfovibrionales | Nematicidal | Extracellular endophytic roots | Heterotrophy, S-reduction | Rodrigues-Diaz et al. (2008) | 1.30 (1.1) | 153 | 0 | 2 | 0.24 | 0.26 | 0.12 | 0 | | 0.34* | 3 | 0.17 |
| Ktedonobacteraceae | Unknown | | CO-oxidation | Webber and King (2010) | 5.12 (5.4) | 320 | 1 | 0 | 0.28 | 0.31 | 0.26* | 1 | 0.51 | 0.32* | 2 | 0.42 |

*(Continued)*

**Table 1 | Continued**

| Clade | Plant symbiosis | Symbiosis location | Free-living metabolism | Reference | Average abundance (SD) | OTU richness | Positive interactions | Negative interactions | Model $r^2$ | Avg corr str (*r*) | Harsh environ up $r^2$ | Corr number harsh env up | Avg corr harsh up (*r*) | Harsh env down $r^2$ | Corr number harsh env down | Avg corr harsh down (r) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Pseudonocardiaceae | N-fixation | Endophytic roots | Heterotrophy, S-oxidation, N-fixation | Reichert et al. (1998), Chen et al. (2009) | 1.14 (1.7) | 24 | 1 | 0 | 0.24 | 0.3 | 0.35* | 2 | 0.44 | 0.18 | 0 | |
| Rhizobiales | N-fixation, pathogenic | Extracellular endophytic | Heterotrophy, N-fixation | Rodrigues-Diaz et al. (2008), Carvalho et al. (2010) | 3.28 (2.3) | 205 | 1 | 0 | 0.22 | 0.3 | 0.2 | 0 | | 0.47* | 2 | 0.35 |
| Rhodospirillales | N-fixation, P-mobilization | Extracellular endophytic leaves, stems, roots | Heterotrophy, phototrophy, N-fixation, S-reduction | Madigan (2005), Rodrigues-Diaz et al. (2008) | 2.25 (2.4) | 119 | 2 | 0 | 0.31 | 0.21 | 0.44* | 2 | 0.47 | 0.2 | 0 | |
| TM7 | Unknown | | Unknown | Marcy et al. (2007) | 0.96 (1.2) | 86 | 1 | 0 | 0.3 | 0.39 | 0.24 | 0 | | 0.33* | 1 | 0.55 |

**Indicates a significant model fit with harshness up- or harshness down-weighted data, ANOVA, $p < 0.004$.*

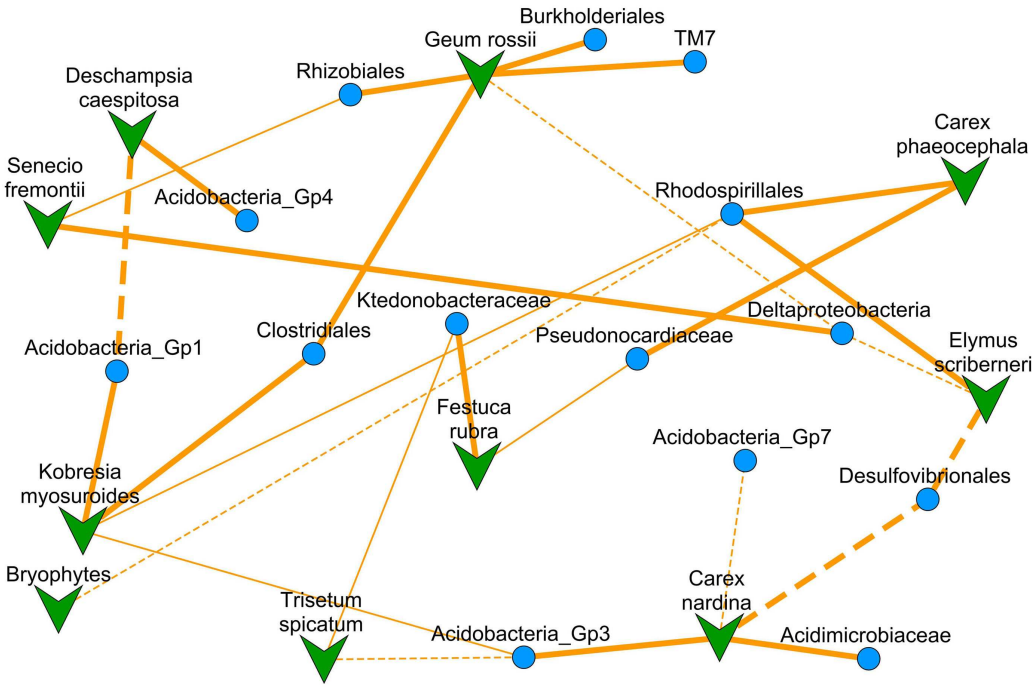


**FIGURE 2 | A NMDS (stress = 0.292) ordinated co-occurrence network based on the correlation matrix (absolute values of Pearson *r*) for 10 plant species abundances and 14 bacterial clade relative abundances (Datasheet S4 in Supplementary Material).** Overlaid are lines designating significant model-based interactions between a plant species and bacterial clade with solid for positive and dashed for negative interactions (thin lines represent two-tailed *t*-test for model coefficient significance, $p < 0.05$; thick lines represent significance after Bonferroni correction for multiple comparisons). Because this is an ordination of the correlation matrix, proximity of points to one another represents higher correlation between their abundances across the subnival landscape.

models for the three most spatially structured clades examined by King et al. (2010) with 21 potential explanatory variables; on average our models explain 28% of variance in clade relative abundance for Rhodospirillales, Rhizobiales, and Acidobacteria Gp4, compared to 40% for the previously published (King et al., 2010) models using plant abundance, soil biogeochemistry, and spatial autocorrelation as predictors.

Six of the 14 bacterial clades (comprising 10% of the total sequences) are related to isolates that are known for forming symbioses with plants (**Table 1**) for N-fixation and/or plant growth promotion: Rhizobiales, Rhodospirillales, Clostridiales, Desulfovibrionales, Pseudonocardiaceae, and Burkholderiales (also pathogenic) in addition to their capacity of free-living heterotrophic functions (**Table 1**). Many of these clades are also related to isolates known to be able to perform rhizosphere-independent soil functions such as sulfur reduction (Rhodospirillales, Desulfovibrionales) and sulfite oxidation (Clostridiales, Pseudonocardiaceae). Two of the clades (comprising 9% of the sequences) lacked support for plant symbiosis capability and displayed a diverse array of metabolic strategies: carbon monoxide oxidation (Ktedonobacteraceae), general heterotrophy/sulfate + iron reducing (Deltaproteobacteria). Five of the remaining six clades (comprising 11% of the total sequences) represent common soil bacteria with no cultured representatives and largely unknown functions (four clades of Acidobacteria, TM7). The last clade, Acidimicrobiaceae, is defined by their autotrophic growth by oxidizing ferrous iron, but has been hypothesized to also inhabit the rhizosphere and root tissue based on culture-independent surveys (Stafford et al., 2005; Qin et al., 2012) and may have the capacity for nitrogen fixation (Buckley et al., 2007).

### *H1: Abundant plants will have more bacteria associated with them than less abundant plants because they represent the most common plant-derived nutrient source*

At first inspection, plant species abundance was not correlated to the number of correlated bacterial clades ($r = 0.45$, two-tailed *t*-test for rho = 0: $p = 0.14$, **Table 2**) or the average correlation strength for clades associated with a particular plant species ($r = 0.25$, two-tailed *t*-test for rho = 0: $p = 0.44$, **Table 2**). However, when plants with no correlated clades were removed from the analysis, there was a significant relationship between plant abundance and average correlation strength for clades associated with a particular plant species ($r = 0.88$, two-tailed *t*-test for rho = 0: $p = 0.004$, **Figure 4B**) and a weakly significant correlation for the number of correlated bacterial clades ($r = 0.67$, one-tailed *t*-test for rho = 0: $p = 0.0275$, **Figure 4A**). The plants with the greatest number of positive associations were *Geum rossii* (four associations), *Kobresia myosuroides* (two associations), and *Carex nardina* (two associations). Abundant plant species lacking significantly correlated bacterial clades were *Bryophytes, Trisetum spicatum, Trifolium nanum, Silene acaulis.*

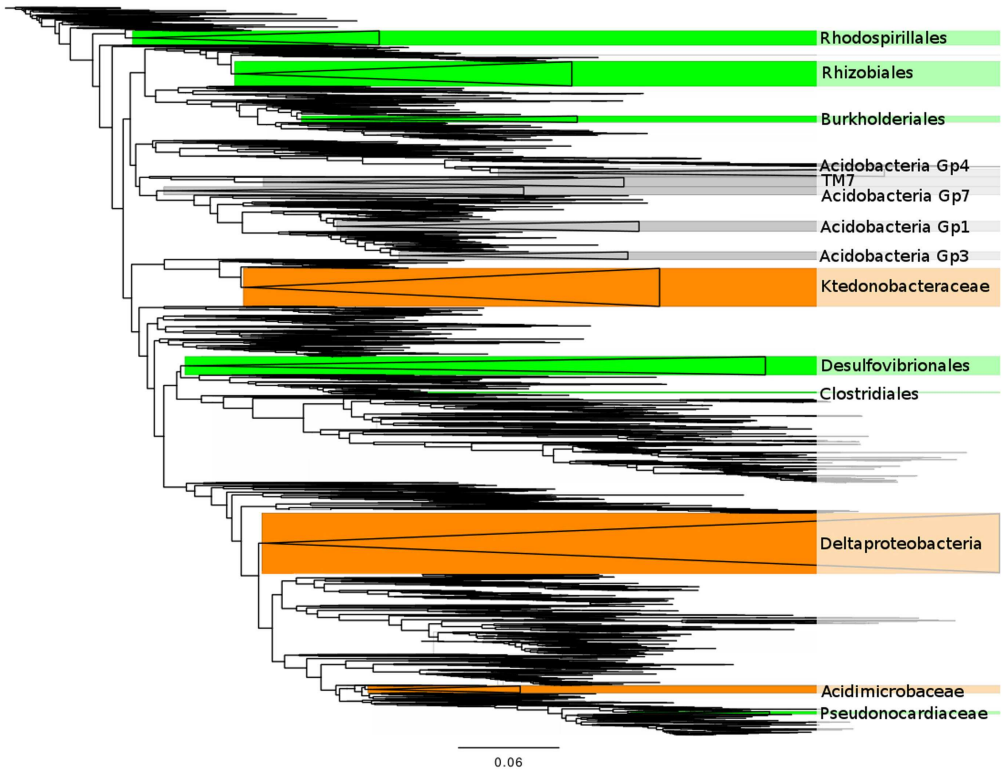


**FIGURE 3 | A phylogenetic tree showing the clades correlated with plant abundance. Green clades are commonly found associated with plants in the scientific literature (Table 1).** Orange clades have no known symbiotic association with plants. Gray clades are only known from environmental sequencing. Wedge size is proportional to the number of 3% OTU clusters in a clade.

**Table 2 | A summary of subnival zone plant species' abundances, number of significant bacterial clade-associations and model fits.**

| Plant species | Average (plants/site) | SD | Positives | Negatives | Avg str corr (*r*) | Correlation with remote index (*r*) | Total Corr remote upweight | Avg corr rm up | Total cor remote downweight | Avg corr rm down |
|---|---|---|---|---|---|---|---|---|---|---|
| *Geum rossii* | 8.3 | 24 | 4 | 0 | 0.44 | −0.2 | 0 | 0 | 4 | 0.60 |
| *Bryophytes* | 7.6 | 13.7 | 0 | 0 | 0 | 0.05 | 1 | 0.52 | 0 | 0 |
| *Deschampsia cespitosa* | 5.7 | 14 | 1 | 1 | 0.39 | −0.27 | 0 | 0 | 2 | 0.50 |
| *Trisetum spicatum* | 3.9 | 5.7 | 0 | 0 | 0 | 0.08 | 0 | 0 | 1 | 0.42 |
| *Kobresia myosuroides* | 3.5 | 15.3 | 2 | 0 | 0.39 | −0.16 | 1 | 0.05 | 5 | 0.41 |
| *Carex nardina* | 3.2 | 5.7 | 2 | 1 | 0.31 | −0.25 | 1 | 0.01 | 2 | 0.43 |
| *Festuca rubra* | 2.9 | 4 | 1 | 0 | 0.32 | 0.4 | 4 | 0.42 | 6 | 0.06 |
| *Trifolium nanum* | 2.7 | 10.6 | 0 | 0 | 0 | −0.04 | 0 | 0 | 0 | 0 |
| *Senecio fremontii* | 2 | 6.3 | 1 | 0 | 0.26 | −0.132 | 0 | 0 | 1 | 0.43 |
| *Silene acaulis* | 1.9 | 6.1 | 0 | 0 | 0 | 0.01 | 0 | 0 | 1 | 0.34 |
| *Cirsium scopulorum* | 1 | 2 | 1 | 1 | 0.25 | 0.05 | 0 | 0 | 0 | 0 |
| *Elymus scribneri* | 0.9 | 2.8 | 2 | 0 | 0.31 | 0.27 | 2 | 0.48 | 0 | 0 |
| *Carex phaeocephala* | 0.9 | 2.9 | 4 | 0 | 0.44 | 0.13 | 3 | 0.40 | 0 | 0 |

***H2: Potentially plant symbiosis capable bacterial clades will have greater average correlation strength with plant species than clades predicted to lack the capacity for symbiosis***

On average, bacterial clades related to strains that were capable of forming symbioses with plants did not differ significantly in their average association strength from bacterial clades without known symbiosis capability (mean $r$ was 0.34 symbiosis capable versus 0.29 incapable, two-tailed $t$-test: $p = 0.63$; **Table 1**). Of the bacterial clades with assignable functional capacities (**Figure 3**, **Table 1**), the Burkholderiales had the strongest association with a

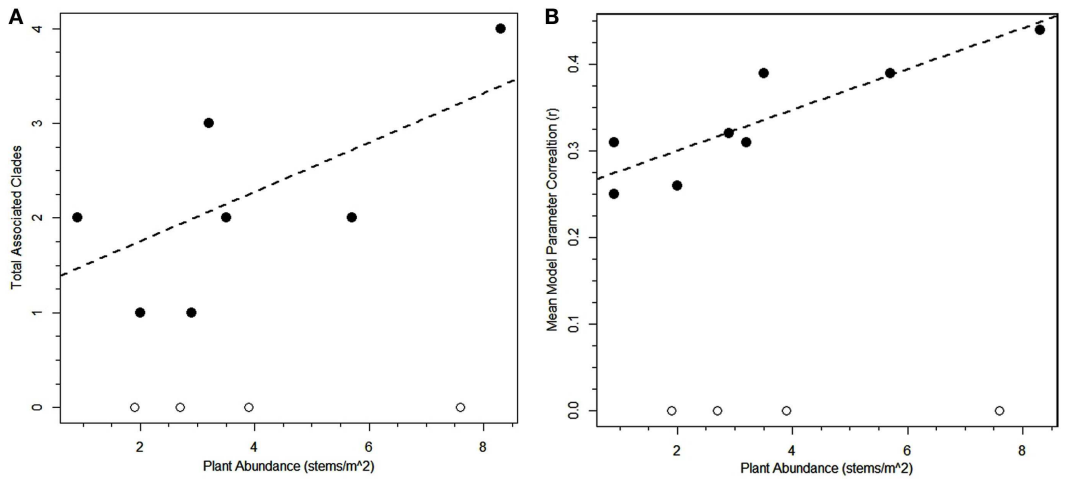


**FIGURE 4 | Regressions of plant abundance versus total number of significant associated bacterial clades (A) or average strength of the correlations for associated bacterial clades (B).** Regression lines are significant if the plant species without any significant associated bacterial clades are excluded from the analysis [$r = 0.67$, one-tailed $t$-test for rho = 0: $p = 0.0275$ **(A)**; $r = 0.88$, two-tailed $t$-test for rho = 0: $p = 0.004$ **(B)**].

plant species (*G. rossii*; **Figure 2**; Datasheet S4 in Supplementary Material). Furthermore, two relatively poorly understood clades, the Acidobacteria Gp4 and the TM7, were similarly strongly associated with plant species (**Table 1**). The primary distinction between the two groups of clades related to strains with known function was that putatively plant symbiosis capable bacteria clades had lower within clade sequence richness than plant-independent clades (mean OTU richness was 93 for plant-dependent versus 411 for plant-independent, two-tailed $t$-test: $p = 0.005$).

### *H3: More remote sites will have fewer but stronger associations between plant species and bacterial clades because facilitation should be more common with increasing environmental harshness*

There were only two bacterial clades that had significant models only in the harshness upweighted dataset the Rhodospirillales, and Pseudonocardiaceae (**Figure 5A**; Datasheet S2 in Supplementary Material; **Table 1**). The Rhodospirillales also increased its association strength with the dicots *E. scriberneri* and *C. phaeocephala* when environmental harshness was upweighted (**Table 1**). Similarly, the Actinobacterial clade, Pseudonocardiaceae, increased its association strength with the plants *F. rubrum* and *C. phaeocephala* when harsh sites were upweighted. On the other hand, 7 of the 14 clades had significant model fits only in less harsh sites (sites proximal to the continuous tundra were upweighted; **Figure 5B**; Datasheet S3 in Supplementary Material; **Table 1**). Although there were fewer bacterial clades significantly co-occurring with plants in harsh portions of the landscape there was no significant difference in the average correlation strength with plant abundances versus clades preferring less harsh locations (0.42 versus 0.44, two-tailed $t$-test, $p = 0.60$).

Plants species had the greatest number of associations in places where they were most abundant (**Table 2**). For example, *G. rossii* decreased in abundance with distance from tundra and its correlations with bacterial clades were entirely dependent on associations in sites proximal to the tundra. The number of plant-bacteria associations was lower in harsher portions of the landscape (22 associations near tundra versus 12 near snowline) but the average correlation strength per plant species did not differ between harshness upweighted and downweighted datasets (0.31 versus 0.40; two-tailed $t$-test $p = 0.42$).

## DISCUSSION

This study is the first demonstration, to our knowledge, of plant species and soil bacterial associations across a heterogeneous landscape with a diverse plant community and indicates that plant-bacteria interactions are a key potential driver of the structure of the subnival ecosystem. In fact, 31% of bacterial sequences in this study fell into clades whose relative abundance was significantly correlated to plant species abundance. This percentage represents the presence in bulk soil material of rhizosphere symbiotes, decomposers that grow particularly well on senesced material from the associated plant, and independent bacteria sharing similar environmental preferences (Mahaffee and Kloepper, 1997; Graff and Conrad, 2006). That the percentage of bacteria correlated with plant species abundance in subnival soil is not greater likely reflects the fact that surveys of bulk soil detect a limited percentage of plant-bacteria symbiotes relative to direct analysis of plant roots (Mahaffee and Kloepper, 1997; Graff and Conrad, 2006). Yet it remains to be seen if studies of bulk soils in less extreme landscapes will find similar percentage of their sequences associated with plant species abundances. Importantly, the associations between plant species and bacteria were not evenly distributed across the subnival environment, but showed clear differences in strength and frequency between environmentally harsh sites close to the snowline and the less harsh sites close to continuous tundra (**Table 1**). The examination of our three hypothesized drivers of community assembly in this landscape (plant abundance, clade potential functional capacity, and environmental harshness) helps place these plant-bacteria co-occurrence patterns for subnival soils in a broader context.

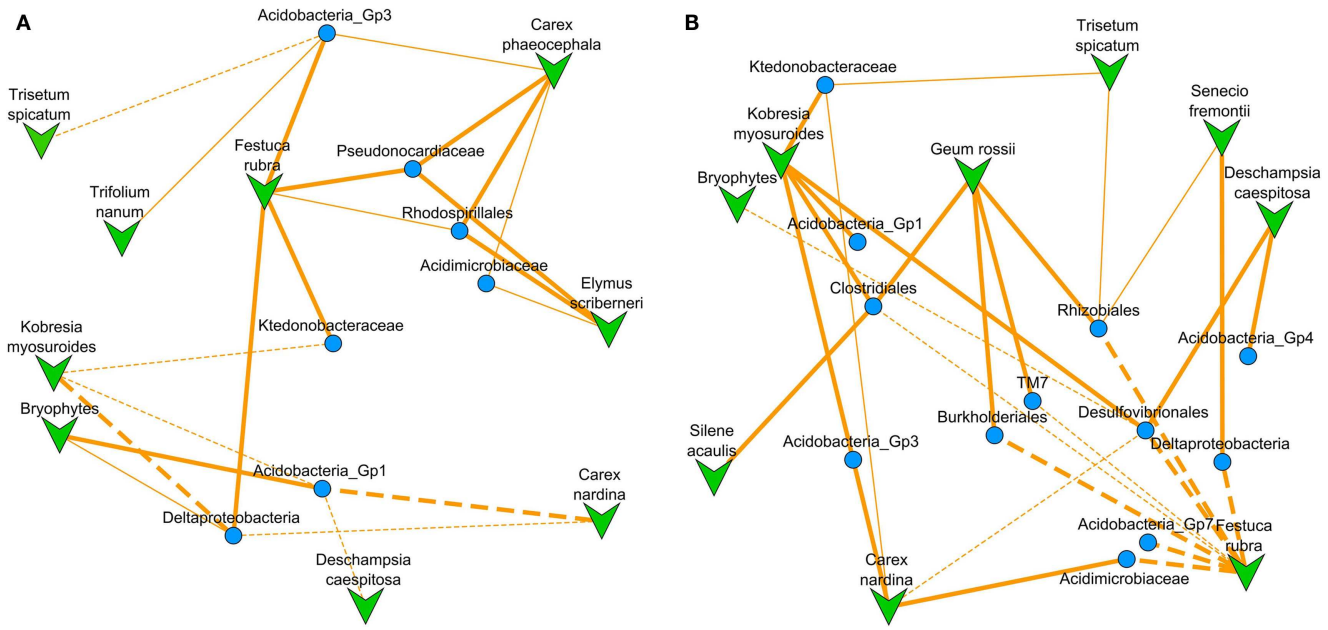


**FIGURE 5 | NMDS ordinated co-occurrence networks based on the correlation matrix after reweighting our clade relative abundances by a harshness metric. (A)** For harshness upweighted there were nine plant species and seven bacterial clades which were significantly co-occurring (stress = 0.227). **(B)** For harshness down-weighted there were nine plant species and 12 bacterial clades which were significantly co-occurring (stress = 0.245). Overlaid are lines designating significant model-based interactions between a plant species and bacterial clade with solid for positive and dashed for negative interactions (thin lines represent two-tailed *t*-test for model coefficient significance, $p < 0.05$; thick lines represent significance after Bonferroni correction for multiple comparisons). Because this is an ordination of the correlation matrix, proximity of points to one another represents higher correlation between their abundances across the subnival landscape.

The first of our hypothesized assembly drivers, plant abundance, was thought to influence the distributions of soil bacteria. Although in general plant abundance was not correlated with the number of associated clades in this subnival landscape, the subset of plants that had significant correlations with bacterial clades showed a strong relationship between abundance and mean clade correlation strength as well as number of clades (**Figure 4**; **Table 2**). This suggests that abundant plants are good predictors of the bulk soil relative abundance of both generalist plant-dependent bacteria and bacteria directly specializing on the micro-environment created by a plant species. An analogous situation occurs in glacial recession landscapes wherein bacterial diversity increases along with successive plant colonization although only a subset are specifically associated with individual plant species (e.g., Schmidt et al., 2008b; Knelman et al., 2012). While the plant with the greatest number of positive associations, *G. rossii*, was indeed the most abundant plant species, *K. myosuroides* and *C. nardina* had the second highest number of positive associations even though they are only sparsely distributed across this subnival landscape. Interestingly, while *G. rossii* is dominant in moist meadow tundra at Niwot Ridge (Bowman et al., 2004), *K. myosuroides* and *Carex* species are dominant in dry meadow tundra ecotypes (Fisk and Schmidt, 1995; Seastedt and Vaccaro, 2001). Dominance is not the only important factor here, however, as the co-dominant with *G. rossii*, *D. caespitosa*, did not have a large number of associated bacteria. Digging deeper, both *G. rossii* and *K. myosuroides* are known to associate with soil microorganism in oligotrophic conditions (Lipson et al., 2002; Bowman et al., 2004) whereas *D. caespitosa* is thought to prefer higher nutrient soils and may have weaker associations with soil microorganisms (Bowman et al., 2004). Thus, bacterial clades may co-occur with plants not just based on the abundant plants in a specific environment but also based on the plant species' level of interaction with soil bacteria. This revised hypothesis, that the life history of abundant plants drives association number, is similar to previous observations that bacterial clades associated with plant community types in less extreme environments (e.g., Berg and Smalla, 2009; Eskelinen et al., 2009; Bezemer et al., 2010) and suggests that the signature of plant dominance on co-occurring soil bacterial clades should have transferability across ecosystems.

Clade functional capacity, our second hypothesized driver of assembly, tests the assumption that previously reported functional niche characteristics for bacterial clades predict patterns co-occurrence with plants (i.e., functional niche assembly, Chase and Leibold, 2003). Six of the eight clades that were related to cultured isolates, representing 10% of the sequences in our study, were closely related to strains previously reported to form mutualistic associations with plants; however, these clades did not have a stronger average correlation with associated plants than the other clades in our study (**Table 1**). However, the two bacterial clades we categorized as likely to be plant-independent were the largest clades in our study (themselves representing 10% of the total sequences) and are known only for their function as generalist soil heterotrophs (**Table 1**). This difference in diversity

between our clades related to previously cultured strains supports the idea that functionally relevant clades (or Operational Taxonomic Units) can be successfully identified using a phylogenetic comparative method to selecting the nodes on a phylogenetic tree of environmental sequences with the greatest correlation to a factor of interest (co-occurrence with plants in this study). Thus, if our assignment of association type is accurate, the strength and likely the specificity of associations may differ within both symbiotic and non-symbiotic soil bacterial guilds; for example, soil heterotrophs have been shown to strongly specialize on a particular plant's litter or non-specifically attack any detritus (van der Heijden et al., 2008), and symbiotic bacteria have been shown to opportunistically infect a plant but also commonly grow as a free-living form (Rodrigues-Diaz et al., 2008). Therefore, although strength of correlation is not a strong predictor of the type of plant-bacterial associations, clades related to plant-symbiotic bacteria may be more likely to correlate with plant species abundances and with a finer taxonomic resolution than clades related to free-living bacterial species.

An additional note on the potential selection for specific bacterial functions is that only one bacterial clade had only negative significant correlations with plants in this subnival ecosystem – the Desulfovibrionales (1.3% of the total sequences). In the context of correlations, negative interactions could mean either inhibition either from disease and competition or an indirect link via differential response to an environmental gradient. For the subnival ecosystem, the presence of a significant area of exposed soils and microbial crusts selects for bacteria that are adapted to the plant-free state (Nemergut et al., 2007; Freeman et al., 2009) and these crust bacteria should show a negative association with abundant plant species (i.e., fewer plants = more crust bacteria). That so few clades displayed this pattern suggests inhibition between bacteria and plants is rare in the subnival zone, which is in agreement with previous hypotheses about facilitation being the primary driver of species interactions in early successional systems (Chapin et al., 1994; Walker and del Moral, 2003).

Termed environmental harshness, the last of our three hypothesized drivers of subnival soil community assembly is based on the theory that abiotic factors create a selective filter for plant-bacterial associations (Weiher and Keddy, 1995; Diaz et al., 1998; Ackerly, 2003) with fewer but stronger associations under harsher conditions (Callaway and Walker, 1997; Reynolds et al., 2003; Seeds and Bishop, 2009). Within the subnival landscape of Niwot Ridge, there were six significant models in the remote-upweighted dataset whereas there were 12 significant models in the remote-downweighted dataset. Moreover there were only two bacterial clades that had a significant model fit in only the most environmentally harsh sites (**Table 1**). This is in agreement with the hypothesis that plant-microbe interactions are less frequent in the more remote portions of the subnival landscape as a result of less plant diversity and low abundance of plant-symbiotic bacteria (Seeds and Bishop, 2009). With these harsh conditions, it is also hypothesized that interactions should be stronger (Reynolds et al., 2003), yet, there was no significant difference in the average correlation strength for clades favoring one end of the harshness gradient or the other. This lower number of harshness upweighted plant-bacteria correlations and similar strength of correlation despite the high correlation of individual plant species with our harshness gradient suggests that environmental filters become more important than biotic filters in harsher environments and is in agreement to what previous work has found at the community scale (Carlson et al., 2012).

Interestingly, both of the bacterial clades that were remote specialists are related to isolates known to garner energy from non-organic sources; members of the Rhodospirillales have demonstrated the ability to perform anaerobic phototrophic sulfate reduction in addition to heterotrophy (Madigan, 2005; Rodrigues-Diaz et al., 2008) and members of the Pseudonocardiaceae have demonstrated the ability to perform hydrogen sulfide oxidation as well as heterotrophy (Reichert et al., 1998; Chen et al., 2009). Because the harshest environmental conditions of the subnival zone are more common in remote portions of the landscape, it may be that these two extremophilic bacterial clades are early colonizers of recently exposed soils and later facilitate plant colonization via their potential to form symbioses or because they improve soil conditions. This phenomenon is similar to what is seen in the primary soils from pyrite weathering (Choler et al., 2001) and experimental work to tease apart the environmental versus biotic association is needed to further explore these patterns.

A caveat with this correlation network approach is that the correlation between a plant species and a bacterial clade may be the result of environmental factors shaping the distribution of both groups independently, a symbiotic/competitive/facilitative interaction, or even a tertiary relationship such as are seen with parasites of a parasite. However, that 75% of the clades to which we could assign hypothetical functions appear to have the potential to form symbioses with plants (six of eight, **Table 1**; **Figure 3**) suggests that we are identifying true plant-bacteria associations. It also suggests that the many of the strongly correlated Acidobacteria or TM7 clades, despite their unknown functional capacities, are associated with plants in this landscape.

## CONCLUSION

We found evidence of three hypothesized assembly drivers between plant species and bulk soil bacteria using a correlational-based analysis along a gradient of environmental harshness in the subnival landscape of Niwot Ridge. First, our findings suggest that the abundance of bacteria-promoting plant species may serve as an indicator or even drive the patterns of plant-associated groups for specific ecosystem types within a landscape. Second, bacterial clades with putative plant symbiosis capacity, as inferred from the close relationship of sequences within a clade to known isolates, were not more likely to be correlated with plant species but have narrower phylogenetic breadth than putative plant-independent clades. Third, our analysis suggests that the influence of environmental harshness on plant-bacterial associations results in fewer plant-bacteria associations in harsh areas than in the less harsh, tundra proximal portion of the landscape. Our results provide the first description of plant-bacteria interactions at the species/clade level and demonstrate an overwhelming number of plant species specific associations with soil bacteria. In addition, facilitative associations appear to be more common than inhibitory associations in the subnival zone. One obvious next step is to test how often these bacteria are indeed found on or within the roots of the plants they co-occur with and if they affect plant growth or survival as opposed to co-occurring due to shared environmental

preferences. If co-occurrence relationships are as important for the ecology of high-alpine systems as our correlation network suggests, then the biotic community's functional resilience is likely sensitive to disturbances affecting either the plant or soil microbial community.

## ACKNOWLEDGMENTS

This work was supported by the NSF Microbial Observatories Program (MCB-0455606). Logistical assistance was provided by the Niwot Ridge LTER site.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Terrestrial_Microbiology/10.3389/fmicb.2012.00347/abstract

**Datasheet S1 | Initial best AIC ranked models predicting bacterial clade relative abundance with plant species abundances.**

**Datasheet S2 | Best AIC ranked models predicting harshness-upweighted bacterial clade relative abundance with plant species abundances.**

**Datasheet S3 | Best AIC ranked models predicting harshness-downweighted bacterial clade relative abundance with plant species abundances.**

**Datasheet S4 | A correlation matrix of plant species abundances and bacterial clade relative abundances (Pearson *r* values).** A single * designates a correlation that is *p*-value $< 0.01$ for a two-tailed *t*-test of rho $= 0$; ** indicates $p < 0.001$; *** indicates $p < 0.0001$. The Bonferroni correction for alpha with 325 comparisons is $p < 0.0002$.

**Datasheet S5 | A combination of two correlation matrices (Pearson *r* values).** The upper right triangle in bold represents correlations between bacterial clades and plant abundances with remote locations upweighted. The lower left triangle represents correlations between bacterial clades and plant abundances with remote locations down-weighted. A single * designates a correlation that is *p*-value $< 0.01$ for a two-tailed *t*-test of rho $= 0$; ** indicates $p < 0.001$; *** indicates $p < 0.0001$. The Bonferroni correction for alpha with 325 comparisons is $p < 0.0002$.

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

*Received: 15 June 2012; accepted: 08 September 2012; published online: 11 October 2012.*

*Citation: King AJ, Farrer EC, Suding KN and Schmidt SK (2012) Co-occurrence patterns of plants and soil bacteria in the high-alpine subnival zone track environmental harshness. Front. Microbio. **3**:347. doi: 10.3389/fmicb.2012.00347*

*This article was submitted to Frontiers in Terrestrial Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

FRONTIERS COMMENTARY
published: 13 August 2014
doi: 10.3389/fmicb.2014.00424

# Changes in community assembly may shift the relationship between biodiversity and ecosystem function

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**Keywords: biodiversity-ecosystem functioning, community assembly theory, niche vs. neutral, stochastic vs. deterministic, rare organisms, biodiversity dilution effect**

Can differences in community assembly alter the relationship between biodiversity and ecosystem function? Pholchan et al. (2013) used a variety of manipulations to change microbial community assembly in sludge reactors and examined the subsequent links between diversity and a rare function, the removal of endocrine disrupting compounds (EDCs). Interestingly, the authors saw no consistent differences between shifts in alpha diversity (e.g., species richness and evenness) and ecosystem function, observing an increase, decrease and no difference in the amount of removal of specific EDCs with increases in diversity. They suggested that differences in community assembly may be driving variation in the relationship between biodiversity and function, a fascinating hypothesis that unites processes in community and ecosystem ecology.

Combinations of four processes affect community assembly: dispersal and diversification add new taxa to communities while selection and drift affect their relative abundances (Vellend, 2010; Nemergut et al., 2013). Particular research emphasis has been placed on assembly processes that are driven by differences between taxa ("niche") compared to those in which any such differences are irrelevant to fitness ("neutral") (Hubbell, 2001). Likewise, researchers have focused on the role of stochasticity, where assembly is more probabilistic vs. determinism, in which randomness does not affect community dynamics. Niche and neutral processes can operate in unison (Adler et al., 2007) and both can be affected by stochastic and deterministic forces (Fox, 2012). Indeed, extensive data demonstrate that a variety of factors, including nutrients, productivity, resource availability, successional stage, and disturbances may affect the relative importance of different community assembly mechanisms (Chase, 2007, 2010; Ferrenberg et al., 2013; Kardol et al., 2013). However, to our knowledge, no studies have directly tested how shifts in community assembly may affect the relationship between biodiversity and ecosystem function.

Of course, a great deal of research has focused on pairwise combinations of the interactions between community assembly, biodiversity and/or function in isolation. First, a large body of work demonstrates links between biodiversity and ecosystem function (Cardinale et al., 2011; Hooper et al., 2012), even for microbial systems (Bell et al., 2005; Hsu and Buckley, 2009; Langenheder et al., 2010; Levine et al., 2011; Jousset et al., 2014). However, the nature and strength of biodiversity ecosystem function (BEF) relationships have been widely debated and strongly depend on the type of function and ecosystem examined (Grime, 1997; Hooper et al., 2005) and the degree of redundancy within the community (Reich et al., 2012; Jousset et al., 2014). These complexities may be heightened for microorganisms due to the extraordinary phylogenetic diversity harbored within microbial communities, and the fact that a typical microbial community contains organisms from within a variety of functional guilds.

Second, it is known that different assembly mechanisms drive biodiversity in distinct ways. For example, spatial or temporal variation in environmental conditions increases biodiversity through niche processes while increases in the diversity of the metacommunity or in the ratio of immigration/emigration rates can increase biodiversity through neutral processes (Vellend, 2010).

Finally, a relatively new topic in the literature relates community assembly and ecosystem function (Fukami et al., 2010; Nemergut et al., 2013). Vital to such a consideration is the relationship between response traits, or traits that can interact with environmental variation to determine species distribution and abundance patterns, and effect traits, or traits that determine the functional roles of different taxa (Naeem and Wright, 2003). When communities are largely structured by niche processes, variation in the environment can directly correlate to effect traits that are linked to selected response traits (Allison, 2012). However, when communities are structured by neutral processes, ecosystem function will primarily depend on effect trait abundances within the metacommunity, dispersal and ecological drift; thus, relationships between variation in the environment and effect traits

can be decoupled (Nemergut et al., 2013). Communities can also be structured by niche-based processes that act on response traits that are unrelated to the effect trait, i.e., the ecosystem process of interest, again resulting in a lack of a relationship between the environment and effect traits (Jiang et al., 2008).

Thus, various studies have examined separate pieces of the assembly-BEF puzzle, but we know of no work that explicitly ties all three factors together. However, given that niche and neutral processes partially underpin the proposed mechanisms driving positive BEF relationships (Loreau and Hector, 2001), examining the links between assembly, biodiversity and ecosystem function simultaneously, rather than in pairwise combinations, may yield new insights into the controls and consequences of biodiversity. For example, niche complementarity occurs when a more diverse community occupies a greater diversity of niches and thus can have greater overall functional efficiency (**Figure 1A**). Alternatively, neutral processes can lead to increases in diversity through increases in immigration, a phenomenon that may be particularly important in early succession (Ferrenberg et al., 2013). This could lead to a sampling effect in which more diverse communities include members with an effect trait of interest as the community equilibrates with the metacommunity, thus having a higher rate of function per capita (**Figure 1B**). Thus, because different assembly processes can lead to communities with different community compositions but with the same level of alpha diversity, assembly may lead to differences in nature of BEF relationships.

Additionally, depending on the starting conditions of the community and the degree of stochasticity, it is also possible that different assembly processes could lead to declines in ecosystem function coincident with increases in biodiversity through changes in the "mass ratio" (Grime, 1998). In early successional communities that contain the effect trait of interest, increases in diversity driven by both niche and neutral process could lead to decreases in the amount of ecosystem function per unit biomass (**Figures 1A,B**). When stochastic immigration processes result in early communities with an effect trait of interest that is present in lower abundance in the metacommunity, increases in diversity driven by immigration could lead to decreases in ecosystem function per unit biomass (**Figure 1B**). The inverse of this

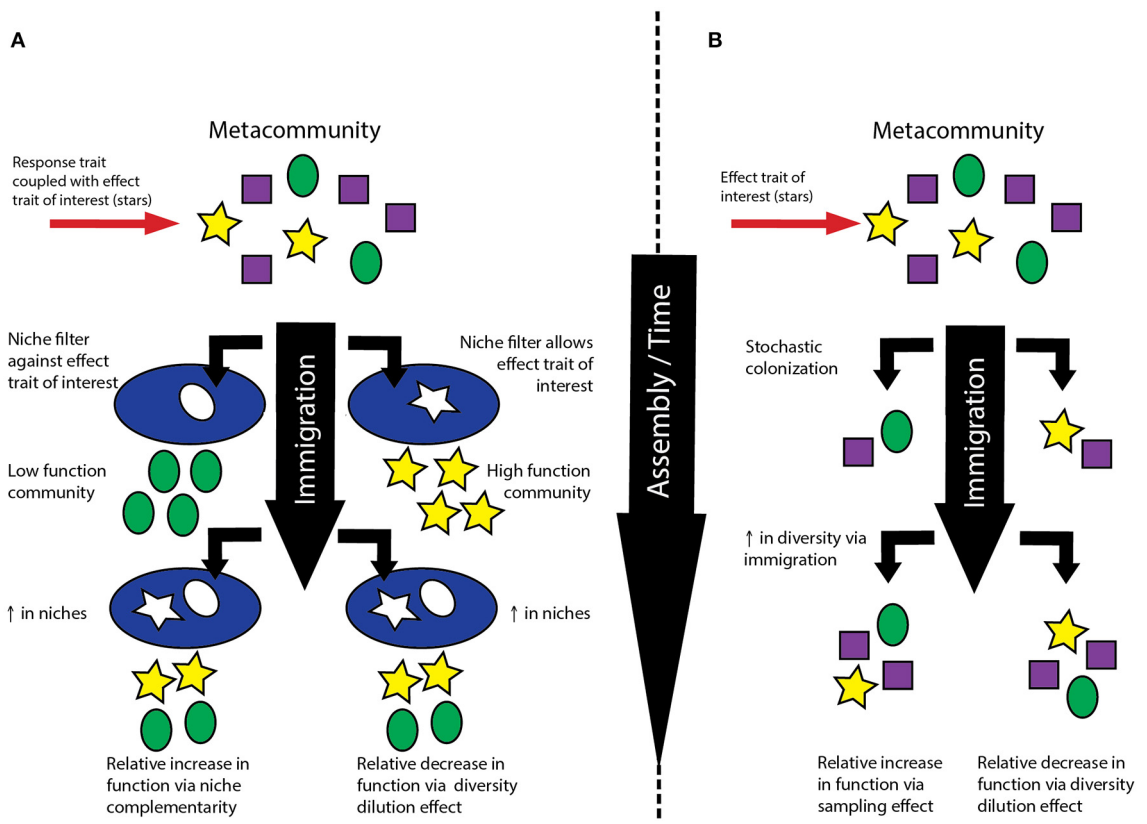


**FIGURE 1 | (A)** Relationship between niche-driven increases in biodiversity and ecosystem function. Shapes represent traits where response and effect traits are directly coupled; the trait of interest is represented as a yellow star. Depending on the initial community, increases in diversity can lead to increases in relative function through niche complementarity or decreases through the biodiversity dilution effect. **(B)** Relationship between stochastic, neutrally-driven increases in biodiversity and ecosystem function. The trait of interest is represented as a yellow star. Increases in diversity can lead to increases in relative function through a sampling effect if the trait of interest is absent from the initial community. However, increases in diversity could also result in decreases in relative function through the biodiversity dilution effect if the trait is present in the initial community. The biodiversity dilution effect may be especially important if the trait is more rare in the metacommunity but stochastic processes result in its appearance in the initial community.

relationship was reflected in the neutral model generated by Pholchan and coworkers, which showed that decreases in diversity could lead to increases in the relative abundance of rare taxa. Likewise, if a coupled response-effect trait of interest is present in an early successional community, niche-driven increases in diversity could also lead to decreases in function per unit biomass (**Figure 1A**). We refer to both of these scenarios as examples of *biodiversity dilution effects*, conceptually akin to the disease dilution effect (Keesing et al., 2006). Indeed, rare ecosystem functions such as EDC removal may be catalyzed by a very select group of organisms, and thus the activity of interest could be related to the presence or absence of specific taxa and particularly sensitive to diversity dilution effects.

Thus, biodiversity dilution effects, differences in response and effect traits, niche complementarity and sampling effects may have interacted in poorly understood ways to produce the lack of a consistent relationship between biodiversity and EDC degradation across the different treatments in the work presented by Pholchan and coworkers. It is important to bear in mind, however, that microbial communities are highly complex and that other factors besides assembly could affect the nature of the BEF relationship. For example, the unique resource requirements of EDC removers may have contributed to the complex relationships between diversity and ecosystem function observed in this study: Chesson (2000) showed that when organisms require highly specialized niches, they can exhibit negative frequency dependence and be more competitive in low abundance. In the research presented by Pholchan and coworkers, increases in diversity were correlated with increases in evenness, which could have affected the competitiveness and thus the overall function of EDC removers. Additionally, some ecosystem processes may be catalyzed by a consortium of organisms acting sequentially and across trophic scales; thus, ecosystem function may not be related to alpha diversity *per se*, but rather to overall community composition. The importance of species-specific traits vs. biodiversity for function has been a subject of debate for decades and appears to depend on the system and function of interest (Grime, 1997). Given the high functional and phylogenetic diversity of microbial communities and our ability to perform comparative metagenomics on a large number of samples, this should be a research priority into the future. As well, the general hypothesis put forward by Pholchan and coworkers connecting assembly processes, biodiversity and ecosystem function should be examined with directed experiments and simulations to better understand the mechanistic details of such links and when and where they may vary.

## ACKNOWLEDGMENTS

The authors wish to acknowledge support from the National Science Foundation through a graduate research fellowship to Joseph E. Knelman and DEB-1258160 to Diana R. Nemergut. We also thank Caroline Tucker and Alan Townsend for helpful discussions.

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

*Received: 21 March 2014; accepted: 27 July 2014; published online: 13 August 2014.*
*Citation: Knelman JE and Nemergut DR (2014) Changes in community assembly may shift the relationship between biodiversity and ecosystem function. Front. Microbiol.* ***5****:424. doi: 10.3389/fmicb.2014.00424*
*This article was submitted to the journal Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

HYPOTHESIS AND THEORY ARTICLE
published: 09 January 2012
doi: 10.3389/fmicb.2011.00273

# Evolutionary history, immigration history, and the extent of diversification in community assembly

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During community assembly, species may accumulate not only by immigration, but also by *in situ* diversification. Diversification has intrigued biologists because its extent varies even among closely related lineages under similar ecological conditions. Recent research has suggested that some of this puzzling variation may be caused by stochastic differences in the history of immigration (relative timing and order of immigration by founding populations), indicating that immigration and diversification may affect community assembly interactively. However, the conditions under which immigration history affects diversification remain unclear. Here we propose the hypothesis that whether or not immigration history influences the extent of diversification depends on the founding populations' prior evolutionary history, using evidence from a bacterial experiment. To create genotypes with different evolutionary histories, replicate populations of *Pseudomonas fluorescens* were allowed to adapt to a novel environment for a short or long period of time (approximately 10 or 100 bacterial generations) with or without exploiters (viral parasites). Each evolved genotype was then introduced to a new habitat either before or after a standard competitor genotype. Most genotypes diversified to a greater extent when introduced before, rather than after, the competitor. However, introduction order did not affect the extent of diversification when the evolved genotype had previously adapted to the environment for a long period of time without exploiters. Diversification of these populations was low regardless of introduction order. These results suggest that the importance of immigration history in diversification can be predicted by the immigrants' evolutionary past. The hypothesis proposed here may be generally applicable in both micro- and macro-organisms.

**Keywords: adaptive peaks, colonization, dispersal, eco-evolutionary dynamics, fitness trade-off, historical contingency, niche pre-emption, priority effect**

## INTRODUCTION

During community assembly, species can accumulate both ecologically, by immigration, and evolutionarily, by *in situ* diversification. Traditionally, these processes have been studied separately by ecologists on one hand and evolutionary biologists on the other. However, an increasing number of recent studies on both micro- and macro-organisms consider immigration and diversification simultaneously (e.g., Hubbell, 2001; Gillespie, 2004; Fukami et al., 2007). Much of this change has been prompted by the growing appreciation of "eco-evolutionary dynamics," where ecological and evolutionary processes operate interactively at the same time scales (Hairston et al., 2005; Schoener, 2011). One idea that is emerging from eco-evolutionary research is that the extent of diversification can be affected by stochastic differences in immigration history, such as the relative timing and order of immigration of competing genotypes (Silvertown, 2004; Fukami et al., 2007; Gillespie and Emerson, 2007; Seehausen, 2007). This interactive effect of immigration and diversification arises because early arriving immigrants pre-empt available niches, thereby suppressing diversification of late-arriving immigrants – an evolutionary "priority effect" (Samuels and Drake, 1997; Chase, 2003; Gillespie, 2004; Silvertown, 2004; Fukami et al., 2007; Gillespie and Emerson, 2007; Seehausen, 2007; Urban and De Meester, 2009). It remains unclear, however, under what conditions immigration history influences the extent of diversification. As a first step toward answering this question, this paper proposes one hypothesis, namely that the importance of immigration history is determined by the degree of prior adaptation of founding populations to the new environment. To provide empirical support for this hypothesis, we present the results of an experiment that involved the plant-colonizing bacterium *Pseudomonas fluorescens* and its viral parasite, phage Φ2.

*Pseudomonas fluorescens* is a useful model system to study diversification. It has been shown to rapidly diversify into spatial niche specialists by *de novo* mutation when propagated in a novel habitat (static vial containing nutrient-rich liquid medium; Rainey and Travisano, 1998). Primary classes of niche specialists that emerge through this adaptive radiation include "smooth morphs" (SM), which resemble the ancestral type and primarily colonize the liquid phase, "wrinkly spreader (WS) morphs," which form a biofilm at the air–liquid interface, and "fuzzy spreader (FS) morphs," which appear to inhabit the bottom of the vial (Rainey and Travisano, 1998). Heritable variation exists within each class, and evolved

morphotypes coexist via negative frequency-dependent selection (Rainey and Travisano, 1998; Fukami et al., 2007; Meyer and Kassen, 2007). Because reproduction in *P. fluorescens* is entirely asexual, genotypes are analogous to species in other organisms (Kassen et al., 2004). Further, previous work has shown strong effects of the presence of phage Φ2 on *P. fluorescens* diversification via reduction in bacterial density and selection for phage resistance (Buckling and Rainey, 2002).

Using *P. fluorescens* and phage Φ2, we examined the effects of two aspects of prior evolutionary history on the role of immigration history in diversification: how long immigrants have previously evolved in an environment similar to the new habitat, and whether or not immigrants have previously evolved in the presence of the exploiters that they encounter in the new habitat. We examined these two aspects because both theory (Wright, 1932; Dobzhansky, 1937; Simpson, 1953; Kauffman, 1989; Whitlock et al., 1995; Schluter, 2000) and observations from the field (Simpson, 1953; Schluter, 2000) suggest that the degree of similarity between the prior environment that immigrants come from and the new environment (both biotic and abiotic) is a key factor affecting diversification in the new environment. In our experiment, we allowed replicate bacterial populations to evolve in a novel environment, with and without phage Φ2, for approximately 10 or 100 generations. We then introduced each of the evolved genotypes into a new habitat either before or after a standard competitor genotype to evaluate the effect of immigration history. We found that whether or not the extent of diversification was influenced by immigration history did depend significantly on the adaptation history of the immigrants prior to immigration. We will discuss possible mechanisms for this effect, and suggest that they may be generally applicable to other systems of both micro- and macro-organisms, with the caveat that results of microbial experiments should not be extrapolated uncritically (Carpenter, 1996; Cadotte et al., 2005).

## MATERIALS AND METHODS

### CREATION OF GENOTYPES WITH DIFFERENT PRIOR EVOLUTIONARY HISTORIES

We propagated six independent replicate lineages of wild type *P. fluorescens* SBW25 (Rainey and Travisano, 1998) at 28°C in static 25-ml universal vials with loose caps containing 6 ml of standard King's B (KB) liquid medium (Rainey and Travisano, 1998; Fukami et al., 2007; Meyer and Kassen, 2007), in the absence of phage (three lineages) and in the presence of phage (three lineages). All of these lineages were initiated with a common isogenic SM population of *P. fluorescens*. Approximately $3 \times 10^5$ particles of phage SBW25Φ2 were also introduced to all appropriate vials at the start of the propagation. Subsequently, 60 μl of culture was transferred to fresh media every 48 h for 30 days. We harvested 1 ml of each replicate at every transfer, starting on day 1. Immediately after each harvest, the samples were stored in 70% glycerol at −80°C. From this collection of evolved SM genotypes, we chose genotypes isolated after 3 days (corresponding to approximately 10 bacterial generations) and 27 days (approximately 100 bacterial generations) of propagation to represent brief and long periods of prior adaptation to the novel environment, respectively. Thus we had four treatments of prior evolutionary history: short or long periods of adaptation, each in either the presence or absence of exploiters. It has been shown under laboratory conditions that *P. fluorescens* evolves to have increased general resistance to phage over time when continuously exposed to phage (Brockhurst et al., 2003).

### MANIPULATION OF PRIOR EVOLUTIONARY HISTORY AND IMMIGRATION HISTORY

Using the same conditions as above, we inoculated fresh vials with an isogenic population of SM genotype isolated from an evolved lineage (one of each of the three replicates for all four of the varied evolutionary histories as described above; hereafter referred to as focal genotype) and that of a common ancestral SM genotype with a neutral *lacZ* genetic marker (Zhang and Rainey, 2007; used as a standard competitor; hereafter referred to as competitor genotype). Use of a *lacZ* lineage as the competitor genotype allowed the origin of each bacterial cell to be determined by observation of colonies developed on KB agar plates supplemented with X-gal (see below).

The following 12 treatments of immigration history were used: (1) focal genotype with 3-day prior history with phage (hereafter called 3W) introduced by itself; (2) focal genotype with 3-day prior history without phage (3WO) introduced by itself; (3) focal genotype with 27-day prior history with phage (27W) introduced by itself; (4) focal genotype with 27-day prior history without phage (27WO) introduced by itself; (5) competitor genotype introduced first, then 3W introduced 24 h later; (6) 3W introduced first, then competitor genotype introduced 24 h later; (7) competitor genotype introduced first, then 3WO introduced 24 h later; (8) 3WO introduced first, then competitor genotype introduced 24 h later; (9) competitor genotype introduced first, then 27W introduced 24 h later; (10) 27W introduced first, then competitor genotype introduced 24 h later; (11) competitor genotype introduced first, then 27WO introduced 24 h later; and (12) 27WO introduced first, then competitor genotype introduced 24 h later.

To initiate these replicates, we separately grew the competitor genotype and the focal genotypes overnight (for 16 h) in liquid KB medium at 28°C in an orbital shaker (at 150 r.p.m.). We then introduced 6 μl of these overnight samples to each appropriate vial. Additionally, all vials were inoculated with phage, as described above. Replicates were destructively harvested every 24 h for 10 days to measure bacterial diversity and abundances (see below). We used a total of 360 vials, i.e., 12 treatments × 3 replicates × 10 destructive harvests.

This experimental design allowed us to test if the effect of immigration history on diversification depended on the prior evolutionary history of the focal genotype. Additionally, inclusion of phage in all replicates enabled us to determine if diversification was affected by prior evolution with the exploiters that the focal genotype would encounter in the new habitat.

### MEASURING BACTERIAL MORPHOTYPE DIVERSITY AND ABUNDANCES

For each harvest (every 24 h), we determined cell densities of different morphotypes by counting colonies after 2 days of growth on KB agar supplemented with 50 μg ml$^{-1}$ 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal). Colony morphotype diversity was measured following the standard methods used in

previous work (Buckling et al., 2003; Meyer and Kassen, 2007). Niche preference of each morphotype was confirmed by observing growth of each genotype in static medium and establishing whether they mainly colonized the liquid phase (SM), the air–liquid interface (WS), or the bottom (FS) of the medium. This measure of diversity is ecologically relevant because colony morphology corresponds to spatial niche use. It should be noted, however, that genetic diversity may have also existed within colony morphotypes, which we did not measure.

Ecological differences among these mutants can be regarded as large as those among species in many systems of animals and plants, for three reasons. First, previous research has identified the genetic bases of the colony morphotypes (Spiers et al., 2002, 2003; Kassen and Rainey, 2004; Spiers and Rainey, 2005), indicating that they reflect genetic variation rather than phenotypic variation within the same genotype. Second, when colonies of a given morphotype were isolated, inoculated into a fresh medium, grown for 2 days, and plated, they produced the same colony morphotype (Fukami et al., 2007), further confirming that the colony morphotypes have genetic bases. Third, colony morphology corresponds to spatial niche differentiation that is of similar or greater ecological magnitude compared to that found among species in many other systems. However, in future research, full genome sequencing of different morphotypes should be helpful in further uncovering the genetic mechanisms underlying diversification in this system.

