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Microbial Activity, Growth, and Mortality in Environmental Assemblages:  
Population and Community Response to Rewetting of a Dry Mediterranean Soil  
and Anaerobic Methane Cycling in Tropical and Boreal Soils

By

Steven Joseph Blazewicz

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Environmental Science, Policy, and Management

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Mary K. Firestone, Chair

Professor John D. Coates

Assistant Adjunct Professor Eoin L. Brodie

Fall 2012



## Abstract

### Microbial Activity, Growth, and Mortality in Environmental Assemblages: Population and Community Response to Rewetting of a Dry Mediterranean Soil and Anaerobic Methane Cycling in Tropical and Boreal Soils

by

Steven Joseph Blazewicz

Doctor of Philosophy in Environmental Science, Policy, and Management

University of California, Berkeley

Professor Mary K. Firestone, Chair

Microbial activities are key to our planet's habitability and sustainability since they play essential roles in shaping and controlling virtually every natural system including our atmosphere, oceans, soils, and every plant and animal. Microbes exist in different metabolic states in these systems: growing, active, dormant, and recently deceased. These metabolic states correspond to different degrees of influence that a microbe can have on its environment. Therefore, to understand the relationships between microbial community patterns and ecosystem functions, it is important to accurately associate microbial identity with concurrent metabolic state. Through my research I strive to improve our understanding of microbial population, community, and process dynamics in soils and how to apply this information to better predict changes in ecosystem function. I applied a combination of community molecular analyses, chemical and physical characterizations, and process measurements to explore microbial mechanisms, interactions, and responses to changes in their environment.

Nucleic acid analysis has proven to be an effective avenue for characterizing the phylogenetic, taxonomic, and functional structure of microbial assemblages, but this approach has limitations when attempting to assess current metabolic state. Ribosomal RNA genes (rRNA genes) have frequently been used to identify microorganisms present in environmental samples regardless of metabolic state, while ribosomal RNA (rRNA) has been widely applied to identify the active fraction of microbes. Chapter 1 re-evaluates utilizing rRNA as an indicator of microbial activity in environmental samples. A growing body of evidence indicates that the general use of rRNA as an indicator of metabolic state in microbial assemblages has serious limitations. This chapter highlights the complex and often contradictory relationships between rRNA, growth, and activity. Potential mechanisms for confounding rRNA patterns are discussed,

including differences in life histories, life strategies, and non-growth activities. Ways in which rRNA data can be used for meaningful characterization of microbial assemblages are presented.

Chapter 2 presents direct measurements of growth, mortality, and survival for bacteria and fungi following the rewetting of dry soil. The rapid stimulation of microbial activity that occurs when a dry soil is rewetted has been well documented and is of great interest due to implications of changing precipitation patterns on soil C dynamics. Many studies have characterized net changes in microbial populations, but gross population dynamics for bacteria and fungi following wet-up are not well understood. Here, DNA stable isotope probing with  $H_2^{18}O$  was coupled with quantitative PCR to characterize new growth, survival, and mortality for bacteria and fungi following the rewetting of a seasonally dried California annual grassland soil. This study documents both net and gross changes that bacterial and fungal populations underwent over the course of 7 days after wet-up. A pulse of non-growth activity appears to immediately follow wet-up followed by linear growth for both bacteria and fungi. Mortality dynamics indicate that dead microbial bodies provide a large pool of available C and nutrients, thus offering insight into possible C sources fueling the  $CO_2$  pulse following wet-up. Results reveal that a vibrant assemblage of growing and dying organisms may comprise a seemingly static microbial community following a change in the environment.

The bacterial growth stimulated by the rewetting of dry soil was further characterized using high throughput sequencing of 16S rRNA genes, and results are presented in Chapter 3. Of the 25 different phyla present in the pre-wet community, members of the Firmicutes Bacillales order were the only detectable early responders with close to a 5% increase in relative abundance from growth in the first 3 h after wet up. The second group of growers detected at 24 h included only Betaproteobacteria and Bacteroidetes. Members of the Burkholderiales order in the Betaproteobacteria phylum were by far the dominant growers during this period with a 21% increase in relative abundance. The highest richness of growing bacteria was detected during the third time-period (between 24-72 h), with significant changes in relative abundance due to growth found in 11 phyla. Nonmetric multidimensional ordination of community composition data through time shows a somewhat cyclical pattern for phylogenetic composition of growing bacteria with composition at 3 hours differing slightly from the pre-wet community, differing greatly at 24 h, and then becoming progressively more similar to the pre-wet community at 72 and 168 h. This suggests a degree of community resilience in response to this abrupt environmental change. However, some net compositional changes were observed following wet-up. Actinobacteria were the most dominant pre-wet phylum, but Proteobacteria became the most dominant phylum by 168 h. This change in composition was likely driven by new growth since Proteobacteria