### STATISTICAL ANALYSES

Data were assessed for normality and log-transformed when necessary prior to analyses. Two-way analysis of variance (ANOVA) was used to test for effects of length of time of prior evolution, prior evolution in the presence of phage, and their interaction on the following response variables: the number of morphotypes observed (time-averaged for days 8–10; **Figure 1**; **Tables 1–3**), differences in the number of morphotypes observed (time-averaged

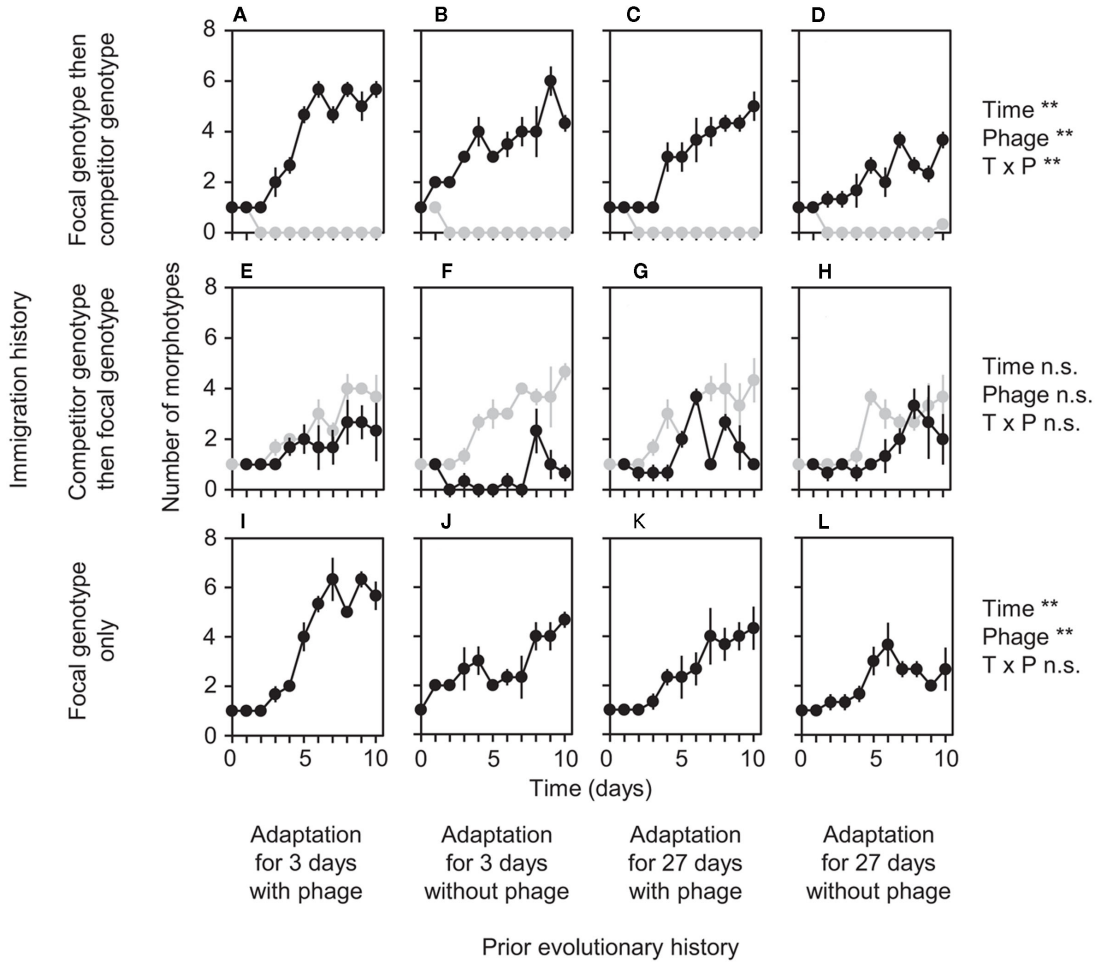


**FIGURE 1 | Effects of prior evolutionary history and immigration history on bacterial diversification.** Black and gray lines indicate focal and competitor genotypes, respectively. Each panel **(A–L)** shows mean number of morphotypes ± SEM ($n = 3$) for the corresponding treatment of immigration history and prior evolutionary history. For each treatment of immigration history, two-way ANOVA results are indicated as follows: n.s. denotes $p > 0.05$, *denotes $p < 0.05$, and **denotes $p < 0.01$ (see **Tables 1–3**). "Time," "Phage," and "T × P" refer to the effect of length of time of prior adaptation, of prior evolution with phage, and of their interaction, respectively.

for days 8–10) between early immigration and late-immigration treatments (**Figure 2**; **Table 4**), and total number of morphotypes descended from both the focal genotype and the competitor genotype (time-averaged for days 0–10; **Figure 4**; **Table 5**). All statistical results were for two-tailed tests with $\alpha = 0.05$. Statistical analyses were performed using JMP v.8 (SAS Institute Inc.).

## RESULTS AND DISCUSSION

Genotypes differing in prior evolutionary history showed significant differences in the degree to which immigration history affected diversification (**Figures 1** and **2**). In three of the four prior-history treatments, after approximately 35 bacterial generations (at days 8–10), the focal genotype diversified to a greater extent when it was introduced before, rather than after, its competitor (compare **Figures 1A** vs. **1E**, **1B** vs. **1F**, and **1C** vs. **1G**, see also **Figure 2**). This result is an expected outcome from previous work (Brockhurst et al., 2007; Fukami et al., 2007). However, when the focal genotype had a long history of adaptation to the laboratory environment in the absence of phage, immigration history did not have a significant effect on the extent of diversification (compare **Figures 1D** vs. **1H**, see also **Figure 2**).

Why did the importance of immigration history depend on prior evolutionary history? To address this question, we examined the effect of prior evolutionary history within each of the two immigration treatments separately, i.e., when the focal genotype was introduced first and when it was introduced after the competitor. When introduced first, the focal genotype almost completely suppressed the competitor genotype, both in diversity (**Figures 1A–D**) and abundance (**Figures 3A–D**), regardless of prior evolutionary history. Therefore, diversification of early immigrants can be considered equivalent to diversification following a single immigration event, simplifying interpretation of results. In fact, when the focal genotype was introduced alone, diversification patterns resembled those observed in the early immigrant treatments (compare **Figures 1A–D** vs. **1I–L**).

We expected that, if introduced alone, genotypes with shorter prior history, and therefore with decreased opportunity for previous adaptation to the novel environment, would diversify more extensively. Two possible mechanisms underlie this expectation. First, both theory (Wright, 1932; Dobzhansky, 1937; Kauffman, 1989; Whitlock et al., 1995) and empirical evidence from *P. fluorescens* (Buckling et al., 2003) indicate that poorly adapted genotypes have a high potential to diversify via ascending of multiple adaptive peaks. If an immigrant is already adapted to a particular peak, it may be difficult to shift to a new peak, consequently hampering diversification via niche specialization (Buckling et al., 2003; Brockhurst et al., 2007). Second, poorly adapted genotypes may be unable to out-compete descendant genotypes, facilitating the descendants' increase in abundance once they arise by mutation. Although our data do not allow us to ascertain which of these two mechanisms (or both) operated, our results are consistent with the expectation that shorter prior adaptation to the environment results in more extensive diversification (compare **Figures 1I** vs. **1K** and **1J** vs. **1L**).

**Table 1 | ANOVA results on the number of morphotypes (time-averaged for days 8–10) for focal genotypes introduced 24 h before the competitor genotype.**

| Factor | df | SS | MS | *F* | *p* |
|---|---|---|---|---|---|
| Time | 1 | 2.99 | 2.99 | 20.10 | 0.002 |
| Phage | 1 | 1.80 | 1.80 | 12.10 | 0.008 |
| Time × phage | 1 | 2.99 | 2.99 | 20.10 | 0.002 |
| Error | 8 | 1.19 | 0.15 | | |

**Table 2 | ANOVA results on the number of morphotypes (time-averaged for days 8–10) for focal genotypes introduced 24 h after the competitor genotype.**

| Factor | df | SS | MS | *F* | *p* |
|---|---|---|---|---|---|
| Time | 1 | 0.59 | 0.59 | 0.60 | 0.461 |
| Phage | 1 | 0.04 | 0.04 | 0.04 | 0.847 |
| Time × phage | 1 | 4.48 | 4.48 | 4.58 | 0.065 |
| Error | 8 | 7.83 | 0.98 | | |

**Table 3 | ANOVA results on the number of morphotypes (time-averaged for days 8–10) for focal genotypes introduced alone.**

| Factor | df | SS | MS | *F* | *p* |
|---|---|---|---|---|---|
| Time | 1 | 8.91 | 8.91 | 20.02 | 0.002 |
| Phage | 1 | 6.75 | 6.75 | 15.17 | 0.005 |
| Time × phage | 1 | 1.00 | 0.00 | 0.00 | 1.000 |
| Error | 8 | 3.56 | 0.45 | | |

We also expected that genotypes that previously evolved in the presence of phage would diversify more than those that did not evolve with phage. There are three possible mechanisms for this expectation. First, previous work has shown that reduction in *P. fluorescens* density by phage results in reduced resource competition, weakening selection pressure for competitive ability (Buckling and Rainey, 2002). Genotypes that previously evolved with phage may therefore be relatively poorly adapted to the laboratory environment, thereby facilitating subsequent diversification as discussed above. Second, genotypes that previously evolved with phage multiplied more quickly (compare **Figures 3I** vs. **3J** and **3K** vs. **3L**), which may have caused more intense competition, resulting in more extensive diversification. Third, previous work has also found the existence of fitness trade-offs in *P. fluorescens* between phage resistance and competitive ability (Brockhurst et al., 2004). With such trade-offs, genotypes that previously evolved with phage may be relatively weak in competition and may not strongly suppress descendant mutants, thereby allowing adaptive radiation. Although we cannot determine which mechanism(s) operated, we found that, as expected, prior evolution with phage led to more extensive diversification (compare **Figures 1I** vs. **1J** and **1K** vs. **1L**).

In contrast to these results for early immigrants, prior evolutionary history did not significantly affect the extent of diversification of late immigrants (**Figures 1E–H**). Diversification of late immigrants was consistently low regardless of prior evolutionary history (**Figures 1E–H**). We hypothesize that any potential effect of prior evolutionary history on diversification was overwhelmed by strong niche pre-emption by the early-arriving competitor genotype (Brockhurst et al., 2007; Fukami et al., 2007), which fared better in both diversity (**Figures 1E–H**) and abundance (**Figures 3E–H**) by virtue of early arrival. If niche pre-emption was important, diversification should be deterministic when overall diversity of all genotypes descended from both immigrants is considered. Our data are consistent with this expectation, with no significant difference in total diversity between prior-history treatments (**Figure 4**).

Taken together, our data, combined with previous evidence (Buckling and Rainey, 2002; Buckling et al., 2003; Brockhurst et al., 2004, 2007; Fukami et al., 2007), provide likely explanations for why the importance of immigration history depended on prior evolutionary history, which can be summarized as follows. The focal genotype usually diversified more extensively when it arrived early, due to niche pre-emption (Brockhurst et al., 2007; Fukami et al., 2007). However, this priority effect did not occur if the focal genotype previously had a long period of adaptation in the absence of phage. Genotypes with this history had such a heavily compromised ability to diversify that the extent of diversification after early arrival was indistinguishable from the consistently low level of diversification after late arrival of any immigrant (**Figures 1D** vs. **1E–H**).

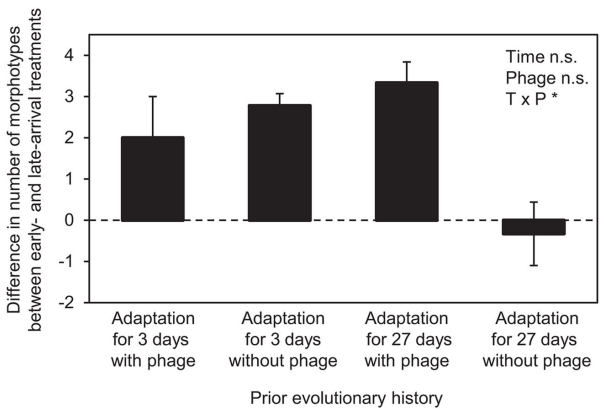


**FIGURE 2 | Comparison of diversification of focal genotypes immigrating early and late.** Data show the difference in the mean number of morphotypes derived from the focal genotype ± SEM ($n = 3$) measured at the end of the experiment (on days 8–10). Two-way ANOVA results are indicated as in **Figure 1** (see **Table 4**).

**Table 4 | ANOVA results on the number of morphotypes (time-averaged for days 8–10) for the difference between early and late arrival of focal genotypes.**

| Factor | df | SS | MS | *F* | *p* |
|---|---|---|---|---|---|
| Time | 1 | 7.79 | 7.79 | 4.97 | 0.056 |
| Phage | 1 | 1.56 | 1.56 | 1.00 | 0.347 |
| Time × phage | 1 | 12.67 | 12.67 | 8.09 | 0.021 |
| Error | 8 | 12.53 | 1.57 | | |

**Table 5 | ANOVA results on the total number of morphotypes from both the competitor genotype and the focal genotype (time-averaged for days 0–10).**

| Factor | df | SS | MS | *F* | *p* |
|---|---|---|---|---|---|
| Time | 1 | 0.23 | 0.23 | 0.08 | 0.785 |
| Phage | 1 | 1.13 | 1.13 | 0.40 | 0.545 |
| Time × phage | 1 | 0.76 | 0.76 | 0.27 | 0.617 |
| Error | 8 | 22.38 | 2.80 | | |

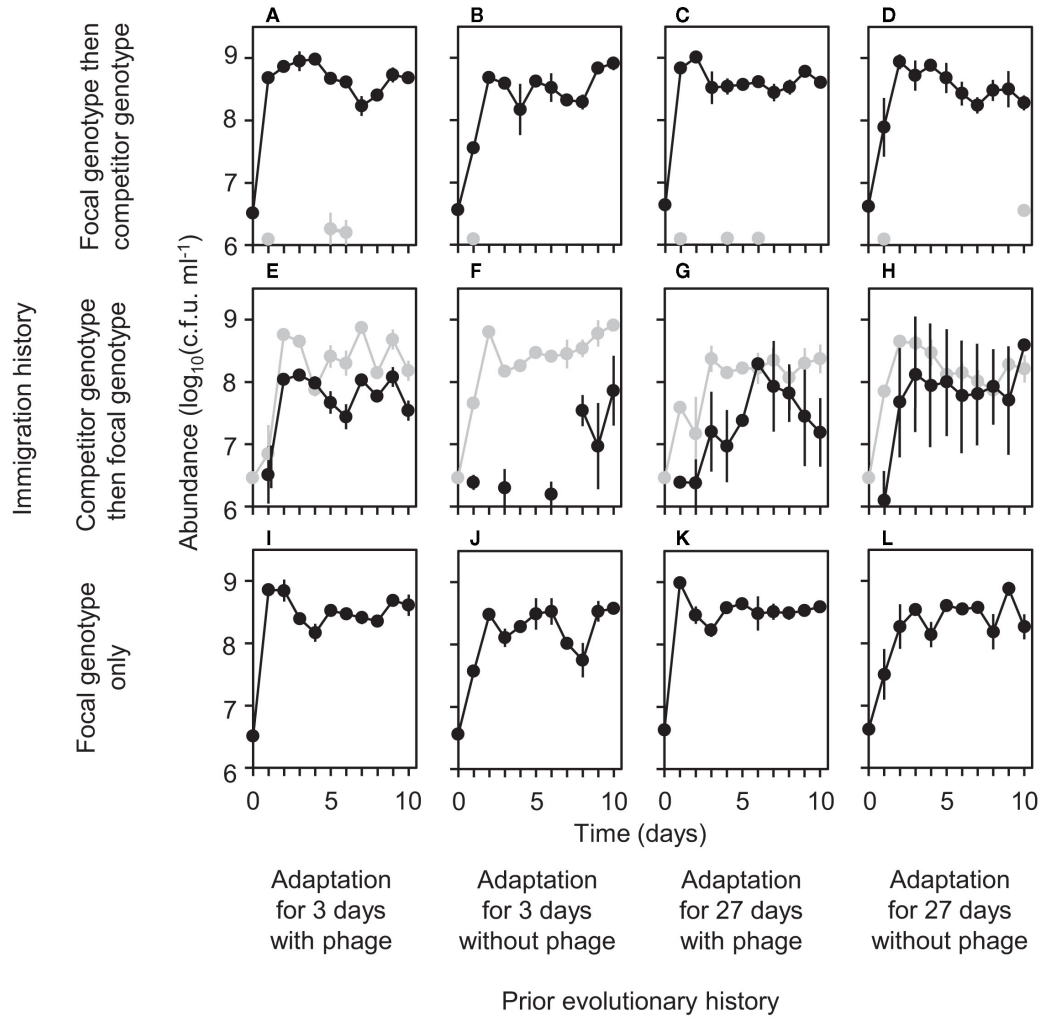


**FIGURE 3 | Effects of prior evolutionary history and immigration history on bacterial abundance.** Black lines indicate focal genotype and gray lines indicate competitor genotype. Each panel **(A–L)** shows mean abundance [$\log_{10}$(cfu ml$^{-1}$)] ± SEM ($n = 3$) for the corresponding treatment of immigration history and prior evolutionary history.

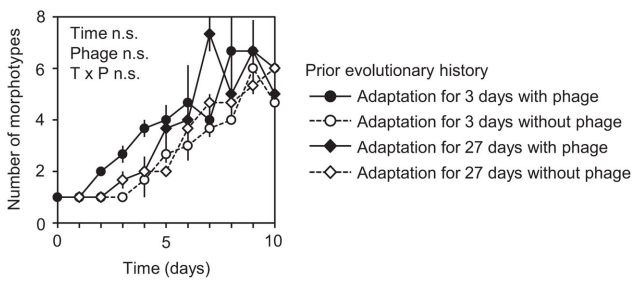


**FIGURE 4 | Temporal change in total number of morphotypes after the competitor genotype was introduced on day 0 and the focal genotype on day 1.** Data show mean number of morphotypes (those derived from focal and competitor genotypes combined) ± SEM ($n = 3$). Two-way ANOVA results are indicated as in **Figure 1** (see **Table 5**).

The mechanisms indicated in our experiment may also explain some of the variation in the extent of diversification in other systems of micro- and macro-organisms, particularly those involving discrete, replicated habitats such as islands, lakes, and mountaintops. Some of the evolutionary mechanisms that influence bacteria, including lateral gene transfer, uptake of foreign DNA from the environment, mobile DNA elements, recombination machinery, and high mutation rates, may make their diversification different from that of macro-organisms. Nevertheless, evolutionary priority effects similar to those we found in *P. fluorescens* may explain variation in diversification in macro-organisms as well. Possible examples include Macaronesian plants (Silvertown, 2004), African lake cichlids (Seehausen, 2007), and Hawaiian *Tetragnatha* spiders (Gillespie, 2004). Further, in some systems where the extent of diversification varies considerably, e.g., Caribbean *Anolis* lizards (Calsbeek and Cox, 2010), mainland source populations appear to have evolved in

the presence of exploiters (predators), whereas islands where founders immigrated and diversified differ in the occurrence of exploiters, indicating the potential for exploiters to affect diversification.

## CONCLUSION

Historical contingency undermines our ability to explain community assembly because it is difficult to ascertain past events in detail (Gould, 1989; Schluter, 2000; Gavrilets and Losos, 2009; Fukami, 2010). Not all aspects of history are equally complicated, however. In many communities of both micro- and macro-organisms, immigration history is not possible to infer, but adaptation history often is through the combined use of geological, biogeographical, and phylogenetic information (Emerson and Gillespie, 2008; Losos and Ricklefs, 2009; Glor, 2010). Thus, if the hypothesis we propose here is correct, it means that the evolutionary importance of historical events that cannot be reconstructed (i.e., subtle differences in early immigration history) may nevertheless be possible to predict by analyzing more tractable aspects of history (i.e., immigrants' prior adaptation history). For this reason, we believe it will be worthwhile to evaluate the generality and mechanistic bases of our hypothesis in greater detail to gain an improved understanding of immigration, diversification, and community assembly.

## ACKNOWLEDGMENTS

We thank Julia Borden, Benjamin Callahan, Whitney Hoehn, Nathan Kim, Christine Kyauk, Katrina Luna, Sharia Mayfield, and Aaron Wacholder for laboratory assistance. We thank Paul Rainey and Xue-Xian Zhang for providing us with *P. fluorescens* SBW25 (wild type) and *P. fluorescens* SBW25 *lacZ*, and Angus Buckling for providing us with phage Φ2. We thank Bertus Beaumont, Benjamin Callahan, Sinead Collins, Rodolfo Dirzo, James Estes, Diana Nemergut, Paul Rainey, Uli Stingl, John Thompson, Susannah Tringe, and David Wardle for comments. Financial support was provided by Stanford University.

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

*Received: 21 September 2011; accepted: 21 December 2011; published online: 09 January 2012.*

*Citation: Knope ML, Forde SE and Fukami T (2012) Evolutionary history, immigration history, and the extent of diversification in community assembly. Front. Microbio.* ***2****:273. doi: 10.3389/fmicb.2011.00273*
*This article was submitted to Frontiers in Evolutionary and Genomic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 24 August 2012
doi: 10.3389/fmicb.2012.00309

# Microbial biogeography of arctic streams: exploring influences of lithology and habitat

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Terminal restriction fragment length polymorphism and 16S rRNA gene sequencing were used to explore the community composition of bacterial communities in biofilms on sediments (epipssamon) and rocks (epilithon) in stream reaches that drain watersheds with contrasting lithologies in the Noatak National Preserve, Alaska. Bacterial community composition varied primarily by stream habitat and secondarily by lithology. Positive correlations were detected between bacterial community structure and nutrients, base cations, and dissolved organic carbon. Our results showed significant differences at the stream habitat, between epipssamon and epilithon bacterial communities, which we expected. Our results also showed significant differences at the landscape scale that could be related to different lithologies and associated stream biogeochemistry. These results provide insight into the bacterial community composition of little known and pristine arctic stream ecosystems and illustrate how differences in the lithology, soils, and vegetation community of the terrestrial environment interact to influence stream bacterial taxonomic richness and composition.

**Keywords: arctic streams, bacterial community composition, microbial community composition, lithology, T-RFLP, 16S rRNA gene sequencing**

## INTRODUCTION

The underlying lithology of watersheds controls the physical structure of landscapes, but also influences their biology by controlling the chemistry of soils (Jenny, 1980), plants (Whittaker, 1960), and water (Hynes, 1975). The composition of bacterial communities may also be sensitive to factors controlled by underlying lithology, particularly in arctic stream ecosystems where water chemistry has been shown to influence the composition of epiphyte and macroinvertebrate communities (Slavik et al., 2004). The few studies that are available demonstrate environmental controls on the variability in stream bacterial communities related to stream water pH (Fierer et al., 2007), organic matter sources (Van Hannen et al., 1999), and available nutrients such as the dissolved forms of organic carbon and inorganic nitrogen and phosphorus (Findlay and Sinsabaugh, 2006). These types of studies guide our understanding of first order controls on community composition, but we have yet to identify whether microbial composition in streams is linked to overarching, watershed controls such as lithology.

Sessile bacteria are ecologically important members of the biota in streams and other aquatic environments. Microbial community composition in aquatic ecosystems are responsive to many different chemical and biological factors including physical variables (i.e., temperature variations, climate, topography, and light availability; Kaplan and Bott, 1989; Autio, 1998) and biogeochemical variables (i.e., the quality and quantity of carbon sources, inorganic nutrients, and electron acceptors; Drever, 2002; Crump et al., 2003; Eiler et al., 2003). Recent studies have also shown that bacterial communities within biofilms are important regulators of stream biogeochemical functions (Sobczak and Findlay, 2002; Hall et al., 2009) and have the potential to generate unique biogeochemical signatures across stream types.

These previous studies suggest, therefore, that the microbial community composition in streams could be influenced by biogeochemical cues from the local landscape but could in turn strongly influence the biogeochemical characteristics observed in streams. It is likely that both of these alternatives interact to varying degrees in different ecosystems, but it would be difficult to test these alternatives in a field study. Nevertheless, few studies have investigated the effect of combined lithological and biogeochemical differences on attached microbial community structure between streams (Takai et al., 2003; Skidmore et al., 2005; Oline, 2006) or the effect of habitat differences within streams (Hullar et al., 2006). This study is targeted to a unique environment where lithology differs over a small-scale and is likely to have a strong influence on the community structure of sessile bacteria, providing the opportunity to detect biogeographical patterns.

We hypothesized that fundamentally different lithologies that support significantly different vegetation communities on land also support significantly different bacterial communities in streams by imparting different biogeochemical characteristics to water, which could be further modified by the lithology-specific bacterial communities. Our objectives were: (1) compare stream bacterial community composition among streams emerging from the three distinctly different lithologies (non-carbonate, NC; complex sedimentary, CS; and ultramafic, UM) that dominate the Noatak National Preserve region of arctic Alaska; (2) compare stream bacterial community composition among two different stream habitat types (sediment vs. epilithon); and (3) determine if

community composition patterns correlate with the biogeochemical characteristics of streams for the three different lithologies. While we expected to find differences at the microhabitat scale due to variations in resources (e.g., light availability and organic matter sources) and hydrologic stressors, we were most interested in detecting differences across lithologies, which may suggest a sensitivity of microbial community composition at the broader, landscape scale. This work adds to a small but growing base of knowledge about microbial biogeography in arctic ecosystems that are currently responding to a rapidly changing climate (Crump et al., 2003, 2007; Skidmore et al., 2005; Galand et al., 2006; Garneau et al., 2006).

## MATERIALS AND METHODS

The Noatak River is in the Noatak National Preserve in Alaska (USA). It is the longest continuous river in the USA National Wild and Scenic Rivers system and the largest mountain-ringed river basin in North America, virtually unaltered by direct and indirect (e.g., nitrogen deposition) human activity (Milner et al., 2005). The lithology of this area is complex (Jorgenson et al., 2002) but includes three important and distinctly different types that were the focus of this study. Ultramafic rocks (basalt, gabbro, peridotite, pyroxenite, dunite) of the Siniktanneyak mountains tend to be high in iron and magnesium with sparse vegetation. Non-carbonate rocks (glaciolacustrine deposits, conglomerate, sandstone, shales) of the Avingyak Hills support acidic, organic-rich soils, and host shrub birch, willow and ericaceous plants. Complex sedimentary rocks (shale, basalt, limestone, and mafic rocks) of the Aniuk mountains support vegetation similar to the non-carbonate lithology. Jorgenson et al. (2002) found that the composition of plant communities differed by lithology, possibly due to variations in soil pH and phytotoxic effects of soluble minerals. Jorgenson et al. (2009) showed NC soils contain higher available soil phosphorus compared to UM soils, suggesting an interaction between vegetation and soils that may influence the nutrient content of soil water that subsequently enters streams.

Samples were collected between July 8 and July 13, 2006 from headwater stream tributaries arising from catchments with uniform and contrasting lithologies. We sampled 15 streams located in the foothills of the Delong Mountains on the northern edge of the Noatak River basin in the vicinity of Feniak Lake (68°14′56.55″N and 158°19′19.90″W, elevation 1,411 feet). Terminal Restriction Fragment Length Polymorphism (T-RFLP) analyses were performed on all streams and 16S clone libraries were constructed from both sediment and epilithon samples from five sites (**Figure 1**).

Replicate first or second-order stream reaches were sampled within each separate lithology: four non-carbonate, five ultramafic, and six complex sedimentary (**Figure 1**). Sediment samples were collected in triplicate along a 25-m reach of each stream. Single epilithon (rock scrub) samples were also collected from cobble-bottom streams, but only from ultramafic and non-carbonate lithologies. Stream water chemistry sampling of the water column took place at the same time as microbial sampling and analysis details are provided in Flinn et al. (2009). Stream biogeochemical variables mentioned in this paper are defined as $NO_3^-$ (nitrate); TDN (total dissolved nitrogen); TDP (total dissolved phosphorus);

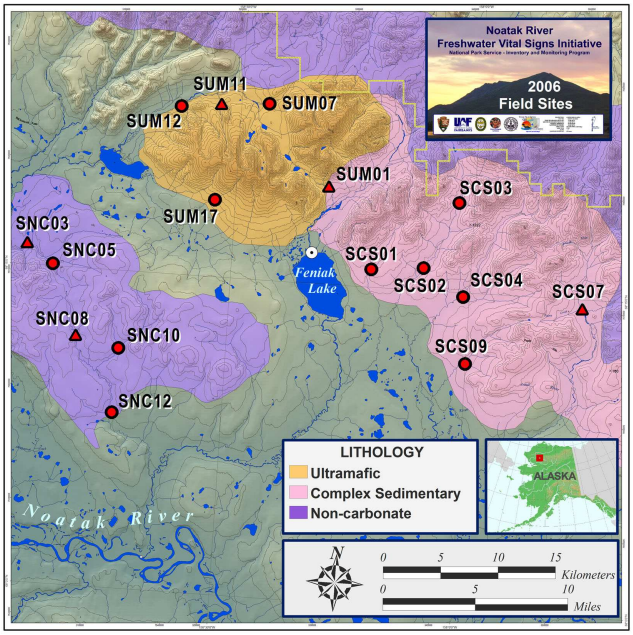


**FIGURE 1 | Study area of Feniak Lake region with stream site locations across contrasting lithologies in the Noatak National Preserve, Alaska (Map credit: Andrew Balser).** T-RFLP analyses were performed on sediment samples at all sites and epilithon samples at a subset of sites. 16S clone libraries were built from sediment and epilithon samples from sites indicated with triangles.

TDN-$NO_3^-$ (total dissolved nitrogen minus nitrate = indication of dissolved organic nitrogen, DON); $NO_3^-$/TDN (nitrate as a proportion of total dissolved nitrogen); TDN/TDP (ratio of total dissolved nitrogen to total dissolved phosphorus); DOC (dissolved organic carbon); and cations (calcium, $Ca^{2+}$; magnesium, $Mg^{2+}$; potassium, $K^+$; and sodium, $Na^+$).

Surface (~3 cm deep) sediment samples for microbial analysis were collected in sterile 15-ml plastic tubes, preserved immediately with sucrose lysis buffer [SLB; 20 mM EDTA, 400 mM NaCl, 0.7 M sucrose, 50 mM Tris (pH 9.0)] in a 1:1 ratio, frozen on dry ice in the field, and stored at −80°C in the laboratory until analysis. Epilithic material from the tops of six submerged rocks in riffle sections of each stream reach was scrubbed with a nylon brush, rinsed into a sterile plastic container with filtered (0.22 μm) stream water, combined, and collected in a 0.22-μm Sterivex filter capsule (Millipore). Filter capsules were removed from syringes, flooded with 1 ml DNA extraction buffer [100 mM Tris (pH 8), 100 mM NaEDTA (pH 8), 100 mM phosphate buffer (pH 8), 1.5 M NaCl, 1% CTAB], frozen immediately on dry ice in the field, and stored at −80°C in the laboratory until analysis.

DNA extractions used the MoBio Power Soil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's protocol with the following modification: a FastPrep Homogenizer and Isolation System (Thermo Fisher Scientific Waltham, MA, USA) was used to shake the MoBio extraction tubes at 4.5 m/s for 30 s to ensure complete cell lysis of bacteria. Extractions were conducted on 500-μl subsamples of streambed

sediment (1:1 sediment:SLB slurry), and on filters of the rock biofilm samples that were removed from the Sterivex capsules.

Terminal restriction fragment length polymorphism analysis was conducted on all sediment (42 total) and epilithon samples (eight total). The 16S rRNA gene was amplified using Illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Piscataway, NJ, USA), 2 μl of DNA, and the following primers: HEX-labeled primer Bac8f and unlabeled primer Univ1492r (Reysenbach and Pace, 1995) obtained from Sigma-Genosys (St. Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA), respectively. The PCR protocol consisted of an initial denaturation at 94˚C for 4 min, followed by 94˚C for 45 s, 54˚C for 20 s, and 72˚C for 2.5 min for 40 cycles, and a final 4 min at 72˚C. Two separate PCR reactions for each DNA sample were pooled and digested separately in triplicate with three restriction enzymes: *Msp*I, *Alu*I, and *Hin*P1I (New England BioLabs, Beverly, MA, USA). Restriction digests (25 μl) consisted of 10 μl PCR product, 1 unit enzyme, 2 μl of 10× reaction buffer 2 (New England BioLabs), and sterile Sigma water (Sigma-Aldrich). PCR products were digested overnight at 37˚C. Fluorescently labeled terminal restriction fragments (T-RFs) were size separated on an ABI Avant Genetic Analyzer 3100 (Applied Biosystems, Foster City, CA, USA) using an internal size standard (BioVentures MapMarker 1000, BioVentures, Inc., Murfreesboro, TN, USA).

Terminal restriction fragment length polymorphism electropherograms were analyzed using GeneMapper software version 3.7 (Applied Biosystems, Foster City, CA, USA). T-RF peaks that differed by less than 0.5 bp were grouped (Dunbar et al., 2001), and peaks > 80 bp with >50 relative fluorescent units were included in the analysis. Triplicate profiles were collapsed into one average profile by including peaks that occurred in two of the three replicate profiles in order to eliminate false peaks arising from dust or bubbles present in the capillaries of the detector. T-RFs of different lengths represent distinct operational taxonomic units (OTUs) but should not be interpreted as specific bacterial species because similar restriction fragment sizes can be produced from different organisms (Liu et al., 1997).

Terminal restriction fragment length polymorphism statistical analyses were performed in DECODA (Database for Ecological Community Data) version 3 (Minchin, 1990). Non-metric multidimensional scaling (NMS; Clarke, 1993) was used for ordination of the T-RFLP data using T-RF length and normalized peak height from all three restriction enzymes as input data. Similarities between samples were based on the Bray–Curtis dissimilarity matrix (Bray and Curtis, 1957), which has been recommended (Rees et al., 2004) and commonly used for T-RFLP data (Denaro et al., 2005; Deslippe et al., 2005; Fierer et al., 2007). Analysis of Similarity (ANOSIM; Clarke and Green, 1988; Clarke, 1993) was used to determine which samples were most closely related with patterns of similarity between bacterial communities using the Gower metric (Gower, 1971). Vector analysis was used to examine correlations between microbial community patterns and corresponding environmental data.

Representative samples with the highest degree of variation using T-RFLP were chosen for more detailed phylogenetic analyses. Clone libraries were prepared with the PCR-amplified 16S rRNA gene from sediment ($n = 5$) and epilithon ($n = 4$) from representative streams within the non-carbonate (two streams), ultramafic (two streams), and complex sedimentary lithologies (one stream, sediment only). The 16S rRNA gene was amplified using primers Bac8f (unlabeled) and Univ1492r (Invitrogen) with the protocol: 94˚C for 2 min, followed by 40 cycles of 94˚C for 30 s, 54˚C for 20 s, and 72˚C for 1.5 min with a final extension of 15 min at 72˚C. To minimize the effects of PCR drift, PCRs were run in triplicate and pooled for each DNA extract. PCR products were run on 0.75% agarose gels, visualized with ethidium bromide, excised with a sterile razor blade, purified with Zymoclean Gel DNA Recovery kit (Zymo Research, Orange, CA, USA), cloned into pCR®2.1 vector using the TA cloning kit (Invitrogen), and transformed into OneShot® Competent Cells (Invitrogen). Transformants were plated on Luria broth (LB) agar medium containing ampicillin, X-gal, and isopropyl-β-D-thiogalactopyranoside (IPTG). Ampicillin-resistant and β-galactosidase-negative clones were randomly selected and grown overnight at 37˚C in LB with ampicillin. Clones were tested for the presence of the 16S rRNA gene inserts by PCR amplification using modified M13 primers: M13Long Forward (5′-CAGGAAACAGCTATGACCATGATTAC-3′) and M13Long Reverse (5′-GTAAAACGACGGCCAGTGAATTGT-3′) designed to the pCR®2.1 vector. An excess of 100 clones for each sample were sequenced using the M13 primer listed above as well as internal 16S primers custom designed for specific clone groups in this study to ensure complete overlap of sequence reads in both directions: 16S-A1F (5′-GTGCCAGCAGCCGCGGTAATAC-3′); 16S-A1R (5′-GTATTACCGCGGCTGCTGGCAC-3′); 16S-B1F (5′-GGTGCTGCATGGCTGTCGTCAGC-3′); 16S-B1R (5′-GCTGACGACAGCCATGCAGCACC-3′); 16S-B2F (5′-GGTGGTGCATGGTTGTCGTCAGC-3′); and16S-B2R (5′-GCTGACGACAACCATGCACCACC-3′). Clones from SedSNC03 were sequenced using the following protocol: 96˚C for 1 min, followed by 25 cycles of 96˚C for 10 s, 50˚C for 5 s, and 60˚C for 4 min. Ready to load sequence reactions were run at Vermont Cancer Center, University of Vermont on an ABI Avant Genetic Analyzer 3100 (Applied Biosystems). PCR products of the other eight clone libraries were cleaned and sent to Agencourt Bioscience Corporation (Beverly, MA, USA) for sequencing.

Clone sequences were assembled and edited using Sequencher version 4.6 (Gene Codes, Ann Arbor, MI, USA) aligned using the Ribosomal Database Project II (RDP) release 9.58 web resource[1] (Brown, 2000; Gutell et al., 2002; Cole et al., 2007), and checked for chimeric sequences using the RDP's CHIMERA_CHECK program based on the Pintail algorithm (Ashelford et al., 2005), Chimera Slayer in the Mothur software package (Schloss et al., 2009), and through manual inspection of aligned sequences in the software package ARB (Ludwig et al., 2004). Twenty-seven chimeric sequences were removed. Sequences were then classified with the RDP Classifier (Wang et al., 2007) and 123 chloroplast sequences (mainly from diatoms) and 11 mitochondria sequences were removed. The remaining 625 sequences were clustered into OTUs (97% sequence similarity) using uclust in the QIIME software package (Caporaso et al., 2010). Sequences have been submitted to GenBank under accession numbers FJ849067–FJ849648.

[1] http://rdp.cme.msu.edu/

**Table 1 | Values of biological, chemical, and physical parameters for study streams by lithology (CS, Complex Sedimentary; NC, Non-carbonate; UM, Ultramafic).**

| Parameters | Lithology | | |
|---|---|---|---|
| | CS ± 1 SE | NC ± 1 SE | UM ± 1 SE |
| Conductivity (μS/cm) | 502.2 ± 140.2 | 168.7 ± 26.1 | 38.8 ± 4.4 |
| pH | 8.8 ± 0.6 | 7.5 ± 0.1 | 7.3 ± 0.1 |
| Temperature (°C) | 7.7 ± 0.2 | 8.5 ± 1.2 | 7.9 ± 0.3 |
| DO (mg/l) | 10.9 ± 0.3 | 10.9 ± 0.7 | 10.9 ± 0.2 |
| **METALS (μg/l)** | | | |
| [Cu] | 0.5 ± 0.04 | 1.5 ± 0.1 | 0.5 ± 0.01 |
| [Al] | 92.3 ± 1.4 | 102.1 ± 1.8 | 95.7 ± 2.7 |
| [Fe] | 12.3 ± 1.1 | 42.3 ± 6.8 | 33.0 ± 10.4 |
| [Si] | 1703 ± 73 | 3176 ± 608 | 2278 ± 134 |
| **CATIONS (mg/l)** | | | |
| $[Ca^{2+}]$ | 38.1 ± 9.0 | 14.0 ± 2.4 | 3.8 ± 0.8 |
| $[Mg^{2+}]$ | 27.4 ± 4.9 | 9.1 ± 1.8 | 1.9 ± 0.6 |
| $[K^{+}]$ | 1.1 ± 0.1 | 0.9 ± 0.02 | 0.9 ± 0.01 |
| $[Na^{+}]$ | 5.1 ± 1.5 | 1.6 ± 0.01 | 1.3 ± 0.01 |
| **NUTRIENTS (μM)** | | | |
| [TDN] | 7.7 ± 0.7 | 17.9 ± 2.6 | 7.3 ± 1.2 |
| $[NO_3^-]$ | 2.8 ± 0.9 | 1.1 ± 0.1 | 4.3 ± 1.4 |
| $[TDN\text{-}NO_3^-] = DON$ | 5.1 ± 1.0 | 16.8 ± 2.6 | 3.0 ± 0.8 |
| $[NO_3^-/TDN]$ | 0.3 ± 0.1 | 0.1 ± 0.01 | 0.6 ± 0.1 |
| [TDP] | 0.2 ± 0.01 | 0.1 ± 0.01 | 0.2 ± 0.02 |
| [TDN/TDP] | 54.8 ± 5.9 | 128.6 ± 19.0 | 53.8 ± 11.4 |
| **BASAL RESOURCES** | | | |
| DOC (mg/l) | 2.8 ± 0.2 | 8.3 ± 0.9 | 2.1 ± 0.2 |
| Benthic Chl-*a* ($\mu g/cm^2$) | 0.3 ± 0.3 | 0.8 ± 1.1 | 0.2 ± 0.1 |

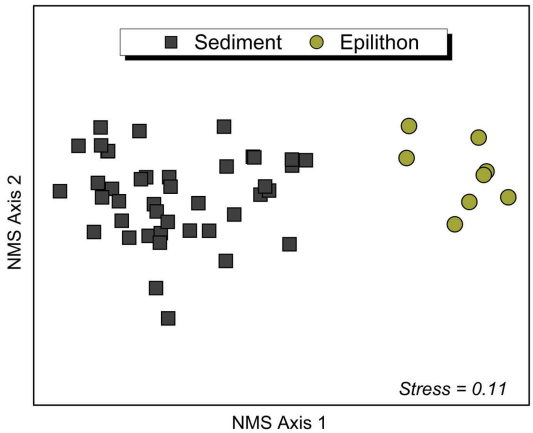


**FIGURE 2 | Non-metric multidimensional scaling (NMS) ordination (first and second of the 3-dimensional solution) of stream sediment bacterial communities (left side, $n = 42$) and epilithon bacterial communities (right side, $n = 8$) based on pairwise similarity estimates (Bray–Curtis).** Points that are close together represent communities with similar bacterial community composition based on the T-RFLP method. The associated normal stress value of the ordination is 0.11, indicating a good approximation of the overall structure of the data in multivariable space.

## RESULTS

In general, study streams were typical ultraoligotrophic foothill tundra and mountain streams. Mountain streams were dominant in the UM lithology, characterized by mountain runoff, unstable substrate, scoured channels, and sparse biota. Tundra streams were dominant in the NC lithology, characterized by tundra runoff, organic peat-lined banks, and moderate biota. All streams were similar in physical structure and several basic water quality parameters including pH (7.3–7.8) and water temperature ranges (5.5–12°C; **Table 1**). Complex sedimentary streams had high electrical conductivity ($502 \pm 140\ \mu S/cm^2$) compared to UM and NC streams ($40 \pm 5$ and $168 \pm 26\ \mu S/cm^2$, respectively). Non-carbonate streams had higher concentrations of some metals (e.g., Al, Fe, and Si), DOC, and TDN. Ultramafic streams had higher concentrations of $NO_3^-$ compared to NC streams resulting in nearly a 10-fold higher proportion of $NO_3^-$ to TDN ($NO_3^-$/TDN).

Non-metric multidimensional scaling and associated ANOSIM analysis of T-RFLP fragments confirmed differences in bacterial community composition between lithology and habitat. The composition of bacterial communities was significantly different between sediment and epilithon habitats (ANOSIM Global $R = 0.98$; $P < 0.0001$), with communities in each habitat clustering together regardless of the lithology of the catchment (**Figure 2**).

The number of phylotypes (i.e., restriction fragments or T-RFs) in sediment samples ranged from 19 to 69 (mean: 51) with an average of 51 for CS, 54 for NC, and 50 for UM. The number of T-RFs in rock biofilm epilithon samples was higher and ranged from 66 to 99 (mean: 79) with an average of 72 for CS, 86 for NC, and 75 for UM.

Bacterial communities in the 42 sediment samples clustered by catchment lithology (ANOSIM Global $R = 0.40$; $P < 0.001$; **Figure 3**). Pairwise comparisons indicate that sediment communities in ultramafic UM streams were significantly different from those in NC and CS streams (UM vs. CS: $R = 0.50$, $P < 0.0001$; UM vs. NC: $R = 0.55$, $P < 0.001$; and NC vs. CS: $R = 0.06$, $P = 0.2$). Vector analysis indicated that DOC, base cations, TDN/TDP, TDN-$NO_3^-$ (DON), and the proportion of $NO_3^-$/TDN were positively correlated with Axis 1 NMS sites scores (Bonferroni-corrected $P > 0.05$ in all cases; **Figure 3**). The UM and NC communities are separated along Axis 1 of the NMS, suggesting that the key biogeochemical differences (e.g., DOC, TDN/TDP, and TDN-$NO_3^-$) may be driving these differences in community composition. Though not shown here, we also detected differences (ANOSIM Global $R = 0.415$, $P = 0.048$) by lithology with the limited epilithon samples ($n = 2$–4 per lithology).

Samples with the greatest difference in microbial communities based on T-RFLP patterns were selected for 16S rRNA gene cloning and sequencing from each of the three lithologies (two NC, two UM, and one CS; indicated on **Figure 1**). Epilithon samples were not analyzed from CS streams because after analyzing one CS sediment library we decided to compare the two most starkly contrasting lithologies (NC and UM). The number of clones for each of these nine libraries ranged from 33 to 87. Sequences were nearly full length extending from *Escherichia coli* positions 28–1491.

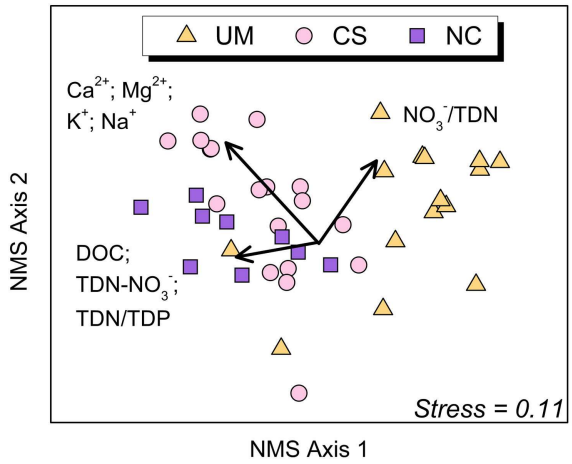


**FIGURE 3 | Non-metric multidimensional scaling (NMS) ordination (first and second of the 3-dimensional solution) of stream sediment bacterial communities ($n = 42$) based on pairwise similarity estimates (Bray–Curtis).** Points that are close together represent communities with similar bacterial community composition based on the T-RFLP method. Groupings show significant (ANOSIM Global $R = 0.40$; $P < 0.001$) separation by lithology (orange triangles = Ultramafic (UM); pink circles = Complex Sedimentary (CS); purple squares = Non-carbonate (NC). The associated normal stress value of the ordination is 0.11, indicating a good approximation of the overall structure of the data in multivariable space. Significant ($P < 0.005$), Bonferonni adjusted biogeochemical variables were overlaid (arrows) showing the degree of correlation with sediment data. Abbreviations: TDN-$NO_3^-$ (total dissolved nitrogen minus nitrate, an indication of dissolved organic nitrogen); TDN/TDP (ratio of total dissolved nitrogen to total dissolved phosphorus); DOC (dissolved organic carbon); Base Cations = $Ca^{2+}$, $Mg^{2+}$, $K^+$, and $Na^+$; and $NO_3^-$/TDN (ratio of nitrate to total dissolved nitrogen).

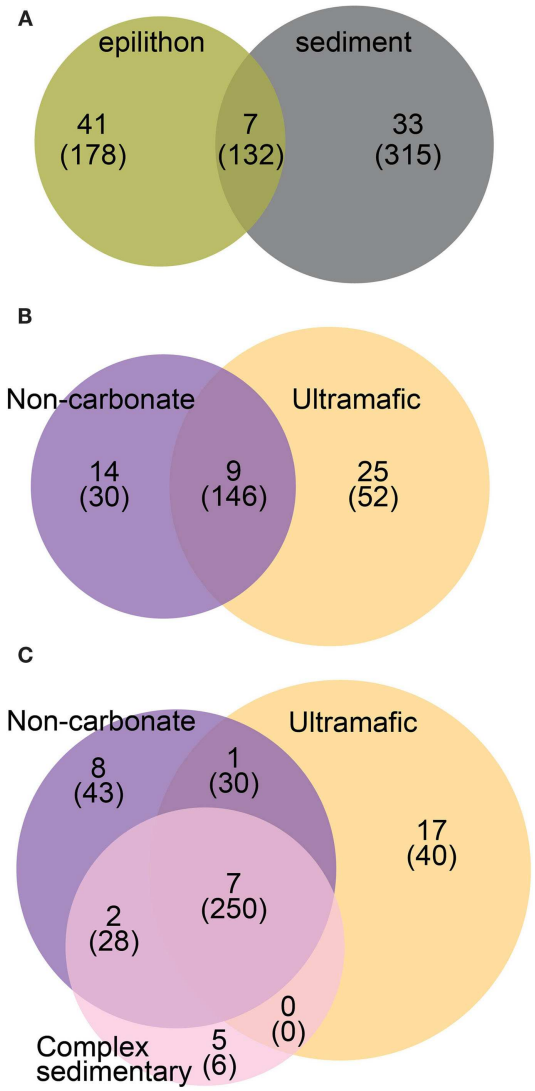


**FIGURE 4 | Venn diagrams representing the overlap between samples at the following levels: (A) All OTUs grouped by habitat; (B) OTUs from epilithon samples grouped by lithology, and (C) OTUs from sediment samples grouped by lithology.** Numbers represent OTUs and parenthetical numbers represent sequences.

Of the 81 bacterial OTUs identified in all clone libraries, most were only found in one of the two habitats (**Figure 4A**). There were seven, nine, and seven cosmopolitan OTUs respectively, in sediment vs. epilithon samples (**Figure 4A**), epilithon samples by lithology (**Figure 4B**), and sediment samples by lithology (**Figure 4C**). There were fewer OTUs that were specific to a lithology while there were more that were specific to stream habitat type. The phylogeny of clone library sequences was different between sediment and epilithon samples (**Figure 5**). Sediment communities were dominated by Gammaproteobacteria (55%) belonging to several different orders, and Firmicutes in the order Bacillales (35%). In contrast, epilithon communities were dominated by Bacteroidetes (41%), Betaproteobacteria (31%), and Cyanobacteria (14%). These communities were consistent within habitat types, except that epilithon in UM streams supported a larger fraction of Deinococci, and sediments in ultramafic streams included Alphaproteobacteria in the order Rhizobiales. All of the taxa detected in the sediment and epilithon samples are widely distributed in the environment, including soil, sediments, seawater, and freshwater.

## DISCUSSION

We hypothesized that inherent lithological characteristics of the Noatak National Preserve would impart a unique chemical composition to stream water, which in turn would introduce differences in bacterial community composition among NC, UM, and CS streams. Streams sampled were geomorphically similar in order, size, slope, and dominant benthic substrate type (cobble and gravel), yet the bacterial communities in these streams differed systematically according to biogeochemical characteristics of the stream water (e.g., DOC, nutrients, and base cations), which is likely a reflection of material inputs from the surrounding lithology and subsequent in-stream modification by biological activity.

Not surprisingly, bacterial communities in stream beds differed primarily by habitat type within streams (sediment vs. epilithon), and secondarily by the lithology of the upstream catchment. This

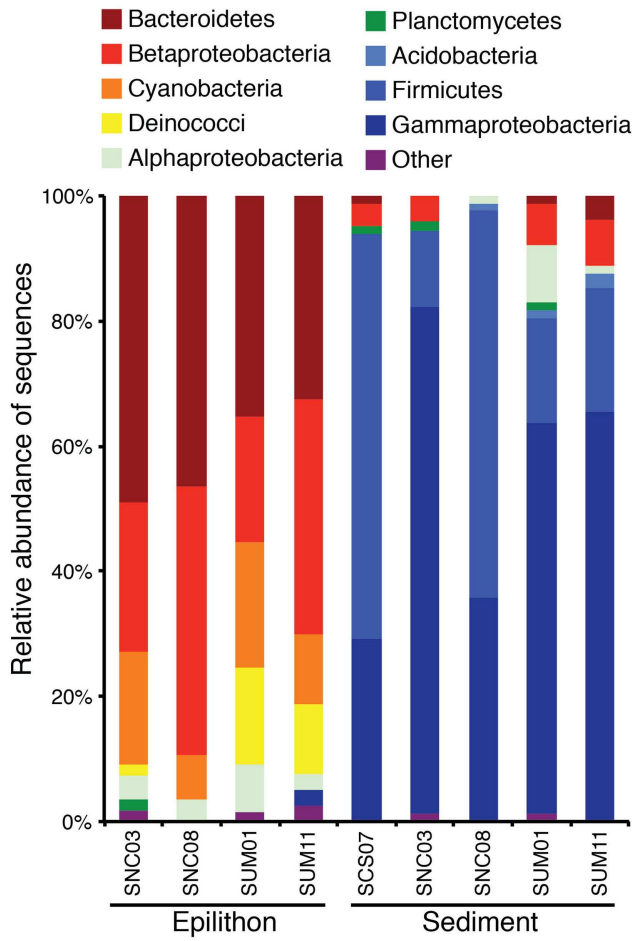


**FIGURE 5 | Phylogenetic composition of clone sequences from stream epilithon and sediment samples in the non-carbonate (SNC), complex sedimentary (SCS), and ultramafic (SUM) lithologies expressed as relative abundance within each library.**

latter observation suggests that the basic lithology of terrestrial environments influences not just the physical and aqueous biogeochemical structure of the environment, but the community composition of microorganisms that inhabit these environments. Sediment communities clustered by lithology with a high degree of separation between UM and NC communities and only partial separation between NC and CS. These trends mirror stream biogeochemistry across the three lithologies in that UM and NC has significantly different biogeochemical characteristics, while the NC and CS are similar. These results add stream sediment and epilithon to a small but growing list of habitats in which lithology of parent material correlates with microbial community composition. This list includes soils (Dunbar et al., 2000; Oline, 2006), glaciers (Skidmore et al., 2005), groundwater, and substratum (Takai et al., 2003).

Vector analysis indicates that certain biogeochemical variables explain NMS ordination of T-RFLP data (**Figure 3**), although causal relationships can only be inferred. Specifically, we observed a positive correlation between base cations and sediment communities from streams with CS lithology, suggesting bacterial community structure may be influenced by the high base cation concentration in CS streams, whereas the scarcity of cations in NC and UM streams may alter the composition of resident bacterial communities. Notably, calcium and magnesium are well known to enhance bacterial adhesion to substrates within the exopolysaccharide matrix of biofilms (Geesey et al., 2000), which may facilitate a niche for a more stable bacterial community in the CS lithology.

Other constituents such as DOC, TDN-$NO_3^-$ (dissolved organic nitrogen), and TDN/TDP were found in highest concentrations in the NC streams, and correlated with NC community composition. Also, the percent proportion $NO_3^-$ of TDN was highest in UM streams, and was positively correlated with Axis 1 and 2 (UM community, **Figure 3**). DOC and TDN were lowest in streams of the CS and UM lithologies and highest in streams of the NC lithology. These trends further suggest that bacterial community composition may be influenced by the abundance or scarcity of resources, a likely consequence of surrounding lithology, soil type, and vegetation cover. Jorgenson et al. (2009) showed NC soils contain higher available soil phosphorus compared to UM soils, suggesting an interaction between vegetation and soils that may influence the nutrient content of soil water to streams in the Noatak region. The NC lithology supports relatively productive vegetation on land, which potentially correlates with higher available phosphorus in soil waters entering the NC streams; this would lead to increased uptake of $NO_3^-$, reduced stream water $NO_3^-$ content, and higher apparent productivity. Conversely, the lack of vegetation in the barren UM lithology may be an indication of the extreme phosphorus limitation of UM streams (indicated by the higher nitrogen concentrations) perhaps due to reduced phosphorus delivery to streams. These streams, in particular the UM streams, are phosphorus limited as are many streams on the North Slope of Alaska (Peterson et al., 1993; Bowden et al., 1994). This important landscape control appears to impart a biogeochemical fingerprint to waterways that shape the resident microbial communities within the streams bounded by unique lithologies.

Higher order controls on microbial community composition such as landscape and climate have been observed in studies that investigated the influence of microbial activity on redox chemistry and mineral processes in natural environments (Nealson and Stahl, 1997; Ehrlich, 1998). Furthermore, other studies have determined that microbial community composition can be correlated with observed aqueous geochemistry in subglacial chemical weathering (Skidmore et al., 2005), stream conductivity, and hydrology (Zeglin et al., 2011), stream water pH, quality of fine benthic organic matter, and quantity of DOC and nitrogen in stream water (Fierer et al., 2007), seasonal changes in temperature, nutrient availability, and light in estuarine biofilms (Moss et al., 2006), and flow heterogeneity (Singer et al., 2010). This study adds to the limited body of work demonstrating landscape influences on microbial community composition.

Clone libraries of 16S rRNA gene sequences confirmed the results of T-RFLP analysis concerning the discrimination of community composition by habitat and lithology and permitted the identification of bacterial taxonomic composition at the stream reach and landscape scale. **Figure 5** shows that at the phylum and class levels, microbial communities are unique to each habitat type,

but show substantial overlap across the two contrasting lithologies within habitat type. Thus, differences in community composition are more pronounced at the habitat scale than at the lithology scale. This difference was also reflected in the average number of phylotypes (T-RFs), which were greater in epilithon than sediment indicating a difference in taxonomic richness at the habitat scale. This result is likely due to the heterotrophic nature of the sediment environment and autotrophic nature of the epilithon environment. Differences between sediment and epilithon communities could also result from different hydrologic stressors. For example, varying flow regimes alter sediment structure via erosion and the redistribution of bacteria, exposing them to different environmental conditions (Hullar et al., 2006). While the epilithic community is not as likely to experience the same degree of disturbance as that found in the sediment, differences in hydrodynamic conditions are known to influence the structure and activity of epilithic biofilms (Battin, 2000; Battin et al., 2003).

Our results are similar to those reported by Hullar et al. (2006), who sampled headwater streams in southeast Pennsylvania and found differences between sediment and rock biofilm communities at the class-level. However, they differ from Hullar et al. (2006) in that we detected a high abundance of Cyanobacteria (14%) exclusively in rock biofilm samples whereas they found that Cyanobacteria comprised the majority (40%) of their sediment-derived sequences and a smaller proportion (25%) of the epilithic-derived sequences. The dominance of Cyanobacteria in the Hullar et al. (2006) sediment samples may be due to the more eutrophic nature of temperate streams they studied compared to the pristine arctic systems we sampled for this study. Moreover, we may have sampled at a greater depth into the sediment layer where light does not penetrate, thus explaining why photosynthetic Cyanobacteria in our sediment samples were not detected.

Ribosomal database project II Classifier was used to identify matches to clones at the phylum and order and class levels when possible. Many members of these phyla from the sediment samples: Acidobacteria; Firmicutes; and Proteobacteria (class: Gammaproteobacteria), are known heterotrophs and have been previously isolated from similarly classified pristine stream bed sediments in forested watersheds (Halda-Alija and Johnston, 1999). Orders within these phyla include: Aeromonadales, Pseudomonadales, and Xanthomonadales, all containing members that are obligately aerobic while Enterobacteriales and Bacillales members are facultatively anaerobic. Specializations of these groups include Enterobacteriaceae species having the ability to reduce nitrate to nitrite and Paenibacillaceae, a nitrogen-fixing group. Interestingly, Enterobacteriaceae were dominant in the relatively high $NO_3^-$ waters of the UM lithology and Paenibacillaceae were found to be dominant in the CS clone library where nitrate and TDN values were exceptionally low in CS streams, potentially explaining the persistence of a nitrogen-fixing bacterium.

Clones from the epilithon samples include the following phyla and classes: Bacteriodetes; Betaproteobacteria; Cyanobacteria; Deinicocci; Alphaproteobacteria; and Planctomycetes. Order members within these phyla (Sphingobacteriales, Burkholderiales, Deinicoccales, and Sphingomonadales) are varied in their function (chemoorganotrophic as well as obligately aerobic). Clones belonging to Sphingomonadales were found exclusively associated with the epilithic community and members of this Order have been isolated from a range of environments, including ultra-oligotrophic waters, in which certain species (e.g., *S. alaskensis*) have been shown to possess physiological characteristics adapted to very low carbon substrate concentrations (Eiler et al., 2003). The presence of Sphingomonadales in epilithon samples may indicate lower availability of carbon sources for bacterial metabolism in this habitat; in contrast to the high loads of particulate and dissolved organic matter associated with stream sediment habitats that are influenced by upwelling areas from the hyporheic zone (Sobczak and Findlay, 2002).

In the past decade, studies on the taxonomic, phylogenetic, and physiological diversity of prokaryotes have begun to provide more comprehensive information about microbial communities and their natural environments, and in particular, whether microbes exhibit biogeographical patterns. Structural geographic patterns detected in microbial communities within stream ecosystems have been attributed to the following factors: geographic distance (<10 km) and connectivity between lakes and streams (Crump et al., 2007); biome-level control in low-order streams (Findlay et al., 2008); variation of chemical characteristics in streams across the southeastern and Midwestern USA (Gao et al., 2005); and landscape-level controls on streams due to biogeochemical factors (Fierer et al., 2007). In general, very few studies have focused on low-order streams (Hullar et al., 2006; Findlay et al., 2008) and none have included streams arising from catchments of single, uniform lithologies, as we have done in this study.

Our results suggest that there are differences in bacterial community composition across differing lithologies that can be related to large-scale linkages between streams and the terrestrial environment and parent material in which they are embedded. In turn, this relationship is reflected in differences in resource availability. Furthermore, the resident microorganisms of sediment and epilithon habitats are composed of significantly different bacterial taxa, indicating the presence of specialized ecological niches at the small-scale within stream ecosystems. Our study of arctic streams using T-RFLP and 16S rRNA gene sequencing indicates that bacterial community composition is influenced by lithological characteristics across the landscape as well as physical characteristics of habitat within an individual stream ecosystem.

## ACKNOWLEDGMENTS

We gratefully acknowledge D. Sanzone, J. Lawler, K. Rattenbury, and T. Whitsell of the National Park Service for logistical support. We thank A. Allen and B. Peterson for field assistance, S. Hermans for helicopter support and G. Druschel for lab space and equipment. L. Zeglin's advice with molecular methods is greatly appreciated. The comments from C. Nelson greatly improved this manuscript. This study relied on funding supported by the National Park Service's Inventory and Monitoring Program and U.S. National Science Foundation DEB-0639790. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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

*Received: 15 May 2012; accepted: 03 August 2012; published online: 24 August 2012.*

*Citation: Larouche JR, Bowden WB, Giordano R, Flinn MB and Crump BC (2012) Microbial biogeography of arctic streams: exploring influences of lithology and habitat. Front. Microbio.* ***3****:309. doi: 10.3389/fmicb.2012.00309*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 04 January 2013
doi: 10.3389/fmicb.2012.00432

# Ecological strategies shape the insurance potential of biodiversity

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Biodiversity is thought to provide insurance for ecosystem functioning under heterogeneous environments; however, such insurance potential is under serious threat following unprecedented loss of biodiversity. One of the key mechanism underlying ecological insurance is that niche differentiation allows asynchronous responses to fluctuating environments, although the role of different ecological strategies (e.g., specialists vs. generalists) has yet to be formally evaluated. We present here a simple experimental study that illustrates how different ecological strategies (i.e., generalists vs. specialists) can shape the biodiversity-insurance relationship. We assembled microcosm of generalists and specialist bacteria over a gradient of salinity and found that, bacterial communities made up of generalists were more productive and more stable over time under environmental fluctuations. We discuss our results in context with simple theoretical predictions and propose future directions for biological insurance theory. We argue that beyond species richness itself, it is essential to incorporate the distribution of ecological strategies across relevant environmental gradients as predictors of the insurance potential of biodiversity in natural ecosystems.

**Keywords: insurance, biodiversity, functioning, evolution, bacterial microcosms**

## INTRODUCTION

Despite ecologists long interest in the role of environmental heterogeneity on the evolution and stability of natural communities (e.g., Hutchinson, 1959; MacArthur and Levins, 1967; Levins, 1968), unprecedented global changes in biodiversity (e.g., McKinney, 1998; Purvis et al., 2000; Duffy, 2003) have prompted a renewed focus in understanding the mechanisms underlying species' responses to increasingly unpredictable natural environments (reviewed by McCann, 2000; Cottingham et al., 2001). The last decade has seen a range of theoretical and empirical studies proposing statistical (e.g., Doak et al., 1998) and biological (e.g., Tilman, 1999; Yachi and Loreau, 1999) mechanisms to explain how biodiversity might determines the stability of natural communities (McCann, 2000; Cottingham et al., 2001). In general, it is well established that more diverse communities should cope better with environmental heterogeneity given that different species will have different responses thus stabilizing the aggregate community properties (Cottingham et al., 2001), although recent synthesis have established that these stabilizing effects may depend on trophic complexity (Jiang and Pu, 2009).

One of these stabilizing mechanisms is the *insurance hypothesis*—species that might be functionally redundant in the ecosystem, increase in numbers in more favorable conditions to compensate for the reduction in performance of the dominant species, thus providing "insurance" for community productivity (Yachi and Loreau, 1999). Biodiversity promotes greater insurance when communities are made up of species that are better performers in different environments (i.e., specialists), so that their responses to environmental fluctuations are asynchronous, hence stabilizing the ecosystem and maximizing productivity (Yachi and Loreau, 1999; Loreau et al., 2003). In variable environments, communities with a greater numbers of species are expected to (1) be more productive because different species are responsible for community productivity under different environmental conditions and (2) be more stable since species compensate each other stabilizing community productivity in time. In practical terms assemblages with higher numbers of species will have higher temporal mean productivity and lower temporal variation in measures such as productivity (measured as the CV; Yachi and Loreau, 1999; Loreau et al., 2003).

The theoretical basis of ecological insurance theory is relatively well established in the literature (Yachi and Loreau, 1999; Norberg et al., 2001; Loreau et al., 2003; Gonzalez et al., 2009), although there is need for further empirical evidence that formally tests its predictions and basic assumptions (Boles et al., 2004; Cooper et al., 2005; Leary and Petchey, 2009; Bouvier et al., 2012). One of the key underlying assumption is that niche differentiation will maximize species' asynchronous responses of species to environmental fluctuations thus insuring community productivity (Yachi and Loreau, 1999). Natural communities are, however, made of species with different degrees of ecological specialization (i.e., specialist and generalist species; Futuyma and Moreno, 1988; Devictor et al., 2010). Until recently, the role of different ecological strategies had been ignored in experiments exploring the relationship between species richness and ecosystem functioning (BEF), even though there is widespread evidence that specialist species have greater extinction risks which makes them more vulnerable to global changes (McKinney and Lockwood, 1999; Devictor et al., 2008). Recent

empirical and theoretical studies have revealed that communities made of generalists are more productive on average because of their superior ability to exploit the environmental heterogeneity, although the slope of the BEF relationship is, actually, higher when assemblages are made solely of specialists due to enhanced niche complementarity (Gravel et al., 2011). Despite this evidence, it is not known whether communities made of specialists are more likely to provide greater insurance for community aggregate properties (e.g., productivity) than generalist species.

Depending on the costs associated with being a generalist, it is likely that assemblages of specialists or generalists have different performances across the range of environmental conditions encountered in a fluctuating environment (sensu Kassen, 2002). The magnitude of the insurance effect of biodiversity should therefore be determined by the distribution of strategies within an assemblage of species. We present here a simple experimental study to investigate the relationship between species richness and functioning (e.g., Bell et al., 2005) in communities made up of specialists or generalists with a different numbers of bacterial strains (i.e., 1, 2, or 4). We used bacterial strains, collected across a natural salinity gradient from fresh water to marine environments, to make up bacterial assemblages with different levels of richness for both generalist and specialist strategies over the salinity gradient (**Figure 1**). The insurance effects were determined by measuring temporal mean community productivity and its temporal variability under fluctuating environments by manipulating salinity in bacterial batch cultures.

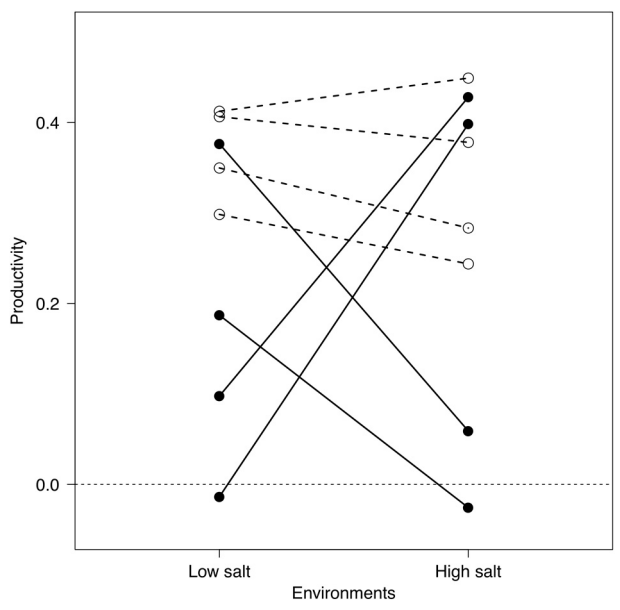


**FIGURE 1 | Reaction norms of generalist (white circles) and specialist (black circles) bacterial strains to different environments.** Reported values indicate the change in optical density after 48 h (as a proxy of productivity) of each strain in monocultures at low- (3 g $l^{-1}$) or high-salt (80 g $l^{-1}$) environments (each symbol indicates the average of 12 microcosms; see "Methods" section).

## METHODS

### BACTERIAL ISOLATION AND NICHE PROFILES

We used a pool of bacterial strains that were collected from a range of locations with different salinity conditions. Salinity is a determinant environmental filter influencing the composition of microbial communities (e.g., Lozupone and Knight, 2007), therefore an appropriate trait to evaluate responses to environmental fluctuations. We isolated marine or freshwater bacterial strains from samples of 50 ml of water taken from coastal lagoons or rivers nearby Montpellier (South of France) in several dates in 2009 and 2010. The salinity of these water samples ranged from freshwater (1.2 g/l) to high salinity (>100 g/l). Freshwater samples were spread on previously autoclaved (20 min at 121°C) LB agar plates; samples from coastal lagoons were spread on marine agar plates (BD Difco Marine Agar, 2216); and high-salt lagoons were spread on MB XS (salinity 50 g/l). All of these plates were grown for 5 days at 20°C. Colonies with distinct morphotypes (i.e., size, shape, and color) were isolated, clean-streaked three times, and frozen in glycerol at −80°C.

Salinity profiles were determined for each strain by measuring the bacterial growth across for a range of salinity between 1.2 g/l and 100 g/l. Marine bacteria (optimum growth at salinity >30 g $l^{-1}$) were grown overnight for 24 h at 20°C in 5 ml MB medium under constant orbital shaking (200 r.p.m) in humid chambers. Freshwater bacteria (optimum growth at salinity <5 g $l^{-1}$) were grown in LB standard medium (Luria–Bertani medium; 5 g $l^{-1}$ yeast extract + 10 g $l^{-1}$ tryptone + 5 g $l^{-1}$ NaCl, autoclaved 20 min at 121°C) under the same culture conditions. Cultures were centrifuged (5 min at 3500 r.p.m.) and the supernatant was completely removed. The cell abundances of each strain were then adjusted to match the mean abundances across all strains that were previously measured using light absorbance at 590 nm on a FLUOstar Optima spectrophotometer (BMG LABTECH) in microplates with 200 µl of overnight cultures of each strain ($n = 3$). This adjustment was done by either diluting or concentrating each overnight using buffered M9 minimal salts (0.1 g $l^{-1}$ $NH_4Cl$, 6 g $l^{-1}$ $Na_2HPO_4$, 3 g $l^{-1}$ $KH_2PO_4$, 0.5 g $l^{-1}$ NaCl). This procedure ensured that all bacterial strains had equivalent initial abundances. Finally, bacterial growth was measured in microplates by transferring 20 µl of overnight cultures of each strain in to 200 µl wells already containing 180 µl of each type of medium across the gradient ($n = 3$). Following initial inoculation, we estimated initial abundances using light absorbance at 590 after 48 h.

From a pool of over 250 potential strains, we identified low-salt specialists, high-salt specialists, and generalists (i.e., similar bacterial productivity at high and low salinities; see **Figure 1**) based on variation between productivity between high- and low-salt medium concentrations. Strains that showed great variation were considered specialists; strains with low variation between the two environments were considered generalists. Finally, we chosen 8 bacterial strains to be used in the experiment (see "Appendix Methods: Model Description" **Table A1** for GenBank accession numbers and provisional taxonomical information) based on their consistent reaction norms across several preliminary trials (**Figure 1**). The specialists' group consisted in bacterial strains that were consistently better performers in either

low- or high-salt environments. The pool of generalists consisted of four strains that had similar performances at high- and low-salt concentration.

### COMMUNITY ASSEMBLAGES

We generated assemblages of bacterial strains with three levels of richness (i.e., 1, 2, and 4 strains) for each of the two experimental groups (i.e., generalists or specialists; **Figure 1**). Bacterial strains were grown overnight and initial abundances were adjusted as described in the previous section. Bacterial strains were assembled in a "master" 96-well, 1-ml sterile microplate for a total of 11 different community types that consisted of four monocultures, six 2-strain cultures and one mixture with all four strains of either generalists or specialists. The relative proportion of different strains in multi-strain assemblages was kept constant (i.e., 2 and 4 strains).

### ENVIRONMENTAL FLUCTUATIONS

We created a fluctuating environment by manipulating the salinity in bacterial batch cultures; at each transfer, we changed the salinity of the *target medium* (that is, the medium with concentration assigned by the salt treatment for that transfer) by transferring cultures between low-($3\,g\,l^{-1}$) and high-salt ($80\,g\,l^{-1}$). The basic media was standard LB medium with 5 g of select yeast extract, 10 g of tryptone and 1l sterile water. We used diluted LB medium with in M9 to get LB ½and added 1.2 g NaCl/l that optimized the growth of these bacterial strains. The salt concentration was manipulated to obtain LB½[3] (LB ½ + 0.24 g NaCl/ 100 ml solution) and LB½ [80] (LB ½ + 7.88 g NaCl/ 100 ml solution). The experiment was ran for 5 transfers that comprised two full environmental fluctuations.

Twelve replicates of each of the 11 combinations were randomly assigned to six 96-well, 0.25 ml sterile microplates previously filled with the two different medium. To account for the potential variability associated with initial conditions (i.e., high or low salt at $t = 0$), we started six replicates at the low-salt concentration and six at the high-salt concentration. Each well was inoculated with 20 µl of each assemblage already containing 180 µl of the target medium. Following initial inoculation, we estimated initial abundances using light absorbance at 590 nm after 48 h. All microplates were incubated at 20°C in humid chambers for 48 h at which point final abundances were estimated. The difference between the initial and final abundances (i.e., after 48) was used to estimate productivity of each assemblage (Gravel et al., 2011; Jousset et al., 2011; Münkemüller et al., 2012). All assemblages were transferred (20 µl) in to new microplates containing 180 µl of the salt medium concentration corresponding to the subsequent transfer. Preliminary trials confirm that this procedure ensures limited modification of the imposed salt concentrations (i.e., <2%). The whole experiment consisted of 2 (strategies) × 11 (assemblages) × 5 (transfers) × 12 (replicates) = 1320 microcosms.

### STATISTICAL ANALYSIS

We tested the effects of diversity and strategies on temporal mean productivity and CV using an unbalanced ANOVA with Diversity and Strategy as fixed factors. All assemblages with the same diversity of strains were pooled together which lead to an unbalance in the number of replicates in each level of diversity due to uneven numbers of possible combinations. ANOVAs were done on log-transformed productivities. In all analyses, we considered a replicate to be the mean productivity (or CV) of each assemblage from all of five time steps pooled together (i.e., $n = 12$). All statistical analyses and data handling were done using R.

## RESULTS

Bacterial diversity had a significant positive effect on temporal mean productivity [**Figure 2A**; $F_{(2,\,522)} = 51.27$; $P < 0.0001$; **Table 1**], although the magnitude of effect was dependent on the strategies making up whether an assemblage was made up of specialists or generalists [Diversity × Strategy interaction: $F_{(2,\,522)} = 5.52$; $P < 0.01$]. In contrast, bacterial diversity had a significant and negative effect on the coefficient of variation for mean productivity [CV; **Figure 2B**; $F_{(2,\,522)} = 12.02$; $P < 0.0001$]. Assemblages made up of generalists were generally more productive [**Figure 2A**; $F_{(1,\,522)} = 77.44.1$; $P < 0.0001$], and significantly less variable over time [**Figure 2B**; $F_{(1,\,522)} = 19.65$; $P < 0.0001$]. *Post-hoc* comparisons showed that there were significant differences in temporal mean productivity between specialists and generalists in assemblages with 1 or 2 strains but no differences in mixtures with 4 strains (Tukey HSD test at $P < 0.05$; **Figure 2A**). The same pattern was found for CV with significant differences in 1 and 2 strain assemblages but not for the more diverse 4 strain assemblages (**Figure 2B**).

**Table 1 | ANOVA of the effects of species richness and ecological strategies mean and temporal variability of productivity.**

| Source | (a) Temporal mean | | | | (b) CV | | |
|---|---|---|---|---|---|---|---|
| | Df | MS | *F* | *P* | MS | *F* | *P* |
| Strategy | 1 | 1.43 | 77.44 | $P < 0.0001$ | 39.37 | 19.65 | $P < 0.0001$ |
| Diversity | 2 | 0.94 | 51.27 | $P < 0.0001$ | 24.07 | 12.02 | $P < 0.0001$ |
| Diversity × Strategy | 2 | 0.10 | 5.52 | $P < 0.01$ | 1.04 | 0.52 | $P > 0.5$ |
| Residuals | 522 | 0.02 | | | 2.00 | | |

*Analysis was done using an unbalanced ANOVA with Strategy and Diversity and main factors. All assemblages with the same species richness were pooled together which lead to an unbalance on the numbers of observations due to the uneven numbers of possible combinations in each level of diversity. The analysis was done using log-transformed productivities. In all analyses, we considered a replicate to be the mean productivity (or CV) across all microcosms of the same assemblage taken from five time steps (i.e., n = 12).*

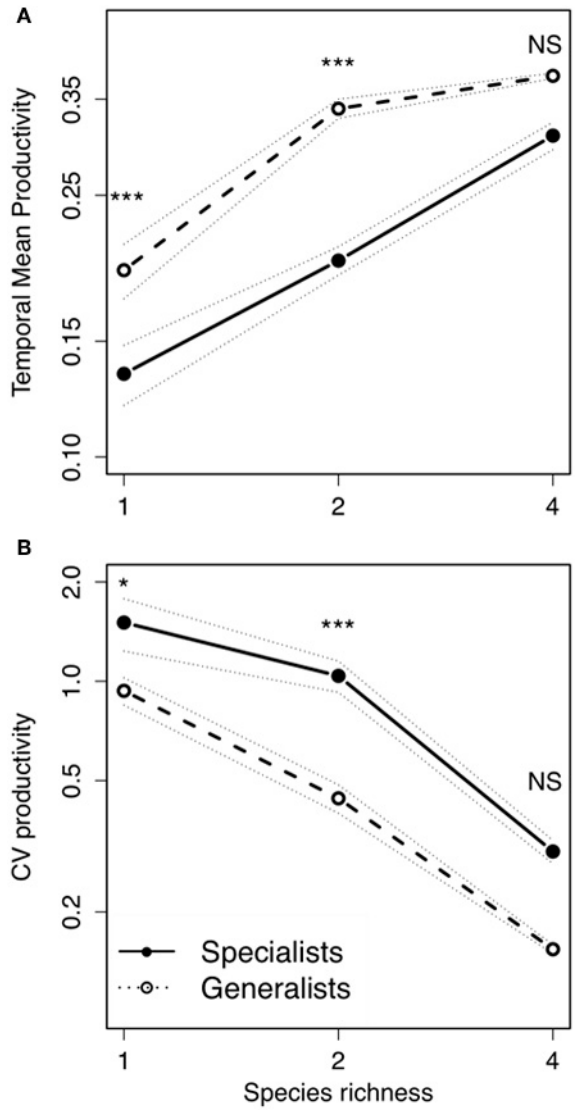


**FIGURE 2 | Bacterial temporal mean productivity and temporal variability in relation to bacterial diversity. (A)** Temporal mean productivity and **(B)** temporal variability (CV) of productivity in fluctuating environments: Each symbol indicates the average of 12 microcosms. Solid lines indicate specialists; and dashed lines indicate generalists. Dotted lines indicate ± standard error of the mean. *Post-hoc* Tukey HSD test to compare means of significant factors with levels of significance: $^{*}P < 0.05$; $^{***}P < 0.001$; NS, $P > 0.05$.

## DISCUSSION

### INSURANCE EFFECTS DEPENDS ON THE ECOLOGICAL STRATEGIES

It is crucial to further investigate the mechanisms involved in the emergence and maintenance of the insurance potential of biodiversity to enable better predictions about whether communities will be able to cope with increasingly pervasive landscape homogenization and global climatic change. In particular, since specialist species in are amongst those considered extremely vulnerable under current extinction scenarios (McKinney and Lockwood, 1999; Devictor et al., 2008). Here we have illustrated how insurance effects of biodiversity can indeed be determined by the ecological strategies within a community. Our microcosm experiment showed that bacterial assemblages made up of generalists were significantly less variable over time than those composed of specialists. The effects of bacterial diversity on productivity and temporal variability were consistent across the two types of strategies; although at higher levels of diversity, there was no longer any difference between specialists and generalists. This suggested that the full insurance potential of specialists was only achieved when all species are present (no saturation), which is consistent with predictions that communities made up of specialists (i.e., greater niche differentiation) should have a steeper influence on the BEF relationship than generalists as a result of greater complementary (Gravel et al., 2011).

Previous studies have revealed that the insurance potential of biodiversity is somewhat contingent on species identity, with responses differing depending on the species present in each community (Leary and Petchey, 2009). Similarly, it has also been shown that the insurance effects may vary depending on competitive interactions between species making up community, with more competitively asymmetrical communities buffering the insurance potential of certain communities (Gonzalez and Descamps-Julien, 2004). Overall, these examples emphasize the importance of determining relative contributions of different species to the insurance potential of each community. In fact, in natural communities there is likely a continuum of strategies between "strict" specialists and generalist and that the distribution of these strategies within ecological communities will shape the ecosystem level response to a varying environment. It is thus essential to expand our understanding of ecological insurance beyond the effects of species diversity (Yachi and Loreau, 1999; Loreau et al., 2003; Gonzalez et al., 2009), by incorporating the distribution of ecological traits across the relevant environmental gradients to better predict of insurance potential of biodiversity. Note that the distribution of strategies within the community is likely to mediate the strength of competitive interactions thus determining potential facilitatory or competitive relationships between species that might, in turn, either enhance or weigh down the insurance potential of biodiversity.

### INSURANCE POTENTIAL: SPECIALISTS vs. GENERALISTS

In our experiment, the assemblages made of generalist species were significantly more productive than those made of specialist species, which would not have been expected if there was a performance "cost" associated with generalization (Kassen, 2002). In fact, we found little or no trade-off between productivity and specialization, as generalists were often the best performer in each environment (**Figure 1**). This result might be due to the nature of the procedures we used to isolate bacterial strains that somehow select for particular genotypes. They also illustrate the need for a more comprehensive understanding of the insurance effect that would encompass both the different ecological strategies but also the strength of the cost associated to each strategies.

Simple hypothetical predictions can be obtained using a phenomological model of the insurance effects. To do so we built a simple model of community dynamics across a continuum of diversity and strategies, varying the strength of the

trade-off between specialization (i.e., niche width) and productivity (**Figure 3A**; see full description of the model and simulations in "Appendix Methods: Model Description"). We generated theoretical predictions of community productivity and insurance potential of biodiversity for assemblages with different levels of species richness and ecological strategies (see **Figures 3B,C**; see also Appendix **Figure A2**). We found that the species richness-temporal variability relationship in assemblages of generalists had higher intercept and slope than those made of specialists (**Figure 3C**; Appendix **Figure A3**). Assemblages made up of generalist species were better at insuring community productivity than assemblages made up of specialists that need greater numbers to maintain the insurance potential. Furthermore, the strength of the specialization-productivity trade-off (i.e., the cost paid by the species for being either generalist or specialist) alters the BEF (i.e., reduces the intercept) relationship in assemblages of generalists (as in Gravel et al., 2011). However, the strength of the specialization-productivity trade-off did not have a major effect on species diversity-temporal variability relationship of both strategies (**Figure A3**), which suggests that the presence/absence of different strategies, rather than the specialization trade-offs, might be determinant for estimating the insurance potential of biodiversity.

More complex models should be used to push our result forward, by including more realistic trade-off curves and different kind of interactions between species; our simple phenomenological model clearly emphasizes the potential for specialist and generalist species to contribute differently to the insurance potential of biodiversity in stabilizing the ecosystems under fluctuations environmental conditions. Our empirical results could not capture the whole range of adaptative strategies we have simulated with our phenomenological model. Our microcosm experiment illustrated a "weak trade-off" scenario that still revealed differences between generalists and specialists in ensuring the stability in productivity throughout the experiment. Future experiments might explore different isolation procedures and investigate other related environmental gradients (e.g., resource availability) that might explain the differences in optimal performance between the different strategies across the different environmental conditions.

## EVOLUTIONARY PERSPECTIVES

We have performed our experiment on ecological time scale (by allowing only 5 batch transfers) but it is likely that a promising future direction will be to include evolutionary response of organisms along environmental gradients. Particularly to consider how an "evolutionary insurance" can emerge in natural communities during diversification and niche differentiation (e.g., Boles et al., 2004). This will imply integrating knowledge on the role of temporal environmental heterogeneity on diversification (Levins, 1968). Generalist or specialist species may be selected depending on the nature of the environmental heterogeneity (e.g., Bell, 1997; Venail et al., 2011), although only particular fluctuations scenarios are likely to promote niche differentiation (e.g., temporal grain; Venail et al., 2011). Moreover, the outcome of local adaptation or selection in complex environments often results

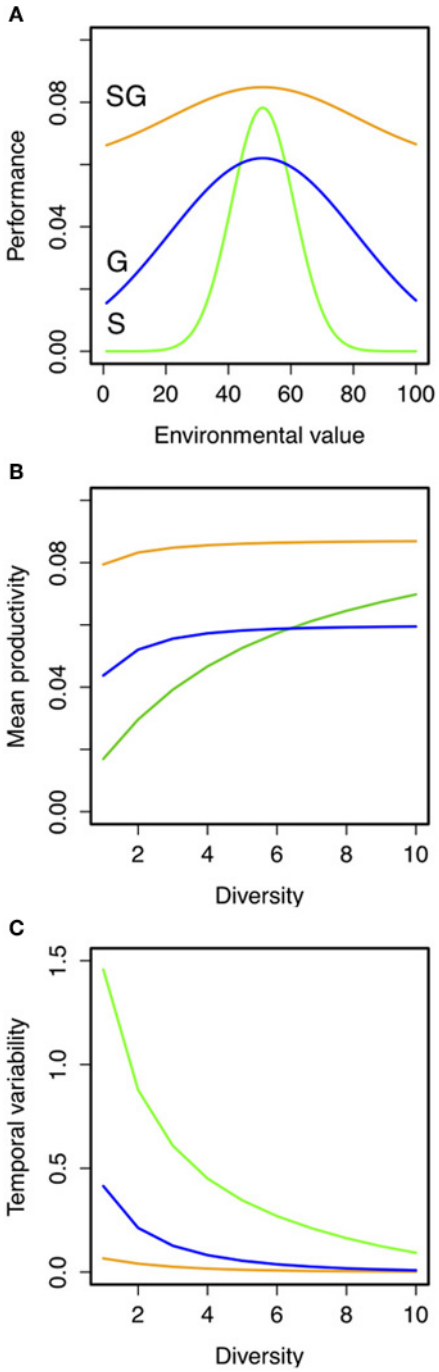


**FIGURE 3 | Hypothetical predictions of the role of ecological strategies (i.e., specialist vs. generalists) on the relationship between species richness, mean, and temporal variability of productivity under fluctuating environments. (A)** Hypothetical niche curves across an environmental gradient for specialist (S, green lines; $\sigma = 10$), generalist (G; blue lines; $\sigma = 40$) or "super" generalist (SG; orange lines; $\sigma = 40$ and maximum productivity of 5; see "Appendix Methods: Model Description" for details). Predictions of **(B)** temporal mean productivity and its **(C)** coefficient of variation were the result of 100 independent simulations for each combination of these parameters. Details on the simulations are given in "Appendix Methods: Model Description" and in Appendix **Figures A1**–**A3**.

neither in specialists nor in generalists but instead in mixtures of overlapping "transient" strategies that might be adapted to certain ranges within an environmental gradient (Barrett et al., 2005). The evolutionary insurance potential of diversity is consequently likely to be the result of different combinations of strategies present at each point during the process of diversification and adaptation. Whether it is driven by strong niche differentiation between specialists or by the evolution of generalists will thus depend on the environmental background in which species evolve. Future research on the insurance will thus have to incorporate the evolutionary history of different species trait diversification to fully understand the potential stabilizing effect of biodiversity on ecosystem functioning (e.g., Gravel et al., 2011).

## AUTHOR CONTRIBUTIONS

Miguel G. Matias designed and performed experiments, analyzed data, and wrote the paper; Marine Combe performed experiments and wrote the paper; Claire Barbera performed experiments; Nicolas Mouquet designed experiments, analyzed data, and wrote the paper. Marine Combe, Claire Barbera, and Nicolas Mouquet collected the bacterial strains.

## ACKNOWLEDGMENTS

Miguel G. Matias, Marine Combe, Claire Barbera, and Nicolas Mouquet were supported by the CNRS and grant ANR-BACH-09-JCJC-0110-01. The authors thank Alison Duncan for providing insightful comments on an earlier version of the manuscript; Cyrille Violle and two anonymous reviewers for comments.

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

*Received: 12 June 2012; paper pending published: 28 June 2012; accepted: 14 December 2012; published online: 04 January 2013.*

*Citation: Matias MG, Combe M, Barbera C and Mouquet N (2013) Ecological strategies shape the insurance potential of biodiversity. Front. Microbio. 3:432. doi: 10.3389/fmicb.2012.00432*

*This article was submitted to Frontiers in Evolutionary and Genomic Microbiology, a specialty of Frontiers in Microbiology.*

## APPENDIX

### APPENDIX METHODS: MODEL DESCRIPTION

We generated theoretical predictions of the role of ecological strategies on the relationship between species richness, mean, and temporal variability of productivity when confronted to a fluctuating environment. These predictions were achieved by (1) generating species niche profiles specialists and generalists with different trade-off strengths and (2) measuring expected community mean productivity and temporal stability of productivity for assemblages of different species richness and combination of strategies.

### PRODUCTIVITY ALONG AN ENVIRONMENTAL GRADIENT

Potential productivity $\Phi_{ik}$ of species $i$ across an environmental gradient $k$ was generated following a modified gaussian function:

$$\Phi_{ik} = \Phi_i \exp\left(\frac{-(E_k/\mu_i)^2}{(2\sigma_i)^2\,\sigma\sqrt{2\times\pi}}\right) \quad (1)$$

where the niche optimum ($\mu_i$) and the environmental value ($E_k$) were set to between 0 and 100 to match the range of salinity used later in the experiment. The parameter σ control for the niche widths (generalists with high σ and specialists with low σ). This function minimizes the differences in overall productivity (sum over the environmental gradient) between the different strategies even though they cannot be made exactly constant (this would be fully true only when this function is integrated between –Inf and +Inf). Examples of the resulting niche curves are shown in main text (**Figure 3B**).

### GENERALIST AND SPECIALISTS' PERFORMANCE TRADE-OFFS

We implemented the trade-off between species performance and specialization (i.e., niche width) following:

$$\Phi_i = \Phi_m \times \left(1 - \left(\frac{\sigma_i}{100}\right)\right)^{\theta} \quad (2)$$

where $\Phi_i$ is productivity of species $i$, $\Phi_m$ is maximum productivity; θ is the trade-off strength; $\sigma_i$ is the niche width of the species $i$ (between 0 and 100). The parameter θ allows us to vary the strength of the trade-off between weak and strong (also called respectively concave and convex) trade-offs (as illustrated in Appendix **Figure A1**).

### BEF SIMULATIONS

We assembled all possible combinations from 1 to 10 species and estimated overall productivity for each community using at each time step the productivity of the best performer (Yachi and Loreau, 1999) and averaging these values over time to calculate both the mean temporal productivity and its coefficient of variation (CV). Using the best performer to approximate community productivity makes the assumption that the best performer (i.e., dominant species) is also the best competitor (at that particular environment or resource level; following Tilman, 1999) and that other species do not contribute significantly to the instantaneous community productivity when they are rare (that is when they are not adapted to local environmental conditions). This assumption holds for calculating community productivity in time as in our model species are ought to be productive only when they are adapted to the environmental conditions (which vary in time). Environmental fluctuations were simulated for 10 time steps. At each time step, a random environmental value was chosen between 0 and 100 and we calculated expected productivity of each assemblage (example of dynamics in Appendix **Figure A2**).

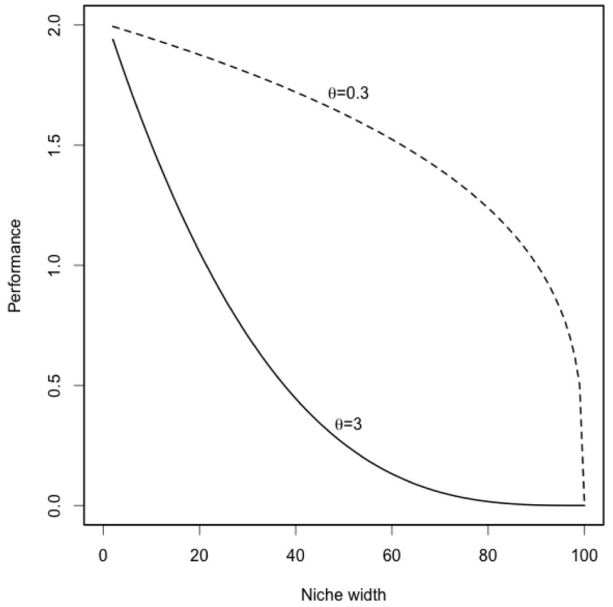


**FIGURE A1 | Trade-off curves between performance and specialization (i.e., niche width).** The parameter niche width determines the ecological strategies of each species (generalists with high σ and specialists with low σ). The parameter θ varies the strength of the trade-off between weak and strong (also called respectively concave and convex) trade-offs (Gravel et al., 2011).

Note that we could have used the sum of the species individual performances within each assemblage but it did not change qualitatively our results (data not shown). Using the mean of species individual performances to calculate a mean temporal productivity would have lead to no relationship between diversity and functioning and thus cannot be used here. Yachi and Loreau (1999) explored the determination of total ecosystem productivity by implementing scenarios of dominance (i.e., total productivity equals the productivity of the best performer) or equivalence (i.e., total productivity is the mean of productivities of all species in mixtures). Equivalence assumes that inter-specific competition is negligible, whilst dominance assumes that the best performer is also the best competitor (at that particular environment or resource level; following the work of Tilman, 1999), which was shown to be a key argument of the insurance hypothesis. These two scenarios are the end points of a continuum and Yachi and Loreau (1999; Figure 2, pp. 1465) have shown that the insurance was operating only mostly in the dominance scenario.

To illustrate contrasting scenarios of specialization, we generated assemblages made of generalists (high niche width: σ = 40),

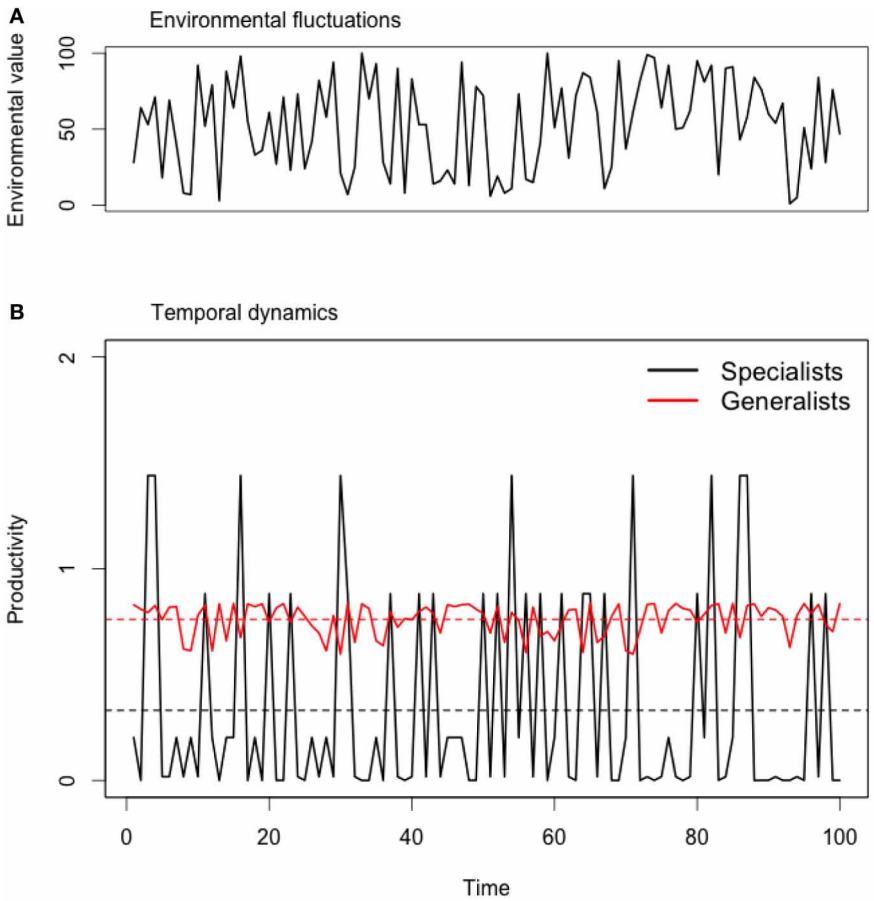


**FIGURE A2 | Temporal dynamics of community productivity of a simulated generalist and specialist species under fluctuating environments. (A)** Sequence of random environmental values ranging between 0 and 100. **(B)** Temporal dynamics of different ecological strategies: specialists ($\sigma = 10$) or generalists ($\sigma = 40$). For the purpose of illustration, we only present simulations for strong trade-offs ($\theta = 2$). In this particular case, specialists are able to perform better under particular environments than generalists but the overall productivity is lower. This effect is illustrated by the lower mean temporal of specialists (black horizontal lines) when compared to generalists (red horizontal lines).

**Table A1 | Taxonomical information and GenBank accession numbers for all bacteria used.**

| Strategy | Strain | Code | Familiy | Genus | Accession number |
|---|---|---|---|---|---|
| Specialists | S1 | S304 | Pseudomonadaceae | Pseudomonas | JX470193 |
| | S2 | S287 | Pseudomonadaceae | Pseudomonas | JX470194 |
| | S3 | S82 | Halomonadaceae | Cobetia | JX470195 |
| | S4 | S172 | Pseudoalteromonadaceae | Pseudoalteromonas | JX470196 |
| Generalists | G1 | S136 | Vibrionaceae | Vibrio | JX470197 |
| | G2 | S328 | Pseudoalteromonadaceae | Pseudoalteromonas | JX470198 |
| | G3 | S239 | Pseudomonadaceae | Pseudomonas | JX470199 |
| | G4 | S241 | Comamonadaceae | Acidovorax | JX470200 |

specialists (low niche width: $\sigma = 10$) or "super" generalists ($\sigma = 40$ with a maximum productivity of 5). These general scenarios were simulated to illustrate different point in the discussion (main text; **Figure 3**). A more comprehensive set of simulation was then performed on the entire range of levels of specialization (i.e., with values of $\sigma$ from 2 to 100) under weak and strong trade offs ($\theta = 0.2$ and $\theta = 2$). Predictions of temporal mean productivity and its CV were averaged over 100 simulations for each combination of these parameters. These general simulations showed how species strategies and the strength of the specialization-productivity trade-off alter the BEF relationship and insurance potential. Specialization leads to a steep BEF relationship with high productivities while generalization leads to weaker relationships as generalists cover the entire environmental ranges (Appendix **Figures A3A**,**B**). As generalization comes to a cost, maximal productivities of

the generalists' assemblages are lower than those of specialists. Assemblages made up of generalists have a greater insurance potential than assemblages made of specialists (lower CV). Although, the extent of this insurance is not affected by the strength of the trade-off (**Figures A3C**,**D**). These results show that the insurance potential is more dependent on the width of the niches (specialist vs. generalist) rather than on the maximal species productivity (linked to the strength of the trade-off).

Note that our model is phenomenological and it does not include elements of species interactions and/or resource dynamics; it only illustrates the expected temporal productivities when the distribution of strategies varies in species assemblages. Future developments are required to build on more complex models of species interactions and resources consumption to disentangling the relative contribution of temporal niche differentiation and species interaction to the potential role of biodiversity as insurance (which was not our aim here).

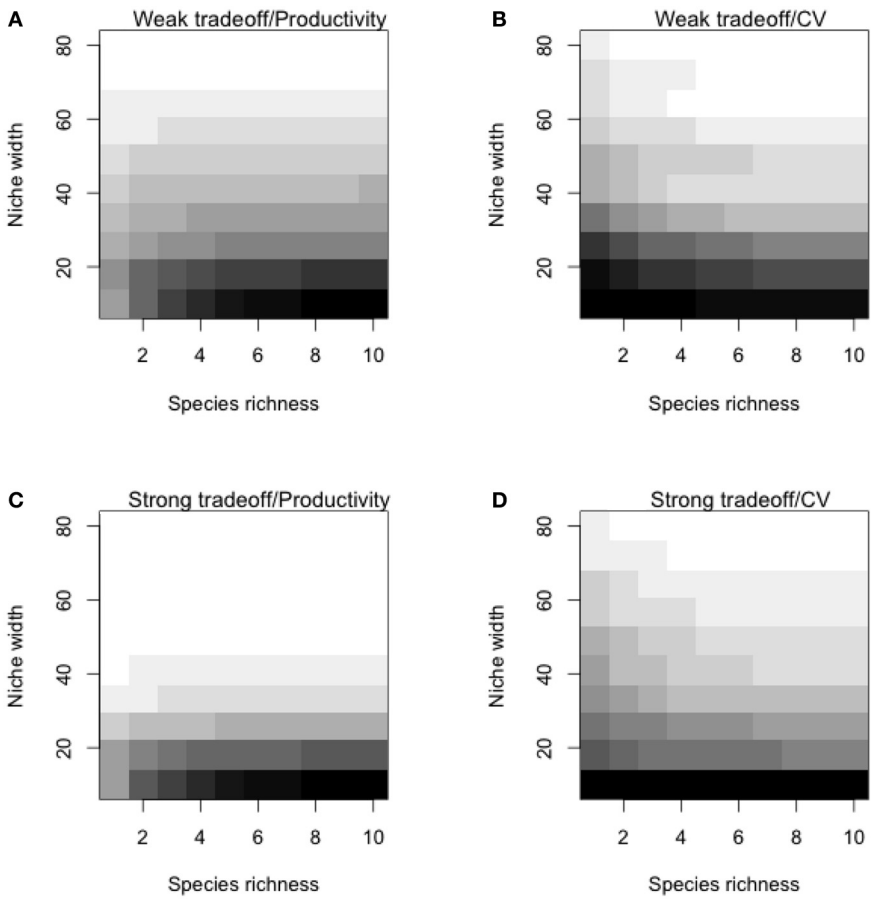


**FIGURE A3 | General theoretical predictions of temporal mean productivity and its coefficient of variation.** Predictions are presented for weak ($\theta = 0.2$; panels **A** and **B**) and strong ($\theta = 2$; panels **C** and **D**) trade-offs. Here, we ran simulations with all combinations of 10 levels of niche width ($\sigma$; from 2 to 100) and 10 levels of species richness (from 1 to 10); for each combination of parameters, we ran 100 independent simulations. Additional parameters are explained in detail in the "Appendix Methods: Model Description." Darker cells indicate greater values of temporal mean productivity or coefficient of variation (CV).

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 05 December 2012
doi: 10.3389/fmicb.2012.00403

# Metagenomic analysis of a southern maritime Antarctic soil

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Our current understanding of Antarctic soils is derived from direct culture on selective media, biodiversity studies based on clone library construction and analysis, quantitative PCR amplification of specific gene sequences and the application of generic microarrays for microbial community analysis. Here, we investigated the biodiversity and functional potential of a soil community at Mars Oasis on Alexander Island in the southern Maritime Antarctic, by applying 454 pyrosequencing technology to a metagenomic library constructed from soil genomic DNA. The results suggest that the commonly cited range of phylotypes used in clone library construction and analysis of 78–730 OTUs (de-replicated to 30–140) provides low coverage of the major groups present (~5%). The vast majority of functional genes (>77%) were for structure, carbohydrate metabolism, and DNA/RNA processing and modification. This study suggests that prokaryotic diversity in Antarctic terrestrial environments appears to be limited at the generic level, with *Proteobacteria*, *Actinobacteria* being common. Cyanobacteria were surprisingly under-represented at 3.4% of sequences, although ~1% of the genes identified were involved in $CO_2$ fixation. At the sequence level there appeared to be much greater heterogeneity, and this might be due to high divergence within the relatively restricted lineages which have successfully colonized Antarctic terrestrial environments.

**Keywords: Antarctica, bacteria, metagenomics, polar, soil, 454 pyrosequencing**

## INTRODUCTION

Antarctic soils have fascinated microbiologists throughout the last century. Expeditions to the continent as far back as 1901 collected soils specifically for bacteriological study. The results of these studies, and later twentieth century research, indicated that Antarctic soils typically harbor low numbers of bacterial taxa (Wynn-Williams, 1996). It is thought that this low taxonomic diversity is associated with increasingly severe environmental conditions, such as restricted water and nutrient availability, and frequent freeze-thaw cycling in summer. These environmental factors also increase in severity with progression from the island archipelagos of the Scotia arc and northern Antarctic Peninsula southwards to the Antarctic continent (Convey, 2001). However, the evidence for this restricted taxonomic diversity has been largely based on data from culture-based studies (e.g., Wynn-Williams, 1983) and, more recently, on data from clone libraries (**Table 1**). These studies have typically only provided levels of coverage of 0.50–0.80 (but see Aislabie et al., 2009), and all have recorded similar levels of biodiversity (at ~30–140 OTUs), all of which could be considered to be relatively low when compared with soils sampled from temperate or tropical environments (Fierer et al., 2003, 2007; Fierer and Jackson, 2006). Many of the dominant bacterial taxa identified by previous studies of Antarctic soil fall into similar taxonomic categories (**Table 2**). However, in recent years, with the advent of metagenomic technology (Handelsman et al., 1998; Hugenholz et al., 1998; Eisen, 2007; Delmont et al., 2011) it is now possible to make a more comprehensive assessment of the scope of the microbial biodiversity present in these soils, and even to determine some of the potential geochemical functions of these microbial communities.

Antarctic soils are of particular interest because chemical analyses has shown that they are relatively low in nutrient content (Lawley et al., 2004; Newsham et al., 2010). This can lead to strong gradients in physicochemical parameters at a wide range of spatial scales; of the order of meters (Chong et al., 2010), kilometers (Chong et al., 2011), or hundreds of kilometers (Yergeau et al., 2007). Antarctic soils also provide extremely good early indications of the potential effects of environmental change. The Antarctic Peninsula, for example, is warming three times faster than the global average (Turner et al., 2005).

Unsurprisingly, an increasing number of studies are beginning to show that the microbial biodiversity associated with these environmental gradients could be much larger than was once thought. Mars Oasis was chosen for this study as preliminary data already exist for this site. It has been suggested to be a potential biodiversity hotspot (Yergeau et al., 2007) and it has unique soil chemistry when compared to the surrounding area (Chong et al., 2011). It is also geographically isolated, being situated 1,000 km from South America on the south-eastern coast of Alexander Island in

**Table 1 | Summary data from a selection of previous studies of Antarctic soil microbial diversity.**

| Reference | Latitude and longitude (place name) | T | S | R | % D | Shannon–Weaver DI | Coverage | Richness |
|---|---|---|---|---|---|---|---|---|
| Aislabie et al. (2006) | S 77° 25′E 163° 41′ (Marble Point) | 728 | 33 | 52–85 | 44–56 | 2.65–3.95 | 0.50–0.52 | n/a |
| | S 77° 31′ E 161° 52′ (Bull Pass) | n/a | n/a | 29–47 | 82–85 | 2.53–3.19 | 0.81–0.83 | n/a |
| | S 77° 31′ E 161° 40′ (Lake Vanda) | n/a | n/a | 47–61 | 67–69 | 3.27–3.32 | 0.70–0.70 | n/a |
| Saul et al. (2005) | S 77° 50′ E 166° 45′ (Scott Base) | 522 | 62 | 56 | n/a | 3.70–3.76 | n/a | n/a |
| Aislabie et al. (2008) | S 77° 55′ E 166° 45′ (Scott Base) | 155 | n/a | 45–51 | 11–18 | n/a | 0.52–0.57 | 46–182 |
| | S 77° 25′ E 163° 41′ (Marble Point) | 131 | n/a | 47–85 | 4–12 | n/a | 0.50 | n/a |
| | S 77° 31′ E 161° 52′ (Bull Pass) | 236 | n/a | 29–47 | 23–24 | n/a | 0.78–0.83 | n/a |
| | S 77° 31′ E 161° 40′ (Lake Vanda) | 211 | n/a | 47–49 | 16–17 | n/a | 0.63–0.70 | n/a |
| | S 77° 19′ E 170° 13′ (Cape Hallett) | 173 | n/a | 26 | 19–27 | n/a | 0.77–0.80 | n/a |
| Aislabie et al. (2009) | S 77° 19′ E 170° 13′ (Cape Hallett) | 580 | 52 | 27–57 | 29–76 | n/a | 0.45–0.78 | 63–256 |
| | S 77° 13′ E 166° 26′ (Cape Bird) | 168 | 11 | 4–19 | 78–99 | n/a | 0.85–0.99 | 5–36 |
| Smith et al. (2006) | S 78° 05′ E 165° 53′ (PENP) | 181 | 61 | n/a | n/a | 1.598 | 0.73 | n/a |
| | S 78° 06′ E 165° 49′ (MVG) | n/a | n/a | n/a | n/a | 1.331 | 0.64 | n/a |
| | S 78° 01′ E 165° 33′ (BIS) | n/a | n/a | n/a | n/a | 1.238 | 0.56 | n/a |
| Taton et al. (2003) | S 77° 37′ E 163° 07′ (Lake Fryxell) | 78 | 16 | 15 | n/a | 2.88 | 0.79 | n/a |
| Niederberger et al. (2008) | S 72° 22′ E 169° 53′ (Luther Vale) | 323 | 323 | n/a | n/a | 3.32–4.04 | n/a | n/a |
| Yergeau et al. (2007) | 54° 15′ S, 36° 30′ W (South Georgia) | 178 | 2111 | 130 | n/a | n/a | n/a | 470 |
| | 60° 43′ S, 45° 38′ W (Signy Island) | 174 | n/a | 128 | n/a | n/a | n/a | 420 |
| | 67° 34′ S, 68° 08′ W (Anchorage Island) | 154 | n/a | 100 | n/a | n/a | n/a | 430 |
| | 71° 19′ S, 68° 18′ W (Fossil Bluff) | 183 | n/a | 60 | n/a | n/a | n/a | 180 |
| | 71° 53′ S, 68° 15′ W (Mars Oasis) | 168 | n/a | 138 | n/a | n/a | n/a | 460 |
| | 72° 03′ S, 68° 31′ W (Coal Nunatak) | 187 | n/a | 40 | n/a | n/a | n/a | 100 |
| | 78° 26′ S, 85° 60′ W (Ellsworth Mountains) | 170 | n/a | 98 | n/a | n/a | n/a | 270 |
| Yergeau et al. (2009) | 60° 43′ S, 45° 38′ W (Signy Island) | 320 | n/a | n/a | n/a | n/a | n/a | 420 |
| | 67° 34′ S, 68° 08′ W (Anchorage Island) | 367 | n/a | n/a | n/a | n/a | n/a | 430 |
| | 71° 19′ S, 68° 18′ W (Fossil Bluff) | 107 | n/a | n/a | n/a | n/a | n/a | 180 |
| | 72° 03′ S, 68° 31′ W (Coal Nunatak) | 160 | n/a | n/a | n/a | n/a | n/a | 100 |

*T, total number of clones; S, number of sequences deposited; R, number of ribotype or phylotype patterns; % D, clones assigned to dominant ribotype (percentage); DI, diversity Index; n/a, not available.*

**Table 2 | Many of the dominant bacterial taxa identified by previous studies on Antarctic soil fall into similar taxonomic categories.**

*Rubrobacter*, *Arthrobacter*, *Acidobacteria*, *Oscillatoria*, *Phormidium*, *Deinococcus*, *Sphingomonas*, *Bacteroides*, *Brevundimonas*, *Chloroflexus*, *Hymenobacter*, *Leptolyngbya*, *Nostoc*, *Pseudonocardia*, *Psychrobacter*, *Rhodococcus*, *Synechococcus*, *Actinobacteria*, *Anabaena*, *Cytophaga*, *Fervidobacterium*, *Friedmanniella*, *Microcoleus*, *Microcystis*, *Nitrosospira*, *Pseudomonas*, *Sphingobacterium*, *Sporosarcosina*, and *Xanthomonas*

the southern Maritime Antarctic, and is isolated by the Antarctic Circumpolar current and prevailing wind direction from the continental interior. Through studies of aerobiological transfer at Rothera (Hughes et al., 2004) and Halley (Pearce et al., 2010) we have some idea of the type of colonist arriving via aerial transfer, and there is relatively little wildlife or human impact at the site. One such study (Newsham et al., 2010) showed no difference between microbial biodiversity across two different parts of the same site at the 97% sequence homology level. However, the effects of alignment quality, distance calculation method, sequence filtering, and region on the analysis of 16S rRNA gene can all influence biodiversity estimates (Schloss, 2010). A re-analysis of data from this study, showed that the biodiversity could be different at each of the two study sites examined depending upon the specific criteria used for sequence differentiation. Hence the site may contain a higher diversity than that shown by clone library analysis alone.

Here, we report the biodiversity and functional potential of the soil community at Mars Oasis, based on the application of 454 pyrosequencing technology to a metagenomic library. It is important to recognize that all techniques in molecular biology impose some degree of bias or selection, and indeed numerous studies have investigated new methods to improve extraction, purification, amplification, and quantification of DNA from soils. In addition, comparative studies have been performed to analyze the efficiency of methods for extraction and purification of soil DNA recovered, and there are a number of excellent reviews in the literature which consider this topic in some detail (for example,

Wintzingerode et al., 1997; Frostegård et al., 1999; Courtois et al., 2001; Martin-Laurent et al., 2001; Feinstein et al., 2009; Delmont et al., 2011; Mahmoudi et al., 2011). For this reason, we do not attempt to provide a systematic analysis of the differentiation based on 16S rDNA. We rather highlight that a polyphasic approach can significantly increase the apparent diversity present and to focus on the relative magnitude and direction of the difference rather than absolute values. This is particularly important for Antarctic soils now, where the total biodiversity was believed to be limited. This view is changing. So the aim of this study was to gain a more comprehensive understanding of the taxonomic diversity of bacteria present in the soil and to determine an initial frequency distribution of potential functional genes. By combining the latter data with analyses of the chemistry of runoff and lake water, we also aimed to try to gain some preliminary insight into the main elements being utilized and cycled by the soil microbial community.

## MATERIALS AND METHODS

### SITE DESCRIPTION AND SAMPLING

Mars Oasis consists of an upper and lower terrace formed from shales. The soil used in this study was collected from the lower terrace, which is situated on a moraine ridge formed by contact between the George VI ice-shelf and Alexander Island. The lower terrace consists of areas of till, fluvial, and lacustrine sediments, with streams and ponds forming during the austral spring and summer. Restricted stands of bryophytes occur on the lower terrace close to meltwater streams and ponds. Lichens are sparse at the site, from which higher animals, including seals and nesting birds, are absent. Mean monthly air and soil (20 mm depth) temperatures at Mars Oasis vary between 1 and 6°C in January and −20 and −15°C in June, respectively. Snow depth at the site is typically ∼2 m in winter, but snow ablates rapidly, usually in November, coinciding with a marked rise in soil water content close to the melt water ponds at the lower site (H. J. Peat, personal communication).

The site was accessed from Rothera Research Station on Adelaide Island by fixed-wing aircraft fitted with skis in December 2004. Samples of moraine soil were collected from an area of level ground at the south-eastern margin of a permanent meltwater pool (71°52.6960′ S; 68°14.9879′ W; **Figure 1**). The soil was collected by inserting four sterile Vacutainers, with their lids removed, to a depth of 5 cm into the soil. Bryophytes were absent from this soil. The lids of the Vacutainers were replaced and the samples placed into re-sealable polythene bags, which were packed in ice in an insulated box. The soils were returned the following day to Rothera Research Station and were frozen at −20°C, prior to their return to the UK at the same temperature.

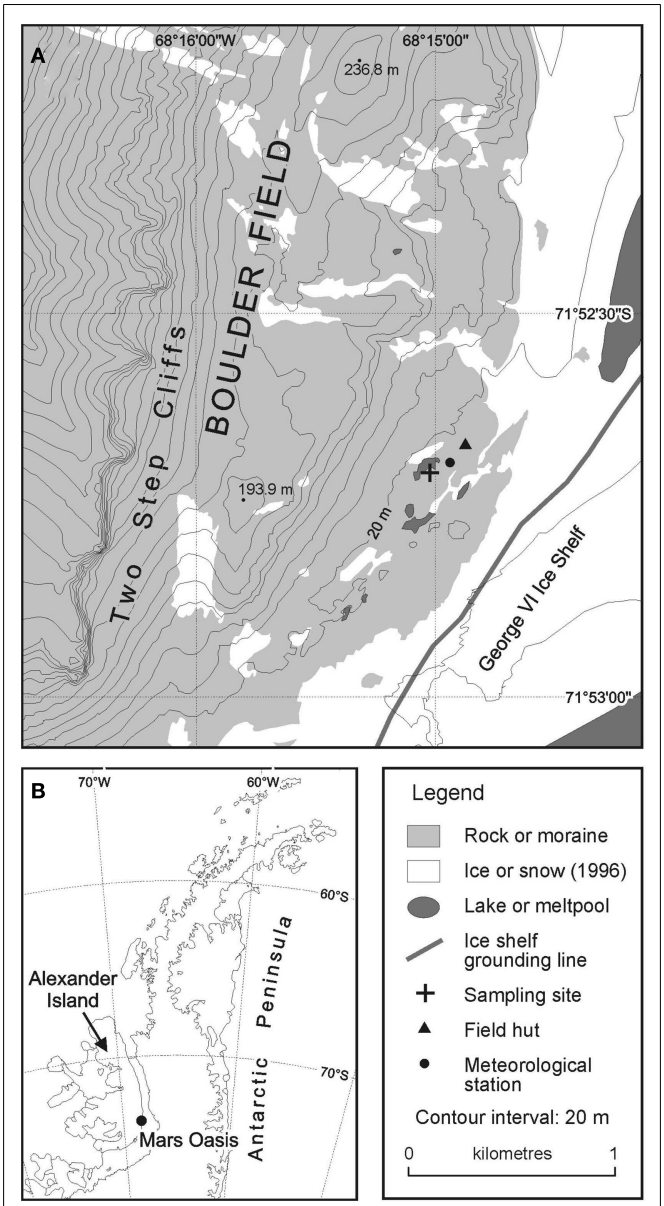


**FIGURE 1 | Location of Mars Oasis (A) on the Antarctic Peninsula (B).**

### METAGENOMIC LIBRARY CONSTRUCTION

The four soil samples (75 g each) were each suspended in 1% SDS solution (25 ml), to which 0.05 g of glucanex and glucanase had been added. The suspensions were vortexed for a few seconds and then incubated at 37°C for 4 h. They were cooled and filtered (1 mm) and 250 μg of RNAase was added to each solution. The solutions were centrifuged six times at 7,500 rpm for 15 min. After each centrifuge run, the supernatant (12 ml) was decanted. Three molar sodium acetate at pH 7.0 (1.2 ml) and ethanol (26.4 ml) was added to each aliquot of the supernatant, which was centrifuged at 10,000 *g* for 10 min. The pellet of DNA was drained and dried for several minutes. TE buffer (0.1 ml) was added to each pellet, which was then incubated at 4°C for 16 h. All of the TE buffer solutions were combined (∼240 μl), mixed with an equal volume of loading buffer, and ran out in a large-welled 1% low melting point agarose gel (Sigma-Aldrich) at 20 V overnight. The gel ran for 48 h, after which a block of agarose containing the target DNA, which had advanced 14–17 mm, was excised from the gel with a sterile scalpel. A size standard was used to select the region of the gel containing 35–45 kbp fragments. The agarose containing the target nucleic acids was then kept at 4°C for 60 h.

The agarose was digested in Gelase according to the manufacturers' instructions and enzymes were then denatured by heating to 60°C for 30 s. DNA was precipitated in three separate aliquots and was re-suspended in TE buffer (4.5 μl). The concentration of DNA ($3 \times 20$ ng μl$^{-1}$ aliquots) was determined by running against known standards in 1.5% agarose gels (1 h at 120 V). The DNA was then end-repaired by combining aliquots (12 μl) on ice with 10 × end-repair buffer (1.85 μl), 2.5 mM dNTP mix (1.85 μl), 10 mM ATP (1.85 μl), and end-repair enzyme mix (0.92 μl). The mixture was incubated at room temperature for 45 min and then at 70°C for 10 min. The DNA, consisting of a solution containing 240 ng of DNA, was then ligated into the pEpiFOS-5 fosmid vector (EpiCentre, Madison, WI, USA) by combining it with sterile water (2 μl), 10 × fast link ligation buffer (3 μl), 10 mM ATP solution (3 μl), fastlink ligase (3 μl), and vector (1 μl). The ligation reaction was then incubated at 4°C for 7 days.

The ligation mix was heated to 70°C for 10 min and the fosmid clones were packaged into lambda phages using MaxPlax lambda packaging extracts according to the manufacturer's instructions (Epicentre, Madison, WI, USA). This process yielded three aliquots (1.025 ml) of cloned cells. The packaged library was transduced into *E. coli* EPI-100, and *E. coli* transformants were selected on LB agar supplemented with 12.5 μg ml$^{-1}$ chloramphenicol. After determining the number of viable cells present, aliquots (200 μl) were spread onto dry Luria broth (100 ml) containing 12.5 μg ml$^{-1}$ chloramphenicol in 47 Petri dishes (150 mm diameter). Chloroformed phage (0.5 ml) was added to EPIFOS cells (5 ml, OD 1.0) and were incubated at 37°C for 20 min. Aliquots (100 μl) were added to each plate, spread, and grown at 37°C for 17 h. Colonies were picked into individual wells of 96 well plates containing Luria broth with 12.5 μg ml$^{-1}$ chloramphenicol (40 μl). The plates were incubated at 37°C for 17 h before sterile glycerol (10% v/v) was added to each well and the plates covered with plastic seals prior to storage at −80°C. All procedures described above took place under a sterile hood.

Quality control was established by end sequencing 20 random fosmids using pEpiFOS™-5 forward and reverse end sequencing primers to ensure environmental DNA had been successfully incorporated, from microorganisms that one might expect to find in this extreme environment. One full fosmid was also sequenced. Primers were used to identify specific sequences from the fosmid library. The metagenomic library was screened using a range of primers for viral (Cyanophage CPS4GC, CPS5 Fuller et al., 1998; Wilson et al., 1999 and Phycodnaviridae AVS1, AVS2 Chen and Suttle, 1995), fungal (ITS1F/ITS4F; White et al., 1990; Gardes and Bruns, 1993), phosphonate (Gilbert et al., 2009), and N cycling (nosZ-F/nosZ-R, nirS1F/nirS6R, and nifHF/nifHRb; Thröback et al., 2004; Rösche and Bothe, 2005) genes. A selection of *E. coli* cells containing fosmids were screened for antibiotic production.

Cells from 25 plates (10% of the total) were combined (to favor depth of sequencing rather than coverage) and cultured in Luria broth with 12.5 μg ml$^{-1}$ chloramphenicol overnight in a shaking incubator at 37°C until an OD of 0.8 was obtained. The cells were centrifuge-concentrated and used to construct a 10,000 Gbp metagenomic library for 454 pyrosequencing. Fosmids were extracted from *E. coli* cells using the QIAGEN Plasmid Midi Kit (QIAGEN Plasmid Midi Kit, Cat. No. 12145. QIAGEN) and then treated with ATP-dependent Exo-nuclease (Plasmid-SafeTM ATP-Dependent Dnase, 10 U μl$^{-1}$ 10,000 U, Cat. No. E3110K, Epicentre). Extracts were sequenced by Macrogen (South Korea) according to the emPCR Method Manual – Lib-L MV (Anon, 2009a) and the Sequencing Method Manual (Anon, 2009b).

## MARS OASIS CLONE LIBRARY RE-ANALYSIS

In clone library based studies, it is common to de-replicate samples through RFLP, or to assign sequences to groups with a predetermined sequence similarity (commonly 97%), for the purposes of comparison across different samples, studies, or environmental gradients. The consequence of this approach is a potential underestimate of the total sequence diversity present in any given sample. To estimate the magnitude of this uncertainty, we selected 21 groupings of OTUs derived from a Mars Oasis clone library study (Newsham et al., 2010) and independently aligned them in CLUSTALW, to determine the levels of variation or similarity within each designated group. In the original study, PCR products were aligned in ClustalW and vector sequences removed. Initially these sequences were grouped according to gross similarity by aligning all sequences in Clustal and generating a single average distance tree based on percentage identities. Groups of sequences and any ungrouped sequences were then analyzed as separate data sets. In this re-analysis, this step was modified so that sequences within each group were only retained in that group if they had ≥97% sequence similarity to other members of the same grouping.

## CHEMISTRY

Samples of runoff, lake water, sediment pore water from lake margins and snow were collected in December 2007. The samples were filtered immediately in the field (1 μ m) and then frozen. Subsamples were stored at ∼1°C in the dark for ∼10 days until pH and $HCO_3^-$ analyses (alkalinity titration using 1 mM HCl) could be made. Thawed samples were analyzed for major ions ($Ca^{2+}$, $Mg^{2+}$, $Na^+$, $K^+$, $Cl^-$, $NO_3^-$, $SO_4^{2-}$) in the UK using Dionex DX90 ion chromatography units, and for $NH_4^+$ using a Skalar Autoanalyzer. Precision errors were <5% according to mid-range standards for all tests.

## DATA ANALYSES

After stringent removal of technical replicates (Gomez-Alvarez et al., 2009) with cd-hit (at 99.5%; Li and Godzik, 2006), vector screening with Lucy (Chou and Holmes, 2001) and MG-RAST (Meyer et al., 2008), and eliminating shorter (<100 bp) reads, 262,086 reads (average length 441 bp) were then analyzed using MG-RAST (Meyer et al., 2008). Searches with a minimum cut-off of $1e^{-05}$, were made against the RDP (Cole et al., 2009), Greengenes (DeSantis et al., 2006), and SEED (Overbeek et al., 2005) databases. An alternate independent OTU analysis was carried out by screening the original set of reads for 16S sequence using both RDP and GenBank (Benson et al., 2005), eliminating redundancy, and selecting those above 90% identity. Sequences were deposited in GenBank accession number SRA060370.

## RESULTS

### METAGENOMIC LIBRARY

#### *Preliminary analysis*

End sequencing gave matches to phototrophs and halotolerant organisms such as *Nocardioides* sp., *Actinobacteria*, *Chlamydomonas reinhardtii*, *Halobacterium* sp., Halophilic archaeon, *Chromohalobacter salexigens*, *Phytophthora sojae*, and a plant pathogen, confirming that genomic DNA incorporated into the metagenomic library was predominantly derived from typical soil micro-organisms. Specific gene probing for phosphonate genes, fungal genes, and phage genes all generated successful amplifications.

#### *Phylogeny from 454 pyrosequencing data*

The total number of sequences containing some taxonomic information was 261,840. Blasting these sequences against the SEED database produced 322 Phyla hits (including bacteria, eukarya, viruses, and archaea). The full phylogeny of these sequences (>1,000 hits) is shown in **Table 3**. The numbers of genera in each class in the 454 phylogeny were (with percentage abundances in parentheses): Proteobacteria 320 genera (48.7%) as: alpha 100 genera (12.2%), beta 46 genera (4.2%), delta 27 genera (4.1%), epsilon 18 genera (0.1%), gamma 129 genera (7.5%), unclassified (0.1%), then Actinobacteria 78 genera (10.6%), Firmicutes 85 genera (7.9%), Bacteroidetes 21 genera (5.9%), Planctomycetes 3 genera (4.7%), Acidobacteria 2 genera (4.0%), Cyanobacteria 26 genera (3.4%), Verrucomicrobia 2 genera (3.1%), and Chloroflexi 9 genera (2.3%).

A rarefaction analysis of the final 454 data matches from the 261,840 sequences yielded 1,160 genera identified (**Figure 2**). An analysis of frequency distribution allowed an assessment of rare diversity, with the most common individual sequence match occurring 5,652 times (**Figure 3**). One hundred sixty-eight sequences occurred only once and 58 only twice. These data gave a Chao estimated sequence number of 1,400 (82.9% coverage) and a coverage estimate (Good, 1953) of 85.52%.

Of the 1,160 genera identified, the top 10 matches were to *Candidatus Solibacter* (5,652), *Burkholderia* (5,405), *Streptomyces* (5,348), *Xanthomonas* (3,685), *Pseudomonas* (3,554), *Sphingomonas* (3,432), *Planctomyces* (3,417), *Bradyrhizobium* (3,308), *Rhodopseudomonas* (3,295), and *Bacillus* (3,279). Other important groups were also present, for example, the *Methylobacteria* (2,562). Only 228 sequences had a single hit and 10,236 sequences in 73 groups were unclassified genera (3.88%).

Of the 3,316 species identified, the top 10 matches were to *Candidatus Solibacter usitatus* (5,652), *Rhodopseudomonas palustris* (3,293), *Rhodopirellula baltica* (2,764), *Gemmatimonas aurantiaca* (2,735), *Sphingomonas wittichii* (2,507), *Candidatus Koribacter versatilis* (2,343), *Salmonella enterica* (2,299), *Sphingopyxis alaskensis* (2,272), *Novosphingobium aromaticivorans* (2,239), and *Chthoniobacter flavus* (2,100). Other important groups were also identified, e.g., bacterium Ellin514 (1,341), which is commonly recorded in polar studies. Only 2,326 sequences in 328 groups were unclassified species (0.82%).

Screening the sequences using the SEED database and selecting for virus sequences gave 494 phage sequences in 28 genera (shown in parentheses). The top 10 phage type occurrences in order of frequency were *Mycobacterium* 107 (10 types), *Burkholderia* 104 (8 types), *Bordetella* 59 (3 types), *Pseudomonas* 51 (7 types), *Enterobacteria* 39 (10 types), *Flavobacterium* 22 (1 type), *Myxococcus* 14 (1 type), *Synechococcus* 11 (2 types), *Prochlorococcus* 9 (3 types), and *Sinorhizobium* 9 (1 type).

**Table 3 | Phylogeny of bacterial sequences derived from 454 pyrosequencing data, with number of sequences in each group (where frequency >1,000).**

| | |
|---|---|
| Candidatus *Solibacter usitatus* | 5,652 |
| *Rhodopseudomonas palustris* | 3,293 |
| *Rhodopirellula baltica* | 2,764 |
| *Gemmatimonas aurantiaca* | 2,735 |
| *Sphingomonas wittichii* | 2,507 |
| Candidatus *Koribacter versatilis* | 2,343 |
| *Salmonella enterica* | 2,299 |
| *Sphingopyxis alaskensis* | 2,272 |
| *Novosphingobium aromaticivorans* | 2,239 |
| *Chthoniobacter flavus* | 2,100 |
| *Pirellula staleyi* | 1,931 |
| *Ruminococcus albus* | 1,889 |
| *Gemmata obscuriglobus* | 1,811 |
| *Planctomyces limnophilus* | 1,692 |
| *Myxococcus xanthus* | 1,624 |
| *Xylella fastidiosa* | 1,601 |
| *Bradyrhizobium japonicum* | 1,571 |
| *Verrucomicrobium spinosum* | 1,519 |
| *Opitutus terrae* | 1,379 |
| *Frankia* sp. | 1,352 |
| Bacterium Ellin514 | 1,341 |
| *Bradyrhizobium* sp. | 1,340 |
| *Xanthomonas campestris* | 1,332 |
| *Erythrobacter* sp. | 1,302 |
| *Spirosoma linguale* | 1,263 |
| *Chitinophaga pinensis* | 1,182 |
| *Blastopirellula marina* | 1,161 |
| *Planctomyces maris* | 1,128 |
| *Escherichia coli* | 1,127 |
| *Synechococcus* sp. | 1,120 |
| *Erythrobacter litoralis* | 1,105 |
| *Sinorhizobium meliloti* | 1,074 |
| *Sorangium cellulosum* | 1,055 |
| *Sphingobium japonicum* | 1,037 |
| *Roseiflexus* sp. RS-1 | 1,019 |

Screening sequences using the SEED database and selecting for Archaea sequences gave 32 Euryarchaeota, 16 Crenarchaeota, and 1 Korarchaeota. The top 10 Archaeal species that were recorded were *Methanosarcina acetivorans* 296 (3 types), *Methanospirillum hungatei* 77 (1 type), *Pyrococcus abyssi* 75 (3 types), *Sulfolobus acidocaldarius* 64 (3 types), *Haloarcula marismortui* 60 (1 type), *Methanococcus maripaludis* 59 (1 type), *Pyrobaculum aerophilum* 55 (4 types), *Methanoculleus marisnigri* 50 (1 type), *Archaeoglobus fulgidus* 48 (1 type), and *Methanosphaerula palustris* 48 (1 type).

Screening sequences using the SEED database and selecting for eukaryotic sequences generated few matches. These included the nematode *Caenorhabditis* sp., the liverwort *Marchantia* sp., the

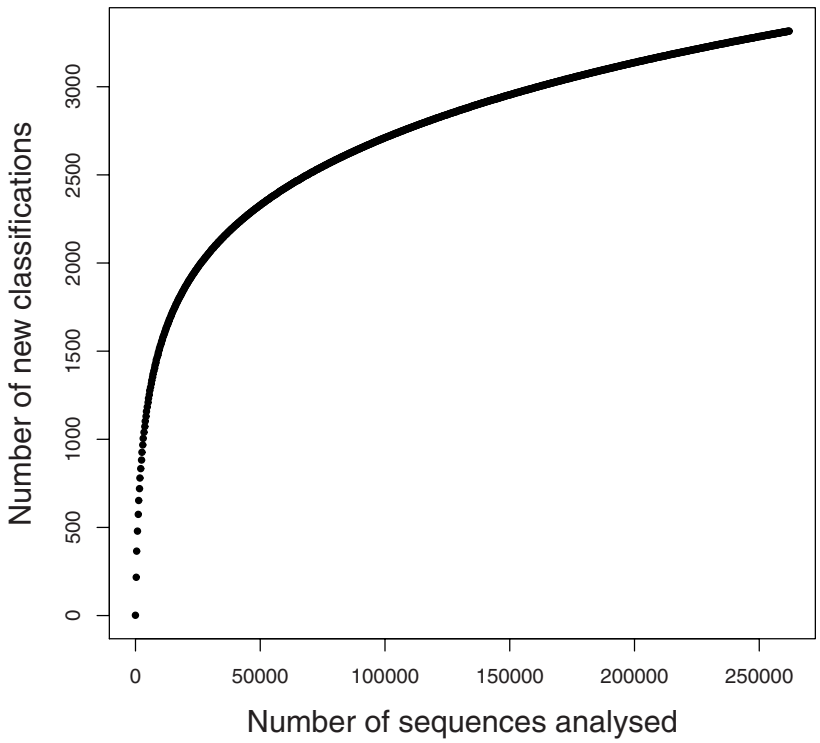


**FIGURE 2 | Rarefaction graph from SEED data.**

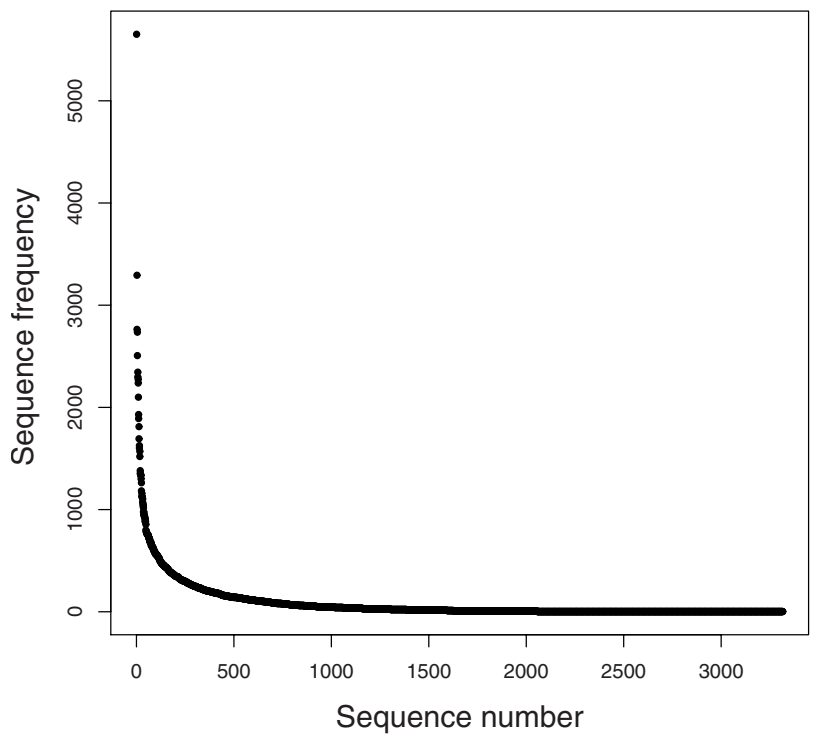


**FIGURE 3 | Rare diversity graph.**

marine diatom *Odontella* sp., the fungi (*Gibberella* sp., *Neurospora* sp., *Magnaporthe* sp., *Schizosaccharomyces* sp., *Saccharomyces* sp., and *Eremothecium* sp.), protozoa (*Mesostigma* sp., *Naegleria* sp., and *Paramecium* sp.), and algae (*Cyanidium* sp., *Cyanidioschyzon* sp., and *Laminaria* sp.).

### *Gene ontology from 454 data*

The gene ontologies derived from the metagenomic data are shown in **Figure 4**. Based on activity class, the most frequently encountered genes were those for clustering-based sub-systems (the precise functions of which are currently unknown), with the next most frequent genes being for carbohydrate metabolism, protein metabolism, amino acids and their derivatives, and cofactors, vitamins, prosthetic groups, and pigments. After these, the next most common genes were for DNA and RNA metabolism, membrane transport, and the cell wall and capsule. Genes for respiration, nucleosides and nucleotides, three classes of potentially ecologically important genes phages, prophages, transposable elements, plasmids, stress response genes, virulence, disease, and defense followed these. After these, four further classes of housekeeping genes (fatty acids, lipids, and isoprenoids; regulation and cell signaling, metabolism of aromatic compounds and cell division and cell cycle) occurred. Finally, ecologically important classes of genes for sulfur, phosphorus, and nitrogen metabolism, motility and chemotaxis, iron acquisition and metabolism, secondary metabolism, potassium metabolism, dormancy and sporulation, and photosynthesis were recorded.

Removing all structural, carbohydrate metabolism, and DNA/RNA processing and modification genes (77% of matches) resulted in 31 classes of protein with >500 matches (10% of

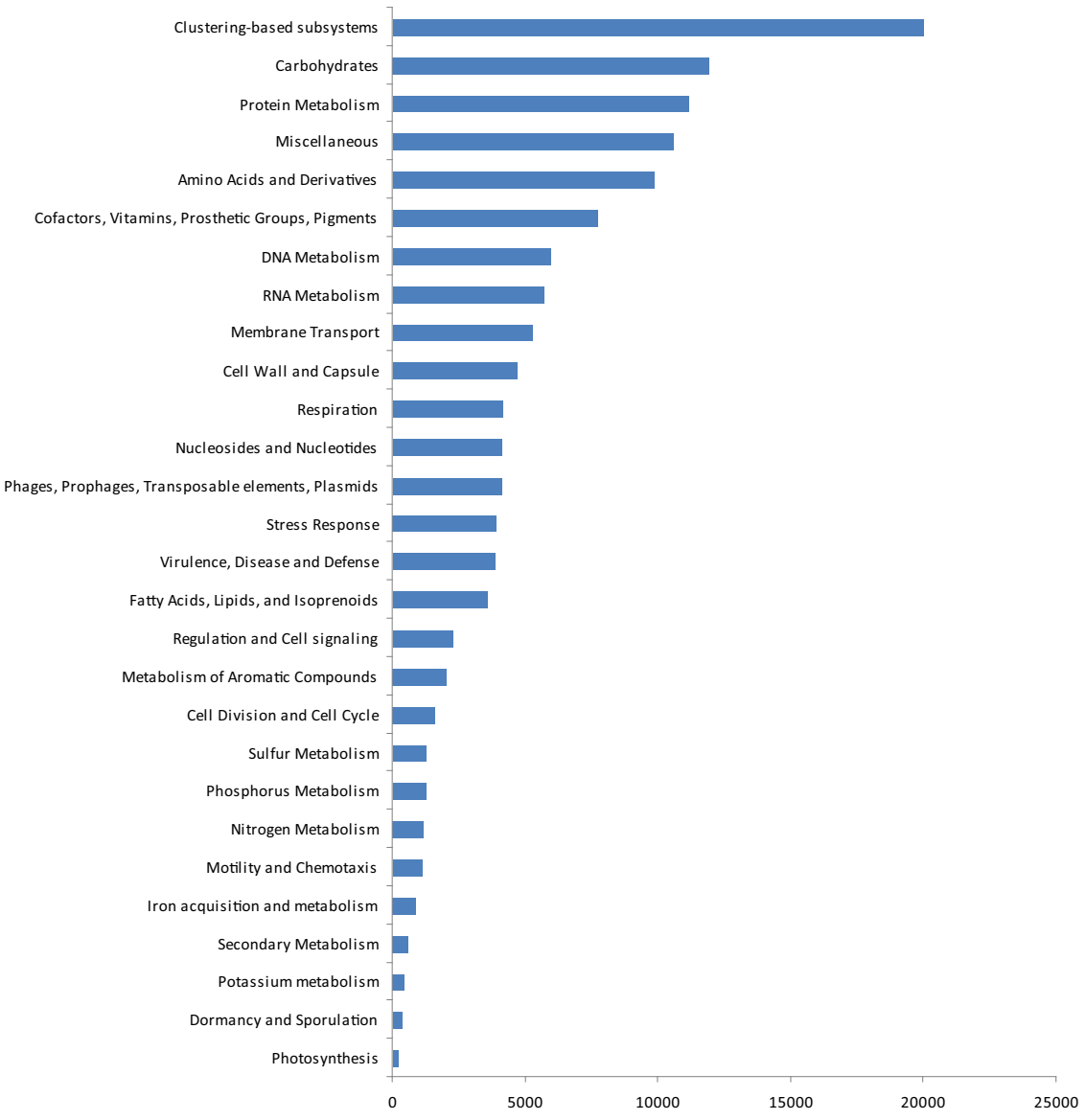


**FIGURE 4 | Gene ontology by function.**

matches). The most common (<6% of all matches, ∼10,000 matches) was to a protein (DUF1446) of unknown function. The next most frequent category (>1%, ∼3,000 matches) contained genes for prophage associated DNA combinational repair protein (2.4%), resistance to antibiotics and toxic compounds (2.4%), one-carbon metabolism (1.5%), and oxidative stress (1.5%). The next category, with 1,500–3,000 matches (0.5–1.0%) contained genes for $CO_2$ fixation (0.86%), flagellar motility (0.7%), phosphate metabolism (0.6%), and phospholipids (0.6%). After these, 1,000–1,500 matches (0.3–0.5%) were for osmotic stress (0.45%), heat shock (0.42%), quinone cofactors (0.38%), Ton and Tol transport systems (0.38%), and ammonia assimilation (0.36%). In the next category, several groups of potentially ecologically important proteins for Antarctic soils were then recorded, 1,000–600 (0.2–0.3%) containing genes for siderophores (0.3%), high affinity phosphate transporter and control of PHO regulon (0.3%), iron acquisition (0.29%), potassium homeostasis (0.29%), organic sulfur assimilation (0.28%), nitrate and nitrite ammonification (0.28%), nucleotidyl-phosphate metabolic clusters (0.28%), inorganic sulfur assimilation (0.27%), bacterial chemotaxis (0.25%), regulation of virulence (0.24%), P uptake in cyanobacteria (0.21%), and pathogenicity islands (0.21%). Finally, <600 hits (0.15–0.2%) occurred to quorum sensing and biofilm formation (0.19%), periplasmic stress (0.19%), and detoxification genes (0.17%). A further 43,168 categories had <500 matches, with 17 classes having a single sequence associated with them.

### *Clone library re-analysis*

Of 43 sequences that had earlier been grouped into 21 sequence types based on a ≥97% cut-off level, pair-wise comparison of sequences within the originally assigned groups showed that only four of these independently the criteria within the group itself (**Table 4**), suggesting that 14 of the original groupings underestimated total diversity.

### *Chemistry*

Chemical data are shown in **Table 5**. Runoff and soil pore water from the margin of the lake at Mars Oasis had pHs of 7.4–7.6. Sulfate and calcium were the dominant ions present in the water (2,700–4,100 μ equivalents $L^{-1}$). Carbonate and magnesium ions were less frequent (450–1,400 μ equivalents $L^{-1}$), followed by sodium, chloride, and potassium (13–333 μ equivalents $L^{-1}$). Finally, ammonium-*N* and nitrate-*N* were the least frequent ions in runoff, and ammonium was infrequent in pore water (each 3–6 μ equivalents $L^{-1}$), but nitrate was relatively frequent in the latter (200 μ equivalents $L^{-1}$).

## DISCUSSION

Studies of bacterial communities from around the world suggest a wide spectrum of taxonomic diversity, from the Amazonian soils, where every sequence sampled could be different (Fierer et al., 2007) to the highly selective Rio Tinto river in Europe, with a relatively restricted biodiversity (Palacios et al., 2008). It is apparent from the current study that the bacterial community diversity in the soil at Mars Oasis lies somewhere between the extremes, with a total of 1,160 genera from 3,318 phylotypes detected in the 454 library. This is an order of magnitude greater than data from clone library studies alone, which have to date recorded 78–730 (de-replicated to 30–140) phylotypes present in Antarctic soils sampled from the sub-Antarctic Islands, the Antarctic Peninsula, and the continent itself (see references in **Table 1**). Of the 1,160 genera recorded in our study, 71 (6.12%) have also been identified by other studies of Antarctic soil biodiversity.

### DIVERSITY AT THE GENUS LEVEL

The most frequent genera in Antarctic soils (identified in >3 studies) are *Rubrobacter*, *Arthrobacter*, *Oscillatoria*, *Sphingomonas*, *Chloroflexus*, *Anabaena*, *Actinobacteria*, *Microcoleus*, *Microcystis*, *Nitrosospira*, *Pseudomonas*, *Fervidobacterium*, *Xanthomonas*,

**Table 4 | Mars Oasis clone library re-analysis.**

| Nominal identification given | Original (≥97% BLAST i.d.) | Sequence number | Sequence pair-wise comparisons (mismatch/sequence length similarity%) | | |
|---|---|---|---|---|---|
| Uncultured bact clone MeCl 62 | 12/657 (98.2%) | 3 | 27/841 (96.8%) | 38/721 (94.7%) | 33/721 (95.4%) |
| Uncultured eukaryote clone | 36/730 (99%) | 3 | 12/769 (98.4%) | 74/802 (90.8%) | 51/760 (93.3%) |
| Uncultured bact clone FRCH17502 | 3/745 (99.6%) | 2 | 20/767 (97.4%) | | |
| Uncultured bact clone LVH3-G7 | 4/715 (99%) | 2 | 4/715 (99.0%) | | |
| Uncultured bacteroidetes AS28 | 209/728 (71.3%) | 2 | 92/725 (87.3%) | | |
| Uncultured bacteroidetes clone | 34/780 (95.6%) | 3 | 317/826 (97.9%) | 25/781 (96.8%) | 29/782 (96.3%) |
| Uncultured cyanobacterium clone | 20/699 (97.14%) | 2 | 20/708 (97.2%) | | |
| Uncultured Micrococcineae | 64/715 (91.1%) | 3 | 23/715 (96.8%) | 48/708 (93.2%) | 53/757 (93.0%) |
| Soil bacterial clone U8 | 24/795 (97.0%) | 2 | 26/801 (96.8%) | | |
| Actinomycetes clone FB-2 A11 | 28/724 (96.1%) | 2 | 24/725 (96.7%) | | |
| Uncultured bacterium clone CM131 | 32/414 (92.3%) | 2 | 33/414 (92.0%) | | |
| Bacterial clone KuyT-IWPB 17 | 26/744 (96.5%) | 2 | 29/744 (96.1%) | | |
| *Solirubrobacter* Gsoil 921 | 33/709 (95.3%) | 3 | 7/710 (99.0%) | 33/701 (95.3%) | 30/767 (96.1%) |
| Uncultured bact 071021-ONK-KR1-12 | 69/812 (91.5%) | 2 | 62/799 (92.2%) | | |
| Uncultured bacterium FACH1766 | 16/805 (98.0%) | 2 | 20/802 (97.5%) | | |
| Uncultured bacterial clone F1-2F-F12 | 163/775 (79.0%) | 2 | 80/774 (89.7%) | | |
| Uncultured Caldilineaceae bacterium | 59/734 (92.0%) | 3 | 30/744 (96.0%) | 48/761 (93.7%) | 48/744 (93.5%) |
| *Anabaena* sp. | 37/756 (92.0%) | 3 | 18/772 (97.7%) | 29/770 (96.2%) | 39/780 (95.0%) |

**Table 5 | pH and ion concentrations of lake runoff, lake water, pore water, and snow at Mars Oasis.**

| Sample | pH | $NH_4^+$ | $Na^+$ | $K^+$ | $Ca^{2+}$ | $Mg^{2+}$ | $HCO_3^-$ | $Cl^-$ | $SO_4^{2-}$ | $NO_3^-$ | $O_2$ | DOC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Runoff | 7.6 | 5.61 | 118 | 12.8 | 2,981 | 784 | 1,392 | 32.6 | 4,082 | 3.33 | 13.0 | 0.35 |
| Lake water (sample 1) | 7.3 | 6.25 | 101 | 14.2 | 1,238 | 259 | 1,771 | 141 | 956 | 2.38 | 12.2 | 6.53 |
| Lake water (sample 2) | 7.4 | 4.09 | 129 | 20.0 | 1,540 | 233 | 1,573 | 108 | 1,310 | 1.80 | 12.9 | 2.43 |
| Lake water (sample 3) | 7.5 | 6.14 | 85.7 | 16.6 | 849 | 115 | 768 | 103 | 781 | 0.86 | 12.7 | 1.15 |
| Lake water (sample 4) | 7.7 | 3.11 | 36.8 | 8.37 | 510 | 64.0 | 1,088 | 26.1 | 165 | 5.92 | 12.6 | m.d. |
| Pore water | 7.4 | 2.91 | 124 | 16.2 | 2,693 | 449 | 652 | 333 | 2,464 | 199 | 7.31 | 5.59 |
| Snow | 5.7 | 2.03 | 16.5 | 12.0 | 46.1 | 3.91 | m.d. | 19.2 | 46.2 | 2.38 | m.d. | 3.64 |

*DOC, dissolved organic carbon; m.d., missing data.*

*Data are expressed in μ equivalents $L^{-1}$ except for dissolved oxygen and DOC (both mg $L^{-1}$) and pH. Data are means of 7, 12, 3, and 4 measurements for runoff, lake water, pore water, and snow, respectively. $PO_4^{3-}$ was below detection limits of 1 μ equivalents $L^{-1}$*

*and Acidobacteria*, *Phormidium*, *Deinococcus*, *Bacteroides*, *Brevundimonas*, *Hymenobacter*, *Leptolyngbya*, *Nostoc*, *Pseudonocardia*, *Psychrobacter*, *Rhodococcus*, *Synechococcus*, *Cytophaga*, *Friedmanniella*, and *Sphingobacterium*. The former group was identified in both clone library based and 454 based studies, indicating that there is some broad agreement with previous studies, whilst the latter group was specifically identified in this study.

#### DIVERSITY AT THE SEQUENCE LEVEL

The 10 Antarctic soil biodiversity studies examined (based upon PCR amplification, cloning, and sequencing of 16S rRNA genes) each reported relatively low prokaryotic biodiversity in Antarctic terrestrial ecosystems. However, estimated coverage in each of these studies ranged from 30–70%, only two of the collectors' curves approached anywhere near saturation and all were based upon the assumption that taxonomic differentiation occurs with <97% sequence similarity over 200–800 bp. Despite 97% sequence similarity being adopted for species differentiation in many biodiversity studies, it has already been demonstrated that 100% sequence similarity in the 16S rRNA gene can be found between isolates with different ecological phenotypes (Peña et al., 2010), and conversely, <97% sequence similarity has been found between isolates which have an identical ecological function. We therefore conducted a detailed re-analysis of 300 sequences (Newsham et al., 2010), and show that at a sequence similarity of 97%, only 7 of the 300 sequences were duplicated in the clone library. This re-analysis showed that the diversity of closely related sequences may have been underestimated by up to 40%. If we add to this an approximate doubling of the total number of genera identified through the addition of 454 data to the combined clone library summary, then there could be a minimum of four times greater diversity than was previously described. So although prokaryotic diversity may indeed be restricted at the generic level, there appears to be high sequence diversity present in the soil at Mars Oasis.

#### BIODIVERSITY

The range of taxa identified extended (indeed increased by five times), rather than contradicted the taxa identified in earlier clone library studies (140 Genera were present in both; 56 from clone library studies only and 1,026 from this 454 study only). A number of key taxa were identified, that have been indicated to be important in other studies of Antarctic soils – these include the Actinobacteria and Cyanobacteria. The taxa identified were also similar to those recovered in airborne samples of the Peninsula region. Hughes et al. (2004) sampled air for a 2-week period above Rothera Station on Adelaide Island, and found a range of microorganisms, including cyanobacteria, actinomycetes, diatom plastids, and other uncultivated bacterial groups. Elsewhere, on the Brunt Ice-Shelf over an isolated scientific research station, on an ice-shelf in continental Antarctica Pearce et al. (2010) found Bacilli, Pseudomonads, and Sphingomonads. In common with other environmental studies, particularly in the polar regions, many of the sequences obtained were from as yet uncultivated organisms. The detected aerial microorganisms were different from those obtained over the Antarctic Peninsula. In both aerobiological studies, a low microbial biodiversity was detected, which included many sequence replicates. In this study, there were also important genera identified that had not been described in a range of selected clone library studies (**Table 6**).

#### GENE ONTOLOGY

The gene ontology data in the present study, when expressed by activity class, gave some potential insights into the presence of functional genes in the soil at Mars Oasis. Although many genes for clustering-based sub-systems were encountered in the 454 library, suggesting the functional coupling of genes whose present purpose is unknown, the data clearly indicated that the microbial community was active, with the presence of many genes for cell division and the cell cycle, cell wall formation, nucleotides and nucleosides, and RNA metabolism. This indicates that there is potential for the soil community at the oasis to express these genes, at least during the austral summer, when temperatures are typically above freezing point during the daytime and liquid water is freely available. There is also the potential for active competition between microbes in the soil, with the presence of antibiotic and toxic compound resistance genes, quorum sensing, and biofilm formation genes and many genes relating to virulence. Given the abundance of *Actinobacteria* in the soil, and particularly genera such as *Streptomyces*, which are active synthesizers of antibiotics, it is unsurprising that many virulence genes were encountered in the soil. Genes found at lower frequencies than expected were those for stress responses (including oxidative stress, osmotic stress, periplasmic stress, cold shock, and detoxification genes), perhaps reflecting the not unfavorable environmental conditions for soil microbial growth at

**Table 6 | Diversity of bacteria (and other groups) identified to the Genus level from Antarctic soil from by 454 sequencing.**

*Abiotrophia*, *Acanthamoeba*, *Acaryochloris*, *Acetitomaculum*, *Acetivibrio*, *Acetobacter*, *Acetohalobium*, *Acholeplasma*, *Achromobacter*, *Acidaminococcus*, *Acidilobus*, *Acidiphilium*, *Acidithiobacillus*, *Acidithiomicrobium*, *Acidobacterium*, *Aciduliprofundum*, *Acorus*, *Actinocorallia*, *Actinomadura*, *Actinoplanes*, *Actinosynnema*, *Acuclavella*, *Acyrthosiphon*, *Adineta*, *Aedes*, *Aerococcus*, *Aeromonas*, *Aeropyrum*, *Afipia*, *Aggregatibacter*, *Agromyces*, *Ahrensia*, *Ailuropoda*, *Ajellomyces*, *Akkermansia*, *Albidiferax*, *Alcanivorax*, *Algoriphagus*, *Alicycliphilus*, *Alicyclobacillus*, *Aliivibrio*, *Alistipes*, *Alkalilimnicola*, *Alkaliphilus*, *Allium*, *Allochromatium*, *Alphabaculovirus*, *Alphapapillomavirus*, *Alternaria*, *Alteromonas*, *Amaranthus*, *Amatitlania*, *Aminobacterium*, *Aminomonas*, *Ammonifex*, *Amplypterus*, *Amycolatopsis*, *Anaerobaculum*, *Anaerococcus*, *Anaerofustis*, *Anaerolinea*, *Anaeromyxobacter*, *Anaerostipes*, *Anaerotruncus*, *Anaplasma*, *Ancylobacter*, *Aneurinibacillus*, *Anopheles*, *Anoplopoma*, *Anoxybacillus*, *Anthopleura*, *Antonospora*, *Aphanizomenon*, *Aphanomyces*, *Apis*, *Apteryx*, *Aquimonas*, *Arabidopsis*, *Arcanobacterium*, *Archaeoglobus*, *Arcobacter*, *Aromatoleum*, *Arsenophonus*, *Artemia*, *Arthroderma*, *Arthrospira*, *Ascidia*, *Asfivirus*, *Aspergillus*, *Asticcacaulis*, *Ateles*, *Atopobium*, *Aulacoseira*, *Aurantimonas*, *Aureobasidium*, *Aureococcus*, *Azorhizobium*, *Azospirillum*, *Babesia*, *Bacteroides*, *Basfia*, *Bathymodiolus*, *Batrachovirus*, *Beggiatoa*, *Beijerinckia*, *Bermanella*, *Beryx*, *Beutenbergia*, *Bilophila*, *Blastocystis*, *Blastomonas*, *Blastopirellula*, *Blattabacterium*, *Blautia*, *Bombyx*, *Bordetella*, *Borrelia*, *Bos*, *Botryotinia*, Bpp-1-like viruses, *Brachybacterium*, *Brachymonas*, *Brachypodium*, *Brachyspira*, *Branchiostoma*, *Brassica*, *Brevibacillus*, *Brevundimonas*, *Bromus*, *Brucella*, *Brugia*, *Bryonia*, *Bulleidia*, *Butyrivibrio*, *Caenorhabditis*, *Caldanaerobacter*, *Calditerrivibrio*, *Caldivirga*, *Caligus*, *Callithrix*, *Camelus*, *Caminibacter*, *Camponotus*, *Campylobacter*, *Canavalia*, *Candida*, Candidatus, *Accumulibacter*, Candidatus, *Amoebophilus*, Candidatus, *Azobacteroides*, Candidatus, *Blochmannia*, Candidatus, *Chloracidobacterium*, Candidatus, *Cloacamonas*, Candidatus, *Desulforudis*, Candidatus, *Hamiltonella*, Candidatus, *Korarchaeum*, Candidatus, *Koribacter*, Candidatus, *Kuenenia*, Candidatus, *Liberibacter*, Candidatus, *Magnetobacterium*, Candidatus, *Micrarchaeum*, Candidatus, *Nitrososphaera*, Candidatus, *Odyssella*, Candidatus, *Parvarchaeum*, Candidatus, *Pelagibacter*, Candidatus, *Phytoplasma*, Candidatus, *Protochlamydia*, Candidatus, *Puniceispirillum*, Candidatus, *Regiella*, Candidatus, *Rhodoluna*, Candidatus, *Solibacter*, Candidatus, *Sulcia*, *Canis*, *Capnocytophaga*, *Capsaspora*, *Capsicum*, *Carassius*, *Cardiobacterium*, *Carnobacterium*, *Catabena*, *Catenibacterium*, *Catenulispora*, *Catharanthus*, *Catonella*, *Cavia*, *Cellulomonas*, *Cellulosilyticum*, *Cellulosimicrobium*, *Cellvibrio*, *Cenarchaeum*, *Chaetoceros*, *Chaetomium*, *Chara*, *Chattonella*, *Chelativorans*, *Chitinophaga*, *Chlamydia*, *Chlamydomonas*, *Chlamydophila*, *Chlorella*, *Chlorobaculum*, *Chlorobium*, *Chloroherpeton*, *Chlorokybus*, *Chlorovirus*, *Chondrus*, *Chrysopathes*, *Chthoniobacter*, *Cicer*, *Ciona*, *Citreicella*, *Citrobacter*, *Citromicrobium Clavibacter*, *Clavispora*, *Coccidioides*, *Cochliobolus*, *Collimonas*, *Collinsella*, *Colossendeis*, *Comamonas*, *Compsopogon*, *Congregibacter*, *Coprinopsis*, *Coprobacillus*, *Coprococcus*, *Coprothermobacter*, *Coptotermes*, *Coraliomargarita*, *Corallina*, *Corynebacterium*, *Coxiella*, *Crassostrea*, *Cricetulus*, *Croceibacter*, *Crocosphaera*, *Cronobacter*, *Crustomastix*, *Crypthecodinium*, *Cryptobacterium*, *Cryptosporidium*, *Cucumis*, *Cucurbita*, *Culex*, *Culicoides*, *Cupriavidus*, *Curvibacter*, *Curvularia*, *Cyanidioschyzon*, *Cyanidium*, *Cyanobium*, *Cyanophora*, *Cylindrospermopsis*, *Cystobacter*, *Cytophaga*, *Dactylosporangium*, *Danio*, *Daphnia*, *Dasypus*, *Daucus*, *Debaryomyces*, *Deferribacter*, *Dehalogenimonas*, *Deinococcus*, *Denitrovibrio*, *Dermacentor*, *Dermacoccus*, *Desulfarculus*, *Desulfatibacillum*, *Desulfobacterium*, *Desulfobulbus*, *Desulfocella*, *Desulfococcus*, *Desulfohalobium*, *Desulfomicrobium*, *Desulfomonile*, *Desulfonatronospira*, *Desulfotomaculum*, *Desulfurispirillum*, *Desulfurivibrio*, *Desulfurococcus*, *Desulfuromonas*, *Dethiobacter*, *Dethiosulfovibrio*, *Dialister*, *Dicathais*, *Dichelobacter*, *Dickeya*, *Dictyoglomus*, *Dictyostelium*, *Dietzia*, *Discophora*, *Dokdonia*, *Dolichospermum*, *Dorea*, *Drosophila*, *Durinskia*, *Dyadobacter*, *Echinops*, *Ectocarpus*, *Edwardsiella*, *Eggerthella*, *Ehrlichia*, *Eikenella*, *Elaeis*, *Eleocharis*, *Eleotris*, *Elizabethkingia*, *Elusimicrobium*, *Elymus*, *Emericella*, *Encephalitozoon*, *Enchytraeus*, *Endoriftia*, *Enhydrobacter*, *Enhygromyxa*, *Ensifer*, *Entamoeba*, *Epiphyas*, *Epulopiscium*, *Equus*, *Eremococcus*, *Eremothecium*, *Erinaceus*, *Erwinia*, *Erysipelothrix*, *Ethanoligenens*, *Eubacterium*, *Euglena*, *Faecalibacterium*, *Felis*, *Ferrimonas*, *Ferroglobus*, *Ferroplasma*, *Filifactor*, *Filobasidiella*, *Finegoldia*, *Flammeovirga*, *Flexithrix*, *Fragilariopsis*, *Francisella*, *Fructobacillus*, *Fulvimarina*, *Fusarium*, *Fusobacterium*, *Gallionella*, *Gallus*, *Gardnerella*, *Gasterosteus*, *Gemella*, *Gemmatimonas*, *Gentiana*, *Geobacillus*, *Giardia*, *Gibberella*, *Glaciecola*, *Glomerella*, *Glossina*, *Gluconacetobacter*, *Glycine*, *Gordonia*, *Gordonibacter*, *Gorilla*, *Gracilaria*, *Gramella*, *Granulicatella*, *Grosmannia*, *Guillardia*, *Haemophilus*, *Hafnia*, *Hahella*, *Haladaptatus*, *Halalkalicoccus*, *Halanaerobium*, *Haliangium*, *Haliotis*, *Haloarcula*, *Halobacterium*, *Haloferax*, *Halogeometricum*, *Halomicrobium*, *Halomonas*, *Haloquadratum*, *Halorhabdus*, *Halorubrum*, *Haloterrigena*, *Halothermothrix*, *Halothiobacillus*, *Harpegnathos*, *Hartmannella*, *Haslea*, *Helianthus*, *Heliobacillus*, *Heliobacterium*, *Heliothis*, *Herbaspirillum*, *Herminiimonas*, *Hirschia*, *Histophilus*, *Hoeflea*, *Holdemania*, *Homo*, *Hordeum*, *Hydra*, *Hydrogenivirga*, *Hydrogenobacter*, *Hydrogenobaculum*, *Hydrogenophaga*, *Hyles*, *Hyperthermus*, *Hyphomicrobium*, *Hyphomonas*, *Ictalurus*, *Ignicoccus*, *Ignisphaera*, *Ilyobacter*, *Ipomoea*, *Isosphaera*, *Ixodes*, *Jackiella*, *Jannaschia*, *Jonesia*, *Jonquetella*, *Kalidium*, *Kangiella*, *Karenia*, *Ketogulonicigenium*, *Kineococcus*, *Kingella*, *Kitasatospora*, *Kluyveromyces*, *Kocuria*, *Kordia*, *Kosmotoga*, *Ktedonobacter*, *Kutzneria*, *Kytococcus*, L5-like viruses, *Labrenzia*, *Laccaria*, *Lachancea*, *Lactobacillus*, *Lactococcus*, Lambda-like viruses, *Laminaria*, *Lamprocystis*, *Laribacter*, *Larimichthys*, *Lautropia*, *Lawsonia*, *Leadbetterella*, *Leclercia*, *Leeuwenhoekiella*, *Legionella*, *Leishmania*, *Lentisphaera*, *Lentivirus*, *Lepeophtheirus*, *Lepidium*, *Leptolyngbya*, *Leptosphaeria*, *Leptospira*, *Leptospirillum*, *Leptothrix*, *Leptotrichia*, *Limnobacter*, *Limnoria*, *Listeria*, *Listonella*, *Loa*, *Lodderomyces*, *Lolium*, *Loxodonta*, *Lunularia*, *Lutiella*, LUZ24-like viruses, *Lymnaea*, *Lysinibacillus*, *Lysobacter*, *Lytechinus*, *Macaca*, *Macrococcus*, *Magnaporthe*, *Magnetococcus*, *Magnetospirillum*, *Malassezia*, *Malus*, *Mannheimia*, *Marchantia*, *Maribacter*, *Maricaulis*, *Marinitoga*, *Marinobacter*, *Mariprofundus*, *Maritimibacter*, *Marivirga*, *Medicago*, *Megamonas*, *Megasphaera*, *Mesembryanthemum*, *Mesorhizobium*, *Mesostigma*, *Metajapyx*, *Metallosphaera*, *Metarhizium*, *Methanobacterium*, *Methanobrevibacter*, *Methanocaldococcus*, *Methanocella*, *Methanococcoides*, *Methanococcus*, *Methanocorpusculum*, *Methanoculleus*, *Methanohalobium*, *Methanohalophilus*, *Methanoplanus*, *Methanopyrus*, *Methanoregula*, *Methanosaeta*, *Methanosarcina*, *Methanosphaera*, *Methanosphaerula*, *Methanospirillum*, *Methanothermobacter*, *Methanothermococcus*, *Methanothermus*, *Methylacidiphilum*, *Methylibium*, *Methylobacter*, *Methylobacterium*, *Methylocapsa*, *Methylocella*, *Methylocystis*, *Methylomonas*, *Methylophaga*, *Methylophilus*, *Methylosinus*, *Methylotenera*, *Methyloversatilis*, *Methylovorus*, *Metridium*, *Meyerozyma*, *Microbacterium*, *Microchaete*, *Micromonas*, *Micromonospora*, *Microtus*, *Mimivirus*, *Mitsuokella*, *Mnemiopsis*, *Mobiluncus*, *Moniliophthora*, *Monocercomonoides*, *Monodelphis*, *Monosiga*, *Moorella*, *Moraxella*, *Mucilaginibacter*,

*(Continued)*

**Table 6 | Continued**

*Mus*, *Mussa*, *Mycobacterium*, *Myotis*, *Myrothecium*, *Myxococcus*, *Myzus*, N15-like viruses, N4-like viruses, *Naegleria*, *Nakamurella*, *Nakaseomyces*, *Nannocystis*, *Nanoarchaeum*, *Nasonia*, *Natranaerobius*, *Natrialba*, *Natronomonas*, *Nautilia*, *Nectria*, *Neisseria*, *Nematostella*, *Neosartorya*, *Nephroselmis*, *Neptuniibacter*, *Neurospora*, *Nicotiana*, *Nitratifractor*, *Nitratiruptor*, *Nitrobacter*, *Nitrococcus*, *Nitrosomonas*, *Nitrosopumilus*, *Nitrospira*, *Nocardiopsis*, *Nonomuraea*, *Nostoc*, *Notechis*, *Novosphingobium*, *Oceanibulbus*, *Oceanicaulis*, *Oceanithermus*, *Oceanobacillus*, *Ochrobactrum*, *Octadecabacter*, *Odontella*, *Oenococcus*, *Oenothera*, *Oikopleura*, *Okibacterium*, *Oligotropha*, *Olsenella*, *Oncorhynchus*, *Opsanus*, *Oribacterium*, *Orientia*, *Ornithobacterium*, *Ornithorhynchus*, *Oryctolagus*, *Oryza*, *Oryzias*, *Oscillochloris*, *Osmerus*, *Ostreococcus*, *Otolemur*, *Ovis*, P1-like viruses, P22-like viruses, P2-like viruses, *Paenibacillus*, *Pagrus*, *Paludibacter*, *Pan*, *Pantoea*, *Parabacteroides*, *Paracoccidioides*, *Paramecium*, *Parascardovia*, *Parvibaculum*, *Parvularcula*, *Pasteurella*, *Paucimonas*, *Paulinella*, *Pectobacterium*, *Pediculus*, *Pediococcus*, *Pelagibacter*, *Pelobacter*, *Pelodictyon*, *Pelotomaculum*, *Penicillium*, *Peperomia*, *Peptoniphilus*, *Peptostreptococcus*, *Perilla*, *Perittia*, *Perkinsus*, *Persephonella*, *Persicobacter*, *Pervagor*, *Petroselinum*, *Petrotoga*, *Pfiesteria*, *Phaeobacter*, *Phaeodactylum*, *Phaeosphaeria*, *Phascolarctobacterium*, *Phenylobacterium*, Phi29-like viruses, PhiC31-like viruses, Phieco32-like viruses, phiKMV-like viruses, phiKZ-like viruses, *Phormidium*, *Phoronis*, *Photobacterium*, *Photorhabdus*, *Physarum*, *Physcomitrella*, *Phytophthora*, *Picea*, *Pichia*, *Picrophilus*, *Picrorhiza*, *Pimelobacter*, *Pinctada*, *Pinus*, *Pisum*, *Planktothrix*, *Planobispora*, *Plasmodium*, *Plesiocystis*, *Pleurotus*, *Ploceus*, *Pneumocystis*, *Podospora*, *Poecilia*, *Polaromonas*, *Polynucleobacter*, *Polysphondylium*, *Pongo*, *Populus*, *Porphyra*, *Porphyrobacter*, *Porphyromonas*, *Postia*, *Potorous*, *Prasinovirus*, *Prauserella*, *Prionoxystus*, *Propionibacterium*, *Prosthecobacter*, *Prosthecochloris*, *Prototheca*, *Providencia*, *Pseudechis*, *Pseudendoclonium*, *Pseudonocardia*, *Pseudoramibacter*, *Pseudovibrio*, *Pseudoxanthomonas*, *Psilotum*, *Psychrobacter*, *Psychroflexus*, *Psychromonas*, *Pteris*, *Puccinia*, *Pyramidobacter*, *Pyrenophora*, *Pyrobaculum*, *Pyrococcus*, *Rahnella*, *Ralstonia*, *Rana*, *Raphidiopsis*, *Rattus*, *Reclinomonas*, *Reinekea*, *Renibacterium*, *Rhadinovirus*, *Rhinoceros*, *Rhodobacter*, *Rhodococcus*, *Rhodomonas*, *Rhodopirellula*, *Rhodopseudomonas*, *Rhodothermus*, *Ricinus*, *Rickettsia*, *Rickettsiella*, *Ricordea*, *Riemerella*, *Robiginitalea*, *Roseburia*, *Roseibium*, *Roseomonas*, *Roseovarius*, *Rothia*, *Rubritalea*, *Ruegeria*, *Ruminococcus*, *Rupicapra*, *Saccharomonospora*, *Saccharomyces*, *Saccharophagus*, *Saccharopolyspora*, *Saccharum*, *Saccoglossus*, *Sagittula*, *Salinibacter*, *Salinispora*, *Salmo*, *Sanguibacter*, *Scardovia*, *Scenedesmus*, *Scheffersomyces*, *Schistocerca*, *Schistosoma*, *Schizophyllum*, *Schizosaccharomyces*, *Sclerotinia*, *Scutigerella*, *Scytonema*, *Sebaldella*, *Segniliparus*, *Selaginella*, *Selenomonas*, *Serratia*, *Shewanella*, *Shigella*, *Shuttleworthia*, *Sideroxydans*, *Simonsiella*, *Simplexvirus*, *Sinorhizobium*, *Slackia*, *Sodalis*, *Solanum*, *Solobacterium*, *Sordaria*, *Sorex*, *Sorghum*, SP6-like viruses, SPbeta-like viruses, *Spermophilus*, *Sphaerotilus*, *Sphingobacterium*, *Sphingobium*, *Sphingopyxis*, *Spinacia*, *Spirochaeta*, *Spirogyra*, *Spirosoma*, *Spisula*, SPO1-like viruses, *Squalus*, *Stackebrandtia*, *Staphylothermus*, *Starkeya*, *Staurastrum*, *Stephos*, *Stigmatella*, *Streptoalloteichus*, *Streptobacillus*, *Streptosporangium*, *Strongylocentrotus*, *Suaeda*, *Subdoligranulum*, *Succinatimonas*, *Sulfitobacter*, *Sulfolobus*, *Sulfuricurvum*, *Sulfurihydrogenibium*, *Sulfurimonas*, *Sulfurospirillum*, *Sulfurovum*, *Sutterella*, *Synechococcus*, *Syntrophobacter*, *Syntrophomonas*, *Syntrophothermus*, *Syntrophus*, T1-like viruses, T4-like viruses, T7-like viruses, *Taenia*, *Taeniopygia*, *Takifugu*, *Talaromyces*, *Teredinibacter*, *Terracoccus*, *Terriglobus*, *Terrimonas*, *Tetrahymena*, *Tetraodon*, *Thalassiosira*, *Thalassobium*, *Thauera*, *Theileria*, *Thermaerobacter*, *Thermanaerovibrio*, *Thermincola*, *Thermoactinomyces*, *Thermoanaerobacter*, *Thermoanaerobacterium*, *Thermobaculum*, *Thermobispora*, *Thermococcus*, *Thermocrinis*, *Thermodesulfovibrio*, *Thermofilum*, *Thermomicrobium*, *Thermomonospora*, *Thermoplasma*, *Thermoproteus*, *Thermosediminibacter*, *Thermosinus*, *Thermosphaera*, *Thermosynechococcus*, *Thermovibrio*, *Thioalkalivibrio*, *Thiobacillus*, *Thiocapsa*, *Thiococcus*, *Thiorhodovibrio*, *Thylacodes*, *Tolumonas*, *Tolypothrix*, *Toxoplasma*, *Toxoptera*, *Trabulsiella*, *Tribolium*, *Trichinella*, *Trichocolea*, *Trichomonas*, *Trichophyton*, *Trichoplax*, *Triglochin*, *Triticum*, *Truepera*, *Trypanosoma*, *Tsukamurella*, *Tuber*, *Tubularia*, *Tupaia*, *Turbo*, *Turicibacter*, *Uncinocarpus*, *Ustilago*, *Vanderwaltozyma*, *Varicellovirus*, *Variovorax*, *Veillonella*, *Verrucomicrobium*, *Verticillium*, *Vicia*, *Victivallis*, *Vigna*, *Vitis*, *Volvox*, VP2-like phages, *Vulcanisaeta*, *Waddlia*, *Weeksella*, *Weissella*, *Wigglesworthia*, *Wolbachia*, *Wolinella*, *Xanthobacter*, *Xenopus*, *Xenorhabdus*, *Xylanimonas*, *Xylaria*, *Yarrowia*, *Yersinia*, *Zea*, *Zingiber*, *Zoophthora*, *Zunongwangia*, *Zygnema*, *Zygosaccharomyces*, *Zymomonas*

Mars Oasis during the summer, and those for photosynthesis. Given the abundance of Cyanobacteria in the lake margin at Mars Oasis (Wynn-Williams, 1996), it was surprising that only ∼1% of the genes in the library encoded for $CO_2$ fixation. Although genes for nitrogen cycling expressed by phyla such as *Acidobacteria* were not found in the library, the use of probes indicated the presence of using *nifH*, *nosZ*, and *nirS* genes in the soil (data not shown). Genes for sulfur, phosphorus, and nitrogen metabolism were all present at about 1%, whilst those for iron acquisition and metabolism were 0.7% and potassium metabolism 0.3%.

The gene ontology data, if expressed as actual function, corroborate the view that the community at Mars Oasis is active during summer, with the potential expression of many genes for cytoskeleton and ribosome formation. The presence of genes for the utilization of lactose and galactose indicate that the microbes in the soil most probably utilize relatively simple sugars for growth: there are few plants at the oasis, and those that are present are bryophytes, which typically do not form complex aromatic molecules such as lignin. Nevertheless, some capacity within the microbial community was found for the assimilation of aromatic compounds, with the presence of genes for the assimilation of peptides, which are known to be of importance to the nitrogen cycle in soils of the northern Maritime Antarctic (Hill et al., 2011).

## SOIL CHEMISTRY

The dominant ion in runoff and soil pore water was sulfate, which is almost certainly derived from the oxidation of sulfide minerals in the local shales. Like the carbonate sources, there were also clear signs of secondary minerals (gypsum and/or anhydrite) contributing to the high $SO_4^{2-}$ concentrations. Furthermore, carbonate precipitates were visible around the base of all larger clasts in the soils (Andre and Hall, 2004), and so dissolution of secondary carbonates will have contributed to the high concentrations of $Ca^{2+}$ and $HCO_3^-$ also present in waters. The major ion geochemistry of surface waters at Mars Oasis therefore seems to be controlled by reactive carbonate and sulfide

mineral phases and the precipitation of secondary salts following the evaporation of sediment pore waters, with coupled pyrite oxidation and carbonate dissolution, and carbonate and anhydrite dissolution and precipitation dominating the rock weathering reactions.

## SUMMARY

Studies during the twentieth century suggested that Antarctic soils are of comparatively low microbial biodiversity (Wynn-Williams, 1996). This is certainly true when most Antarctic soils are compared to temperate or tropical soils (Fierer and Jackson, 2006; Fierer et al., 2007). It appears from the current study that prokaryotic diversity in soil at Mars Oasis is limited at the generic level, with the frequent occurrence of *Actinobacteria* and Cyanobacteria. However, at the sequence level, there appears to be much greater heterogeneity than was previously thought, perhaps owing to high divergence within the relatively restricted lineages that have successfully colonized Antarctic terrestrial environments. However, the process of grouping sequences can have an impact. Furthermore, by grouping the sequences based upon genera-level identification, you do lose the distinction of possible species and strain level diversity. As more studies on the microbial diversity present in Antarctic soil using molecular techniques become available, particularly those using mass sequencing on soils sampled from transect studies along the Antarctic Peninsula, it will become clear whether Antarctic terrestrial prokaryotic diversity is higher than was originally thought, and whether potential biodiversity hot spots, such as Mars Oasis, occur in this region (Yergeau et al., 2007).

## ACKNOWLEDGMENTS

Funding was provided by the Natural Environment Research Council through the British Antarctic Survey's Long Term Monitoring and Survey programme. Mark Laidlaw provided field assistance and the BAS Operations and Logistics group arranged transport to and from Mars Oasis. We would also like to thank the reviewers for helpful comments which improved the manuscript.

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

*Received: 24 June 2012; accepted: 02 November 2012; published online: 05 December 2012.*

*Citation: Pearce DA, Newsham KK, Thorne MAS, Calvo-Bado L, Krsek M, Laskaris P, Hodson A and Wellington EM (2012) Metagenomic analysis of a southern maritime Antarctic soil. Front. Microbio.* ***3****:403. doi: 10.3389/fmicb.2012.00403*

*This article was submitted to Frontiers in Extreme Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 01 May 2013
doi: 10.3389/fmicb.2013.00068

# Microbial community assembly, theory and rare functions

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Views of community assembly have traditionally been based on the contrasting perspectives of the deterministic niche paradigm and stochastic neutral models. This study sought to determine if we could use empirical interventions conceived from a niche and neutral perspective to change the diversity and evenness of the microbial community within a reactor treating wastewater and to see if there was any associated change in the removal of endocrine disrupting compounds (EDCs). The systematic removal of EDCs and micropollutants from biological treatment systems is a major challenge for environmental engineers. We manipulated pairs of bioreactors in an experiment in which "niche" (temporal variation in resource concentration and resource complexity) and "neutral" (community size and immigration) attributes were changed and the effect on the detectable diversity and the removal of steroidal estrogens was evaluated. The effects of manipulations on diversity suggested that both niche and neutral processes are important in community assembly. We found that temporal variation in environmental conditions increased diversity but resource complexity did not. Larger communities had greater diversity but attempting to increase immigration by adding soil had the opposite effect. The effects of the manipulations on EDC removal efficiency were complex. Decreases in diversity, which were associated with a decrease in evenness, were associated with an increase in EDC removal. A simple generalized neutral model (calibrated with parameters typical of wastewater treatment plants) showed that decreases in diversity should lead to the increase in abundance of some ostensibly taxa rare. We conclude that neither niche and neutral perspectives nor the effect of diversity on putative rare functions can be properly understood by naïve qualitative observations. Instead, the relative importance of the key microbial mechanisms must be determined and, ideally, expressed mathematically.

**Keywords: endocrine-disrupting chemicals, steroidal estrogens, theories of microbial community assembly, wastewater treatment**

## INTRODUCTION

Engineers create naturally assembled microbial communities in engineered systems that render ecosystem services for the use and benefit of mankind. The development of working engineered systems has typically run far ahead of microbial ecology with engineers using a mixture of trial and error and Monod kinetics to develop and design processes (Curtis et al., 2003). However, this approach has limitations and is subject to diminishing returns that can make it difficult for engineers to transcend current practices. Microbial ecology has, on occasion, led to the development of novel processes (van der Star et al., 2011). However, to break new ground and reliably engineer new functions, we believe it is necessary to understand what controls microbial diversity and the relationships between diversity and function. The rules that govern engineered systems should be the same as those that govern natural ones, for the microbe is unaware of the distinction. Consequently, ample inspiration for those seeking those rules in natural or engineered microbial systems can be found in the classical ecological literature, which is rich in theory.

With respect to community assembly, microbial ecologists can borrow from two opposing, but not mutually exclusive, perspectives. Classically, microbial assembly processes have been considered to be deterministic in which the presence of microbes reflects the presence or absence of that organism's niche (Chase and Leibold, 2003). Microbial ecologists routinely and uncontroversially interpret their work in this context. Alternatively, one can view the formation of a community as a stochastic process in which microbes colonize an environment and change within it at random (Bell, 2001; Hubbell, 2001). While the more recent introduction of the stochastic or neutral community models (NCM) in general ecology have been controversial, in essence, they are reworkings of the widely accepted theory of island biogeography (MacArthur and Wilson, 1967). Neutral theory has been successfully applied to microbial communities (Sloan et al., 2006, 2007), including the successful prediction of a taxa volume curve (Woodcock et al., 2007), a central feature of the theory of island biogeography. In reality, the two perspectives are not mutually exclusive because birth, death, and immigration, which are central

to NCM, are ineluctable features of microbial life whose rates are affected by differences between species. Indeed, niche and neutral processes have been combined in a dynamic microbial NCM describing a Californian wastewater treatment system (Ofiteru et al., 2010).

The relationship between diversity and function is also important. Many classical ecologists assert that higher levels of diversity promote improved ecosystem functioning (Tilman et al., 1997; Hooper et al., 2005). In naturally occurring microbial communities, evenness and the number of taxa are confounded (a more even distribution means more taxa are observed; Curtis et al., 2006). Two important studies have used pure cultures so that the findings with respect to richness (the number of taxa) and evenness, and the co-occurrence of specific taxa are not confounded. Bell et al. (2005) used artificial communities to demonstrate that greater taxonomic richness led to greater productivity in microcosms degrading leaf litter. (Curtis et al., 2006). Wittebolle et al. (2009) were able to hold diversity constant whilst manipulating evenness to establish that uneven communities exhibited reduced functionality and less resistance to stress. However, the range of taxa used in these highly controlled studies is far below those found in real microbial communities. Less controlled studies have suggested that the benefits of increasing diversity plateau relatively quickly and that most of the extraordinary diversity in microbial communities is redundant (Franklin and Mills, 2006).

Nevertheless, it is also possible that the rare taxa may indeed have important functions. In particular, they may be responsible for the degradation of low concentrations of environmentally or economically important chemicals. For example, rare microbes may be involved in the metabolism of estrogen, compounds that disrupt the endocrine systems of animals and aquatic biota, leading to abnormalities in reproductive structure and function. There are two major types of estrogens; (i) natural estrogens, i.e., 17-estradiol (E2), estrone (E1), and estriol (E3), which are mainly excreted from humans and mammals; and (ii) synthetic estrogens, such as 17 α-ethinyloestradiol (EE2), which is the main ingredient in oral contraceptive pills. Both synthetic and natural forms occur in wastewater and their removal in biological treatment systems is poorly understood and highly unpredictable (Sumpter, 2009). The relative abundance of taxa responsible for the degradation of even the best removed estrogens, e.g., E1, are indeed rare, representing less than 2% of the microbial community (Zang et al., 2008; Thayanukul et al., 2010).

There have been various attempts to explain differences in estrogen removal with respect to differences in the design and operational parameters of biological treatment systems (Vader et al., 2000; Joss et al., 2004; Clara et al., 2005; Koh et al., 2008; McAdam et al., 2010). These studies infer, but do not demonstrate, that competitive niche mechanisms govern this function and stochastic mechanisms have not been considered.

A deeper understanding of the relationship between community assembly and rare functions would permit us to control endocrine disrupting compound (EDC) removal in engineered biological systems and predict the fate of micropollutants in natural microbial systems. Meeting this challenge is both intrinsically interesting and economically important. We have sought to address this by using both niche and neutral concepts to manipulate the microbial diversity of a reactor and evaluating the associated changes in EDC removal in a partial factorial designed experiment. The putatively neutral interventions were the organic loading rate and the addition of soil. The loading rate controls the number of individuals, more individuals should mean more diversity. Adding soil should increase the immigration rate, and thus the diversity. The putative niche interventions were the range of sugars in the feed and temporal variation in feed concentration associated with batch feeding. We reasoned that feed with more sugars would have more niches as would the change in feed concentration in time. We could not unequivocally support simple hypotheses about the relationship between niche or neutral effects and diversity and function. Both approaches could increase or decrease diversity and increases in diversity did not always lead to increases in EDC removal. We found that the counter intuitive results can be interpreted in the context of neutral theory.

## MATERIALS AND METHODS

The experimental design employed has been described in detail by Pholchan et al. (2010), where the effect of microbial community diversity on the engineering functions of carbon and nitrogen removal was addressed. Briefly, a two level fractional factorial design was undertaken, which required the performance of eight experimental runs. We sought to change the diversity of the reactors by manipulating the following variables: types of feed (simple, a single sugar, and complex, multiple sugars), types of feeding regime (sequencing batch reactor, or SBR, and completely stirred tank reactor, or CSTR), organic loading rate (low and high) and the addition of exogenous bacteria to promote immigration (by addition of soil and non-addition of soil). The factorial analysis permitted the determination of the joint effects of these four independent parameters as: the strength of the effects, coefficients, standard deviation of coefficients and associated probabilities. The responses in this study were expressed as percentages of EDC removal, whilst the microbial diversity was tentatively inferred from the number of bands on denaturing gradient gel electrophoresis (DGGE) gels. The data obtained were analyzed using Minitab (version 14, Minitab Inc., USA), based on the analysis of variance (ANOVA).

Two laboratory-scale SBR and two CSTR reactors were constructed each with a working volume of 17 l and fed with synthetic sewage with two types of carbon source (single carbon source/complex carbon source) at two different strengths (600/1,200 mg chemical oxygen demand (COD/l). The carbon sources (mg/l) were, for the simple waste, glucose (600) and for the complex waste: glucose (78.1), sucrose (148), lactose (148), fructose (78.1), and starch (12.7). The concentration of each sugar used in the simple and complex carbon source was calculated to render a total COD of 600 mg/l. The remaining constituents, in mg/l, were peptone (10.0), $KH_2PO_4$ (40.0), $NH_4Cl$ (153), $CaCl_2$ (13.9), $MnSO_4$ (0.28), $ZnCl_2$ (0.21), $CuSO_4$ (0.25), and $MgCl_2$ (36.2). The energy calculations were based on the bomb-calorimeter data of Southgate (1981). Soils used in this study were collected from the Rivington series at the University of Newcastle's Cockle Park Farm (Northumberland, UK). To separate indigenous bacteria

from soil, plant roots were removed and soils (100 g per reactor) were suspended in 250 ml of sterile distilled water with sodium cholate (0.1% (w/v)). The soil suspension was shaken for 15 min with glass balls (35 × 45 mm, BHD Chemical Ltd., England) before being placed into an ultrasonicator for 1 min. The solution was sieved and the dislodged cells were separated from soil particles by centrifuging at 500 × *g* for 1 min. Bacterial suspensions (150 ml) were added to each reactor daily. This is equivalent to adding ∼$6 \times 10^6$ bacterial cells per ml or about 1–5% of the bacterial total count. The seeded soil organisms were calculated to be below the detection limit for denaturing gel electrophoresis (Muyzer et al., 1993).

The influent and effluent were collected every three days for physical and chemical analysis. Physical and chemical tests (i.e., pH, temperature, Alkalinity, COD, total organic carbon (TOC), total kjeldahl nitrogen (TKN), $NO_2^-$, $NO_3^-$, $PO_4^{3-}$, mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), $SV_{30}$, sludge volume index (SVI), and capillary suction time (CST)) were conducted according to the Standard Methods (Clesceri et al., 1998). A biomass sample was taken from the aeration tank of each reactor every week, preserved immediately in 50% ethanol and stored at −20°C prior to the molecular analysis. Changes in the microbial community were monitored by performing DGGE of polymerase chain reaction (PCR)-amplified 16S rRNA gene segments from the bacterial community. The method is described in detail elsewhere (Pholchan et al., 2010). DNA was extracted using the Fast-DNA SPIN for soil kit (Q-Bio gene, Cambridge, UK). The total bacterial community was analyzed using PCR amplification with the classical primers 2 and 3, which contained a GC clamp (Muyzer et al., 1993). DGGE bands were visualized under an UV transilluminator with the program Quantity One (Bio-Rad) and DGGE banding patterns were analyzed using the Bionumerics software (Applied Mathematics) and PAST statistical software (Hammer and Harper, 2006).

The initial concentration of each estrogen studied (i.e., E1, E2, E3, and EE2) in the feed was set to an equivalent of 1 μg/l in all experiments. This is a typical concentration found in the wastewaters in processes that combine returned water from the sludge dewatering process with the raw wastewater (Matsui et al., 2000) and has been used in previous laboratory-scale studies (Li et al., 2005). Estrogens E1, E2, and EE2 (Sigma-Aldrich, UK), were separately dissolved in acetone to the concentration of 1.0 mg/ml and stored at −20°C. Prior to dosing the feed, a mixed solution of all estrogens was prepared by diluting the stock solutions in distilled water to the desired concentration. The EDCs concentrations were measured by gas chromatography mass spectrometry (GC/MS).

The method used to determine concentrations of estrogens was modified by Pholchan et al. (2008) from the method originally described by Nakamura et al. (2001). Mirex (Sigma-Aldrich, UK) was used as an internal standard along with 17α estradiol (Sigma-Aldrich, UK), which was used as a surrogate standard. The method comprised solid phase extraction and derivatization before analysis using a Hewlett–Packard 6890 GC split/splitless injector (260°C) linked to a Hewlett–Packard 5973 Mass Spectrometer with negative-ion chemical ionization mode. All reagents (GC-MS grade) and chemical standards were supplied by Sigma-Aldrich, UK.

Before the estrogens were added, the reactors were all inoculated with 4 l of sludge from the aeration tank of a municipal wastewater treatment plant (Tudhoe Mill Wastewater Treatment Plant, Spennymoor, Durham). At the start of each experimental run, the reactors were re-inoculated in a similar fashion. The Tudhoe Mill wastewater treatment plant was designed to achieve both organic carbon and nitrogen removal. Immediately after delivery to the laboratory, the sludge was kept aerated before being seeded into the reactor within 1–2 h. The MLSS and MLVSS of the seed were approximately 3050 and 2130 mg/l, respectively. Each experimental run lasted for approximately 60 days from the start of the addition of 1μg/l estrogens to the feed. All reactors were operated at room temperature (20 ± 2°C). The pH of the mixed liquor was maintained between seven and eight. The reactors were aerated, to ensure that the dissolved oxygen concentration was ≥2 mg/l. A sludge age of 10 days was maintained for all reactors by wasting excess sludge four times per day.

Community assembly was modeled as described previously (Sloan et al., 2007) using a simple neutral model executed in Matlab, where the source term, Θ, was set to 176 to illustrate lower diversity and 1760 to illustrate higher diversity. The immigration parameter ($m$) was set to $3.2 \times 10^{-9}$ and the total number of individuals in the reactors ($N_T$) was set to $1.7 \times 10^{13}$ (equivalent to 17 l with $10^9$ individuals/ml). The model is illustrative as we could not parameterise the model using the data available. The parameters for the higher diversity and immigration were chosen on the basis of earlier simulations of wastewater treatment plants (Curtis et al., 2006). The lower source diversity was chosen arbitrarily to illustrate the effect of low diversity clearly. In reality the putative difference in diversity would have been less pronounced.

## RESULTS

The manipulations had explicit effects on bacterial diversity: every intervention had a statistically significant effect on the number of bands detected (**Table 1**; **Figure 1**). However, the effects themselves were often counterintuitive. As expected, an increased organic load (more biomass) was associated with an increase in the diversity. Similarly a SBR (with temporal gradients as organic carbon and ammonia are utilized during the batch process) had more diversity than a homogenous stirred tank reactor. However, the use of more sugars in the feed (more nutritional complexity, more niches) was associated with a decrease in the observed richness. Similarly, the addition of soil (putatively increasing immigration) was associated with a decrease in the number of bands detected. The rather complex picture of the main effects is further complicated by the significant interactions between the experimental variables (**Table 1**; **Figure 1**). For example, the effect of the reactor format seemed to be partially dependant on the organic loading rate (**Table 1**; **Figure 1**).

The interventions did have statistically significant effects on the removal of the EDCs (**Table 2**; **Figure 2**). However the effects were inconsistent between compounds and incongruent with our expectations about diversity and function. Thus adding soil, which apparently decreased putative richness, appeared to increase the removal of E1 and E3 and had no effect on E2 and EE2. Likewise the

**Table 1 | Calculated direction and significance of the main effects and their interactions on number of bands.**

| Term | Effect | Coefficient | SE of coefficient | *P* value |
|---|---|---|---|---|
| Constant | | 23.188 | 0.2421 | 0.000 |
| Main factors | | | | 0.000 |
| Type of carbon source | −1.875 | −0.937 | 0.2421 | 0.005 |
| Organic loading rate | 1.125 | 0.563 | *0.2421* | *0.049* |
| Type of feeding regime | −3.625 | −1.813 | 0.2421 | 0.000 |
| Adding soil | −2.625 | −1.313 | 0.2421 | 0.001 |
| Interaction of two factors | | | | 0.000 |
| Type of carbon source × organic loading rate | 3.375 | 1.688 | 0.2421 | 0.000 |
| Type of carbon source × type of feeding regime | −1.875 | −0.937 | 0.2421 | 0.005 |
| Type of carbon source × adding soil | 8.125 | 4.062 | 0.2421 | 0.000 |
| $S = 0.9682$ | $R^2 = 0.9826$ | | $R^2_{(adj)} = 0.9673$ | |

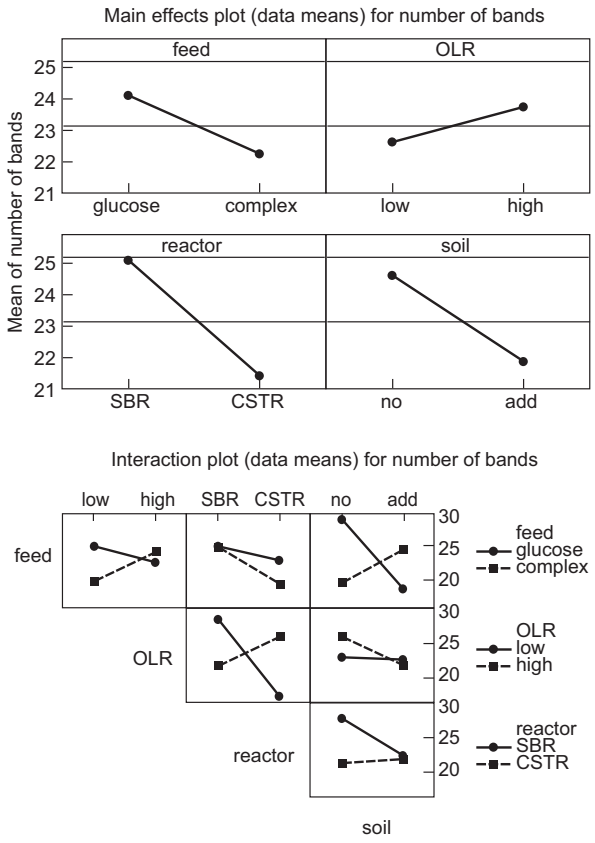


**FIGURE 1 | Main effects plots (data means) on the number of bands: OLR, organic loading rate; SBR, sequencing batch reactor; CSTR, completely stirred tank reactor; no, no added soil; add, soil added to reactor.**

more complex feeds, which also ostensibly decreased the number of detectable bands, apparently increased the removal of E1, E2, and E3, but not EE2. By contrast the increase in diversity associated with an increased organic loading rate coincided with a decrease in E3 removal and had no change in the removal of E1, E2, and EE2. Only in the case of the SBR was the increased diversity associated with an increase in the removal of all the steroidal estrogens tested.

The counter intuitive result for resource complexity led us to examine the waste composition in more detail, in particular the energy of the wastes. The complex and the simple wastewater were designed to have the same COD. However, assuming a free energy of 3.75, 3.95, and 4.15 kj/g for monosaccharides (glucose, fructose) for disaccharides (sucrose, lactose,) and starch, respectively, we can see that the free energy of the complex waste provided by the carbohydrates was 1.8 kj/l, while the free energy of the simple waste provided by the carbohydrates was 20% higher, i.e., 2.2 kj/l.

The counter intuitive observations of EDC removal in relation to diversity led us to consider the evenness of the DGGE gels. Although DGGE data cannot be considered truly quantitative, we assessed the evenness of the communities in the samples using the Berger–Parker index (the proportional abundance of the most abundant type, based on band intensities). The evenness assessed as the reciprocal of this index (**Figure 3**) was proportional to the number of bands observed ($R^2 = 0.74$; $p = 0.006$). Having demonstrated that greater observed evenness is associated with greater observed richness we used a simple neutral model to demonstrate how decreasing diversity might increase or decrease the removal of EDCs. Since such compounds were present in concentrations $\leq$1 μg/l they were not thought to affect the abundance of the taxa responsible for their removal. We can therefore assume that the EDC degraders are present at random at an abundance dictated by their proportional abundance in the metacommunity.

The evenness of the microbial community was changed by making arbitrary changes in the size of the source community term. As shown in **Figure 4**, reducing the microbial diversity paradoxically increases the abundance of many taxa that would be considered to be relatively rare.

## DISCUSSION

An appropriately designed statistical trial was unable to unequivocally support simple hypotheses about niche or neutral effects and

**Table 2 | Calculated direction and significance of the main effects and their interactions on the % removal of EDC in the reactors.**

| Term | Effect | Coefficient | SE of coefficient | *P* value |
|---|---|---|---|---|
| **A: estrone (E1)** | | | | |
| Constant | | 96.2750 | 0.3311 | 0.000 |
| Main factors | | | | 0.001 |
| Type of carbon source | 1.7250 | 0.8625 | 0.3311 | 0.031 |
| Organic loading rate | −0.050 | −0.0250 | 0.3311 | 0.942 |
| Type of feeding regime | −1.500 | −0.7500 | 0.3311 | 0.053 |
| Adding soil | *4.9750* | *2.4875* | 0.3311 | *0.000* |
| Interaction of two factors | | | | 0.095 |
| Type of carbon source × organic loading rate | 0.9250 | 0.4625 | 0.3311 | 0.200 |
| Type of carbon source × type of feeding regime | −0.7250 | −0.3625 | 0.3311 | 0.305 |
| Type of carbon source × adding soil | −1.6000 | −0.8000 | 0.3311 | 0.042 |
| $S = 1.3243$ | | $R^2 = 0.9063$ | | $R^2_{(adj)} = 0.8243$ |
| **B: 17-beta-estradiol (E2)** | | | | |
| Constant | | 96.8188 | 0.1851 | 0.000 |
| Main factors | | | | 0.002 |
| Type of carbon source | 1.7625 | 0.8821 | 0.1851 | 0.001 |
| Organic loading rate | 0.4625 | 0.2313 | 0.1851 | 0.247 |
| Type of feeding regime | −1.7125 | −0.8563 | 0.1851 | 0.002 |
| Adding soil | −0.0625 | −0.0313 | 0.1851 | 0.870 |
| Interaction of two factors | | | | 0.043 |
| Type of carbon source × organic loading rate | −1.2625 | −0.6313 | 0.1851 | 0.009 |
| Type of carbon source × type of feeding regime | −0.4375 | −0.2188 | 0.1851 | 0.271 |
| Type of carbon source × adding soil | −0.0875 | −0.0437 | 0.1851 | 0.819 |
| $S = 0.7404$ | | $R^2 = 0.8801$ | | $R^2_{(adj)} = 0.7753$ |
| **C: estriol (E3)** | | | | |
| Constant | | 96.494 | 0.2292 | 0.000 |
| Main factors | | | | 0.000 |
| Type of carbon source | 2.863 | 1.431 | 0.2292 | 0.000 |
| Organic loading rate | −1.463 | −0.731 | 0.2292 | 0.013 |
| Type of feeding regime | −2.838 | −1.419 | 0.2292 | 0.000 |
| Adding soil | *4.738* | *2.369* | 0.2292 | *0.000* |
| Interaction of two factors | | | 0.2292 | 0.000 |
| Type of carbon source × organic loading rate | 1.113 | 0.556 | 0.2292 | 0.041 |
| Type of carbon source × type of feeding regime | 0.387 | 0.194 | 0.2292 | 0.423 |
| Type of carbon source × adding soil | −3.588 | −1.794 | 0.2292 | 0.000 |
| $S = 0.9139$ | | $R^2 = 0.9704$ | | $R^2_{(adj)} = 0.9445$ |
| **D: 17-alpha-ethynyloestradiol (EE2)** | | | | |
| Constant | | 81.056 | 1.228 | 0.000 |
| Main factors | | | | 0.062 |
| Type of carbon source | 4.863 | 2.431 | 1.228 | 0.083 |
| Organic loading rate | 1.712 | 0.856 | 1.228 | 0.506 |
| Type of feeding regime | −7.513 | −3.756 | 1.228 | 0.016 |
| Adding soil | 1.137 | 0.569 | 1.228 | 0.656 |
| Interaction of two factors | | | | 0.110 |
| Type of carbon source × organic loading rate | 4.612 | 2.306 | 1.228 | 0.097 |
| Type of carbon source × type of feeding regime | −5.313 | −2.656 | 1.228 | 0.063 |
| Type of carbon source × adding soil | 0.987 | 0.494 | 1.228 | 0.698 |
| $S = 4.9140$ | | $R^2 = 0.7362$ | | $R^2_{(adj)} = 0.5054$ |

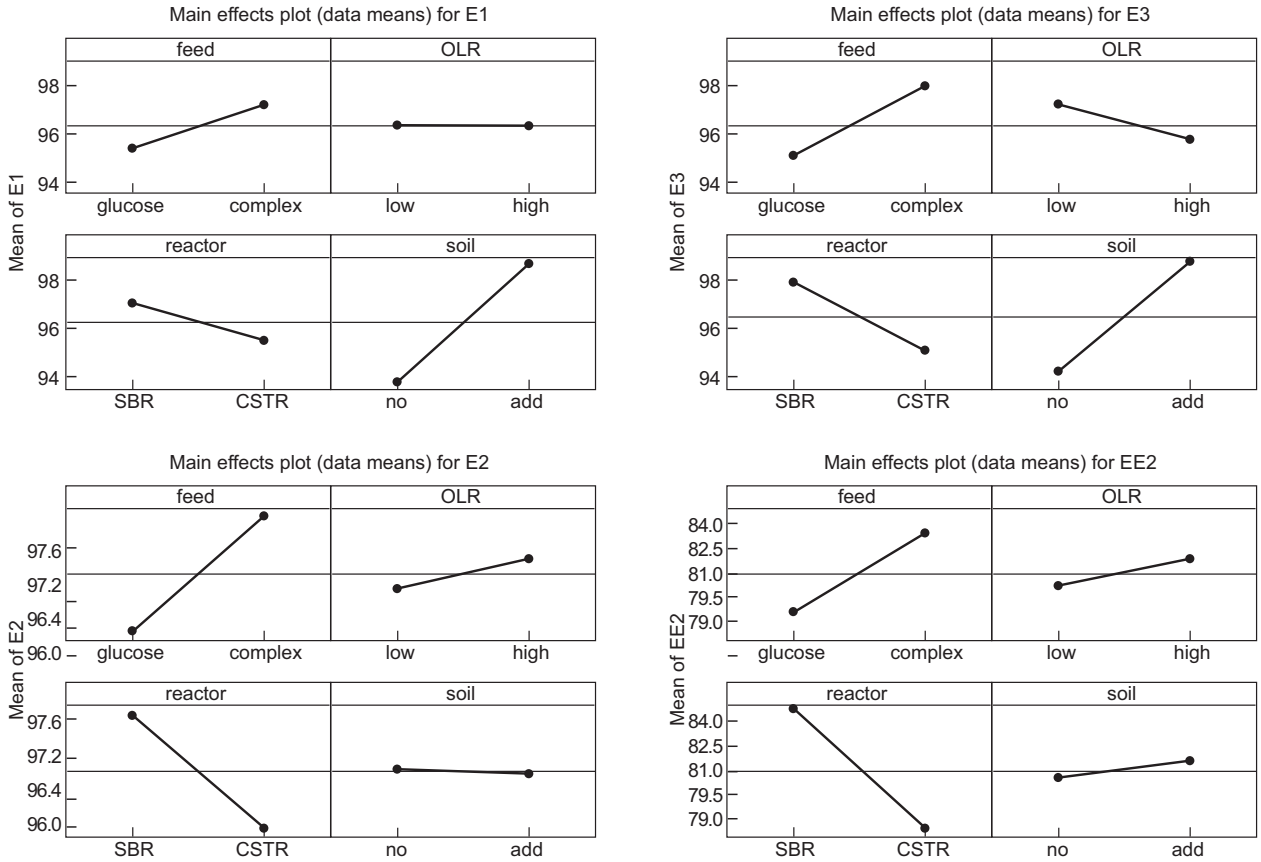


**FIGURE 2 | Main effects plots (data means) on the removal of endocrine disrupting compounds.**

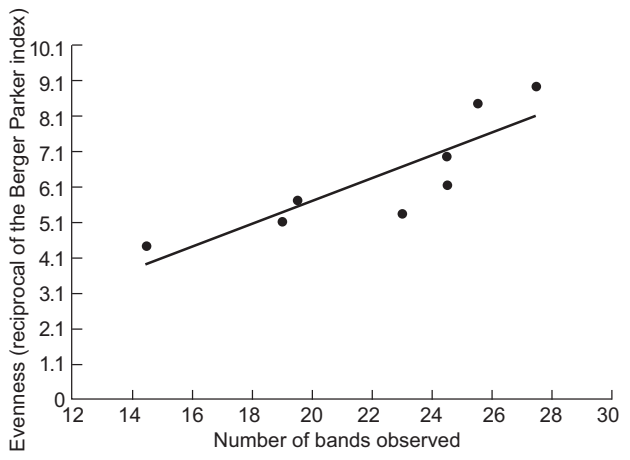


**FIGURE 3 | The relationship between the evenness of the bands on DGGE and the number of the bands.** The Berger–Parker index is the proportional abundance of the most abundant taxon.

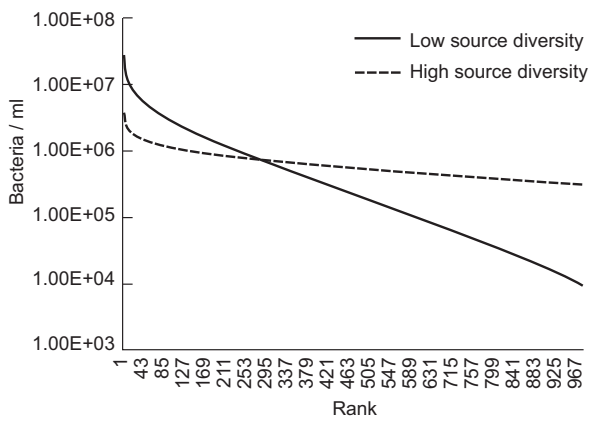


**FIGURE 4 | Results of evenness and diversity using a simple neutral model executed where a source term, or Θ, was set at 176 to illustrate lower diversity and 1760 to illustrate higher diversity.** The immigration parameter ($m$) was set at $3.2 \times 10^{-9}$ and the number of individuals ($N_T$) was set at $1.7 \times 10^{13}$ (equivalent to 17 l with $10^9$ individuals/ml).

the relationship between diversity and function. Consequently, the primary conclusion of this paper is that one cannot necessarily use unguided or untested intuition to make inferences about the diversity in microbial communities or how that diversity affects putatively rare functions. However, the failures are potentially more interesting than successes. For failure implies the existence

of unsuspected mechanisms and reinforces the importance of experimentation in microbial ecology.

We are confident that the counter-intuitive nature of our findings cannot be attributed to the limitations of DGGE. Fingerprinting methods like DGGE are regarded as inferior to the new generation of sequencing techniques. It is often forgotten that DGGE, like next generation sequencing, uses a large sample size (about 10,000 individuals). Studies with next generation sequencing have suggested this sample size is enough to make valid comparisons between datasets. The weakness of DGGE is that it can only detect those taxa that occur above an abundance threshold of about 1% of the community under consideration (Muyzer et al., 1993). The implicit assumption in this study is that a change in diversity is associated with a change in the number of taxa above this threshold. The association between evenness and the number of taxa detected supports this assumption. Moreover, some large-scale patterns first detected with fingerprinting methodologies (Fierer and Jackson, 2006) have been found to be substantially unaltered when re-examined with larger datasets (Lauber et al., 2009).

Thus it is gratifying, but not interesting, that creating temporal variation (using a SBR) and increasing the number of individuals (by increasing the organic load) both appeared to increase diversity. Both results seem intuitively reasonable from a niche and neutral perspective, respectively. That both perspectives appear valid strengthens our assertion, supported by recent modeling work, that niche effects will overlay neutral ones (Ofiteru et al., 2010). However, it is this relatively bland interpretation that emphasizes the challenge and importance of attempting to quantify the relative importance of these mechanisms, which we are unable to do with data in this format.

The failure of immigration to increase diversity is not, on reflection, surprising. The organisms in the soil were at or below the detection limit for DGGE and so will not have been directly detectable unless they increased in abundance in the system. Ecologists recognize that it is difficult to "invade" mature communities and have formulated the monopolization hypothesis to account for this phenomenon (De Meester et al., 2002). There is evidence that immigration occurs at low rates in microbial communities subject to high rates of dispersal (Curtis et al., 2006; Baptista et al., in preparation). This phenomenon may well underlie the poor reputation of bio-augmentation in many quarters. The slight drop in the diversity associated with the addition of soil is harder to interpret from a neutral perspective. There are possible niche-based explanations. For example, the decreased diversity in the glucose treatment could be a result of competition from fewer, but more competitive species from the soil. Alternatively, it might be attributable the small quantity of sodium cholate used to suspend the soil.

However, the failure of a feed with a more complex chemical composition to support greater diversity is problematic. We do not believe this is an artifact, as we have also recently failed to increase diversity by increasing resource complexity in a bioelectrochemical cell (Heidrich, submitted). We are unable to conceive of a simple niche-based explanation though we admit that multiple resource can have complex effects (Lendenmann and Egli, 1998). A NCM requires that we invoke either a more diverse source community, higher rate of migration or a larger local community. We have no reason to invoke a higher immigration rate in glucose fed reactors. However, the biomass was slightly and significantly (Pholchan et al., 2010) higher with glucose (which has a slightly higher free energy) than with complex wastes, even though the COD of the feeds were the same. This suggests that the yields are higher for organisms growing on glucose than on complex wastes. The larger amount of biomass could lead to a higher diversity. However, we have previously postulated that groups of organisms with higher yields will also have higher evolution rates and thus higher source diversities (Curtis et al., 2008). To separate these two possibilities it would be necessary to fit a neutral model to our findings. This is not possible with the data we have generated.

To interpret the ostensibly contradictory and counterintuitive findings with respect to diversity and EDC removal, we must recall that the functions rendered by a microbial community will depend on the abundance of the organisms performing that function and the environment encountered. It is clear from the simple community assembly model that a drop in evenness in a microbial community, which would be associated with a drop in observed richness, could also lead to an increase in the abundance of certain taxa. This would explain why EDC removal increased when diversity decreased in reactors fed with complex wastes or dosed with a soil suspension. The corollary that increases in diversity lead to a reduction in EDC removal was (i) perceptible at higher organic loading rates where the removal of E3 was lower (with no effect being seen with E1, E2, or EE2) but (ii) absent in higher diversity SBRs. However, SBRs are effectively plug flow reactors and thus far more hydraulically efficient than CSTRs. Consequently, the former (SBRs) may achieve higher effluent quality than the latter (CSTRs), even with an inferior removal rate.

We conclude that it is not possible to make simple blanket statements about the relationship between putative rare functions and diversity. This supports earlier insightful suggestion that ecologists should not always assume that increased biodiversity always means improved function (Jiang et al., 2008) and warn against extending findings on biomass and diversity (Tilman et al., 1997) to other functions.

If our interpretation is correct, this relationship may well depend on the proportional abundance of the organisms with that function in the metacommunity, the size of the local community and the immigration rates that connect the former and the latter. It will thus be predictable using neutral theory, but not intuitively obvious and, of course, subject to other environmental constraints. The application of this approach would require that we eschew the nebulous term rare and define the proportional abundance, and possibly activity, of the taxa responsible for the removal of a given micropollutant. This is a study of a series of pilot plants seeded from just one "mother" plant and we cannot be sure how generalizable our findings are. However, neutral theory (if applicable) would allow us to determine "a priori" how generalizable our findings by relating the probability of a function occurring to the proportional abundance of the organisms with that function in the metacommunity.

The niche and neutral perspectives are not mutually exclusive. One might also explain the patterns in these relatively rare functions by invoking the appearance and disappearance of niches for the EDC degrading organisms. However, we have no evidence to

support this perspective which, if true, could mean the presence and absence of functions is situation bound.

In summary we suggest that, in this system, an increase in evenness equates with a decrease in abundance of some abundant taxa and an increase in abundance in some rare taxa. This is why a change in evenness can lead to a decrease in the removal of some EDC and an increase in others. Unless and until we know the proportional abundance of the EDC removing taxa we will not be able to determine the effect of diversity on EDC removal. Unless we know the mechanism governing the diversity (niche or neutral or both) we will not be able to predict EDC removal.

## ACKNOWLEDGMENTS

We thank Diana Nemergut and the excellent anonymous reviewers of this manuscript for their thoughtful and perspicacious comments.

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

*Received: 08 August 2012; accepted: 11 March 2013; published online: 01 May 2013.*

*Citation: Pholchan MK, Baptista JC, Davenport RJ, Sloan WT and Curtis TP (2013) Microbial community assembly, theory and rare functions. Front. Microbiol.* ***4****:68. doi: 10.3389/fmicb.2013.00068*

*This article was submitted to Frontiers in Evolutionary and Genomic Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

ORIGINAL RESEARCH ARTICLE
published: 12 June 2012
doi: 10.3389/fmicb.2012.00209

# Relating phylogenetic and functional diversity among denitrifiers and quantifying their capacity to predict community functioning

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Genetic diversity of phylogenetic or functional markers is widely used as a proxy of microbial diversity. However, it remains unclear to what extent functional diversity (FD), gene sequence diversity and community functioning are linked. For a range of denitrifying bacteria, we analyzed the relationships between (i) the similarity of functional traits evaluated from metabolic profiles (BIOLOG plates) or from $N_2O$ accumulation patterns on different carbon sources and (ii) the similarity of phylogenetic (16S rRNA gene) or functional (*nir* gene) markers. We also calculated different proxies for the diversity of denitrifier community based on taxa richness, phylogenetic (16S rRNA gene) or functional similarities (based either on metabolic profiles or $N_2O$ accumulation patterns), and evaluated their performance in inferring the functioning of assembled denitrifying communities. For individual strains, the variation in the 16S rRNA gene sequence was weakly correlated with the variation in metabolic patterns ($\rho = 0.35$) and was not related to $N_2O$ accumulation. The latter was correlated with the similarity of nitrite reductase residues. When nir genes were analyzed separately, the similarity in amino acids coded by the *nirS* genes was highly correlated with the observed patterns of $N_2O$ accumulation ($\rho = 0.62$), whereas *nirK* amino acid residues were unrelated to $N_2O$ accumulation. For bacterial assemblages, phylogenetic diversity (average similarity among species in a community) and mean community dissimilarity (average distance between species) calculated using 16S rRNA gene sequences, and FD measures associated with metabolic profiles, poorly predicted the variation in the functioning of assembled communities ($\leq$15%). In contrast, the proxies of FD based on $N_2O$ accumulation patterns performed better and explained from 23 to 42% of the variation in denitrification. Amongst those, community niche was the best metric, indicating the importance of complementarity for resources in the context of bacterial community functioning.

**Keywords: biodiversity proxies, carbon sources, denitrification, functional traits, phylogenetic diversity**

## INTRODUCTION

As biodiversity is threatened by global changes, understanding the relationship between diversity and the functioning of ecosystems or biological communities has received increasing attention (Hooper et al., 2005). Microbial communities are key drivers of soil ecosystem processes. Yet, as 10 g of soil might contain $10^4$–$10^6$ distinct taxa (Torsvik and Ovreas, 2002; Gans et al., 2005), a complete understanding of microbial diversity is still a challenge. Despite this overwhelming prokaryotic diversity, the potential loss of diversity at the microbial scale is also of concern. In this context, it remains unclear to what extent microbial community structure, especially when based on the 16S rRNA gene as phylogenetic marker, can be used to explain differences in ecosystem processes related to carbon and nitrogen cycles (Wertz et al., 2006; Fierer et al., 2007; Le Roux et al., 2008; Philippot et al., 2010). Overall, from a biodiversity-ecosystem functioning point of view, it remains unclear the extent to which microbial community composition can be used to predict its impact on ecosystem processes and how community composition should be characterized in this context.

Given the high degree of redundancy observed for microorganisms, changes in microbial community structure might not lead to changes microbial community function. Functional redundancy is indeed commonplace and especially for broad functions such as those associated with carbon metabolism, community composition may not influence community functioning (Wohl et al., 2004; Wertz et al., 2006). In contrast, it has been recently shown that differences in microbial community structure led to changes in mineralization rates (Strickland et al., 2009). Contradictory results have also been observed for other less broad functions, such as denitrification in soils (Cavigelli and Robertson, 2000; Wertz et al., 2006; Salles et al., 2009). This inconsistency in results might be due to differences in the methodological approaches and genes targeted to predict the effect of community structure on ecosystem functioning. For some processes, the genetic diversity of the phylogenetic marker is not related to the functional traits

that influence the functioning of the ecosystem (Jones et al., 2008; Baelum et al., 2010). In that case, taxa identity might not provide enough information on whether an organism is able to carry out a given process, at what rates or under which environmental conditions. For instance, physiological studies have shown that the enzymatic activities among species from different bacterial classes might be more comparable than between species belonging to the same genus (Carlson and Ingraham, 1983) whereas very similar taxa can strongly differ from a functional point of view (Salles et al., 2009). Therefore, in order to better predict community functioning, it is crucial to understand the relationship between microbial phylogeny and physiology (Allison and Martiny, 2008).

In the context of bacteria associated with the nitrogen cycle, the congruence between taxonomic phylogenies, which are mostly based on 16S rRNA gene sequences, and the phylogenies based on functional gene sequences is process-dependent. For instance, ammonium oxidation is carried out by a relatively small number of prokaryotic taxa and experimental evidence suggests a congruence in the phylogeny of ribosomal (16S rRNA) and *amo* genes for both bacterial and archaeal ammonia oxidizers (Prosser and Nicol, 2008). Among denitrifiers, the enzyme nitrite reductase, which mediates the reduction of nitrite to nitric oxide, is present in all denitrifying bacterial species. Furthermore, two functionally redundant but structurally distinct nitrite reductases are found among denitrifiers: a copper (Cu-Nir) and a cytochrome *cd1* (Cd-Nir) nitrite reductase, coded by the *nirK* and *nirS* genes, respectively. In contrast to nitrifiers, denitrifying bacteria are phylogenetic diverse and are distributed over 60 genera (Zumft, 1997). When comparing the phylogeny of the *nir* genes with the 16S rRNA gene, it was shown that only the *nirS* and 16S rRNA gene phylogeny was congruent, suggesting that *nirK* might have been mainly acquired through horizontal gene transfer (Heylen et al., 2006).

The use of either phylogenetic or functional genes has improved our knowledge about the ecology of microbial functional groups. For instance, a good congruence seems to exist between the phylogeny (at the genus level) and functional traits of bacterial nitrite oxidizers (Attard et al., 2010). In the case of denitrification, the use of functional genes might provide evidence that *nirK*- and *nirS*-harboring bacteria are ecologically distinct. For instance, a study focusing on a short-term restoration chronosequence indicated that the abundance of the major *nirS* populations vary similarly with time after disturbance, since sites that were restored at the same time shared higher similarities in *nirS* communities. Conversely, *nirK* populations were characterized by three independent response groups, suggesting higher sensitivity to environmental gradients (Smith and Ogram, 2008). Furthermore, it has been proposed that *nirK* denitrifiers respond to a range of environmental parameters, whereas denitrifiers harboring *nirS* are mainly driven by nitrate availability (Jones and Hallin, 2010). The ecologically distinct role of *nirK* and *nirS* communities has been also observed in the rhizosphere of grain legumes where *nirK* and not *nirS* gene transcripts could be detected (Sharma et al., 2005), and in cropping systems where changes in denitrification were related to the abundance of *nirK*- rather than *nirS*-harboring bacteria (Attard et al., 2011).

Despite our increasing knowledge about the ecological distribution of these functional groups and the mechanisms underlying community assembly, an issue that remains open is to what extent the information based on the genetic diversity of phylogenetic or functional markers reflect the diversity of traits among functional groups, and furthermore, how both these markers ultimately influence ecosystem functioning. In this study we used denitrifiers as a model group to address these relationships. We focused on denitrification because it is a function performed by taxonomically diverse species, whose activity is regulated in particular by the quality and quantity of organic compounds. Moreover, the two key functional genes coding for nitrite reductase (*nirK* and *nirS*) could provide different results due to their different functionalities and to the importance of horizontal transfer for *nirK*. In order to test the extent to which functional similarity (similarity of functional traits) is linked to the variation in the sequence of phylogenetic or functional marker genes, we analyzed a set of 29 denitrifying strains according to the following attributes. Genetic diversity was calculated for the phylogenetic (16S rRNA) and functional markers (nir). Additionally, functional trait diversity was determined according to the metabolic profiles of the strains by measuring their activity over a range of 95 carbon sources under denitrifying conditions and measuring their $N_2O$ accumulation patterns in presence of six different carbon sources. We then assessed to what extent the genetic similarities (16S rRNA gene or nir genes) could be used to infer the similarity in functional traits. In this context, we hypothesize that the 16S rRNA phylogenetic marker would be weakly correlated with the similarity in metabolic profiles, but not correlated with the $N_2O$ accumulation patterns. Moreover, we predict that the genetic diversity of the functional markers (nir genes) would correlate with the $N_2O$ accumulation patterns. Under this hypothesis, we also predicted that this correlation could be weaker for genes highly submitted to horizontal transfer, such as *nirK*.

In addition, to examine the link between bacterial diversity and denitrification, we calculated a range of diversity proxies widely used in general ecology (Heemsbergen et al., 2004; Petchey and Gaston, 2006; Cadotte et al., 2009; Petchey et al., 2009; Mouchet et al., 2010). We also determined which diversity proxies, based on taxa richness, phylogenetic (16S rRNA gene) or functional similarities (based either on metabolic profiles or $N_2O$ accumulation patterns), were more relevant for predicting the effect of bacterial diversity on denitrification. For that, we used recently published data (Salles et al., 2009) on denitrification rates for a range of assembled denitrifying bacterial communities. We hypothesize that diversity proxies that take into account functional attributes would be the best predictors of community functioning. The observed relationships between the functional traits and phylogenetic/genetic relatedness and a range of diversity proxies widely used in microbial ecology are discussed in the context of predicting the effect of bacterial diversity on community functioning.

## MATERIALS AND METHODS

### TAXONOMIC AFFILIATION OF STRAINS

A set of 29 denitrifying bacterial strains commonly occurring in the soil or plant rhizosphere were chosen (**Table 1**). The taxonomic identity of the strains was verified by amplifying 16S rRNA gene with primer set pA (5′ AGA GTT TGA TCC TGG CTC AG 3′) and pH (5′ AAG GAG GTG ATC CAG CCG CA 3′), which amplifies a fragment of approximately 1500 bp, corresponding to

**Table 1 | List of the denitrifying strains used in this work, indicating which functional and genetic aspects were investigated for each one.**

| Species classification | | | Functional information | | | Genetic information | | |
|---|---|---|---|---|---|---|---|---|
| **Phylum** | **Class** | **Species** | **Code** | **BIOLOG[a]** | **$N_2O$[b]** | **16S rRNA gene[c]** | **nir gene[d]** | |
| Proteobacteria | α | *Azospirillum brasilense* | G30 | ✓ | nd | HQ288925 | HQ288906 | *nirS* |
| | α | *Azospirillum lipoferum* TVV3 | A5 | ✓ | ✓ | HQ288929 | HQ288913 | *nirK* |
| | α | *Sinorhizobium* sp. | A1 | ✓ | ✓ | HQ288937 | HQ288915 | *nirK* |
| | α | *Ochrobactrum* sp. | A6 | ✓ | ✓ | HQ288924 | HQ288907 | *nirK* |
| | α | *Ochrobactrum* sp. | A10 | ✓ | nd | HQ288942 | HQ288908 | *nirK* |
| | α | *Ochrobactrum* sp. | A13 | ✓ | nd | HQ288933 | HQ288917 | *nirK* |
| | α | *Ochrobactrum* sp. | A22 | ✓ | ✓ | HQ288936 | HQ288914 | *nirK* |
| | α | *Ochrobactrum* sp. | A3 | ✓ | nd | HQ288931 | HQ288916 | *nirK* |
| | α | *Ochrobactrum* sp. | A17 | ✓ | ✓ | HQ288935 | HQ288911 | *nirK* |
| | α | *Paracoccus denitrificans* ATCC19367 | G11 | ✓ | ✓ | HQ288921 | HQ288897 | *nirS* |
| | β | *Achromobacter xylosoxidans* DSM30026 | G32 | ✓ | ✓ | HQ288922 | nd | nd |
| | β | *Achromobacter xylosoxidans* DSM2402 | G33 | ✓ | nd | HQ288926 | HQ288910 | *nirK* |
| | β | *Burkholderia* sp. G4 | G7 | ✓ | ✓ | HQ288947 | nd | nd |
| | β | *Burkholderia vietnamiensis* TVV75 | G8 | ✓ | ✓ | HQ288948 | nd | nd |
| | γ | *Citrobacter braakii* | A8 | ✓ | nd | HQ288930 | HQ288909 | *nirK* |
| | γ | *Citrobacter* sp. Hg31 | A7 | ✓ | ✓ | HQ288923 | HQ288919 | *nirK* |
| | γ | *Klebsiella pneumoniae* | A18 | ✓ | ✓ | HQ288920 | nd | nd |
| | γ | *Pseudomonas aeruginosa* ATCC27853 | G14 | ✓ | ✓ | HQ288928 | HQ288898 | *nirS* |
| | γ | *Pseudomonas aeruginosa* CIP | G16 | ✓ | ✓ | HQ288946 | HQ288905 | *nirS* |
| | γ | *Pseudomonas aeruginosa* ATCC15691 | A9 | ✓ | ✓ | HQ288943 | HQ288896 | *nirS* |
| | γ | *Pseudomonas aeruginosa* | A11 | ✓ | ✓ | HQ288940 | HQ288901 | *nirS* |
| | γ | *Pseudomonas aeruginosa* PAO1 | A12 | ✓ | ✓ | HQ288939 | HQ288902 | *nirS* |
| | γ | *Pseudomonas fluorescens* CHA0 | A15 | ✓ | ✓ | HQ288938 | HQ288918 | *nirK* |
| | γ | *Pseudomonas fluorescens* ATCC 17386 | A14 | ✓ | ✓ | HQ288944 | HQ288912 | *nirK* |
| | γ | *Pseudomonas stutzeri* ATCC11607 | A16 | ✓ | ✓ | HQ288945 | HQ288899 | *nirS* |
| | γ | *Pseudomonas stutzeri* Zobell | A24 | ✓ | ✓ | HQ288941 | HQ288904 | *nirS* |
| Firmicutes | bacilli | *Bacillus cereus* | A19 | ✓ | ✓ | HQ288927 | nd | nd |
| | bacilli | *Bacillus cereus* | A21 | ✓ | nd | HQ288934 | HQ288900 | *nirS* |
| | bacilli | *Bacillus weihenstephanensis* | A20 | ✓ | ✓ | HQ288932 | HQ288903 | *nirS* |

[a] *Metabolic profiles were performed with BIOLOG AN plates under denitrifying conditions.*

[b] *$N_2O$ accumulation values (μg N-$N_2O$ ml culture$^{-1}$) obtained on each of the six carbon sources, 66 h after the inoculation.*

[c] *Accession number corresponding to the almost full-length of the 16S rRNA gene sequence.*

[d] *Accession number corresponding to the partial sequences of the genes coding for nitrite reductase and the type of gene found. nirK: the copper (Cu-Nir) nitrite reductase; nirS, cytochrome; cd1 (Cd-Nir) nitrite reductase.*

✓, *analysis performed; nd, not determined.*

almost full-length gene sequence (Bruce et al., 1992). Details on the amplification procedure of the 16S rRNA gene are given as Appendix.

PCR products were purified using a PCR purification kit (Macherey Nagel, Düren, Germany) and cloned into the pGEM T-Easy vector system (Promega Ltd., Southampton, UK) and inserted in JM109 supercompetent *E. coli* cells (Stratagene Inc., Maidstone, UK). Clones were sequenced by LGC genomics using ABI 3730-XL Sequencer (LGC genomics, Berlin, Germany). The taxonomic affiliation of the strains was assigned by comparing the obtained sequences against public databases (Blastn, NCBI)[1]. The similarity between the 16S rRNA gene sequences was determined by creating a pairwise distance matrix based on the Jukes and Cantor substitution model (Ribosomal database project, release 10; Jukes and Cantor, 1969; Cole et al., 2007).

### CHARACTERIZATION OF THE NITRITE REDUCTASE IDENTITY

Partial sequences of the genes *nirK* and *nirS*, coding for the copper (Cu-Nir) or cytochrome *cd1* (Cd-Nir) nitrite reductase respectively, were amplified from the denitrifying bacterial strains. The *nirK* genes were amplified using the primers Copper 583F and Copper 909R (Liu et al., 2003), generating a fragment of 358 bp. A 425-bp fragment from the *nirS* gene was amplified by using the primers cd3aF and R3cd (Throback et al., 2004). Details on amplification procedure for *nirK* and *nirS* genes are given as Appendix. Both *nirK* and *nirS* fragments were cloned and sequenced as described above for 16S rRNA gene.

[1] http://www.ncbi.nlm.nih.gov/

The nucleotide sequences were aligned and translated to amino acid sequences using BioEdit[2]. Differences among amino acid residues between pairs of *nirK* or *nirS* denitrifying bacterial strains were measured between pairs of *nirK*- or *nirS*-harboring denitrifying bacterial strains, using Gonnet matrix (Gonnet et al., 1992) implemented in the software DNASTAR (Madison, USA).

#### DETERMINATION OF METABOLIC PROFILES

BIOLOG AN plates (AES Chemunex, Bruz, France) were inoculated with strains listed on **Table 1**. Cultures representing the pre-inoculum were grown under anaerobic conditions on NB medium containing 20 mM $KNO_3$ for 3 days at 28°C and 200 rpm. Cells from pre-inoculum were harvested by centrifugation at 15°C for 15 min at 10,000 *g*, and resuspended in fresh NB medium containing 20 mM $KNO_3$ to obtain $OD_{560} = 0.1$. These fresh cell suspensions that represented the inocula were grown for 5 h under the same conditions mentioned above. After this period, cells were harvested by centrifugation as previously described, washed twice in PBS and starved for 2 h at room temperature. The inocula were then centrifuged and resuspended in PBS containing 20 mM $KNO_3$. Cell density was adjusted to $OD_{560} = 0.2$ and 150 µl of cell suspension was transferred to each well of BIOLOG AN plates (AES Chemunex, Bruz, France). Plates were placed in anaerobic incubation bags (Merck, Fontenay-sous-Bois, France). The atmosphere inside the bags was replaced by $N_2$ and the bags were sealed with two anaeroclips (Merck). Anaerotest strip (Merck) was used as an indicator for the absence of $O_2$ inside the bags. Plates were incubated at 28°C for 2 days and subsequently analyzed with a spectrophotometer (Xenius, SAFAS, Monaco) at 550 nm. The data were normalized by dividing the absorbance of each well by the total absorbance of the plate. The whole analysis was performed in duplicate, meaning that two individual colonies from each strain were used to inoculate two separate pre-inocula.

A matrix representing the metabolic profiles (relative activity values on each of the 95 carbon sources) of the denitrifying strains was used to calculate the functional trait similarity among strains. We used either Euclidean distance model or Bray Curtis similarity (square root transformed data), implemented in the software PRIMER (version 6, PRIMER-E Ltd., Plymouth, UK). Two distance measures were used to evaluate to what extend strain relatedness would be dependent on the distance applied to calculate functional trait similarity.

#### QUANTIFYING $N_2O$ PRODUCTION IN PRESENCE OF DIFFERENT CARBON SOURCES

$N_2O$ accumulation patterns on individual carbon sources were measured for a set of 22 bacterial strains (**Table 1**). Six out of the 95 carbon sources found in the BIOLOG plates were chosen, according to their ability to discriminate the selected strains (Salles et al., 2009) and their common occurrence in bulk or rhizosphere soil. Bacterial cells were inoculated in microcosms consisting of 150-ml plasma flasks sealed with rubber stoppers and containing 50 ml of minimal medium M9 (Sambrook et al., 1989) supplemented with 0.02 M $KNO_3$ and one carbon source (1.6 mg C $L^{-1}$): D-cellobiose, maltose, L-fucose, L-malic acid, L-glutamine, or fumaric acid. We replaced the atmosphere of the flasks by a 90:10 mixture of He-$C_2H_2$ to provide anaerobic conditions for denitrification and to inhibit of $N_2O$-reductase activity, allowing us to quantify the amount of $N_2O$ produced by the bacterial strains. Flasks were incubated at 28°C and 160 rpm. The bacterial inoculum consisted of cells at the exponential growth phase, growing anaerobically on the same medium containing all six carbon sources (each source was present at 0.27 mg C $L^{-1}$). Before inoculation, cells were harvested by centrifugation, washed with sterile PBS buffer and let to starvation for 2 h at room temperature on PBS. Bacteria were inoculated in the microcosms to obtain a final cell density of $OD_{560} = 0.002$.

In order to quantify $N_2O$ accumulation, the head space of the flasks was sampled 10 times during a period of 3 days (0, 11, 15, 19, 23, 37, 43, 46, 61, and 66 h after inoculation) and immediately analyzed by injecting 100 µl of collected gas directly in a gas chromatograph (P200 MicroGC, Agilent technology, Massy, France).

This experiment was carried out in duplicate. The $N_2O$ production by each individual strain was determined as microgram N-$N_2O$ per milliliter 66 h after inoculation, when all the strains had attained the lag phase. For each culture, the number of cells was measured by flow cytometry as detailed in the Section "Appendix." A matrix containing the average $N_2O$ accumulation patterns (µgN-$N_2O$ ml culture$^{-1}$) for each source and each strain was used to determine the functional similarity among strains, as previously indicated for metabolic profiles, using Euclidean distance model. We analyzed both raw data and data normalized by dividing each value by the sum of values obtained on each of the six carbon sources for a given strain.

#### COMPUTATION OF DIVERSITY PROXIES FOR BACTERIAL ASSEMBLAGES

The communities described by Salles et al. (2009) refer to a set of 16 denitrifying bacterial strains that were assembled in different levels of richness (*S*), varying from 1 to 8. For each richness level, community composition also varied, ranging from 24 communities with two strains, 12 communities of four strains and 8 communities with eight strains. All 16 strains were also grown in monocultures, in duplicates.

Phylogenetic diversity (PD) was calculated using similarity distances based on 16S rRNA gene sequences. Briefly, PD was calculated for each one of the bacterial communities mentioned above, by taking into account all possible pairwise combinations among strains in a given community and averaging the computed similarity distances (Jukes and Cantor substitution model). Considering that PD is dependent on species richness, we also used another diversity proxy, mean community dissimilarity (Diss) that is less affected by the number of species in a community. Thus, the 16S rRNA gene was used to calculate Diss, defined as the average distance between species (Heemsbergen et al., 2004; Jousset et al., 2011). Distances were calculated using the Maximum Composite Likelihood model, implemented in MEGA (version 5.2).

Functional diversity (FD) proxies based on physiological data were calculated using similarities obtained from metabolic profiles (BIOLOG) or $N_2O$ accumulation patterns. Two methods were applied to infer FD: one based on distance measures and

[2]http://www.mbio.ncsu.edu/BioEdit/bioedit.html

another on dendrograms. For the distance-based method, we used the measure of Functional Attribute Distance (FAD; Walker et al., 1999; Mouchet et al., 2010), which represents the mean distance between species present in a community (Cadotte et al., 2009; Petchey et al., 2009). The dendrogram-based method relies on the FD measure (Petchey and Gaston, 2002) and corresponds to the total branch length from a functional dendrogram, which connects members of a given community together (Cadotte et al., 2009; Petchey et al., 2009). FAD was determined by using Euclidean distance to calculate the pairwise distance between species. FD was generated using the functions "dist()," "hclust()," and "Xtree()" implemented in R (version 2.13.0; Petchey and Gaston, 2002).

We also used the measure Community Niche (CN) measure (Salles et al., 2009), which is based on the performance ($N_2O$ accumulation) of individual species when they are growing on single carbon sources. Briefly, given a community of several strains growing on a mixture of $n$ C sources, CN aggregates the best performances (here highest $N_2O$ accumulation) observed on individual C sources (Salles et al., 2009). Suppose a community composed of denitrifying species A and B, growing on a mixture of resources 1, 2, and 3. When growing on individual resources, species A has higher denitrification rate than species B for resource 1 ($D_{A1} > D_{B1}$). For the other two resources, species B performs better ($D_{A2} < D_{B2}$; $D_{A3} < D_{B3}$) Thus, the CN of this two-species community represents the sum of the highest performance on each resource ($CN_{AB} = D_{A1} + D_{B2} + D_{B3}$).

### DATA ANALYSIS

Principal components analyses (PCA) and redundancy analysis (RDA) based on the metabolic profiles or $N_2O$ accumulation patterns were performed with CANOCO software (version 4.52, Wageningen, The Netherlands).

The relationships between functional and (phylo)genetic similarities were tested using a non-parametric form of Mantel test (RELATE), implemented in PRIMER-E software package (version 6). The similarity matrices obtained based on metabolic profiles or $N_2O$ production patterns were compared against the matrices based on genetic information (16S rRNA gene sequence or nitrite reductase identity) using a rank correlation coefficient (here Spearman coefficient) and significance level obtained by a permutation test (5000 permutations).

Standard regressions were used to test for relationships between community functioning ($N_2O$ accumulation) and different diversity proxies. The strength, the form and significance of the relationships were assessed by regression, implemented on the SPSS software (release 16.0.2).

In order to compare strains in terms of their functional traits, we used similarity metrics based on Euclidean distance because they provide the distance between strains in a $n$-dimensional space ($n = 95$ or 6, for metabolic profiles and $N_2O$ accumulation, respectively). Since the choice of the similarity coefficient is likely to influence our results, we also computed the similarities using the Bray Curtis coefficient. Although the correlation coefficient values varied according to the similarity measure used, the overall patterns were the same and our conclusions still hold (data not shown).

## RESULTS

### METABOLIC PROFILES

The analyses of the metabolic profiles of the 29 denitrifying bacterial strains, determined with BIOLOG plates under denitrifying conditions, showed that strains varied mainly in their ability to utilize aromatic compounds, carbohydrates, carboxylic acids, amino acids, and alcohols (**Figure 1**). Redundancy analyses performed with the same data set indicated that all classes of substrates had a significant effect ($P < 0.006$) on the metabolic profiles, except for amines ($P = 0.054$) and polymers ($P = 0.066$). In order to determine the relative contribution of each class of substrates, we used variance partitioning to control for the effect of individual classes when all other classes are defined as covariables in a constrained analysis (Leps and Smilauer, 2003). From the amount of variability explained by the ordination model when all classes are considered (63.5%), 29% could be assigned to the classes of substrates, as follows: aromatic compounds, 5.9%; carbohydrates, 5.1%; organic acids, 4.7%; amino acids, 4.2%; alcohol, 3.6%; amines, 3.1%; polymers, 2.4%, whereas the remaining variation was attributed to the variance shared between individual substrates.

When considering each phylogenetic class separately (**Figure 1**), the γ-proteobacteria were functionally diverse, with representatives having very different metabolic profiles. All but two pseudomonads were able to utilize organic acid compounds. The non-pseudomonads strains differed from the other class member by their ability to use aromatic compounds, carbohydrates, and polymers and by their low metabolic activity on organic acids. The α-proteobacteria clustered closely together, except for strain G30, which showed the most divergent metabolic profiles

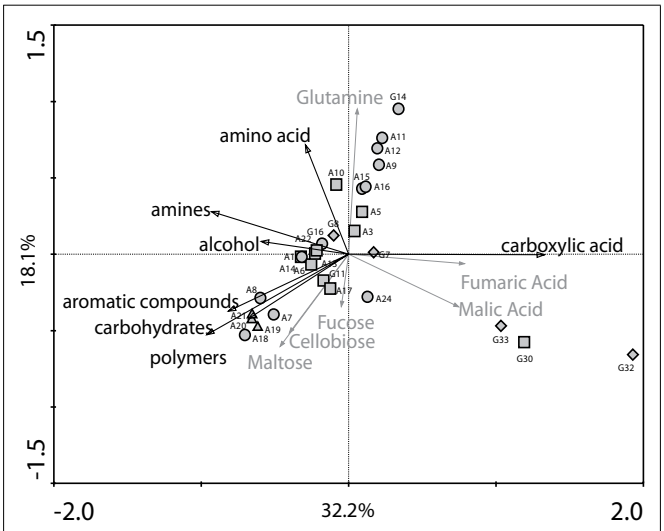


**FIGURE 1 | Principal components analysis showing the metabolic diversity of 29 denitrifying bacterial strains, as determined by metabolic profiles using BIOLOG AN plates under denitrifying conditions.** For each strain, the label corresponds to the code provided in **Table 1**. Symbols correspond to the phylogenetic groups of bacterial strains studied. Squares: α-proteobacteria; circles: γ-proteobacteria; diamonds: β-proteobacteria; triangles: Bacilli. Black arrows indicate the classes of carbon substrates found in the BIOLOG; gray arrows indicate the six selected individual carbons substrates used in the microcosm experiments.

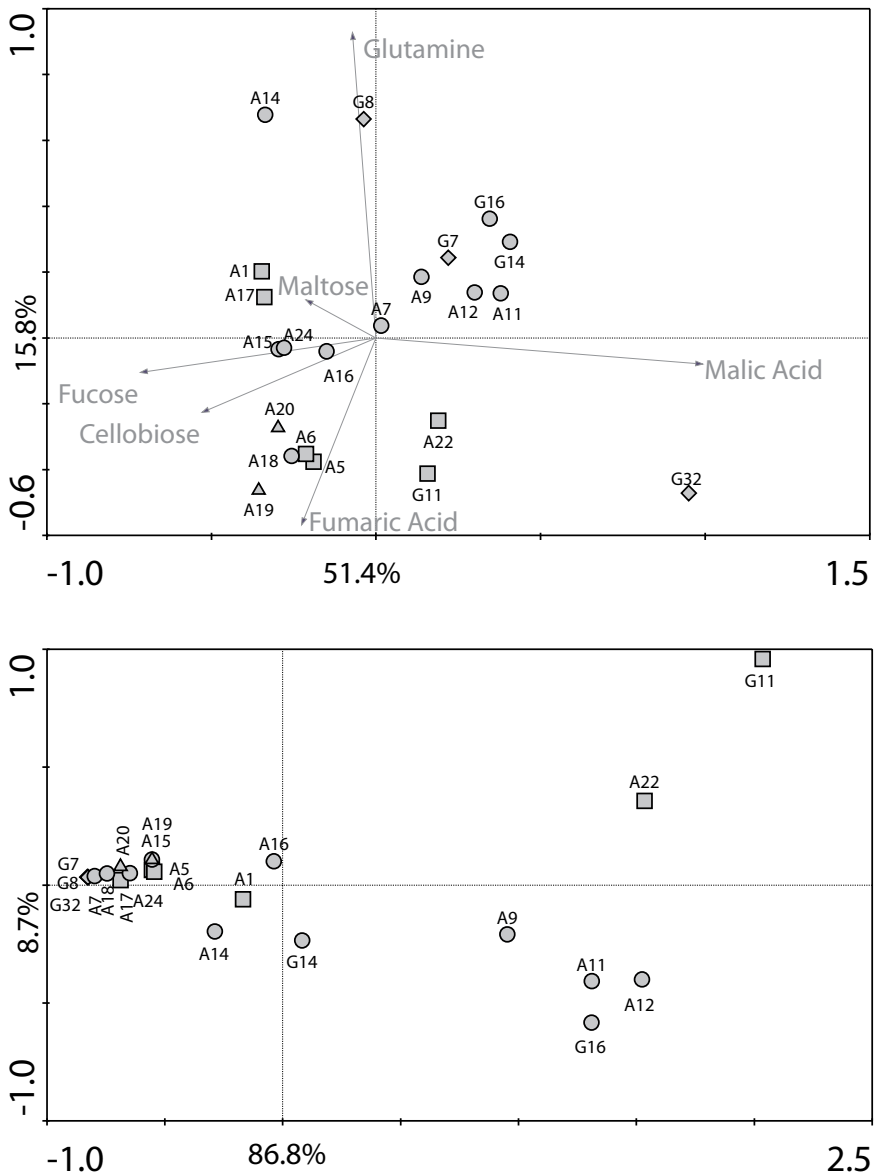


**FIGURE 2 | Principal components analyses performed on (top) normalized and (bottom) non-normalized $N_2O$ accumulation values ($\mu$gN-$N_2O$ ml culture$^{-1}$) obtained for each strain on each carbon source, 66 h after the inoculation.** Normalized data (for each strain, each accumulation value is divided by the sum of accumulation values observed on the six carbon sources) emphasize differences in patterns of resource use utilization whereas raw data enhance the differences in actual levels of $N_2O$ accumulation. Data were obtained from 22 denitrifying bacterial strains. Symbols as in **Figure 1**.

together with two strains from the *Achromobacter xylosoxidans* (β-proteobacteria class). Species belonging to the genus *Ochrobactrum* showed a wide variety of profiles, the only widespread characteristic being their low efficiency in using organic acids. The two representatives of the genus *Azospirillum* were quite distinct in their metabolic profiles, varying mainly in the use of amino and organic acids. The two genera belonging to the β-proteobacteria class differed greatly among each other, mainly regarding the use of sources belonging to the carboxylic acid class. The three representatives of the Bacilli class clustered very close together and could be identified by their ability to use aromatic compounds, carbohydrates, and polymers.

## $N_2O$ ACCUMULATION PATTERNS

The effect of individual carbon sources on the $N_2O$ accumulation patterns by individual strains was characterized by PCA analysis. As seen on **Figure 2** (top), when using normalized values, closely related strains tended to exhibit similar patterns with a number of exceptions. More specifically, *P. aeruginosa* strains showed similar $N_2O$ accumulation when growing on glutamine but varied in their ability to produce $N_2O$ using one of the carboxylic acids (malic acid). Similarly, the two *Burkholderia* species varied mainly in their response to malic acid and glutamine. The functioning of *P. fluorescens* and *P. stutzeri* strains was alike, with $N_2O$ accumulation varying mainly in relation to fucose and cellobiose, except for one

strain of *P. fluorescens* that used glutamine. Fumaric acid and cellobiose induced the largest variation in the $N_2O$ accumulation observed between the Bacilli species. The metabolic profiles of the three *Ochrobactrum* strains were different, varying mainly in the use of glutamine and malic acid. In particular, these strains varied in the absolute values of $N_2O$ production (**Figure 2**, bottom). The majority of *P. aeruginosa* strains showed high $N_2O$ production. The highest $N_2O$ accumulation values were observed for one strain of *Ochrobactrum* and for *Paracoccus denitrificans*.

### RELATIONSHIP BETWEEN THE SIMILARITY OF METABOLIC PROFILES AND SIMILARITY OF PATTERNS OF $N_2O$ ACCUMULATION AND (PHYLO)GENETIC RELATEDNESS

A significant correlation was observed between the metabolic profiles and phylogenetic relatedness between strains (**Figure 3**). The similarity of 16S rRNA gene sequence computed using the Euclidian distance model explained a significant but modest fraction of the variability observed in the metabolic profiles obtained on BIOLOG ($\rho = 0.35$; $P = 0.002$). Interestingly, when focusing on bacterial classes, higher correlations were observed between the genetic distance based on the 16S rRNA gene sequences and the percentage of the variation in metabolic profiles ($\rho = 0.48$, $P = 0.005$ and $\rho = 0.71$, $P = 0.003$ for γ-proteobacteria and α-proteobacteria, respectively; **Table 2**). Considering that the metabolic profiles showed an uneven response across strains, we performed the same analysis using a matrix based on Bray Curtis coefficient instead of Euclidian distance. Although the Spearman correlation coefficients changed slightly, the conclusions were the same (all strains: $\rho = 0.35$, $P = 0.001$; γ-proteobacteria: $\rho = 0.57$, $P = 0.004$; α-proteobacteria: $\rho = 0.69$, $P = 0.003$).

The similarity of $N_2O$ accumulation patterns among strains could not be explained by the similarity of the 16S rRNA gene, even when considering α-and γ-proteobacterial classes separately (**Figure 4A**). Computing the pattern in the $N_2O$ accumulation per cell (i.e., patterns of specific activity) led to similar results, as no significant relationships were observed (**Figure 4B**; **Table 2**). In contrast, the identity of the nitrite reductase residues was significantly correlated with the patterns of $N_2O$ accumulation among all strains ($\rho = 0.45$, $P = 0.004$; **Table 2**; **Figure 5A**). When considering the identity of amino acid residues obtained from the two genes coding for nitrite reductase (*nirK* and *nirS*) separately, the similarity in amino acids coded by the *nirS* genes was highly correlated with the observed pattern of $N_2O$ accumulation ($\rho = 0.62$, $P = 0.003$; **Table 2**), whereas that associated with *nirK* gene was unrelated to $N_2O$ accumulation pattern ($P = 0.735$; **Table 2**; **Figure 5B**). The similarity of the patterns of $N_2O$ production per cell and the identity of the nitrite reductase residues was just above the significance level ($P = 0.053$; **Table 2**). However, when analyzing the *nirK* and *nirS* residues separately, no correlation was found between the patterns of $N_2O$ production per cell and the identity of each type of nir residues ($P_{nirK} = 0.505$ and $P_{nirS} = 0.407$; **Table 2**).

### RELATIONSHIPS BETWEEN DIVERSITY PROXIES AND FUNCTIONING FOR BACTERIAL ASSEMBLAGES

Species richness, PD and phylogenetic dissimilarity (Diss) explained similar, low percentages of variation in community functioning (from 10 to 14%; **Figures 6A–C**). FD measures associated with metabolic diversity either lead to non-significant relationships with community functioning ($FD_{BIOLOG}$, **Figure 6D**) or explained a similar percentage of variation in functioning as measures associated with the phylogenetic marker ($FAD_{BIOLOG}$, **Figure 6E**). FD measures associated with $N_2O$ production ($FD_{N_2O}$ and $FAD_{N_2O}$) both had a better explanatory power (23–26%) of the variation in community functioning than measures based on metabolic diversity or phylogenetic marker (**Figures 6F,G**). Amongst all the diversity proxies, the CN index, which accounts for complementary effects among strains rather than diversity *per se*, was the best predictor of community functioning and explained 42% of the variation in $N_2O$ production (**Figure 6H**).

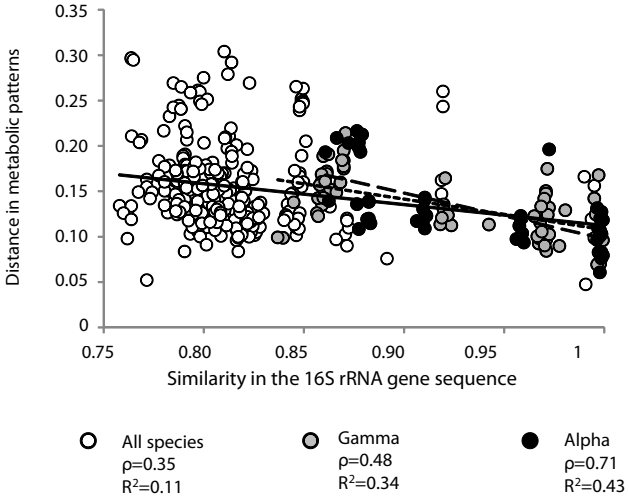


**FIGURE 3 | Pairwise comparisons of the distance in metabolic patterns and similarity between 16S rRNA gene sequence for (○) the 29 denitrifying bacterial strains, and among denitrifying bacteria from (●) α- and (◎) γ-proteobacteria classes.** Metabolic profiles were determined based on carbon utilization patterns on 95 carbon sources under denitrifying conditions (BIOLOG). Comparisons were performed using a non-parametric form of Mantel test and Spearman coefficient (ρ). Significance level was checked using a permutation test (5000 permutations). Linear regression lines are also indicated on the graph with associated $r^2$ values. All correlations were significant ($P < 0.005$).

## DISCUSSION

Many studies evaluating the effects of biodiversity on ecosystem functioning have focused on species richness, despite the fact that ecosystem functioning is not governed by the phylogenetic content of its organisms but rather by the functional traits of the individuals present, the distribution and abundance of these individuals, and their biological activity (Hooper et al., 2002; Naeem and Wright, 2003; Giller et al., 2004; Salles et al., 2009). In this context, the choice of relevant functional traits for macro-organisms has been compared to the search for the holy grail (Lavorel and Garnier, 2002). When considering microorganisms, one could argue that this search for appropriate functional traits represents a more feasible task, as the functional genes coding for the well-studied functions such as denitrification are mostly known (Philippot and Hallin, 2005; Philippot et al., 2007). Defining the groups involved

**Table 2 | Relationship between (phylo) genetic similarity and functional trait distances among denitrifying strains, as determined by Mantel test.**

| (Phylo)genetic information | Functional trait information[3] | Number of species analyzed | *P* value | Rho |
|---|---|---|---|---|
| 16S rRNA gene[1] | BIOLOG profiles | 29 | 0.002 | 0.35 |
| | | 10 (only α-proteobacteria) | 0.003 | 0.71 |
| | | 12 (only γ-proteobacteria) | 0.005 | 0.48 |
| | $N_2O$ accumulation patterns | 22 | 0.716 | ns |
| | | 6 (only α-proteobacteria) | 0.364 | ns |
| | | 11 (only γ-proteobacteria) | 0.062 | ns |
| | Specific activity patterns | 18 | 0.959 | ns |
| | | 5 (only α-proteobacteria) | 0.200 | ns |
| | | 9 (only γ-proteobacteria) | 0.632 | ns |
| Nir[2] | $N_2O$ accumulation patterns | 17 | 0.004 | 0.45 |
| | | 8 (only *nir*k gene) | 0.735 | ns |
| | | 9 (only *nir*S gene) | 0.003 | 0.62 |
| | Specific activity patterns | 14 | 0.053 | ns |
| | | 6 (only *nir*k gene) | 0.505 | ns |
| | | 8 (only *nir*S gene) | 0.407 | ns |

*[1]Similarity between the 16S rRNA gene sequences was determined by creating a pairwise distance matrix based on the Jukes and Cantor substitution model.*

*[2]Similarities in amino acid residues were measured between pairs of nirK- or nirS-harboring denitrifying bacterial species, using Gonnet matrix.*

*[3]Variation in functional patterns between strains were computed based on Euclidean distance.*

*The significance of the relationship was determined using the Spearman correlation coefficient and 5000 permutations.*

in each process sets a good foundation toward the quest for the functional "micro grail," even though the sheer microbial diversity might still limit this quest. Indeed such an approach has been extensively used in microbial ecology, and the diversity within functional groups has been assessed by quantifying the genetic diversity of either phylogenetic markers such as 16S rRNA gene (e.g., Stephen et al., 1996) or functional markers such as key functional genes involved in the process of interest (e.g., Poly et al., 2008). Despite the usefulness of these approaches, the issues that remain open are (i) to what extent does the genetic variation observed for phylogenetic or functional markers correspond to the variation of functional traits within microbial groups, and (ii) what effect does this genetic variation, functional or phylogenetic, and diversity of functional traits have on community and ecosystem functioning. We have addressed these issues for a range of denitrifying bacteria using two approaches. Firstly, by looking at the relationship between the similarity of functional traits evaluated from metabolic profiles that were obtained on BIOLOG plates or from $N_2O$ accumulation patterns on different C sources, and the similarity of phylogenetic (16S rRNA gene) or functional (*nir* gene) markers. Secondly, we computed proxies of phylogenetic diversity and FD among denitrifying bacterial assemblages, which were then used to assess how well they could explain the functioning (denitrification) of assembled communities.

#### PHYLOGENETIC SIGNAL AND PHYSIOLOGICAL TRAITS

The relationship between ecological and phylogenetic similarities defines the phylogenetic signal (Losos, 2008), and this notion was supported in our metabolic profile data: closely related strains were able to utilize carbon substrates in a similar fashion, whereas distantly related strains exhibited a greater variation in their metabolic resemblance. However, the strength of the phylogenetic signal to explain metabolic profiles was low, as the Mantel test exhibited a $\rho = 0.35$ when considering the whole set of strains. In fact, in some cases, unrelated strains shared higher similarities in their metabolic profiles (as shown by smaller Euclidean distances) than closely related ones. Similar conclusions have been recently reported for natural soil microbial communities, from which 39% of the catabolic profiles could be explained by the phylogenetic content of the communities, when considering two sites (Fierer et al., 2012). Interestingly, our findings indicate a higher consistency in terms of metabolic profiles among members of the α-proteobacteria class, which showed higher phylogenetic signal in metabolic profiles when compared to γ-proteobacteria class. This is consistent with previous results reporting that there is an ecological coherence of bacterial groups at deeper branches of bacterial taxonomy, such as bacterial classes (Fierer et al., 2007; Philippot et al., 2010). For instance, the comparison between the metabolic plasticity of soil bacterial communities facing addition of different C compounds and their phylogeny suggests that closely related species do not always use resources in a similar manner, although some bacterial families seem to be more consistent in their carbon metabolism (Goldfarb et al., 2011). Our results are consistent with the conclusion of Cohan (2006) who stated that bacterial systematics fails to provide species labels allowing predictions about the biology of the members of a given bacterial species, given the great diversity in the metabolic capabilities of closely related strains. Actually, our results indicate that the use of phylogeny to infer bacterial community composition or diversity might not be meaningful from an ecosystem function perspective.

This conclusion is even stronger if phylogenetic traits are used to infer denitrification (here, $N_2O$ production), a process known to be weakly related to phylogeny (Philippot and Hallin, 2005, see section below). Indeed, we observed no phylogenetic signal when evaluating the patterns of $N_2O$ production, even after correcting

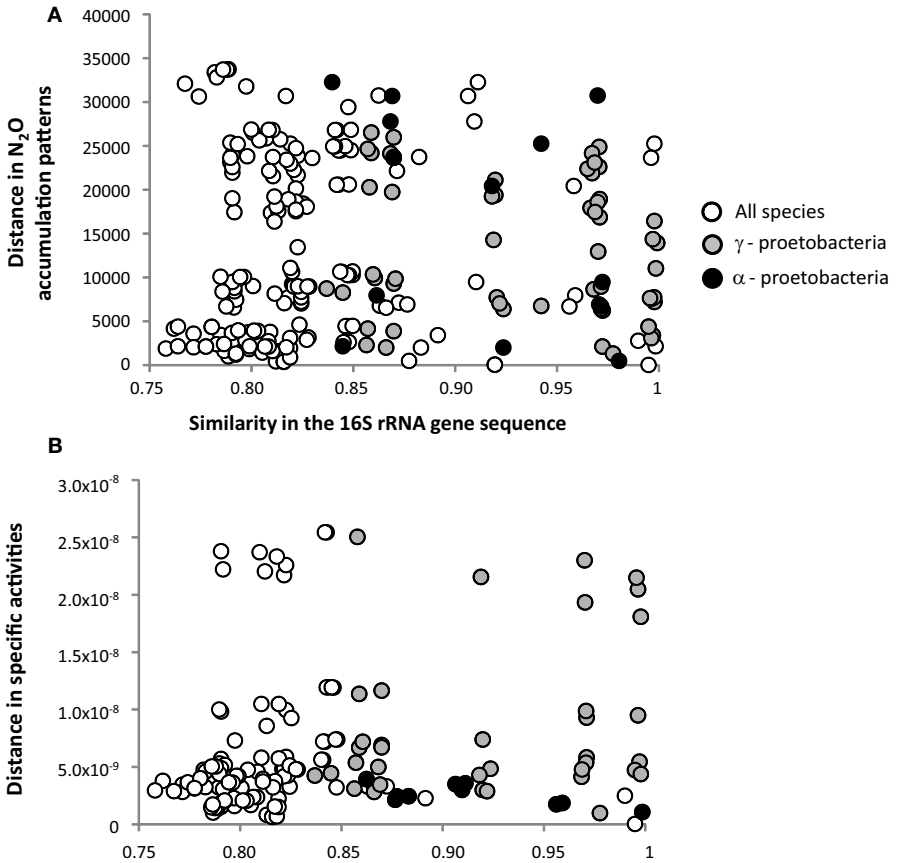


**FIGURE 4 | Pairwise comparisons of the distance between $N_2O$ accumulation patterns (A) or between specific activity patterns (B) and similarity between 16S rRNA gene sequences (○) among the set of 22 denitrifying bacterial strains, and among denitrifying bacteria from (●) α- and (○) γ-proteobacteria classes.** Comparisons were performed using a non-parametric form of Mantel test and Spearman coefficient (ρ). Significance level was checked using a permutation test (5000 permutations). Linear regression lines are also indicated on the graph with associated $r^2$ values. All correlations were non-significant (see **Table 2** for *P* values).

these values for the number of cells (an indication of specific activity).

### FUNCTIONAL MARKERS AND $N_2O$ PRODUCTION

In order to evaluate the diversity of denitrifying bacterial strains in environmental sample, most studies use the genetic diversity of sequences of key genes involved in the denitrification step of interest, rather than phylogenetic markers (Patra et al., 2005; Sharma et al., 2005; Heylen et al., 2006; Smith and Ogram, 2008; Hallin et al., 2009). In our study, the patterns of $N_2O$ accumulations (for a range of carbon sources) did not show a phylogenetic signal but were highly correlated with the identity of nitrite reductase residues. Interestingly, the patterns differed among nitrite reductase genes. The variation in the identity of the partial NirS protein was highly correlated with the variation observed for $N_2O$ accumulation profiles for *nirS*-harboring bacteria; in contrast, the variation observed for $N_2O$ accumulation profiles was not significantly correlated with variation in the identity of the NirK protein for *nirK*-harboring bacteria. It has been suggested that the *nirS* gene is less prone to horizontal gene transfer than *nirK* (Heylen et al., 2006; Jones et al., 2008), which could explain the higher congruency observed between functional gene sequence similarity and functional trait similarity for *nirS*-harboring bacteria. These findings have great implications for studies that use nitrite reductase genes to link community composition and denitrification rates, indicating that although the genetic diversity associated with both *nirK* and *nirS* can be used to study shifts in the composition of denitrifying communities, the changes in the genetic structure of nirK-harboring denitrifier communities are not well related to changes in functional traits relevant for denitrification.

In our study we have opted for focusing on *nir* genes, since those are present in all denitrifying species (Mahne and Tiedje, 1995). One could argue that our analyses were performed on partial amino acid sequences from both proteins (116 and 143 amino acid residues from NirK and NirS, respectively) and that the use of the whole protein could partly influence our results. Nevertheless, the stretch of DNA coding for nitrite reductase used here corresponds to those commonly used to study the diversity of denitrifiers (Heylen et al., 2006; Smith and Ogram, 2008;

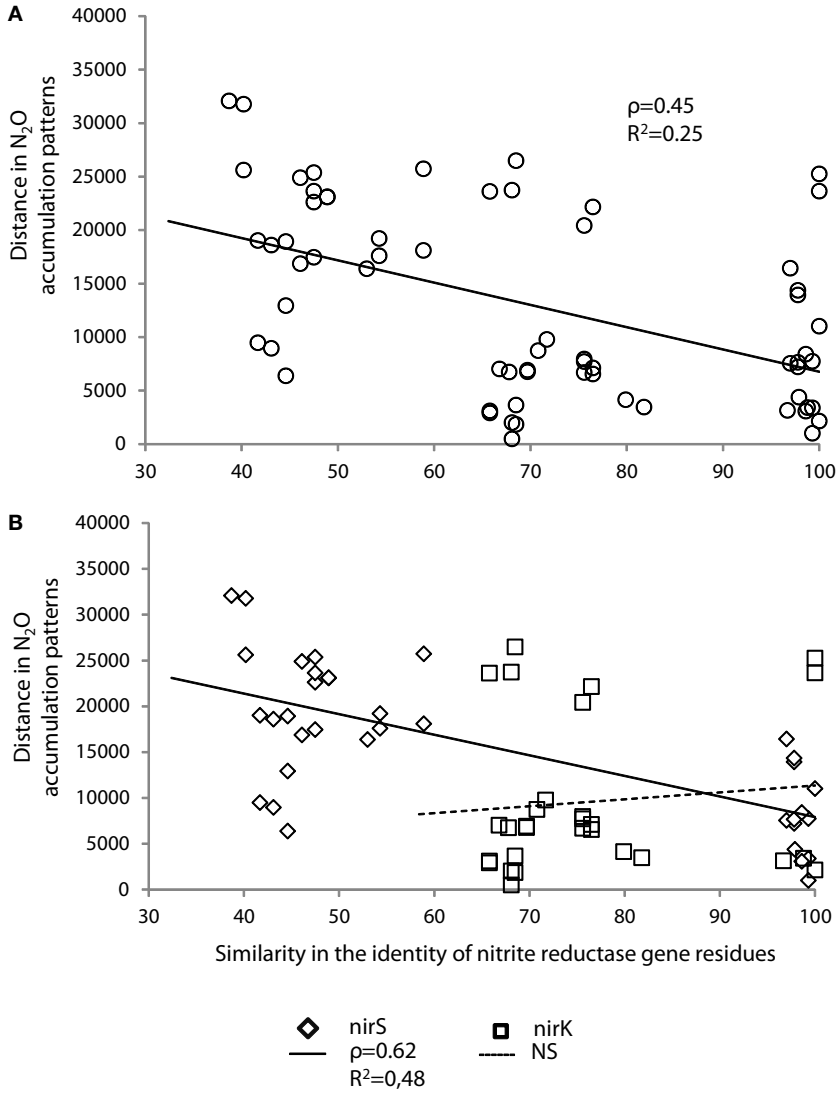


**FIGURE 5 | Pairwise comparisons of the distance between $N_2O$ accumulation patterns and the similarity in the identity of nitrite reductase residues (A) considering *nirK*- and *nirS*-harboring bacteria simultaneously or (B) distinguishing *nirK*- and *nirS*-harboring bacteria.** Comparisons were performed using a non-parametric form of Mantel test and Spearman coefficient (ρ). Significance level was checked using a permutation test (5000 permutations). The correlations were significant ($P = 0.004$) when considering both genes simultaneously or only *nirS* ($P = 0.003$), but non-significant when considering only *nirK* ($P = 0.735$). Linear regression lines are also indicated on the graph with associated $r^2$ values (correlations were significant except for *nirK* for which $P = 0.690$).

Hallin et al., 2009). Additionally, considering that other genes involved in the denitrification processes do not always explain denitrification patterns in a similar fashion as *nir* gene (Kandeler et al., 2006; Hallin et al., 2009) it would be interesting to determine how the identity of genes such as *nar*, *nor*, or *nos*, would predict denitrification rates. These comparisons might indicate the most appropriate functional marker to describe the structure of denitrifying communities. Moreover, our results show that the regulation mechanisms of targeted functions and the evolutionary history of bacterial taxa have to be accounted for to steer us towards our quest for the functional grail in microbial ecology.

## CHARACTERIZING DENITRIFIER DIVERSITY FROM A FUNCTIONAL PERSPECTIVE

The quest to understand and predict the effects of biodiversity on ecosystem functioning has led to the development of a range of diversity measures. These have been used to quantify the extent to which the different aspects of biodiversity, such as species richness, phylogenetic distance or FD, influence ecosystem processes, and services (Petchey and Gaston, 2002; Petchey, 2004; Mouillot, 2007; Cadotte et al., 2009; Mouchet et al., 2010). Likewise, the need to predict microbial-mediated ecosystem processes has spurred on the development of a range of methodological approaches that focus on the genetic information

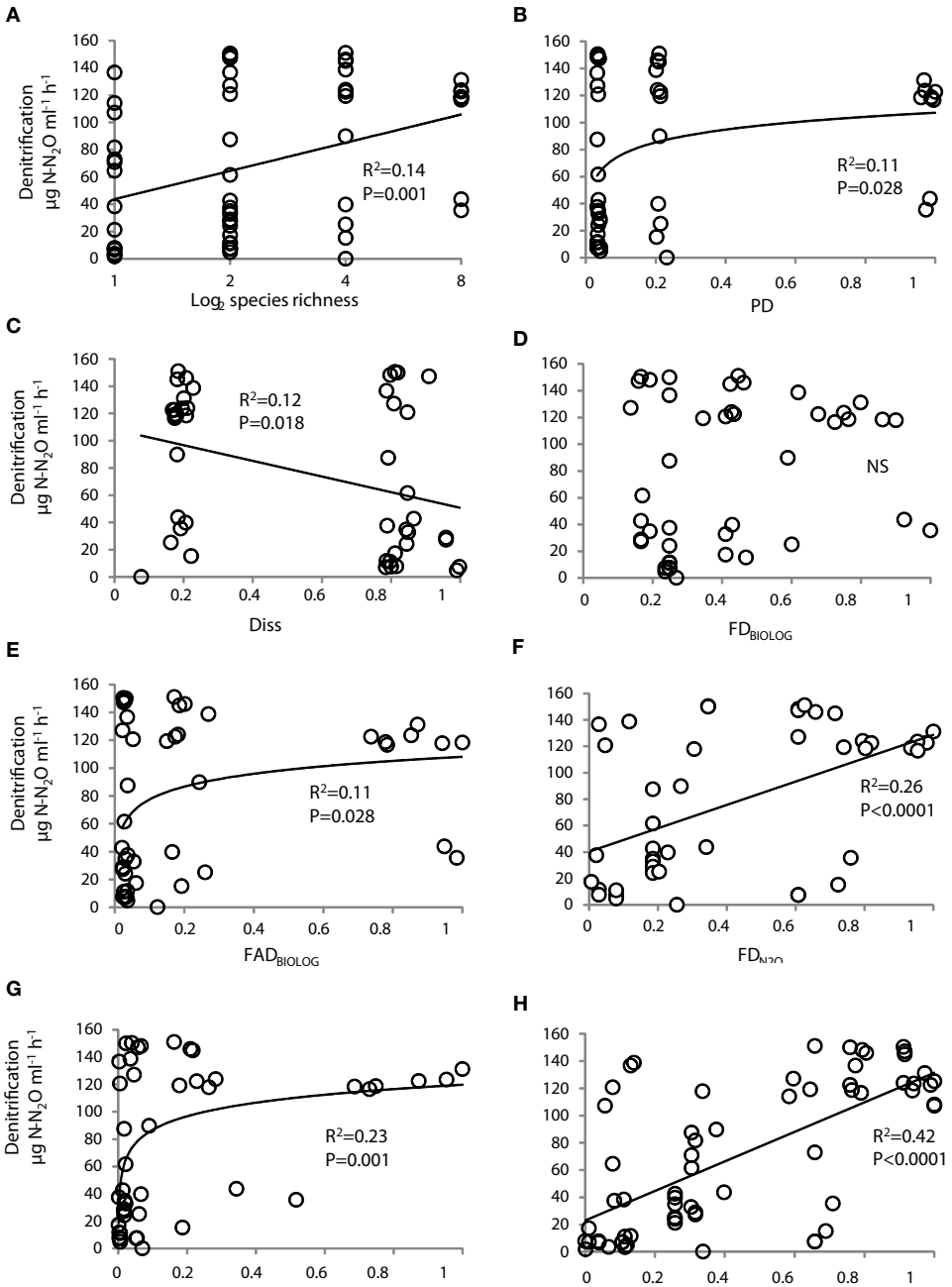


**FIGURE 6 | Relationship between denitrifier community functioning ($N_2O$ production) and indexes of community diversity.** The diversity measures were based on the number of species in the community [species richness **(A)**]; the diversity of 16S rRNA gene sequences [phylogenetic diversity, PD **(B)**]; dissimilarity in 16S rRNA gene sequences [Diss **(C)**]; the functional diversity based on metabolic profiles [$FD_{BIOLOG}$ **(D)**]; the functional attribute diversity based on metabolic profiles [$FAD_{BIOLOG}$ **(E)**]; the functional diversity based on patterns of $N_2O$ accumulation [$FD_{N_2O}$, **(F)**]; the functional attribute diversity based on patterns of $N_2O$ accumulation [$FAD_{N_2O}$, **(G)**]; and the functional niche occupied by each community [CN **(H)**]. **(A,H)** were drawn from Salles et al. (2009). All relationships were significant, except for $FD_{BIOLOG}$ **(D)**. PD, FADs, FDs, and community niche were standardized by dividing them by the maximum values observed across all communities. Diss, CN, and FD represent linear regressions; the remaining figures show a logarithmic regression. S, Species richness; PD, phylogenetic diversity; Diss, Dissimilarity; CN, Community niche; FAD, Functional Attribute diversity; FD, functional diversity

contained on phylogenetic or functional gene markers, and also on physiological trait like metabolic patterns, substrate induced respiration, and enzyme activities, to mention a few. However, a range of proxies is rarely systematically studied, especially for a range of bacterial species that can then be examined as assembled communities. This limits our ability to infer which

diversity proxies are the most useful to explain community functioning.

We tackled these issues by calculating indexes based on phylogenetic and functional diversity that are often used to predict the effect of the diversity of higher organisms on ecosystem processes (Petchey and Gaston, 2002; Petchey, 2004; Cadotte et al., 2009) for a range of denitrifying bacterial strains. Furthermore, we inferred how valuable each one of them was for predicting community functioning (denitrification), by relating these indexes to the level of community functioning reported by Salles et al. (2009) for denitrifying bacterial assemblages.

Despite the fact that diversity metrics based on phylogenetic distance or the number of species in a given community are often poorly related to community functioning in general (Hooper et al., 2005) and microbial (Salles et al., 2009) ecology, they are often used to assess microbial diversity in environmental samples. When used to predict the functioning of denitrifying bacterial assemblages, both the metrics based on phylogenetic marker (PD and Diss) and on species richness (S) performed poorly. Considering the issues discussed in the previous section, the results observed for PD were not surprising, and probably reflect the low phylogenetic signal associated with denitrification. The weak predictive power of species richness should be carefully considered, especially in experiments addressing the relationship between microbial diversity and functioning, in which species richness is often the explanatory variable considered (Bärlocher and Corkum, 2003; Setälä and McLean, 2004; Wohl et al., 2004; Bell et al., 2005; Tiunov and Scheu, 2005; Jiang, 2007).

Contrary to measures based on phylogenetic distance or species richness, measures associated with functional traits are more meaningful in the biodiversity-ecosystem functioning context, as they integrate organismal traits that directly influence a given process. We therefore determined functional diversity for two types of traits, metabolic diversity and $N_2O$ production patterns, each one quantifying a different aspect of denitrifier functionality. For both trait types, we used different metrics based on multivariate strategy, FAD and FD, and compared their ability to predict denitrification to the one of CN (Salles et al., 2009). Interestingly, the diversity measures based on metabolic diversity explained no or little variation in denitrification, whereas those based on $N_2O$ production patterns performed better. This suggests that both the choice of traits and diversity metric(s) are important to properly infer community functioning. More specifically, a diversity measure based on overall carbon metabolism ($FAD_{BIOLOG}$) poorly predicted denitrification, whereas applying the same diversity metric but based on $N_2O$ production patterns doubled its explanatory power, highlighting the importance of selecting for the appropriate traits. Functional diversity metrics also differ in their explanatory power. For instance, multivariate diversity measures based on $N_2O$ production patterns ($FD_{N_2O}$ and $FAD_{N_2O}$) explained the denitrification rates equally well, which could be partially explained by the degree of correlation between them ($R = 0.63$). However, their performance remained inferior to CN. This could be attributed to the fact that CN reflects the complementarity effect among strains (accounting for the performance of each strain on single carbon sources, it considers the sum of the highest performance on each individual carbon source possible for a given community) rather than diversity *per se*, as calculated for $FAD_{N_2O}$ and $FD_{N_2O}$. Thus, the superior performance of CN as compared to the other proxies presented here supports previous evidence (Hooper et al., 2005) of the importance of complementarity for resource use among taxa in the context of bacterial community functioning.

The information retained in the phylogenetic marker might be ecologically meaningful for studies focusing on microbial community composition and distribution. However, our results confirm the often cited hypothesis that, for broad functions such as denitrification, the diversity of functional gene sequences are better predictors of functioning than the diversity of sequences of phylogenetic markers (Philippot and Hallin, 2005). Moreover, our results suggest that characterizing the genetic diversity of *nir* gene fragments, as is often done to analyze the relationship between the diversity and functioning of denitrifiers, might be more meaningful for *nirS*- than *nirK*-harboring communities. Further studies focusing on the whole denitrification machinery and considering full gene sequences are necessary to confirm our hypothesis. Nevertheless, we provide evidence that findings based on the relationship between *nir*K genetic diversity and denitrification rates should be considered with caution. More generally, our results show that when analyzing the link between the diversity and functioning of microbial communities, indexes based on the diversity of phylogenetic or functional marker genes, or on functional trait diversity, might be useful. But they might remain inferior to indexes that more explicitly reflect complementarity effects among populations rather than the diversity *per se*. Overall, our results spur the use of diversity indexes based on relevant functional traits and the development of diversity proxies that integrate complementarity effect.

## ACKNOWLEDGMENTS

We thank G. Mouchiroud and Michèle Weiss (UMR 5534) for use of the flow cytometer, and the graduate students who helped in the characterization of specific activities. Joana Falcão Salles was supported by a post-doctoral grant from the Institute National de la Recherche Agronomique (INRA).

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

*Received: 28 February 2012; accepted: 22 May 2012; published online: 12 June 2012.*

*Citation: Salles JF, Le Roux X and Poly F (2012) Relating phylogenetic and functional diversity among denitrifiers and quantifying their capacity to predict community functioning. Front. Microbio.* ***3****:209. doi: 10.3389/fmicb.2012.00209*

*This article was submitted to Frontiers in Terrestrial Microbiology, a specialty of Frontiers in Microbiology.*

# APPENDIX

## MATERIALS AND METHODS

### *Characterization of taxonomic affiliation of strains*

In order to amplify the 16S rRNA gene, genomic DNA was isolated from the strains using the DNeasy Tissue Kit (QIAGEN, Courtaboeuf, France), following the manufacturer instructions. One microliter of genomic DNA was used as template in a 20-μl-PCR reaction containing 200 μM dNTP, 0.5 μM of each primer, 0.5 U Phusion hot start high fidelity DNA polymerase (Ozyme, St Quentin en Yvelines, France) and 1 × Phusion GC buffer (1.5 mM MgCl2). PCR program consisted of 30 s at 98˚C, followed by 30 cycles at 98˚C for 10 s, annealing at 68˚C for 15 s, and elongation at 72˚C for 45 s. Additionally, a final elongation step at 72˚C for 7 min was performed.

### *Characterization of nitrite reductase identity*

The *nirK* genes were amplified using the primers Copper 583F and Copper 909R (Liu et al., 2003), generating a fragment of 358 bp, in a 20-μl-PCR reaction mix containing 1 μl of genomic DNA as template, consisting of 200 μM dNTP, 0.5 μM of each primer, 0.5 U Phusion hot start high fidelity DNA polymerase (Ozyme, St Quentin en Yvelines, France) and 1 × Phusion HF buffer (1.5 mM $MgCl_2$). An initial denaturation step of 30 s at 98˚C was used, followed by 30 cycles of 98˚C for 10 s, annealing at 62˚C for 15 s, and elongation at 72˚C for 15 s. The final elongation step was extended for 7 min at 72˚C.

For *nirS* gene, amplification was carried out in a 50-μl-PCR reaction mix containing 1 μl of genomic DNA as template, consisting of 200 μM dNTP, 0.5 μM of each primer, 10 ng/μl of T4 gene protein (Qbiogene, Carlsbad, USA), 1.5 mM $MgCl_2$, 1 U of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Cergy Pontoise, France) and 1 × buffer. Amplification was carried out with an initial denaturation step of 2 min at 94˚C, followed by 35 cycles of 94˚C for 30 s, annealing at 57˚C for 60 s, and elongation at 72˚C for 60 s. The final elongation step was extended for 10 min at 72˚C.

### *Determining the cell density for denitrifying bacterial strains*

In order to count the number of cells, 100 μl of the cultures were harvested immediately after collecting gas samples (see Materials and Methods). Cells were fixed by mixing with equal volume of 98% ethanol, and stored at −20˚C. For counting, 15–30 μl of cells were mixed with PBS buffer containing 0.1% BSA to obtain a final volume of 300 μl. The cell suspension was sonicated for 10 s to limit cell aggregates and 6 μl of fluorescent beads (AlignFlow, Fisher Scientific, Illkirch, France) were added. The cell suspensions were then counted using a flow cytometer (BD FACSCalibur, Becton–Dickinson, San Jose, CA, USA). Two measurements were performed for each cell suspension. As mentioned in Materials and Methods, this experiment was carried out in duplicate. Cell counts were performed for 18 strains, up to 46 h after inoculation, when all the strains were in exponential phase.

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 26 September 2012
doi: 10.3389/fmicb.2012.00348

# Microbial control over carbon cycling in soil

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A major thrust of terrestrial microbial ecology is focused on understanding when and how the composition of the microbial community affects the functioning of biogeochemical processes at the ecosystem scale (meters-to-kilometers and days-to-years). While research has demonstrated these linkages for physiologically and phylogenetically "narrow" processes such as trace gas emissions and nitrification, there is less conclusive evidence that microbial community composition influences the "broad" processes of decomposition and organic matter (OM) turnover in soil. In this paper, we consider how soil microbial community structure influences C cycling. We consider the phylogenetic level at which microbes form meaningful guilds, based on overall life history strategies, and suggest that these are associated with deep evolutionary divergences, while much of the species-level diversity probably reflects functional redundancy. We then consider under what conditions it is possible for differences among microbes to affect process dynamics, and argue that while microbial community structure may be important in the rate of OM breakdown in the rhizosphere and in detritus, it is likely not important in the mineral soil. In mineral soil, physical access to occluded or sorbed substrates is the rate-limiting process. Microbial community influences on OM turnover in mineral soils are based on how organisms allocate the C they take up – not only do the fates of the molecules differ, but they can affect the soil system differently as well. For example, extracellular enzymes and extracellular polysaccharides can be key controls on soil structure and function. How microbes allocate C may also be particularly important for understanding the long-term fate of C in soil – is it sequestered or not?

**Keywords: microbial communities, carbon, diversity, litter, roots, soil**

Interest in how the composition of soil microbial communities governs the functioning of soil and ecosystem processes goes back to the dawn of microbiology with workers such as Pasteur and Winogradsky. However, the way we think about such issues has evolved – the focus is no longer identifying organisms with a capacity to carry out a function, e.g., characterizing the traits of specific nitrifiers (Waksman, 1927; Meyer, 1993) but on how varying the composition of a group affects the dynamics of the process it carries out – for example, how differences in the nitrifiers present affect nitrification kinetics (Braker and Conrad, 2011). This shift in the nature of the questions has been driven by three factors. The first is the development of techniques that allow us to characterize the identities of microbes *in situ* (16S and 18S rDNA), their potential (functional genes), and their physiological state (e.g., RNA/DNA ratios; stable isotope probing; BrDU incorporation; Roux-Michollet et al., 2010; Morales and Holben, 2011). The second is the growing interest in integrating evolutionary and ecological theory into microbial ecology to better understand microbial systems (Jiang, 2007; Prosser et al., 2007; Peay et al., 2008; Fierer et al., 2009; Locey, 2010). The third is the societal and scientific need to better understand and model important processes that influence ecosystem functions and the global climate system (Schimel and Gulledge, 1998; Reid, 2011).

In some ways, the thinking in microbial ecology has paralleled the development of plant ecology – questions have gone from "who's there?," analogous to the work of Joseph Banks and other naturalists in the late eighteenth and nineteenth centuries, to "why are they there?," and "what are they doing?," analogous to Tansley and Clements in the early 20th centuries. A question central to much of modern microbial ecology is "does who's there matter?"

As microbial ecologists, we might wish the answer to this question to be "yes," but in fact, this is not certain (Prosser, 2012). It is likely that for some processes the composition of the community matters, while for others it does not, and that the answer changes with physical and phylogenetic scale. Schimel (1995) postulated that "At a small enough scale, microbial community structure *must* be a dominant control on ecological processes, but as we move up in scale toward the ecosystem and integrate across many individual communities, the influence of individual community structures decreases." That paper posed the question "Is there some minimal scale necessary to adequately explain ecosystem processes at which microbial community structure still has a measurable influence on the nature and rates of those processes?"

From that question grew the argument that "narrow" processes – those that involve a specific physiological pathway or which are carried out by a phylogenetically constrained group of organisms – might be sensitive to the composition of the guild of microorganisms carrying it out, even at the ecosystem level (Schimel, 1995; Groffman and Bohlen, 1999). Examples of such processes include specific plant–microbe interactions (e.g.,

N-fixation, mycorrhizae, and pathogens) and trace gas emissions, notably $N_2O$ and $CH_4$. Both the conceptual arguments and the research to back them up are increasingly well developed for such processes. For example, the ratio of $N_2O/N_2$ produced by denitrification (Bakken et al., 2012; Salles et al., 2012), the rate of nitrification and the speed with which it responds to fertilization (Isobe et al., 2011), and the sensitivity of methanogenesis to $NH_4^+$ (Bodelier et al., 2000) are all sensitive to the composition of the community of organisms carrying them out.

However, these narrow processes are generally niche players in overall biogeochemical cycles. They are often important, either for ecosystem functioning (e.g., nitrification or sulfate reduction) or for global systems (e.g., $N_2O$ and $CH_4$ fluxes), but typically engage only a small fraction of the total microbial community and are responsible for a limited portion of the total cycle of the involved element. Most microorganisms in soil are aerobic heterotrophs involved in the "broad" or "aggregate" processes (*sensu*; Schimel et al., 2005); these are the processes that are carried out by a wide range of organisms or that we measure as a single process but are actually the sum of multiple distinct processes (e.g., soil respiration). Broad processes are responsible for the largest flows of C in soil systems: decomposition and C storage.

In this paper, therefore, we will focus on the microbial role in these large flows associated with the soil C cycle. We will briefly discuss our evolving understanding of the nature and causes of microbial diversity in soil to consider the level of phylogeny that might define meaningful functional groups for addressing "who's there" questions in C-cycling research. We will then discuss the circumstances where microbial community structure might regulate the processing of organic matter (OM) in soil, and some areas where we see a particular need for advancing this research.

## CAUSES AND NATURE OF MICROBIAL DIVERSITY IN SOIL

Microbial diversity in soil is high. Typical soil samples contain many thousands of individual taxa (commonly described as "operational taxonomic units"; OTU's) of Bacteria, Archaea, and Fungi. Some estimates suggest there can be more than $10^6$ individual species-level OTUs in a single soil (Fierer et al., 2007). This poses two central, but related, questions to microbial ecologists: how there can be such great diversity, and does it have any functional significance? (Prosser, 2012).

Classical theories of biodiversity are grounded in the concept of the niche and competitive exclusion: two species cannot stably coexist in a single niche. Thus, each species *must* have some functional differentiation (Clark, 2010). Niche-based theory, however, struggles with the high biodiversity of plants, which compete for a limited suite of resources (light, water, mineral nutrients); yet there may be hundreds of species within some habitats.

To explain such anomalous patterns of high biodiversity, alternative ideas have developed. Neutral theory argues that species can coexist within a niche when the variation in fitness among individuals is as great as among species (Hubbell, 2001); but species can also coexist when competition among individuals is as intense as among species (Clark, 2010; Clark et al., 2011; Beckage et al., 2012). Such dynamics allow functionally overlapping taxa to coexist, especially when the environment is highly variable, and when organisms are sessile; conditions that likely hold true for many soil microbes (Sloan et al., 2006; Dumbrell et al., 2010; Fierer and Lennon, 2011; Prosser, 2012).

Growing evidence suggests that niche and functional differentiation explain patterns of diversity at high levels of microbial phylogeny (e.g., families and phyla) and is associated with life-history strategies (Fierer et al., 2007; Philippot et al., 2010). However, it has been hard to identify meaningful functional differentiation within more finely defined groups (e.g., within genera; Philippot et al., 2010; Prosser, 2012). This conclusion, if true, will affect how we study microbial community composition; for example, how deeply to sequence communities to analyze their structure in terms of ecologically meaningful groups. It takes far fewer sequences to quantify a community to family than to species (Barberán et al., 2012).

In soil, organisms must adapt to a complex array of substrates, physical/chemical conditions, and biotic interactions, each of which may affect community composition. Some organisms specialize on particular substrates; for example, fungi that grow best on sucrose vs. cellulose vs. lignin vs. tannin–protein complexes (Hanson et al., 2008). In other cases, organisms appear to respond to specific environmental variables. For example, $O_2$ (Bodegom et al., 2001), moisture (Lennon et al., 2012), pH (Fierer and Jackson, 2006) and even varying levels of these parameters (DeAngelis et al., 2010) can select for specific organisms. In some cases, this selection operates at high phylogenetic levels – e.g., pH controls the relative growth of fungi vs. bacteria (Rousk et al., 2009). In other cases, selection operates at family or genus. For example, within the phylum Glomeromycota (the arbuscular mycorrhizal fungi), soil pH may select more strongly than host plant for the specific taxa present (Dumbrell et al., 2010); within the bacteria, *Clostridium* spp. are obligate anaerobes while most *Bacillus* spp. are aerobes, yet both genera are closely aligned within the Firmicutes.

However, the dominant environments that we can identify in soil are rarely defined by single characteristics (e.g., pH alone), but by combinations of characteristics that organisms must deal with in synchrony. To adapt to an environment with a suite of co-occurring conditions, an organism requires a suite of complementary traits – a life history strategy. For example, litter decomposers (e.g., many basidiomycete fungi) rely on extracellular enzymes to cut plant polymers into oligomers and monomers that may be taken up and metabolized, but they must also deal with litter drying out frequently (Schimel et al., 1999) and with the high C/N stoichiometry typical of leaf litter. Some bacteria are "rhizobacteria" that appear to depend on specific exudates released by plant roots (DeAngelis et al., 2009; Remenant et al., 2009) and are adapted to the complex environment of the rhizosphere (Bertin et al., 2003). The Acidobacteria appear to be stress tolerant oligotrophs (Fierer et al., 2007, 2011) while the Bacteroidetes and the β-Proteobacteria appear to be copiotrophs that require adequate moisture (Lennon et al., 2012).

Evolving a successful life history strategy implies deep evolutionary patterns and may explain why we observe meaningful functional groups or guilds of microbes, and that they are defined primarily at high phylogenetic levels – families or phyla rather than at species or genera (Moorhead and Sinsabaugh, 2006; Fierer et al., 2007; Philippot et al., 2010; Follows and Dutkiewicz, 2011). For example, the ability to retain high levels of rRNA through drought

and to respond quickly to rewetting appears to be a function of bacterial phylum, with Actinobacteria and Verrucomicrobia being rapid responders, while Firmicutes were intermediate; Proteobacterial responses however differed at the class level (Placella et al., 2012). At the species level, there generally appears to be substantial functional redundancy (Prosser, 2012).

This conclusion is reinforced by the ubiquity of horizontal gene transfer (HGT) among bacteria. HGT allows organisms to transfer the genes to carry out specific processes (Kurland et al., 2003). However, HGT is most common among closely related taxa (Kurland et al., 2003), and for pathways that are simple and require few enzymes. Thus, HGT is unlikely to break down functional barriers at high levels of phylogenetic difference and so is unlikely to transfer major life-history strategies that require complex gene networks or rearranging core physiological pathways (Kurland et al., 2003). For example, while the genes for nitrogenase appears to have been transferred across taxa multiple times (Falkowski et al., 2008), transferring the full suite of genes required to form N-fixing nodules in legumes appears to have happened perhaps twice in history (Chen et al., 2003). Denitrification has spread widely across the bacterial world because it requires only a slight modification to the terminal end of the electron transport chain, branching electrons off from cytochrome *b* to one of several nitrogen reductases. It is an easy physiology to maintain as an alternate to aerobic respiration. In contrast, because the redox potential for sulfate reduction is so much higher than that of $O_2$, organisms cannot merely insert sulfate reductase in place of cytochrome *o*; sulfate reducers have an entirely different electron transport system (Rabus et al., 2006), making it difficult for an aerobe to become a $SO_4^=$ reducer through HGT. The ability to carry out specific biodegradation reactions has been transferred frequently (Liang et al., 2012), but a rhizobacterium is not likely to become a litter decomposer overnight.

Much soil diversity may actually reflect beta diversity – a diversity of habitats within a landscape, rather than diversity within a habitat. This argues that soil is really a complex landscape with repeatable and definable microhabitats, each of which might have more constrained diversity. No one considers it surprising that California has > 3,000 native plant species because the State spans from alpine tundra to conifer forests to arid scrubland and desert, each with its own array of species. Does soil have analogous distinct communities? Rhizospheres select for discrete and reproducible communities, based on both the chemical nature of plant rhizodeposits (Paterson et al., 2007; DeAngelis et al., 2009; Dennis et al., 2010) and the physical environment created by roots (e.g., altered $O_2$, pH, and water availability; da Rocha et al., 2009; Hinsinger et al., 2009). Soil aggregates may also select for specific microbial groups; for example, Acidobacteria may be common in macroaggregates but not the inner microaggregate (Mummey et al., 2006). Communities may also vary based on the size pores they inhabit (Ruamps et al., 2011).

However, even if physical structure does not create repeated defined habitats that select for specific communities analogous to grassland or alpine plant communities, the physical complexity of the microbial landscape and a lack of connectivity between pores may reduce competitive interactions among taxa and allow greater overall diversity (Görres et al., 1999; Dechesne et al., 2008) analogously to how different valleys within a single mountain range may have somewhat different flora. Within a single soil, decreasing pore connectivity by reducing water content can increase bacterial species richness (Carson et al., 2010). We still only poorly understand how soil structure creates habitats and niches and how it regulates interactions to control diversity and community composition. This remains an important research area (Schmidt et al., 2011; Dungait et al., 2012).

Questions remain, however, about how many distinct niches can exist in soil, and about the level of phylogenetic resolution at which meaningful niche selection and partitioning acts. As we explore the functional significance of community composition, we need to further develop our understanding of the nature of microbial diversity and the phylogenetic levels at which distinct life-history strategies emerge and how these translate into meaningful microbial functional groups and thence into meaningful functions. We also need to better understand the "microbial landscape" and how the physical structuring of the soil system interacts with microbial communities to regulate the processes that control ecosystem functioning.

## CONSEQUENCES OF SOIL COMMUNITY COMPOSITION

As different groups of microorganisms have distinct functional traits with the potential to influence the processes they carry out (e.g., exoenzyme producers vs. "cheaters"; Allison, 2005), it raises the question of where and how those differences might be expressed in the environment. Just because organisms' traits differ does not mean that they necessarily function differently. There are several necessary conditions for soil microbial community composition to affect ecological processes.

1. Organisms must differ in their functional traits.
2. Biological reactions must be either:
   a. The rate limiting step in a reaction sequence or,
   b. The fate-controlling step – i.e., at a branch point that channels substrates into pathways with different fates.

The first criterion is the basis of the concept of "physiologically broad" within "broad vs. narrow" theory: if all organisms carry out a process in the same way it can not matter which is active for process function. Community composition can only affect processes if organisms vary in how or when they function (Schimel, 1995; Allison and Martiny, 2008).

Even if the organisms present in a community do vary in their functional characteristics, being different is not enough to control the dynamics of C cycling. The organismal biology must also be what controls the process – either in terms of how fast a reaction proceeds or where it proceeds to: what are the products.

### RATE LIMITING STEP: CATABOLISM VS. ACCESS?

Most OM transformations involve multiple steps with different inherent kinetics. For microbial community composition to play a role in controlling such a transformation, the slowest, rate limiting step, must be biological. In soil, that is not necessarily the case; rather abiotic processes can be rate-limiting (Kemmitt et al., 2008). For example, in dry and sunny ecosystems, photodegradation of aboveground litter can potentially oxidize plant-C all the way to $CO_2$ (Austin and Vivanco, 2006). Photodegradation, however,

generally accounts for only a limited amount of litter breakdown (Brandt et al., 2010). The more common way for abiotic processes to regulate OM turnover is through physical mechanisms that limit microbial access to substrate (Stevenson, 1982; Pingnatello, 1999; Kemmitt et al., 2008; Dungait et al., 2012). Thus, in considering the potential role of microbial community composition in a biogeochemical process, the first question is whether microbes have physical access to the substrate (**Figure 1**).

In fresh unprotected detritus, microbial access is not generally a constraint on decomposition. The exception is wood; it can take some time for fungal hyphae to penetrate into a log (Barker, 2008). In mineral soils, however, the situation is different. Mineral soils contain the bulk of OM in the total soil profile, and possibly in an entire ecosystem (Jobbágy and Jackson, 2000). Much of this is in protected forms, either occluded in aggregates or sorbed on mineral surfaces (**Figure 1**; Krull et al., 2003). The rate at which this C can be metabolized is limited by microbes' ability to access it (Six et al., 2004; John et al., 2005). The importance of physical protection has long been recognized as a control on soil OM (SOM) turnover (Yoo et al., 2011), but increasingly researchers have been recognizing the role of physical space and the structure of the "microbial landscape" as a specific control on the dynamics of microbial communities and of their function as well (Dungait et al., 2012).

Microbes in mineral soils are constrained by the pore networks that they live in (Young and Ritz, 2005; Donnell et al., 2007). As soils dry, bacteria become effectively immobile (Wang and Or, 2010) and must rely on diffusion to supply resources. Yet, soil is a "sticky" environment, and substrate diffusion can be slowed or prevented when molecules interact with electrically charged clay particles or OM that coats particles (Carrington et al., 2012). In other words, life in soil is like life in a chromatography column. The interactions of microbes and substrates with the physical matrix regulates how, or even whether, soil C is utilized; the critical processes are sorption/desorption, diffusion, and transport (**Figure 1**; Ekschmitt et al., 2005).

For example, in a California grassland soil, Xiang et al. (2008) showed that in deep soils (1 meter), multiple dry/wet cycles increased total respiration and microbial biomass by more than 500%; the C respired came from a pool with an estimated

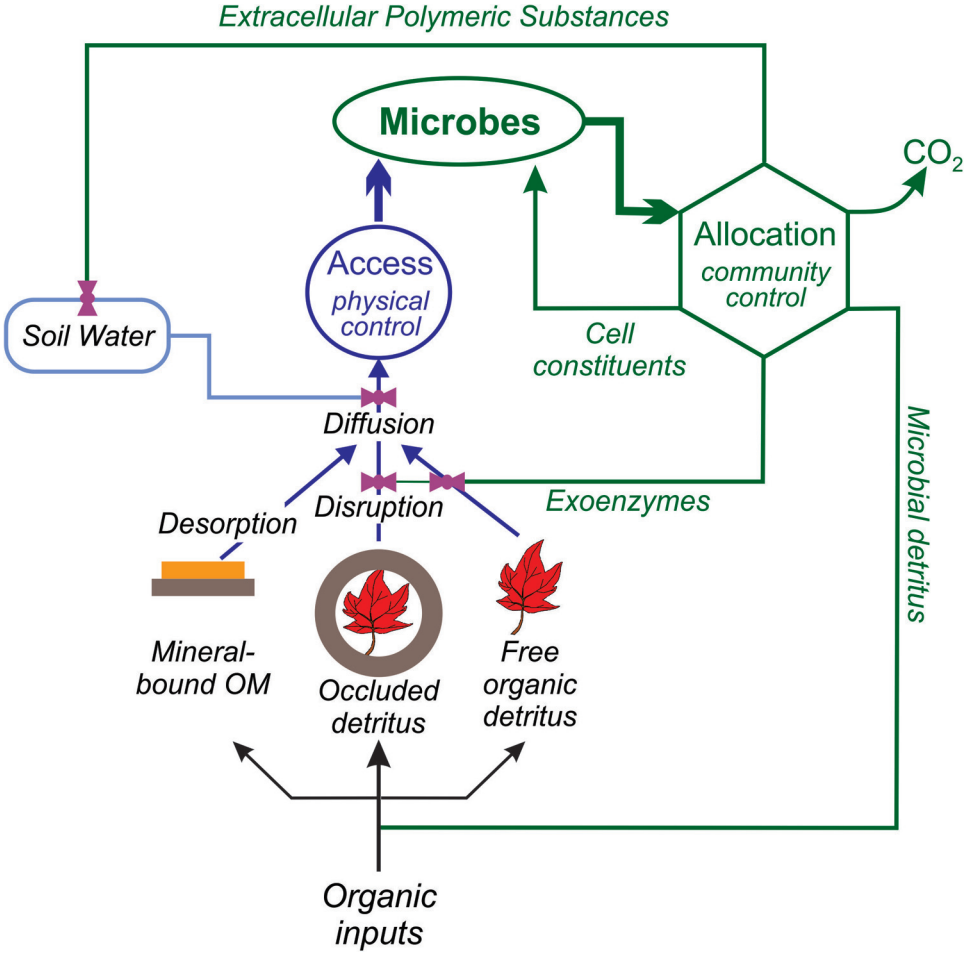


**FIGURE 1 | Microbial C cycling: the relative roles of physical access to soil C pools and of microbial allocation patterns in regulating overall soil C dynamics.**

turnover time of 600–800 years (Schimel et al., 2011). This pool of chemically labile OM was only metabolized following rewetting either because rewetting caused it to desorb, or because the flow of water redistributed C and so overcame diffusion limitations.

A meta-analysis of studies evaluating microbial respiration responses to water stress (Manzoni et al., 2012) showed that across a wide range of soils, the relationship between water potential and relative respiration is linear with a consistent threshold value at which respiration effectively stops (ca. −14 MPa); this value was similar to "the water potentials where soil diffusion becomes impaired." They concluded that the only mechanism that could reasonably explain the observed relationship was diffusion control on substrate supply.

In contrast, when aggregates occlude organic detritus (Six et al., 2004), two steps are required for microbes to process the material – first a physical step of aggregate disruption, and then possibly a second step in which exoenzymes break up the polymers (Navarro-García et al., 2011). The aggregate disruption step still limits overall metabolism of the material, but once that step is overcome, there may still be a delay before material is processed and metabolized.

The conclusion that OM breakdown in mineral soils is not limited by the catabolic capacity of the OM, but rather physical factors that limit microbial access to it, is reinforced by our changing understanding of the chemical structure of SOM. The classical model of SOM was based on humic/fulvic acids, and what might be described as the "snowflake" theory of SOM, in which humic materials were thought to be large polymers (Sutton and Sposito, 2005), molecules so complex it was possible no two were identical. In such a model, molecules require extracellular enzymes to fragment them, but with no repeated structures, enzymes could not be high affinity "latch-and-key" hydrolytic enzymes but instead must be non-specific oxidative "shotgun" enzymes that produce high-energy radicals or peroxides. Thus, microbes would rely on chance to generate fragments that they can take up and metabolize (Stevenson, 1982). In this vision, SOM molecules are inherently resistant and dependent on specialized exoenzymes; decomposition is therefore potentially sensitive to the organisms involved, and whether they have the necessary traits to be able to process humic molecules.

The old humic model of SOM, however, is increasingly considered obsolete (Schmidt et al., 2011), and is being replaced by a conceptual model in which SOM is made up of aggregates of small, but chemically defined molecules (Sutton and Sposito, 2005; Schmidt et al., 2011). These small molecules can also form layers on clay surfaces, leading to a model that has been called the "onion layering" model (Sollins et al., 2006). In this new model, individual constituent molecules are small enough that they can be taken up by microorganisms and are often simple enough that they can be channeled into metabolic pathways that are common among microbes. There should therefore be a lot of redundancy among microbes' ability to use such compounds, making it unlikely that their metabolism would be limited by a lack of appropriate enzymes. Yet, the bulk mineral-phase carbon in soils is frequently thousands of years old (Trumbore, 2009). Because such long turnover times cannot be explained by chemical recalcitrance (Dungait et al., 2012), they are unlikely to result from biological constraints and are presumably insensitive to microbial community composition.

## FATE CONTROLLING STEP: ALLOCATION

Although we argue that for the protected OM in mineral soils, the composition of the microbial community is not important in controlling the *rate* at which SOM is processed, that does not mean community composition is not important in the *fate* of this material. While a molecule's accessibility to a microbe is controlled by physical processes, once a microbe has taken it up, its fate is entirely under the control of that organism (**Figure 1**). Ultimately therefore, while catabolic potentials have limited role in controlling the turnover of mineral SOM, anabolic processes are unquestionably important (Liang et al., 2011). What do microbes do with the carbon they access? How do they allocate it? How do microbial C transformations further affect the composition of SOM? These *are* sensitive to microbial community structure.

The sensitivity of SOM turnover to anabolism is recognized implicitly even in large-scale biogeochemical models; although these models often lack any microbial carbon pool, and even when such a pool exists it is usually just a C reservoir, rather than a driver of decomposition (Manzoni and Porporato, 2009). In these models, when C moves from one pool to another, some is lost as $CO_2$; the proportion of C-moved relative to C-lost is essentially equivalent to carbon use efficiency (CUE). In physiology, CUE is the fraction of substrate that is taken up that is assimilated immediately into microbial biomass (Allison et al., 2010). With simple compounds, the immediate CUE – usually measured by a short-term assay with an isotopically labeled substrate – is more a function of molecular structure than microbial community composition (Sugai and Schimel, 1993). The magnitude of CUE, however, is a function of time and physiological condition; stressed microbes can have a higher maintenance energy demand which functionally reduces CUE (Allison et al., 2010). Thus, how CUE and C-turnover respond to stress may be a function of microbial life history strategy and stress tolerance.

Microbes, however, do more with substrate than just convert it to "biomass." Rather, they synthesize a variety of products that affect the functioning of ecosystems. A select few of these include:

A. Extracellular enzymes
B. Extracellular polysaccharides
C. Cell wall polymers: amino sugar-based peptidoglycan and chitin
D. Stress response compounds: osmolytes, cryoprotectants, chaperones etc. (Schimel et al., 2007).

The fate of each group of compounds is different in soil, and patterns of allocation and production vary across microorganisms (Harris, 1981; Lennon et al., 2012). For example, the microbial products present in decomposing aspen litter differed dramatically with the site the litter was decomposed in (i.e., microbial inoculum), even though decomposition rates did not differ significantly (Wallenstein et al., 2010). Substantial differences among allocation patterns are associated with microbes' life-history strategies and hence with their phylogeny. Further, the ways in which microbes allocate C can influence soil structure and function and so alter microbial habitats and overall soil functioning.

### *Extracellular enzymes*

For those organisms that rely on detritus (plant, animal, or microbial) for their resource supply, extracellular enzymes are required to break down polymers (Sinsabaugh, 1994). Although several simple theoretical models of decomposition include a single enzyme pool (e.g., Schimel and Weintraub, 2003), in reality a suite of different enzymes are required, including substrate-specific enzymes targeting C, N, and P (e.g., cellulolytic, proteolytic, and phosphatase), and non-specific oxidative enzymes such as laccase and peroxidase (Caldwell, 2005; Sinsabaugh et al., 2009). For exoenzyme-producing decomposers, enzymes may be the first priority for C-allocation to ensure resource supply (Schimel and Weintraub, 2003). The overall microbial community is able to shift allocation among these different groups of enzymes to match production to resource demand; enzymes are selectively produced to increase the supply of the most limiting element (Sinsabaugh and Moorhead, 1994; Sinsabaugh et al., 2009) and to target the most available substrates (Sistla and Schimel, 2012). It remains unclear how much of this is due to physiological plasticity of individual organisms or reflects shifts in the composition of the microbial community. The products of exoenzyme breakdown become available to other organisms including other microbes, so called "cheaters" (Allison, 2005) or "opportunists" (Moorhead and Sinsabaugh, 2006), and to plant roots (Schimel and Bennett, 2004). Some microbial groups are dominant producers (e.g., Basidiomycetes) while cheaters appear to dominate in groups such as the β-Proteobacteria and Bacteroidetes (Fierer et al., 2007).

### *Extracellular polysaccharides*

Another important class of extracellular materials is polysaccharides (Holden, 2011; Sutherland, 2001). Some microorganisms can embed themselves in a matrix of extracellular polymeric materials (EPS) that are mostly polysaccharide but also contain DNA and protein (Or et al., 2007; Jiao et al., 2010; Holden, 2011). EPS is hygroscopic (Chenu, 1993; Henao and Mazeau, 2009), facilitating prolonged cellular hydration and nutrient resupply in drying soils (Or et al., 2007). EPS can bridge between microbes and their substrates and allow them to survive in dry soils. EPS can also protect exoenzymes (Sutherland, 2001; Holden, 2011), and can either promote (Chenu and Roberson, 1996) or constrain (Holden et al., 1997) C diffusion to microbes. EPS can alter soil structure to mediate water retention (Chenu and Roberson, 1996), hydraulic conductivity (Henao and Mazeau, 2009; Rosenzweig et al., 2009), and aggregate structure (Roberson et al., 1995; Park et al., 2007). By promoting aggregate formation, microbes can create favorable growth environments, either in the interior of macroaggregates, which may have increased water content, proximity to substrates, and physical protection from predators (Görres et al., 1999; Neher et al., 1999; Six et al., 2006), or in macropores that offer easier access to diffusing substrate (Ruamps et al., 2011). Aggregate interiors, however, can also be a constrained growth environment when accessible substrates are depleted, or if intra-aggregate pore size is small enough to prevent colonization (Chenu et al., 2001).

Microorganisms' ability to produce EPS appears correlated with their ability to grow at low water potentials (Lennon et al., 2012) and so appears to be part of a deeply rooted life-history strategy that includes the ability to tolerate low $O_2$ (associated with being in a saturated biofilm) and a longer lag before starting exponential growth. This strategy is concentrated in a subset of phyla, notably the Firmicutes (Lennon et al., 2012). Microorganisms can also produce other chemicals that directly affect soil conditions and structure, including a variety of proteins such as hydrophobins, glomalin (produced by arbuscular mycorrhizal fungi in the Glomales), and chaplins (produced by Actinomycetes; Rillig et al., 2007).

### *Cell wall polymers*

Within a cell, microbes also have the ability to shift resource allocation among different pools; these allocation patterns affect the functioning of the cell itself and potentially that of the overall soil system. Cell wall materials are thought to be potentially important sources of C and N for long-term stabilization (Liang et al., 2011). The proportion of chitin, peptidoglycan, lipids, and other cell-wall and outer-membrane components depend on the ratio of Fungi:Bacteria:Archaea, as well as the proportion of Gram-positive:Gram-negative bacteria within the bacterial community. Amino sugars, which both bacteria and fungi use as components of their cell walls (peptidoglycan and chitin, respectively) become important constituents in soil organic nitrogen pools (Dai et al., 2002; Kögel-Knabner, 2002; Joergensen and Wichern, 2008), yet are produced differentially by the different groups of organisms.

### *Stress response compounds*

It has been argued that microorganisms physiologically acclimate to survive stresses such as low water potential or freezing by accumulating cytoplasmic constituents such as osmolytes or cryoprotectants (Schimel et al., 2007). These molecules would have to be lost or transformed rapidly when the stress ended (rewetting or thaw). While there are consistently flushes of C and N upon rewetting and thaw, recent research suggests that in soil the "osmolyte theory" for how microbes tolerate drought may be incorrect (Boot et al., 2012; Kakumanu et al., 2012) and that the substrate flushes may be instead be associated with mobilization of non-biomass SOM (Miller et al., 2005; Xiang et al., 2008). This may not be the case for freezing, however, as the organisms that are active at low temperature appear distinct from those active under warmer conditions (McMahon et al., 2011) and the nature of OM processing and nutrient balance shifts between summer and winter (Schimel and Mikan, 2005). Soil freezing may select for anaerobes (Miller et al., 2007). How the production of such material varies across the microbial world remains unclear.

## SCALING UP IN TIME: DO MICROBIAL COMMUNITY INFLUENCES SHIFT WITH TIME SCALE?

The importance of microbial community composition may vary with the time scale being considered, but not necessarily in an intuitive way. Generally, we assume that scales of time and space are linked – it makes no sense to think about the global carbon cycle *this second*, nor does it make sense to ask what carbon in a single square centimeter will be doing over the next century. Rather we assume that questions framed at fine spatial scales will also target short time scales – studies on soil cores rarely last longer than months. In considering spatial scales, Schimel (1995) suggested that there should be a continuum in the influence of microbial

community structure, with maximal influence at the finest spatial scales, with influence fading out at progressively larger spatial scales. We suggest that this is reasonable, but the same logic might not apply to time scales.

As the scale of focus changes, the nature of the processes that are most relevant change, as do the intellectual models that we use for considering them. For example at the pore-scale, we focus on patterns of microbial growth and substrate diffusion; reaction-diffusion models are a core intellectual paradigm for understanding this scale (Or et al., 2007; Holden, 2011); these emphasize access and catabolism. A new approach in microbial ecology is Dynamic Energy Budget modeling, which focuses on how organisms allocate resources under different environmental conditions and how that translates into population growth and turnover (Klanjscek et al., 2012). At the ecosystem scale, studies frequently focus on a seasonal to interannual time scale and emphasize plant production and decomposition (the life and death histories of plants) – box-and-flow biogeochemical models are the dominant paradigm (Manzoni and Porporato, 2009) and at this scale it is difficult to show any substantial influence of microbial community composition on C cycling. Larger scales become the domain of Earth System models, but these generally have a biogeochemistry core that is based on ecosystem-scale models (Clark et al., 2011). One might therefore argue that at these larger scales, the specific dynamics of microbes would be even less important in regulating the key processes. But at the decade-and-up times scales relevant to the global climate system, plant production and litter decomposition become tightly balanced, and overall C storage and loss become increasingly a function of the big, slow pools – e.g., stabilized OM in the mineral soil (Jobbágy and Jackson, 2000). Thus, to evaluate the processes that control C-sequestration, it is not the rate of microbial growth or of litter decay that matter most – it is the production of stabilized materials, which are a small fraction of the total (Liang et al., 2011). These products result from specific anabolic pathways of microbes and on patterns of microbial community composition that are reasonably stable across time (Grandy et al., 2009). Thus, as we focus on understanding soil C dynamics overlong time-scales, the allocation patterns of specific groups of microbes that regulate the fate of OM in mineral soils becomes increasingly important.

## SCALING UP IN SPACE: WHERE IN THE SOIL ARE COMMUNITY INFLUENCES LIKELY IMPORTANT?

We have briefly discussed perspectives on microbial diversity and the ways it may affect C cycling in soil systems. We have argued that our perspectives shift among time scales and specific processes, and that the roles of microbial community composition in controlling these processes shift as well. We also need to consider how these dynamics play out in different compartments of the soil system, because community influences may act differently among them. As a simple breakdown of the dominant zones within the soil landscape, we can consider (A) rhizosphere, (B) aboveground litter, (C) dead roots, and (D) mineral soil (**Figure 2**). We hypothesize that the composition of the microbial community plays a significant role in controlling C cycling in the rhizosphere and in organic detritus (litter or dead roots), but for distinctly different reasons. In the rhizosphere, microbial community composition is regulated by the specific substrates and chemical signals released by the plant root, and by the specific physical and biotic environment created by the plant root in terms of $O_2$, pH, and other chemical variables (Jaeger et al., 1999; Hinsinger et al., 2009). These select for a distinct group of microbes, some of which act as plant growth-promoting rhizobacteria or as pathogens and so have powerful feedbacks to plant growth and C cycling. In physically unprotected organic detritus, chemical structures remain complex and specific to the plant, microbe, or animal that produced them; exoenzyme breakdown is necessary for microbes to metabolize them. Thus, their breakdown remains under biological control and sensitive to the specific identities of the decomposers present. Extensive work has been done on aboveground litter, linking chemistry and organisms, and decomposition dynamics. The community present in litter alters decomposition kinetics, often the community native to the litter's home site is more effective than communities from other sites; the "home-field advantage" (Sinsabaugh and Moorhead, 1994; Schimel et al., 1999; Aneja et al., 2004; Craine et al., 2007; Strickland et al., 2009; Baumann et al., 2011; Freschet et al., 2011; Baldrian et al., 2012; Schneider et al., 2012).

In contrast, comparatively little research has been done on dead roots. This is surprising because typically at least half of ecosystem net primary productivity goes into belowground structures (Chapin III et al., 2002), and root litter is likely a greater source of stabilized soil C than is aboveground litter (Rasse et al., 2005; Schmidt et al., 2011; Carrington et al., 2012). The chemistry of root litter differs from that of foliar litter (Harmon et al., 2009), and differs in decomposition dynamics. For example, while high C/N foliar litter inevitably shows a phase of N-immobilization early in decomposition, this is not the case for roots, which begin to mineralize N in parallel with mass loss (Parton et al., 2007). Relatively few studies have evaluated the microbiology of root decomposition compared to those on leaf litter (e.g., Fisk et al., 2010; Baumann et al., 2011). Because root litter is such an important source of soil C and because so little has been done on the relationships between chemistry and microbiology in dead roots, we consider this to be the most important compartment for microbial study in the soil system.

In mineral soils, as we have discussed, the influence of microbial community composition on the rate of breakdown of SOM is likely to be limited. Rather, community composition may more strongly reflect the physical environment and substrate access patterns. However, the distribution of major phylogenetic groups may control the fate of that material. Here, we need continued study on SOM chemistry to better understand the factors that regulate microbial access to substrate and we need increased study on microbial processing and production of new materials to better understand how they regulate the physical structure of soil and the long-term fate of soil C.

## CONCLUSION

In their effects on soil C cycling, the influences of microbial communities appear to be associated with life history patterns that are deeply rooted in microbial phylogeny – functional groups appear at the level of families or phyla rather than species or genera. Even accepting that soil microbes have different life history strategies and comprise different functional groups however, for those

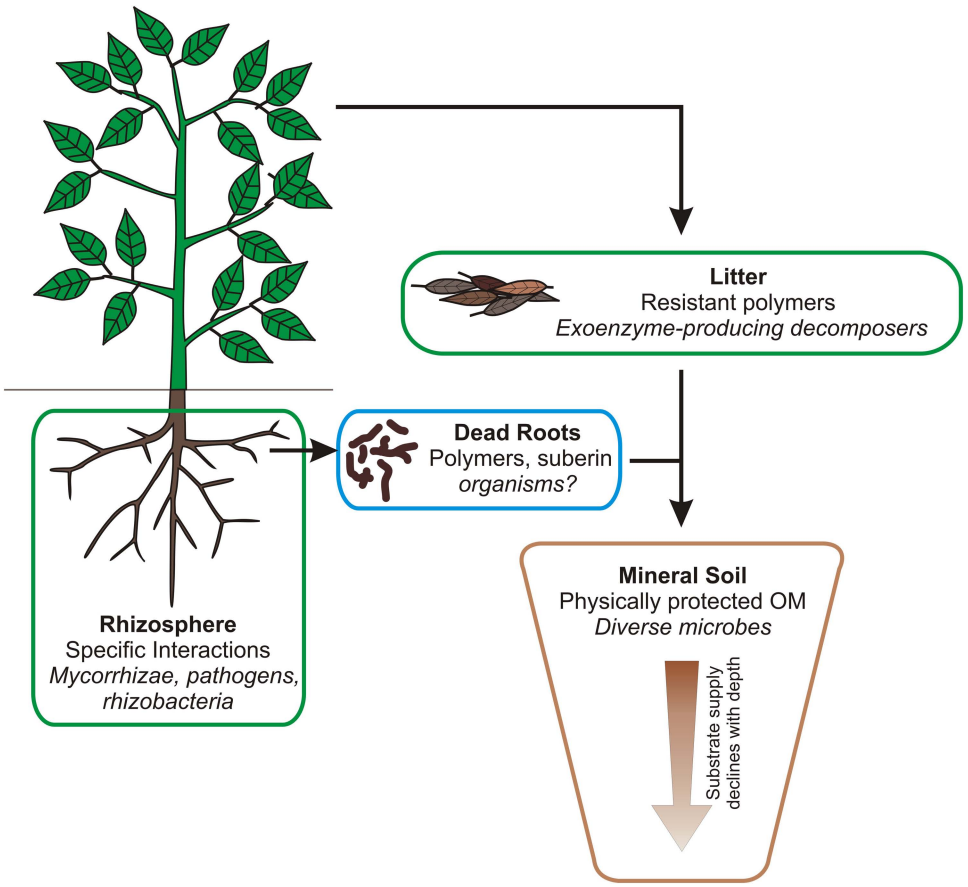


**FIGURE 2 | The main zones in soil, the characteristics that regulate microbial functioning within each zone, and the dominant guilds of microbes present.** In litter and the rhizosphere (outlined in green), microbial community composition likely affects both the rate of processes and the fate of C. In mineral soil (outlined in brown), microbial community composition likely only controls the fate of C. In dead roots, community composition probably regulates both rate and fate, but little research has been done on this compartment.

differences to influence ecosystem C dynamics, those organisms must carry out steps in OM processing that are rate-limiting in overall OM breakdown, which requires that they have physical access to the material, or they must control the fate of that material, synthesizing alternative products with important characteristics for ecosystem function. The specific compounds produced likely affect the nature of soil processes most strongly at either the shortest or the longest time scales, but least strongly at the interannual "ecosystem" scale that dominates much biogeochemical study. In the rhizosphere and in detritus, community composition likely influences C-cycling rates, while in the mineral soil, it may primarily influence the fate of C, while physical processes controlling microbial access to C regulate turnover rate. The largest uncertainty about the role of community composition probably exists for dead roots. These may constitute the largest source of C that is sequestered in the soil and so represent the biggest long-term input yet is the least studied part of the system.

## ACKNOWLEDGMENTS

We thank Dr. Patricia Holden for contributing to the thinking that led to this paper, and to three reviewers who offered valuable criticism and suggestion that substantially improved this paper. We thank the U.S. National Science Foundation Ecosystem Science Program for supporting this effort, both through grants from the Ecosystem Science Program (DEB-0444712; DEB-1145875) and for supporting the Enzymes in the Environment Research Coordination Network, which supported the workshop "Incorporating Enzymes and Microbial Physiology into Biogeochemical Models" in Ft. Collins Co in May 2012; participants at the workshop offered valuable insights.

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

*Received: 25 June 2012; accepted: 09 September 2012; published online: 26 September 2012.*

*Citation: Schimel JP and Schaeffer SM (2012) Microbial control over carbon cycling in soil. Front. Microbio.* ***3****:348. doi: 10.3389/fmicb.2012.00348*

*This article was submitted to Frontiers in Terrestrial Microbiology, a specialty of Frontiers in Microbiology.*

frontiers in
MICROBIOLOGY

REVIEW ARTICLE
published: 19 December 2012
doi: 10.3389/fmicb.2012.00417

# Fundamentals of microbial community resistance and resilience

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Microbial communities are at the heart of all ecosystems, and yet microbial community behavior in disturbed environments remains difficult to measure and predict. Understanding the drivers of microbial community stability, including resistance (insensitivity to disturbance) and resilience (the rate of recovery after disturbance) is important for predicting community response to disturbance. Here, we provide an overview of the concepts of stability that are relevant for microbial communities. First, we highlight insights from ecology that are useful for defining and measuring stability. To determine whether general disturbance responses exist for microbial communities, we next examine representative studies from the literature that investigated community responses to press (long-term) and pulse (short-term) disturbances in a variety of habitats. Then we discuss the biological features of individual microorganisms, of microbial populations, and of microbial communities that may govern overall community stability. We conclude with thoughts about the unique insights that systems perspectives – informed by meta-omics data – may provide about microbial community stability.

**Keywords: microbial ecology, disturbance, stability, sensitivity, structure-function, perturbation, community structure, time series**

## INTRODUCTION

In habitats as diverse as soil and the human body, key ecosystem processes are driven by microbial communities – local assemblages of microorganisms that interact with each other and their environment (Konopka, 2009). Thus, microbiology research in biomedical, environmental, agricultural, and bioenergy contexts shares a common challenge: to predict how functions and composition of microbial communities respond to disturbances (Robinson et al., 2010a; Gonzalez et al., 2011a,b; Costello et al., 2012).

Here, we introduce a breadth of topics that provide insight into the responses of microbial communities to disturbance. We first highlight key concepts from ecology that are useful in thinking about microbial stability, pointing readers to an extensive literature on the subject of disturbance and community stability. We then summarize the current state of knowledge about resistance and resilience of microbial communities inhabiting a variety of ecosystems, emphasizing overarching trends gleaned from a review of 247 representative studies. We next provide a synthesis of the properties of individual microorganisms, populations, and communities that influence microbial community stability. Finally, we discuss insights into stability that may emerge from a systems-level perspective – describing microbial communities as networks of genes, transcripts, proteins, and metabolite signals.

## KEY CONCEPTS FROM ECOLOGY

### DEFINING DISTURBANCE

Disturbances are causal events that either (1) alter the immediate environment and have possible repercussions for a community or (2) directly alter a community (Rykiel, 1985; Glasby and Underwood, 1996). After disturbance, community members may die (mortality) or change in their relative abundances (Rykiel, 1985). A disturbance can be difficult to define, as its definition depends on scale and context. Disturbances occur at various spatial and temporal scales (Paine et al., 1998) with different frequencies (number of occurrences per unit time), intensities (magnitude of the disturbance), extents (proportion of the ecosystem affected), and periodicities (regularity of occurrences; Grimm and Wissel, 1997). Disturbances may also be defined relative to the disturbance regime of an ecosystem, such as a fire or flooding cycle.

Disturbances are often classified as *pulses* or *presses* depending on their duration (Bender et al., 1984). In general, pulse disturbances are relatively discrete, short-term events, whereas

presses are long-term or continuous (**Figure 1**). However, these time scales may differ depending on the generation time of the community of interest. For instance, a tree falling in a forest may create a press disturbance to the underlying soil microorganisms, whereas the same event might be considered a pulse disturbance to nearby understory vegetation. Though the distinction between pulse and press disturbances has received much attention in the ecology literature, there is less discussion of patterns of microbial community responses to pulses and presses. However, microbial community responses to pulses and presses are important to consider in the context of global climate change. With global changes, pulse disturbances (e.g., extreme weather events) are expected to increase in frequency, and ongoing press disturbances are expected to continue (e.g., atmospheric increases in carbon dioxide, ocean acidification; Intergovernmental Panel on Climate Change, 2007). Therefore, throughout this work, we discuss microbial community responses in both pulse and press disturbance scenarios.

## DEFINING STABILITY

Disturbance and community stability are necessarily related, as stability is defined as a community's response to disturbance (Rykiel, 1985). Here, we adopt definitions most similar to Pimm (1984), in which stability is comprised of resistance and resilience (**Table 1**), two quantifiable metrics that are useful for comparing community disturbance responses and have precedent in the microbial ecology literature (e.g., Allison and Martiny, 2008). However, readers should be aware that the ecology literature includes many definitions of stability, and a full examination of these definitions is available elsewhere (Grimm and Wissel, 1997). Here, resistance is defined as the degree to which a community is insensitive to a disturbance, and resilience is the rate at which a community returns to a pre-disturbance condition (Pimm, 1984). A related concept, sensitivity, is the inverse of resistance and defined as the degree of community change following a disturbance. Both resistance and resilience are usually quantified in relation to a community's level of intrinsic variability, sometimes referred to as the "normal operating range" (van Straalen, 2002). There are many methods in the literature [see a recent summary by Griffiths and Philippot (2012)] for comparing resistance or resilience across communities (Orwin and Wardle, 2004).

A community's stability can be investigated in terms of functional or compositional parameters. In microbial ecology, many studies also focus on the degree to which functional and compositional stability are related. This may depend in large part on the particular function of interest (Schimel, 1995). For functions that are carried out by many taxa (Schimel, 1995), i.e., communities harboring a high degree of functional redundancy, changes in community composition may not correspond with changes in functional rates (Allison and Martiny, 2008). Alternatively, for functions performed by only a few taxa (for example, in situations of ecological coherence of closely related taxa, Philippot et al., 2010), the sensitivity and resilience of this function may closely follow changes in the abundance of those taxa. Notably, estimates of resistance and resilience for the same microbial community may have different values depending on whether compositional or functional responses are measured and on which functions are used to assess stability.

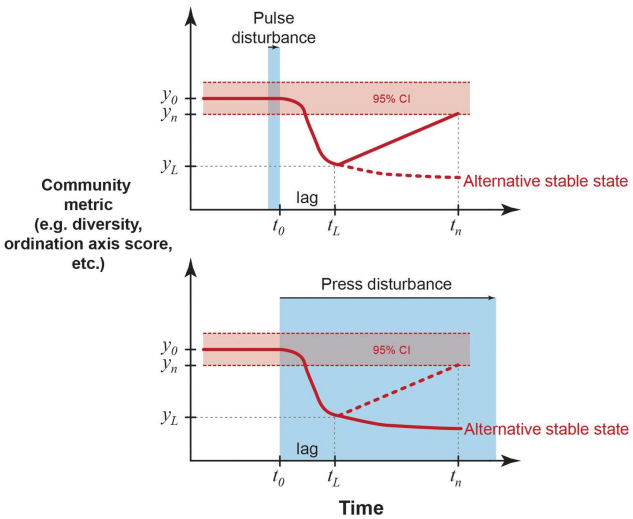


**FIGURE 1 | Examples of quantitative definitions of resistance and resilience from ecology (Westman, 1978; Orwin and Wardle, 2004; Suding et al., 2004).** A microbial community parameter of interest has a mean value of $y_0$ and temporal variance, illustrated here by a 95% confidence interval around the mean (though other quantifications of variance, such as standard deviation or variance ratios may be used). A pulse disturbance ends (or a press disturbance begins) at time $t_0$ and the parameter changes by $|y_0 - y_L|$ after a time lag $t_L - t_0$. Resistance (RS) is an index of the magnitude of this change.

$$RS = 1 - \frac{2\,|y_0 - y_L|}{y_0 + |y_0 - y_L|} \quad (1)$$

Resilience (RL) is an index of the rate of return to $y_0$ after the lag period,

$$RL = \left[\frac{2\,|y_0 - y_L|}{|y_0 - y_L| + |y_0 - y_n|} - 1\right] \div (t_n - t_L) \quad (2)$$

where $y_n$ is the parameter value at measurement time $t_n$. A parameter is "recovered" when it is statistically indistinguishable from the pre-disturbance mean. Alternatively, the parameter may not recover and instead may stabilize at a new mean value representing an alternative stable state. This possibility is more likely in response to a press disturbance. Further, RS and RL could be related to normalized parameters describing the disturbance (e.g., intensity, duration, frequency of the stressor in relation to the pre-disturbance mean and variance), which is useful for cross-system comparisons.

One perspective of stability, sometimes referred to as "ecological resilience," relies on the existence of many stable states in which a community may reside (e.g., Holling, 1973, 1996; Botton et al., 2006). For instance, a community may shift to a new stable state when subjected to a press disturbance (**Figure 1**). The existence of multiple equilibria can also be illustrated by the concept of a stability landscape (Beisner et al., 2003; Scheffer and Carpenter, 2003; Collie et al., 2004; Folke et al., 2004), which can be used to conceptualize microbial community responses to disturbance (Blodau and Knorr, 2006; Mao-Jones et al., 2010; Bürgmann et al., 2011; Seto and Iwasa, 2011). In a visualization of a stability landscape, a ball represents a community that can exist in one of many different equilibrium states (basins) within the stability landscape (Beisner et al., 2003; Scheffer and Carpenter, 2003; **Figure 2**). A disturbance

**Table 1 | Common terms for disturbances, community responses, and community outcomes.**

| | |
|---|---|
| **DISTURBANCE TERMS** | |
| Disturbance | A causal event that causes a discrete change in the physical or chemical environment that has anticipated effects on a community (Rykiel, 1985; Glasby and Underwood, 1996) |
| Press disturbance | A continuous disturbance that may arise sharply but reaches a constant level that is maintained over a long period of time (Lake, 2000) |
| Pulse disturbance | A short-term, often intense disturbance that rapidly decreases in severity over a short period of time (Lake, 2000) |
| **COMMUNITY TERMS** | |
| Community | An assemblage of microorganisms that live in the same locality and potentially interact with each other or with the environment (Konopka, 2009) |
| Metacommunity | Within a regional landscape, a set of local communities whose members are linked by dispersal (Wilson, 1992; Logue et al., 2011) |
| **COMMUNITY RESPONSE TERMS** | |
| Stability | The tendency of a community to return to a mean condition after a disturbance (Pimm, 1984); includes the components of resistance and resilience<br>Ecological stability can be measured in many ways, including the persistence of populations through time, constancy of ecological attributes through time, resistance to a disturbance, or resilience after a disturbance (Worm and Duffy, 2003) |
| Resistance | The degree to which a community withstands change in the face of disturbance (Pimm, 1984; Allison and Martiny, 2008). Inverse of *sensitivity* |
| Sensitivity | The degree to which a community changes in response to disturbance, the inverse of resistance |
| Resilience | The rate at which a microbial community returns to its original composition after being disturbed (Allison and Martiny, 2008). Commonly referred to as community recovery. Inverse of return time |
| **COMMUNITY OUTCOMES** | |
| Stable state | A condition where a community returns to its original composition or function following a disturbance (Beisner et al., 2003). Also known as community equilibrium or an attractor |
| Alternative stable state | A condition where a community moves to a different but stable composition or function following a disturbance. One of multiple, non-transitory stable states in which a community can exist (Beisner et al., 2003) |
| Regime shift | A large change in community composition arising from a shift between alternative stable states (Scheffer and Carpenter, 2003) |

event is analogous to applying a force to the ball within its basin. A community may resist the disturbance, which is represented by the ball remaining in its basin. Alternatively, the community could change but exhibit resilience, which is represented by the ball moving outward from the basin but then returning to its original location (**Figure 2A**). If resistance and resilience are low or the disturbance strong enough, the community may shift to an alternative equilibrium (also called alternative stable state), represented by the ball moving into a new basin. Once in an alternative equilibrium, the community's return to the previous composition or function may be difficult (Botton et al., 2006). Moreover, environmental conditions shape the stability landscape (**Figure 2B**). Thus, if a press disturbance permanently alters the stability landscape, this will have implications for community stability and the likelihood of community shifts to alternative stable states.

Studies of alternative stable states and regime shifts in microbial systems remain rare (Botton et al., 2006), though the conceptual framework is gaining popularity, especially among researchers interested in the human gut microbiome (Lozupone et al., 2012), as the existence of alternative stable states may provide explanation as to the immense variability observed within and among individual gut microbial communities. There is also evidence of alternative stable states in the vaginal microbiome, where eight "super-groups" of distinct microbial assemblages have been detected across hundreds of healthy women (Zhou et al., 2007). Additionally, there are a few concrete examples of microbial communities that exhibited regime shifts. For instance, increased influx of groundwater triggered a functional regime shift from iron-reduction to sulfate-reduction in anoxic sediments of mine drainage lakes (Blodau and Knorr, 2006), and operational changes triggered a compositional and functional regime shift in a sequencing batch reactor for nitrogen elimination from urine (Bürgmann et al., 2011). Furthermore, regime shifts in microbial communities may have far-reaching consequences for ecosystems, as suggested by theoretical models of coral reef microbial communities that shift composition from antibiotic-producers to pathogens (Mao-Jones et al., 2010). As time series studies are extended to include more disturbance events, alternative stable states may be detected for other microbial communities. In the example in **Figure 2**, microbial community composition and function are mapped using multivariate ordination to visualize a stability landscape.

## MEASURING STABILITY: COMMUNITY RESPONSE TO DISTURBANCE

Ecologists have long considered how to quantify resistance and resilience of communities and their functions. One experimental design that specifically addresses the impact of a disturbance is "before-after-control-impact" (BACI; Stewart-Oaten et al., 1992; Smith et al., 1993; Ellis and Schneider, 1997; Stewart-Oaten and Bence, 2001; Fraterrigo and Rusak, 2008). But, BACI has known limitations [discussed in Underwood (1994)], including violation

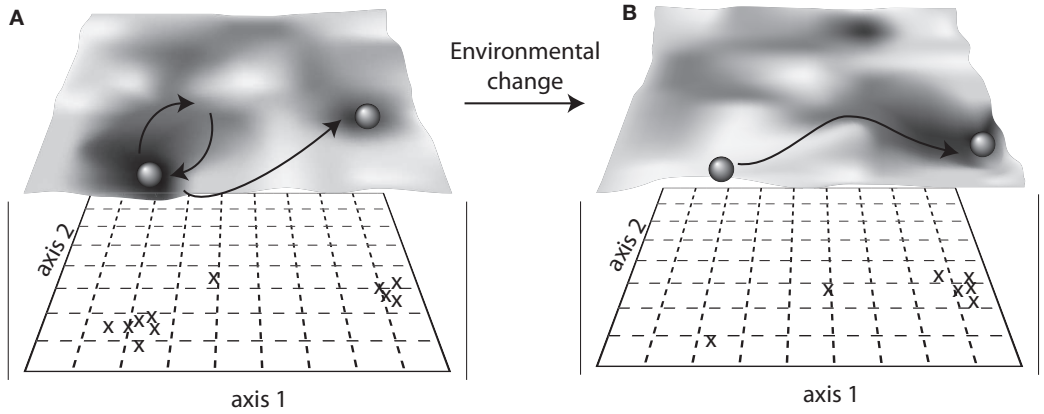


**FIGURE 2 | Alternative equilibria, also called alternative stable states, visualized with a stability landscape.** Here, changes in community composition are assessed using axis scores from an ordination (e.g., principal coordinates analysis (PCoA) of Bray–Curtis similarities) before **(A)** and after **(B)** environmental change. The overlay "terrain" of the landscape shows the different stable states as basins, and the community is represented as a ball that is either maintained in its original basin or displaced to a new basin after a disturbance. Community resilience is represented by the slope of the basin walls, showing a rate of return to the original stable state.

of assumptions of non-independence of samples collected over time. Thus, multivariate autoregressive moving-average models, which remove temporal autocorrelation, have been applied to understand the contribution of species and environmental interactions to the stability of communities (e.g., Ives et al., 2003). Additionally, Bayesian approaches such as dynamic linear models (DLM) account for the autocorrelation of time series data, and estimate the sensitivity of data to disturbance (e.g., Carpenter and Brock, 2006). Techniques such as DLM also make projections about future behavior of a response variable based on pre-disturbance data distribution (sometimes called intervention). There are many other techniques that quantify temporal variability to assess the impact of disturbance and measure resilience (e.g., Underwood, 1994; Ives, 1995; Ives et al., 2000; Fraterrigo and Rusak, 2008). Methods for handling temporal datasets (e.g., Lennon, 2011) will become increasingly useful to microbiologists as more and longer time series of microbial communities become available. However, the species-rich nature of many microbial datasets, especially those generated using high-throughput sequencing, present computational challenges that will likely require new methods of statistical analysis (Gonzalez et al., 2011a).

When measuring stability, it is important to distinguish between responses to pulse and press disturbances, as recovery may be quantified by slightly different methods (Glasby and Underwood, 1996). Ideally, resilience to a press disturbance should be determined after the community composition or function reaches its maximum deviation from the expected mean (**Figure 1**). With a press, there is often more uncertainty about when the disturbance has caused the maximal change in the community, especially if it is unknown when the press disturbance began and whether it has ceased. Therefore, when to establish the baseline for measuring resilience after a press disturbance is less obvious, and therefore a major research challenge. By contrast, response to a pulse can be defined immediately after the pulse ends, although there may be a time lag before the disturbance response is completed.

Additionally, experimental settings allow pulse and press responses to be compared directly and described relative to one another. For example, tropical soil microbial communities exposed to fluctuating (pulse) anoxic-to-oxygenic conditions were compared to those exposed to continuous (press) anoxic or oxygenic conditions (DeAngelis et al., 2010). This pulse-press comparison revealed that communities exposed to repeated redox fluctuations were more diverse and more active (assessed by their RNA to DNA ratio) than communities exposed to a constant condition. Furthermore, when microbial communities are monitored long-term, pulse and press responses also may be compared *post hoc*. In these cases, community stability could serve as an indicator of unmeasured or unobserved pulse and press disturbances: short-term variability around a baseline (equilibrium) may be a sign of pulses while gradual shifts in the baseline may be a sign of presses (Shade et al., 2012).

## COMMUNITY INVASIBILITY AS AN INDICATOR OF STABILITY

Invasion, the successful establishment of a non-native organism in a community (Litchman, 2010), can provide an indicator for both compositional and functional stability. Invasion is unique in that it can be considered both a cause and consequence of disturbance. In studies of communities with larger organisms, a well-known consequence of community disturbance is reduced resistance to invasion by alien species (called "niche opportunity"; Shea and Chesson, 2002), but its parallel use as a functional indicator of stability in microbial communities has been limited, with a few exceptions (e.g., Robinson et al., 2010b). Community invasion does, however, have a long history in microbial ecology in the context of agricultural inoculants and veterinary and clinical probiotics. For example, a century ago, Ilya Metchnikoff explored invasion of his own gut microbiome by lactobacilli consumed in sour milk, and found that the lactobacilli did not invade his gut community and needed to be replenished frequently to obtain the salubrious effects on his health that he reported (Metchnikoff, 1908; Schmalstieg and Goldman, 2008). A century of study of

probiotics revealed the challenge of establishing new strains of bacteria in the mammalian gut, with many studies documenting disappearance of introduced strains within hours of entrance into the gastrointestinal tracts of pigs (e.g., Gardiner et al., 2004) and humans (Robins-Browne and Levine, 1981; Ventura and Perozzi, 2011). These studies contributed to the broadly held sense that microbial communities are resistant to invasion, embodied in the concept of "colonization resistance" of the human microbiome (Savage, 1977; Hopkins and Macfarlane, 2003; Johnson-Henry et al., 2008; Britton and Young, 2012).

## KNOWNS AND UNKNOWNS ABOUT MICROBIAL COMMUNITY STABILITY: AN UPDATED INVESTIGATION OF THE LITERATURE CONSIDERING RESPONSES TO PULSE AND PRESS DISTURBANCES

Recent studies have reported that, in general, soil microbial communities are not resistant to disturbances, as measured by composition, and that even within several years many communities fail to recover entirely (Allison and Martiny, 2008). To extend this analysis to non-soil communities, we explored the literature for studies investigating microbial community stability in the face of disturbance (see Appendix). We considered 247 studies, and these studies included a total of 378 investigations of soil, marine and freshwater, engineered (e.g., wastewater treatment, bioreactors), and host-associated (gut) systems to discern patterns of stability that may be broadly applicable to microbial communities. From this exploration, we chose representative examples from the literature to illustrate key points, as our search was not intended to be exhaustive. We focused our comparisons on microbial community responses to pulse and press disturbances.

Investigations of microbial community stability generally fell into two broad classes: *observations*, which typically involved *in situ,* large-scale disturbances (e.g., deforestation, typhoons, temperature changes), and *designed experiments* that usually involved small-scale disturbances (e.g., nutrient amendment, temperature alterations, or fumigation, **Figure 3A**). Resistance and resilience were assessed either based on microbial community composition or function (Allison and Martiny, 2008; Little et al., 2008), which are sometimes linked (Balvanera et al., 2006; Cardinale et al., 2006). Many studies (23%) measured only microbial composition, assessed by multivariate analysis of molecular fingerprints or 16S rRNA gene sequences, as the response variable to assess stability. Some studies (18%) measured community functions (respiration, biomass production, or activity of extracellular enzymes). Finally, a large number of studies (58%) measured both community composition and function.

From our examination of 310 experimental and 68 observational investigations of microbial responses to disturbances, 82% reported sensitivity to disturbance, either in composition (26%), function (21%), or both (35%, **Figure 3B**). This is in agreement with previous findings for soil communities (Allison and Martiny, 2008), and suggests that most microbial communities may be sensitive to disturbances. One caveat to this finding is that it may be more difficult to publish results of experiments in which communities did not change when challenged with a disturbance, and so this finding may reflect a potential bias in the literature. A habitat-by-habitat summary of sensitivity to disturbance is given in **Figures A1A,B** in Appendix. Though we considered microbial communities from many habitats, soil communities were most represented, affirming that the majority of disturbance investigations in microbial ecology are from soil habitats.

Only a few investigations explicitly measured resilience (**Figure 3C**). Of those 148 investigations that reported community sensitivity to disturbance and also examined recovery, only a small fraction reported return to pre-disturbance composition (13%), function (8%), or both (2%). However, it was unclear whether resilience was not observed in some investigations because of biases in sampling intensity or duration after the disturbance (**Figure A2** in Appendix) or because the communities were not, in fact, resilient. The normal variability of microbial communities in the absence of any disturbance event was also often unreported. Without *a priori* knowledge of community turnover, it may be difficult to inform post-disturbance sampling intensity or duration. Thus, knowledge of baseline microbial community stability as well as post-disturbance dynamics remains limited for many habitats and contexts.

Our results suggest that microbial communities are equally sensitive to pulse and press disturbances (**Figure 3B**). Drawing on the subset of investigations that assessed resilience, our results hint that microbial communities may be more resilient after pulse disturbances than after press disturbances (**Figure 3C**). Recovery from pulse disturbances was reported more often for microbial community function than for composition, while recovery from press disturbances was approximately the same for both function and composition. As more disturbance studies become available, further work will be needed to compare resilience quantitatively across press and pulse disturbances, as different disturbance types (chemical, biological, physical, combination) were differently represented within pulse and press investigations (**Figures A1C,D** in Appendix). For example, though physical disturbance types were represented approximately equally in both pulse and press investigations, press disturbances included a larger representation of chemical disturbance types than pulse.

Additionally, the literature survey results draw attention to our current knowledge gaps regarding microbial community responses to pulse and press disturbances. Specifically, there is limited understanding of microbial responses to biological pulse disturbances and to disturbance combinations for both pulses and presses. First, investigations of microbial responses to biological disturbance types were rare, especially in pulse disturbance scenarios (**Figures A1C,D** in Appendix). Examples of pulse biological disturbances include phytoplankton blooms, which not only impact neighboring microbial communities but also have implications for both heterotrophic and autotrophic contributions to global carbon cycling (e.g., Teeling et al., 2012). Therefore, understanding pulse biological disturbances remains an important gap to fill. Second, investigations of disturbance combinations were also uncommon among the literature surveyed, though pulse disturbances included a larger representation of disturbance combinations than press (**Figures A1C,D** in Appendix). Compounded disturbances include those that occur simultaneously or within the recovery time of a preceding disturbance. Because compounded disturbances may lead to regime shifts (e.g., Paine et al.,

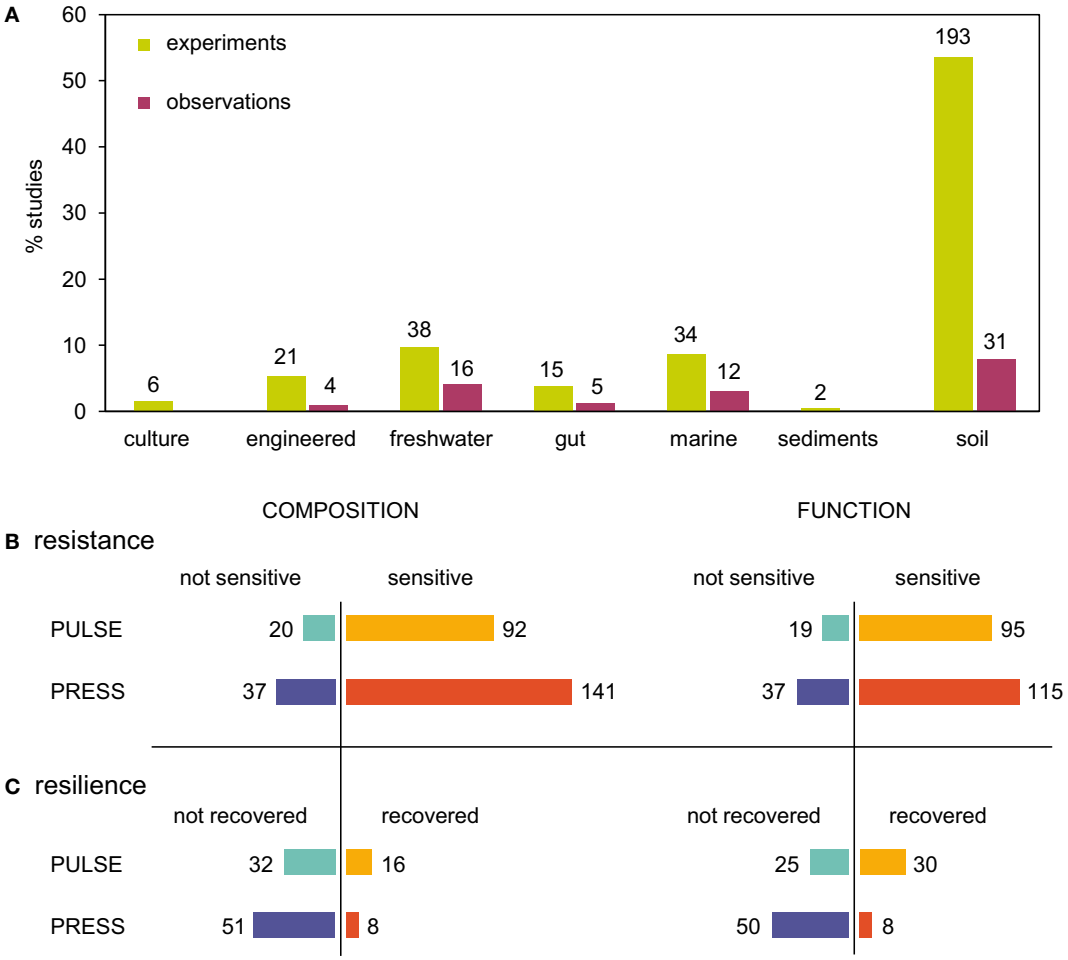


**FIGURE 3 | Summary of a literature survey of microbial community responses to pulse and press disturbances.** The survey included studies that investigated changes in microbial community structure after biological, chemical, or physical disturbances. **(A)** Representation of investigations across ecosystem types, and by whether the investigation was a designed experiment or opportune *in situ* observations after a disturbance. There were 378 total investigations from 247 total studies, as some studies investigated more than one disturbance or measured more than one function, and some studies did not report either. **(B)** Resistance was determined by sensitivity (change in composition or function after disturbance). Some investigations measured both composition and function, and were included in both charts. **(C)** If a community was sensitive to disturbance, resilience was measured as recovery to pre-disturbance composition or function. Many investigations that reported community sensitivity did not assess recovery.

1998), studying microbial community responses to compounded disturbances is increasingly important in face of global climate change.

It is interesting that though many studies measured functional responses (63%), there has been limited conceptual development on the role of pulse and press disturbances in driving relationships between microbial community composition and function. The presence of functionally redundant species in microbial communities has been suggested to increase functional resilience (e.g., Allison and Martiny, 2008); however, the degree of functional redundancy among microorganisms remains controversial. Diversity – function relationships could be probed by asking whether press and pulse disturbances select for different community memberships (see Biological Features That Contribute to Microbial Resistance and Resilience). Applying combinations of press and pulse disturbances to microbial communities could create gradients in community diversity that may clarify the role of disturbance in driving diversity-function relationships.

The results of our literature survey reveal that we have much to learn about the nature of change and recovery for microbial communities from many habitats. Conceptual progress across disciplinary boundaries within microbial ecology could be achieved by cross-system comparison of stability. However, we currently lack a common framework and standard format of reporting compositional and functional responses to disturbance, which inhibits more quantitative cross-system comparisons. There has been recent progress to standardize microbial community data in the new biological observation matrix (.biom taxa table; see biom-format.org), as used by concerted efforts to collect and curate large microbial datasets, such as by the Earth Microbiome Project (Gilbert et al., 2010). This and similar efforts will support development of disturbance theory for microbial ecology.

## BIOLOGICAL FEATURES THAT CONTRIBUTE TO MICROBIAL RESISTANCE AND RESILIENCE

Survival of individual cells is a prerequisite for population-level persistence, which is a prerequisite for community-level recovery (**Figure 4**). In this section, we explicitly focus on compositional, taxon-based resistance and resilience, but make connections to functional resistance and resilience where possible. We hypothesize that there are biological attributes that are of greater importance for microbial community resistance and resilience under pulse disturbance scenarios (orange circles, **Figure 4**), while other attributes are generally important for both pulse and press disturbances (purple circles).

### INDIVIDUAL PROPERTIES: PLASTICITY, STRESS TOLERANCE, AND DORMANCY

#### *Plasticity and stress response*

Resistance to compositional change in the face of disturbances is enhanced if a microbial community contains many individuals that have versatile physiologies, or physiological plasticity (Evans and Hofmann, 2012). Bacteria often navigate environmental change by expressing a range of metabolic capabilities (e.g., Meyer et al., 2004; Swingley et al., 2007), and therefore the existing community can confront new conditions through gene expression by individual cells. From an evolutionary standpoint, adaptive gene expression refers to natural selection acting on gene expression (Whitehead and Crawford, 2006), and this phenomenon has been observed, for example, in a yeast model of experimental evolution (Ferea et al., 1999). Furthermore, mixotrophy, or the ability to use many different carbon and energy sources, may be a common phenomenon in natural microbial communities (Eiler, 2006), and provides further support for the notion of individual flexibility in fluctuating environments. Cellular stress responses also provide protection for individual cells from damaging physical factors such as reactive oxygen species, temperature, and ultraviolet light (e.g., Craig, 1985; Ziegelhoffer and Donohue, 2009; Kolowrat et al., 2010). Stress protection in *E. coli* is associated with a progressive decrease in nutritional competence, or the breadth and range of carbon and nutrient resources that a cell can use (Ferenci, 2005; Ferenci and Spira, 2007), which ultimately may reduce the population's ability to confront other environmental changes. Therefore,

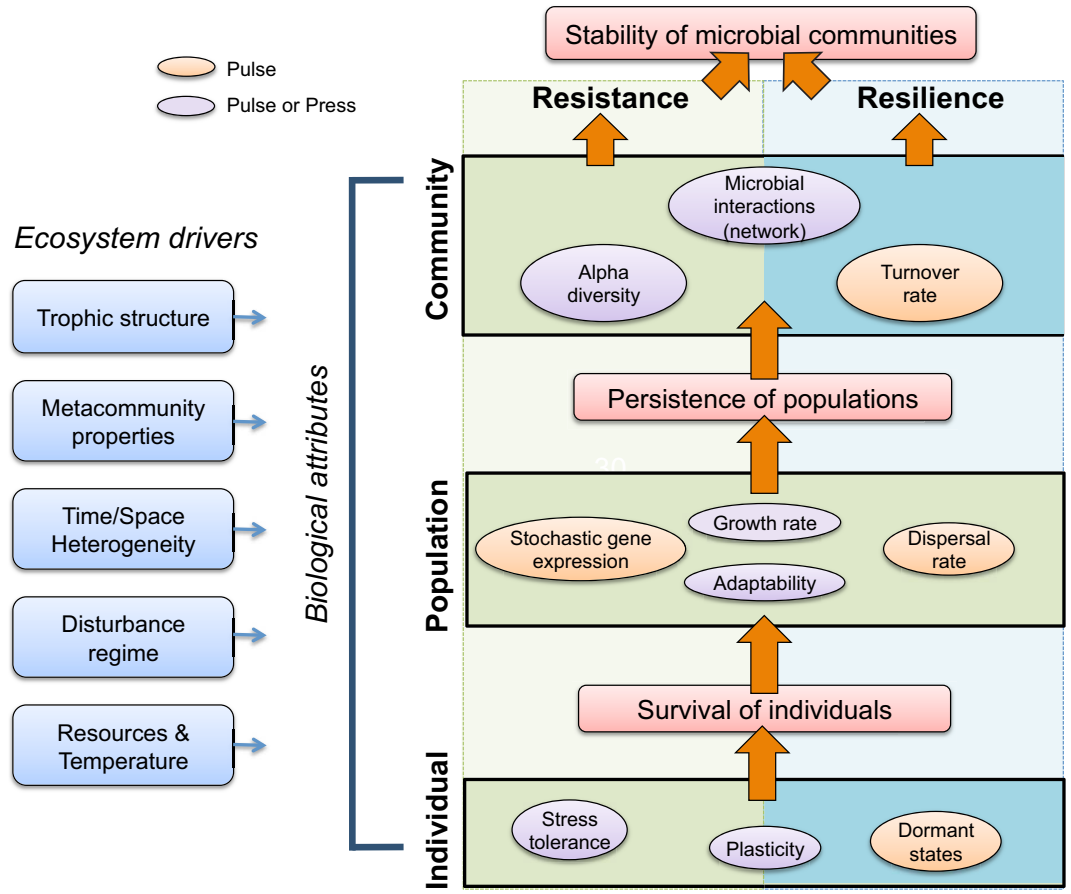


**FIGURE 4 | Conceptual model of biological and ecosystem properties governing microbial community resistance to and resilience after disturbance.** Stability of microbial communities in the face of disturbances is influenced by individual-, population-, and community-level biological attributes that contribute to community resistance (left, green background) and/or resilience (right, blue background), or both (center). Individuals withstand or survive disturbances and promote persistence of populations, which in turn promote overall community stability (orange arrows). Ecosystem drivers (leftmost blue boxes), such as trophic structure and disturbance regime, shape biological attributes, and also contribute to resistance and resilience. Finally, we hypothesize that biological attributes will be differently advantageous given a pulse (orange or purple) or press (purple) disturbance.

there may be fitness costs to microbial stress responses that are manifested in seemingly unrelated metabolic pathways, or costs of maximizing protection against one stress over another. Stress tolerance can contribute to microbial community resistance to pulse or press disturbances, but will depend on the intensity and duration of the disturbance relative to an individual's levels of stress tolerance (**Figure 4**).

Compositional stability is mediated, in part, by the ability of individual microorganisms to respond to, accommodate, and exploit environmental change. This highlights a difference between microbial communities and communities of larger organisms: prokaryotes have a degree of physiological plasticity that is unparalleled in the eukaryotic world. The unique ability to shift to an entirely different lifestyle within a short time (as in the classic example of *Rhodobacter sphaeroides*, which can grow anaerobically as a phototroph but also grow aerobically as a chemoheterotroph) is likely to increase the compositional stability while simultaneously reducing the functional stability. Current ecological theory based on plants and animals may not accommodate the enormous physiological plasticity of prokaryotes, which may necessitate development of new principles applicable to microbial communities. We hypothesize that physiological plasticity can contribute to a microbial community's resistance and resilience to either pulse or press disturbances. But, similar to stress tolerance, the contribution of plasticity to community stability will depend on the intensity and duration of the disturbance relative to an individual's physiological response.

### *Dormancy*

Dormancy is a bet-hedging strategy that allows organisms to enter a reduced state of metabolic activity (Jones and Lennon, 2010); see Lennon and Jones, 2011 for a recent review). A substantial fraction of community members may be dormant or inactive at any given moment (e.g., Pedrós-Alío, 2006; Jones and Lennon, 2010). Dormancy strategies may be common among communities living in temporally dynamic environments, promoting overall compositional stability in fluctuating conditions. Furthermore, the proportion of inactive taxa (and, inactive individuals within a taxon) may signify important environmental differences among communities from similar habitats, as shown, for example, in gut microbial communities from humans with and without irritable bowel syndrome (Rehman et al., 2010).

Dormancy has most likely evolved across the tree of life as a means for contending with temporarily variable environments. It is an advantageous strategy under unpredictable conditions because it allows individuals to maximize their long-term, geometric fitness (de Jong et al., 2011). Recent studies suggest that, in a wide range of ecosystems, a substantial fraction of microbial communities may be metabolically inactive (Lennon and Jones, 2011). This observation has important implications for the resistance and resilience of microbial communities. First, on a population-level, dormancy may prevent the extinction of certain taxa from a system. For example, active individuals of *E. coli* succumb when exposed to certain antibiotics. However, the population can ultimately be rescued, not necessarily by the survival of mutants, but rather by subpopulations of dormant persister cells that become reactivated when antibiotic effects are attenuated (Lewis, 2006). Evidence suggests that microorganisms may use dormancy in a variety of other situations as well, for example when challenged by unfavorable temperatures (Whitesides and Oliver, 1997), starvation (del Giorgio and Gasol, 2008), or predator-induced mortality (Pearl et al., 2008). Second, dormancy has the potential to affect the stability of communities and ecosystem processes. Dormant individuals can be long-lived and contribute to seed banks. It is well documented that seed banks can maintain species diversity (Chesson, 2000), and this diversity may directly contribute to stability of microbial communities via niche complementation and/or functional redundancy (Loreau and Hector, 2001; Petchey and Gaston, 2002). Seed banks may also aid in the recovery of microbial communities from severe disturbance events. For example, dormant seeds often contribute to the reestablishment of plant communities following fire, flooding, or wind storms (Bond and Midgley, 2001). In many cases, microbial communities show similar signs of rapid recovery following catastrophic disturbances (Jones et al., 2008; Shade et al., 2012). Although dormancy may contribute to the stability of these communities, this remains to be tested.

We propose that dormancy is more important for maintaining community stability under pulse disturbance scenarios (**Figure 4**), as dormancy would be advantageous in a temporarily disturbed ecosystem, but likely less so in a continuously disturbed ecosystem. The exception to this would be a long-lived seed bank of dormant cells that is sustained beyond the effects of a press disturbance. Also, depending on the specific ecosystem changes caused by a press disturbance, environmental cues for "waking" from dormancy (Epstein, 2009) may be altered or absent if a disturbance is continuous.

## POPULATION PROPERTIES: ADAPTATION, GROWTH RATE, STOCHASTIC EXPRESSION, AND DISPERSAL

### *Evolutionary adaptation*

Microorganisms generally feature rapid growth, high population densities, and high mutation rates and are capable of recombination via lateral gene transfer, which facilitates response to disturbance events (e.g., Lenski and Bennett, 1993). As such, disturbance often can provide selection pressure that drives diversification (Travisano and Rainey, 1998; Cohan, 2002). A number of studies have shown that rapid evolution of population traits can influence the temporal dynamics of microbial communities. For example, microbial predator-prey dynamics are strongly affected by selection (Yoshida et al., 2003), especially for traits that provide defense against predators (Little and Currie, 2008). Also, rapid evolution can counterbalance the top-down effects of a novel predator on nutrient cycling. For example, viruses introduced into continuous cultures of the picocyanobacterium *Synechococcus* dramatically reduced population densities, thereby increasing availability of the growth-limiting resource, phosphorus. However, *Synechococcus* population densities rebounded with the growth of a virus-resistant *Synechococcus* mutant. Host resistance coincided with reduced nutrient availability (Lennon and Martiny, 2008). Together these studies suggest that rapid evolution is an important mechanism of compositional and functional resistance and resilience to certain disturbances, and is particularly important for response to press disturbances (Cohan, 2002). By contrast, there

may be advantages of bet-hedging strategies, such as phenotypic plasticity or dormancy, to contend with disturbance in systems that experience transient pulse disturbances. Adaptability is likely important for microbial community resistance and resilience given repeated pulse disturbances (such as in a disturbance regime, e.g., fire or flooding), as well as press disturbances (**Figure 4**).

#### *Growth rate*

A tradeoff between growth rate and resource use efficiency (and hence competitive ability) may underlie the capacity of microbial populations to respond to disturbance (Stevenson and Schmidt, 2004). Enhanced growth rate is accompanied by a higher rate of synthesis of ribosomal components and is faster in microorganisms with more copies of rRNA-encoding genes, reducing the response time to favorable growth conditions for such organisms. When pulse disturbances are followed by favorable growth conditions, the fastest responders will multiply and alter community composition (Klappenbach et al., 2000), resulting in low resistance. By contrast, microorganisms with fewer copies of rRNA-encoding genes maximize efficiency of resource use (progeny per mole substrate; Lee et al., 2009), and may increase community resistance to press disturbances that result in long-term resource limitation. Genes other than rRNA genes distinguish bacteria optimized to grow at high nutrient (copiotrophic) and low nutrient (oligotrophic) conditions (Lauro et al., 2009). While the identity of rapid responders may be idiosyncratic across environments, quantifying the capacity of a microbial community for rapid growth or efficient resource utilization could inform hypotheses regarding each community's compositional responses to pulse and press disturbances. Growth rate likely is important for microbial community resilience from pulse disturbances, as a few surviving individuals could grow quickly to recover to pre-disturbance population sizes after a sudden pulse, mortality-inducing disturbance, especially if the disturbance makes new resources available.

The relative growth rates of interacting microbial communities also can have implications for microbial resilience and resistance to press disturbances. A recent study compared the drought responses of a fungal-based food web in grassland soil with that of a bacterial-based food web in agricultural soil (de Vries et al., 2012). The results of this study suggested that relatively slower-growing fungi were more resistant to drought, but less resilient, while relatively faster-growing bacteria were less resistant but more resilient. The authors built structural models to assess the impact of fungal and bacterial drought responses on microarthropod (grazers of bacteria and fungi) richness, soil respiration, nitrogen dioxide production, and nitrogen leaching. This study demonstrated that press disturbances that alter microbial food webs may also influence soil resource availability. It also showed that the stability of microbial food webs was contingent, at least in part, on the ratio of slower-growing fungi to faster-growing bacteria.

#### *Stochastic gene expression*

Another means by which a microbial population can respond quickly to environmental change is through stochastic gene expression (Avery, 2006; Raj and van Oudenaarden, 2008), a process that samples multiple phenotypes and hence, like dormancy, is also considered a bet-hedging strategy. The presence of persister cells – dormant variants within a bacterial population that are tolerant to antibiotics (e.g., Lewis, 2010) – is one example of an alternative phenotype that increases fitness in an environment experiencing a transient selective pressure. Stochastic gene expression is among the multiple pathways that can lead to the formation of persisters (Lewis, 2010), a phenomenon that may have many parallels in non-pathogenic microorganisms. Stochastic gene expression may be key for microbial community resistance to pulse disturbances, as it offers a short-term strategy for survival of individuals that can re-populate a community after disturbance.

#### *Dispersal and immigration*

The large population sizes and rapid dispersal abilities (e.g., Finlay, 2002) of microorganisms can play an important role in community recovery after disturbance. Dispersal is a key feature of metacommunity theory, which recognizes communities as collections of interacting local communities linked by the movement of individuals in heterogeneous landscapes (e.g., Leibold et al., 2004; Logue et al., 2011). Disturbances initiate iterations of community re-assembly by killing or inactivating local resident taxa, releasing resources, and creating empty niches. These empty niches can be filled by local taxa (resistant taxa or taxa retrieved from seed banks) or by immigrants that arrive from other localities within a metacommunity.

Colonists dispersed from nearby localities (patches) can "reseed" microbial populations that have become locally extinct after disturbance, thereby facilitating community resilience (**Figure 4**). For example, freshwater bacterial communities disturbed by a pulse salinity increase were both compositionally and functionally resilient because of continuous dispersal of microorganisms from an undisturbed source community (Baho et al., 2012). As another example, protozoan and rotifer population densities were more resilient after a recurring pulse disturbance (replacing 99% of a mesocosm's contents with sterile media) when the disturbed mesocosm was connected to an undisturbed one (Altermatt et al., 2011a) than when the disturbed mesocosm had no refuge.

On the other hand, if disturbances are wide-spread such that they affect entire regions (e.g., climate effects, such as heat waves), dispersal could promote the dissemination of disturbance-tolerant taxa among localities (Eggers et al., 2012), thereby changing the dominant membership of a community and decreasing overall community resilience at both local and regional scales. For example, marine microalgae communities disturbed by a simulated heat wave were sensitive but not resilient because of a shift in community dominance toward a temperature-tolerant species. This species also became prevalent in other patches connected by dispersal, even though the conditions there were less suitable for it (Eggers et al., 2012).

Furthermore, niches opened by disturbance events are subject to stochastic colonization events by dispersed microorganisms. "*Priority effects*" refers to the impact that successful early colonizers may have on community re-assembly after disturbance, which can affect the likelihood of colonization by subsequently dispersed microorganisms (e.g., Shulman et al., 1983). Early post-disturbance colonizers that adapt rapidly to local conditions may

persist long-term, out-competing native community members and impeding community resilience (Urban and De Meester, 2009).

Together these studies and others demonstrate that microbial dispersal, in interaction with biological attributes of local resident populations (plasticity, dormancy, or evolution) and disturbance characteristics, can have important implications for the resilience of microbial communities.

## COMMUNITY PROPERTIES: DIVERSITY, TURNOVER, AND EMERGENT PROPERTIES

### *Diversity in all of its forms*

In general, diversity is thought to influence how communities respond to disturbance. Aspects of alpha diversity, such as species richness and evenness, have been shown to enhance the functional resilience of communities of larger organisms (Allison, 2004; Downing and Leibold, 2010; Van Ruijven and Berendse, 2010), whereas evidence about the impact of richness and evenness on microbial community resilience is mixed (e.g., Griffiths et al., 2000a; Wertz et al., 2007; Wittebolle et al., 2009; van Elsas et al., 2012).

One challenge lies in clarifying the functional and compositional responses to disturbances of diverse communities. The underlying mechanisms behind a positive relationship between taxon diversity and resilience may be related to a buffering effect, called the *Insurance Hypothesis* (Yachi and Loreau, 1999). More genetically diverse communities are more likely to contain taxa with complementary response traits (e.g., Tilman et al., 1997, 2006; Lavorel and Garnier, 2002; Elmqvist et al., 2003) and the ability for rapid compensatory growth after a disturbance (e.g., Flöder et al., 2010), which may promote resilience. Also, rare microbial taxa (potentially below the limit of detection, and therefore not counted in estimates of community diversity) may quickly respond to altered environmental conditions and become abundant. This is exemplified by the case of a rare *Vibrio* species that was below detection in the majority of the time points over a 6-year study in the Western English Channel, but then bloomed to become a prevalent member of the community at one time point (Caporaso et al., 2011). Co-occurrence networks were used to find that the *Vibrio* bloom was correlated to a bloom of a diatom species (Gilbert et al., 2012). Though models are constantly improved for predicting conditions for microbial blooms (Larsen et al., 2012), niche spaces for many environmental microorganisms remain uncharacterized, which further veils the relationship between compositional and functional diversity and microbial community stability.

Here we discuss a few examples (of many in the literature) of the impact of diversity on microbial community stability. In an early experiment to tease apart the relations between diversity and community functions, Griffiths et al. (2000b) examined the impact of soil microbial diversity on functional stability using a range of intensities of soil fumigation followed by a disturbance, either a heat shock (pulse disturbance) or the addition of the heavy metal copper (II) sulfate (press disturbance). The results indicated that microbial production (rate of generation of biomass, here measured as thymidine incorporation) was not affected, but specific functions, such as nitrification, decreased when diversity was lower. The lower-diversity communities were less functionally resistant (measured as grass residue decomposition rate) to the press disturbance and unable to recover, but were sometimes more resistant to the pulse disturbance than the higher-diversity communities. The control community, which was not fumigated and had the highest diversity, was often the most resilient to both pulse and press disturbances.

Building on the Griffiths et al. (2000b) results, the relationship between species richness and stability was investigated for denitrifiers and nitrite oxidizers (Wertz et al., 2007), two specialized functional groups of soil microorganisms important for nitrogen cycling. In this work, microbial abundance (and, as an extension, richness) was altered by inoculating soil microcosms with different dilutions of microbial cells from non-sterile soil. After a period of incubation, the microcosms were subjected to a pulse heating disturbance. Denaturing gel gradient electrophoresis fingerprints of the denitrifiers and the nitrite oxidizers were coupled with measurements of the two processes, and each responded differently to heating. Though both processes were sensitive, denitrification was resilient after 3 months, while nitrite oxidation did not completely recover. The response of richness to heating was variable across dilutions, and richness did not recover to pre-disturbance levels after 3 months. These results suggest that a community may recover in function even if diversity remains altered after disturbance, and that the initial richness may not necessarily impact functional recovery.

A microcosm experiment was performed to specifically examine the role of initial community evenness (equitability of taxa abundances) on functional stability of denitrifiers subjected to a press increase in salinity and temperature (Wittebolle et al., 2009). The authors found that high initial evenness was important for functional stability of microbial communities. However, the communities were not observed after the disturbance ceased to assess resilience of function or evenness. This work demonstrated that aspects of diversity other than species richness can play a role for community functional stability. Given that many environmental microbial communities are characteristically uneven because they contain a large number of rare taxa (sometimes referred to as the "rare biosphere," e.g., Casamayor et al., 2001; Sogin et al., 2006), the implications of small differences in evenness among generally uneven communities may be of interest for further investigation.

A very recent experiment demonstrated the impact of diversity on stability by creating synthetic combinations of soil isolates, rather than confronting the complexity and unknown organisms of the natural soil (van Elsas et al., 2012). With constructed mixtures containing various numbers of random representatives of a collection of cultured isolates, the authors demonstrated a highly significant correlation between species richness of the community and its resistance to invasion by an *E. coli* strain (van Elsas et al., 2012).

Together, these studies and others reveal the complex relationships between microbial community diversity, function, and stability. Multiple aspects of diversity (richness, evenness) can affect microbial functional resistance and resilience, and general and specific community functions may have different overall responses to pulse disturbance. Importantly, these studies suggest that there may not be a "one-size-fits-all" response of microbial diversity and function to disturbance. For diversity-stability relations, more

work must be done to understand system-specific trends before it will be possible to determine which patterns, if any, are general across microbial systems.

### *Compositional turnover*

Community turnover is the replacement and substitution of community members along an environmental gradient or with time (Wilson and Shmida, 1984). Turnover is directed by the growth of populations, and partially determines how quickly a community recovers from a pulse disturbance (**Figure 4**). High-throughput fingerprinting and sequencing tools that enable experiments involving longer time series demonstrate that many microbial communities have clear trajectories. For example, successional patterns have been observed in tree leaf bacterial communities over the growing season (Redford and Fierer, 2009; Redford et al., 2010), in the human gut after antibiotic treatment (Antonopoulos et al., 2009; Dethlefsen and Relman, 2011), and in the infant gut during the first two and a half years of development (Koenig et al., 2011). Also, seasonal trajectories are common in freshwater, marine, and sediment systems (Fuhrman et al., 2006; Christian and Lind, 2007; Shade et al., 2007; Nelson, 2008; Andersson et al., 2009; Crump et al., 2009; Gilbert et al., 2009, 2010). Together these studies provide insight into the temporal scale on which community turnover and disturbance responses may be anticipated in similar systems, but turnover is unknown for many other habitats. Further investigation is needed to describe, quantify, and compare turnover within and across habitats.

Species-time relationships, which quantify the accumulation of new species in a community with time, provide a method to calculate community turnover (White et al., 2006). There is preliminary evidence that species-time relationships of community turnover may be common among microbial communities from very different environments. For example, microbial communities in streams (Portillo et al., 2012), on leaf surfaces (Redford et al., 2010), across a set of newly deglaciated soils (Nemergut et al., 2007), and in bioreactors (Van Der Gast et al., 2008), have exhibited species-time relationships with turnover rates comparable to those of larger organisms. If most microbial communities display a characteristic turnover rate, as observed for communities of larger organisms (White et al., 2006), variation around the expected range of community turnover rates may be a reasonable starting point for comparing microbial community responses to disturbance.

Similarity-decay (Nekola and White, 1999) is a common method for understanding changes in microbial community structure over space (e.g., Horner-Devine et al., 2004; Jones et al., 2012), but has also been applied over time in communities of larger organisms (Korhonen et al., 2010). For temporal similarity-decay, all pairs of community resemblance (*a.k.a.* similarity or distance) are regressed against time (or space) between community observations. The slope of this regression is analogous to a turnover rate, but has additional utility from species-time relationships because the resemblance metric can be chosen to include properties of community composition (e.g., Sørensen), structure (e.g., Bray–Curtis), and phylogenetic representation (e.g., UniFrac distance, based on the underlying calculation of phylogenetic diversity, Faith, 1992). Similarity-decay has been applied to understand temporal dynamics of microbial communities (e.g., Wittebolle et al., 2008; Bürgmann et al., 2011). As one example, similarity-decay was used to compare resilience of lake microbial communities across treatments in an experiment designed to separate the environmental drivers of oxygen and nutrients from the physical process of water column mixing, an important seasonal disturbance to temperate lake bacterial communities (Shade et al., 2011). In this study, similarity-decay relationships were used in part to quantify the relative robustness of the communities to these environmental disturbances, such that the hypolimnion community was found to be the most sensitive to oxygen addition, but also the most resilient. Though similarity-decay clearly is useful as a baseline descriptor of temporal community turnover, this study demonstrated the additional utility of similarity-decay for comparing microbial community resilience when challenged with different disturbances.

### *Emergent properties: microbial communities as networks*

Interactions between community members, including competition and mutualism (Little et al., 2008), are also important in determining community response to disturbances. For example, a mesocosm experiment with protist communities demonstrated that competitive interactions and disturbance characteristics together determined the community disturbance response, where competition between species became increasingly important in driving extinction as the intensity of disturbance was amplified (Violle et al., 2010). This trend was observed despite the fact that the species were chosen for the experiment because they collectively represented a wide range of competitive ability and disturbance tolerance. Thus, the community response was more complex than the sum of the individual species' traits, and instead hinged on interspecific interactions.

As time series analyses of microbial communities become increasingly available, we will be better able to quantify complex interactions among community members. For instance, recently developed statistical tools enable investigation of microbial interactions by studying co-occurrences of microbial taxa through time. Networks can be built from co-occurrences, with nodes representing taxa or operational taxonomic units (OTUs) and connecting edges representing correlation over time (e.g., Ruan et al., 2006). These networks assess effects of disturbance on community dynamics (Montoya et al., 2006). Positive co-occurrence may indicate common preferred environmental conditions or cooperative activities (facilitation and/or syntrophy). Similarly, negative correlations may represent the outcome of competition (i.e., displacement) or negative interactions, such as allelopathy or predation (Fuhrman, 2009). Co-occurrence networks are gaining popularity in microbial ecology (Faust and Raes, 2012), and recently have been applied to observational studies of marine and soil systems (Steele et al., 2011; Barberan et al., 2012; Eiler et al., 2012; Gilbert et al., 2012). Network analysis applied to controlled experiments can provide insight into the associations between community members that are lost, gained, or maintained after a disturbance (Shade et al., 2010). Networks can also be used to discover "core" community members shared across communities from similar habitats (Shade and Handelsman, 2012), serving as a method to identify taxa shared across localities within a larger metacommunity.

Theoretical and empirical work on food webs indicate that the robustness of such networks is affected by several attributes, such

as the strength of links among species (Montoya et al., 2006). In particular, architectural properties of trophic networks affect the relationship between network complexity and stability (May, 1974; Pimm, 1984; McCann et al., 1998; Rozdilsky and Stone, 2001). For example, in mutualistic networks, compositional diversity and connectivity lead to higher resilience, whereas these properties destabilize trophic networks (Thébault and Fontaine, 2010). Microbial communities are probably affected by both mutualistic and trophic interactions, and thus may provide a unique system for exploring the relationship between network attributes and community stability. Though we hypothesize that microbial interactions and the emergent network properties of a microbial community likely contribute to both resistance and resilience to pulse and press disturbances (**Figure 4**), the exact mechanisms underlying this stability often are unknown, and may be challenging to unravel for many species-rich microbial communities that maintain both mutualistic and trophic interactions among their members.

## OUTLOOK: COMMUNITIES AS SYSTEMS OF GENES AND THEIR FUNCTIONS

Microbial ecology is in a unique position in the larger field of ecology. Since the inception of community ecology, studies of the nature of communities at all taxonomic levels have been challenged by common difficulties. Producing a complete census, controlling variables, sampling completeness, and accounting for low abundance members are typical problems that have confronted all community ecologists, including microbial ecologists. However, the advent of meta-omics has provided microbiologists with the tools to address each of these challenges in new ways (Gilbert et al., 2010; Teeling et al., 2012). Portraits of a community's genes, gene expression, and metabolite production can be represented in a single sample, providing insight into system-level stability. Consequently, microbial ecologists are in a position to elucidate global principles in a manner that is not easily available in the broader field of ecology.

For example, time series of 16S rRNA, shotgun metagenomics, meta-transcriptomics, and meta-metabolomics data will allow researchers to quantify the number of functions shared across taxa from the same community, identify the taxa that are expressing genes for those functions at a given time point, and determine the functional output (in terms of number, abundance, and composition of molecules) from those transcripts. Analyzing this suite of information through time and in response to disturbances will provide quantitative insight as to how often and under what scenarios microbial community structure and function are linked, and whether those linkages are relevant for ecosystem processes. Applying this suite of tools to carefully designed disturbance experiments will additionally help to unravel mechanisms of community stability into different habitats. It will also provide key insights into defining ecologically relevant taxonomic and functional units for microorganisms. Thus, with information-rich datasets, precisely collected time series, and thoughtfully designed experiments (Knight et al., 2012), microbial ecologists are poised to test fundamental hypotheses in ecology, and to move forward in predicting stability of microbial communities in the face of novel disturbances.

## ACKNOWLEDGMENTS

We thank the ISME13 2010 roundtable selection committee for providing discussion space for the topic of microbial resilience. Ashley Shade is a Gordon and Betty Moore Foundation Fellow of the Life Sciences Research Foundation. This work was supported in part by the National Science Foundation (IOS-1041557) and the National Institutes of Health (7RC1DK086831-02) grants to Jo Handelsman. Contributions from David H. Huber were supported by USDA-NIFA-AFRI grant no. 2010-38821-21603 and the West Virginia State University Gus R. Douglass Land-Grant Institute. Silke Langenheder thanks the Swedish Research Council Formas for support. Contributions from Thomas M. Schmidt were supported in part by the U.S. National Science Foundation (MCB-0731913) and the Long-term Ecological Research Program. Contributions from Jay T. Lennon were supported in part by the National Research Initiative Grants (2011-67019-30225) from the USDA National Institute of Food and Agriculture and the National Science Foundation (DEB-0842441). Steven D. Allison's contribution was supported by the Office of Science (BER), US Department of Energy, and the NSF Advancing Theory in Biology program. This is KBS contribution #1604.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Fractal_Physiology/10.3389/fphys.2012.00417/abstract

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

*Received: 01 October 2012; paper pending published: 14 October 2012; accepted: 19 November 2012; published online: 19 December 2012.*

*Citation: Shade A, Peter H, Allison SD, Baho DL, Berga M, Bürgmann H, Huber DH, Langenheder S, Lennon JT, Martiny JBH, Matulich KL, Schmidt TM and Handelsman J (2012) Fundamentals of microbial community resistance and resilience. Front. Microbio.* ***3****:417. doi: 10.3389/fmicb.2012.00417*

*This article was submitted to Frontiers in Aquatic Microbiology, a specialty of Frontiers in Microbiology.*

## APPENDIX

### CRITERIA FOR THE LITERATURE ANALYSIS

To understand what is known about microbial community resilience, we conducted a review of the literature. Studies were searched using ISI's Web of Knowledge with the following search strings: perturb∗, fluctuat∗, disturb∗, robust∗, resilien∗, resist∗ AND "community structure OR diversity OR species composition" AND microb∗, bact∗. Studies were considered if they adhered to the following criteria: (1) explicitly addressed communities (including all aspects of diversity). Papers dealing with single species only were not included; (2) contained empirical data, either from observations or experiments. Conceptual or review papers were excluded.

Papers meeting these criteria were categorized as experimental (manipulation of environmental conditions) or observational (documented responses to naturally occurring disturbances). Information was recorded regarding study organisms, habitat, study duration, sampling frequency, community profiling method used, experimental setup, and dispersal limitations, and classification of the disturbance as press (stressor did not return to pre-disturbance conditions) or pulse (stressor ceased to pre-disturbance conditions). Results were summarized as the presence or absence of changes in community composition or function, and whether there was recovery to the pre-disturbance state. If available, we also noted the post-disturbance recovery time (resilience).

The literature search (18th of January 2012) yielded 3,312 unique references. 352 references were obtained after filtering according to the three primary criteria, and useful data could be extracted from 247 publications. Experimental studies included 310 entries in 196 publications, while observational studies included 68 entries in 51 publications. See **Figure A1** for a detailed comparison of disturbance effects on community composition and function in different habitats and press and pulse disturbance broken up by different disturbance types.

Approximately eighty percent of the investigations included measures of microbial function, such as respiration, biomass production, or the activity of extracellular enzymes. The effects of dispersal on resistance and resilience were only rarely explicitly addressed (Altermatt et al., 2011b; Baho et al., 2012), however approximately one third of the studies used open systems or did not exclude dispersal. Study durations varied greatly, ranging from less than 1 day (Cleveland et al., 2007) to decades (Spiegelberger et al., 2006). In contrast to the rather short-term duration of experimental studies (median: 70 days), observational studies focused on long-term effects (median: 645 days). However, in both cases there was a significant negative relationship between experimental duration and sampling frequency (**Figure A2**), reflecting an astonishing congruency of expected effect sizes of resistance and resilience dynamics in microbial communities.

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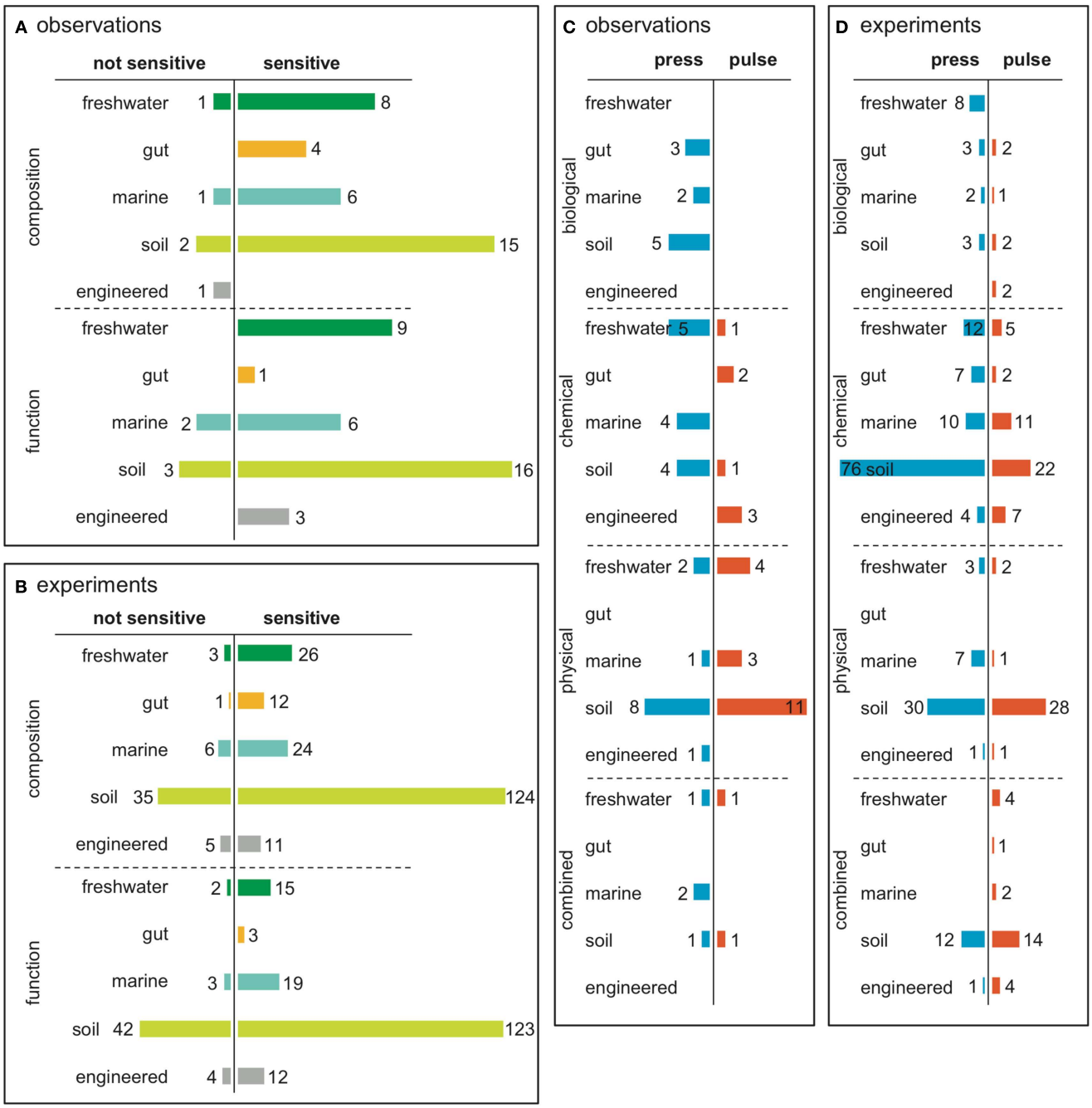


**FIGURE A1 | Summary of the literature survey of microbial community sensitivity to disturbances, grouped by habitat.** There were 378 total investigations gleaned from 247 total studies, as some studies investigated more than one disturbance or measured more than one function. Note that a few studies that considered uncommonly investigated habitats (one wetland, two sediment, six culture-based, and one leaf/detritus) are not shown in this figure. Investigations were classified as either **(A–C)** observations or **(B–D)** experiments. **(A,B)** shows the sensitivity of microbial communities by observations and experiments, and **(C,D)** shows the distribution of different disturbance types between press and pulse disturbance studies. Of the investigations included, 220 investigations reported press disturbances, and 148 were pulses, four did not report press or pulse (NA), and six reported combined press and pulse. Note the lack of observational studies investigating pulsed biological disturbance (e.g., blooms) and the general shortage of work on combined effects of biological, chemical, and physical disturbance.

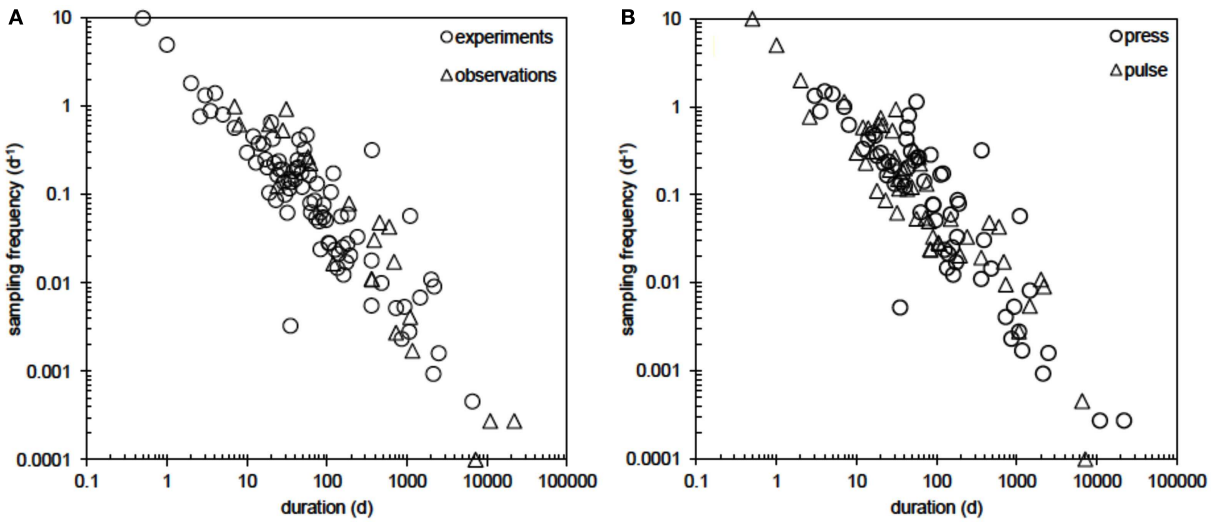


**FIGURE A2 | The impact of sampling frequency and duration on our knowledge of microbial disturbance responses in observational and experimental studies (A) and in studies applying press and pulse disturbances (B).** Experiments apparently well cover the temporal scales of disturbances anticipated by observational studies **(A)** and surprisingly there is no pronounced bias toward short-term studies with a high sampling frequency in pulse disturbance studies **(B)**. However, the relationship between study duration and sampling frequency **(A,B)** indicates logistic and/or conceptual constraints, which may limit our ability to predict microbial community responses to disturbance. "d" is days.