were found to grow in abundance for most of the incubation while Actinobacteria only grew during two later time periods and with smaller increases in relative abundance. Sequential growth patterns found at the phylum and order level suggest that ecologically coherent response was observable at a high taxonomic level.

Chapter 4 shifts emphasis to anaerobic oxidation of methane (AOM) in Tropical and Boreal soils. AOM is a considerable sink for the greenhouse gas methane (CH<sub>4</sub>) in marine systems, but the importance of this process in terrestrial systems is less clear. Lowland boreal soils and wet tropical soils are two hotspots for CH<sub>4</sub> cycling, yet AOM has been essentially uncharacterized in these systems. We investigated AOM in soils from sites in Alaska and Puerto Rico. Isotope tracers were utilized *in vitro* to enable the simultaneous quantification of CH<sub>4</sub> production and consumption without use of biological inhibitors. Boreal peat soil and tropical mineral soil oxidized small but significant quantities of CH<sub>4</sub> to CO<sub>2</sub> under anoxic conditions. Potential AOM rates were  $21 \pm 2 \text{ nmol g}_{\text{dw}}^{-1} \text{ d}^{-1}$  and  $2.9 \pm 0.5 \text{ nmol g}_{\text{dw}}^{-1} \text{ d}^{-1}$  for the boreal and tropical soils, respectively. The addition of terminal electron acceptors (NO<sub>3</sub><sup>-</sup>, Fe(III), and SO<sub>4</sub><sup>2-</sup>) inhibited AOM and methanogenesis in both soils. In all incubations, CH<sub>4</sub> production occurred simultaneously with AOM, and CH<sub>4</sub> production rates were always greater than AOM rates. There was a strong correlation between the quantity of CH<sub>4</sub> produced and the amount of CH<sub>4</sub> oxidized under anoxic conditions. CH<sub>4</sub> oxidation under anoxic conditions was biological and likely mediated by methanogenic archaea. While only a small percentage of the total CH<sub>4</sub> produced in these soils was oxidized under anoxic conditions (0.3% and 0.8% for Alaskan and Puerto Rican soils), this process is important to understand since it could play a measurable role in controlling net CH<sub>4</sub> flux.

*This dissertation is dedicated in love and gratitude  
to my closest friend, Amy Scher.  
On to our next adventure...*

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If someone asked me a decade ago whether or not I would ever pursue a doctoral degree, I would have likely just laughed it off. Working as a mechanic in a small mountain town without even a bachelor's degree, this thought wasn't even a seed in my mind at the time. But life has a way of taking strange and unexpected twists and turns. In my previous (pre-graduate school) life, I loved much about my daily existence, my home, and the beautiful Arizona wilderness that I shared with my Wife, Amy. But, once I was awakened to the microbial life that pervades our planet, it was clear that I needed to know more. Much sacrifice was necessary to move to a new town for graduate school – selling our home, saying goodbye to our close friends, moving into a tiny, moldy, leaky basement apartment because this was all we could afford in Berkeley. I believe this whole process has been hardest for Amy. In addition to leaving everything she knew and loved, she was plopped in a town virtually all alone, due to my new extensive work hours, while having to weather my neurotic tendencies that became periodically exaggerated due to the pressures associated with a rigorous graduate program. Yet, throughout this whole process she has always supported me and encouraged me to follow my heart. I know that without her support I would not have completed this journey. I will never be able to put my appreciation into words, but I want to let you know that I understand how much you have given to me. Thank you.

*“Truth be told, the planet’s most victorious organisms have always been microscopic. In all the encounters between Davids and Goliaths, was there ever a time when a Goliath won?”*

- Leon Trotsky Trout

# 1

## **Re-evaluating rRNA as an Indicator of Environmental Microbial Activity: Limitations and Uses**

### **Abstract**

Microbes exist in a range of metabolic states (e.g. dormant, active, growing) and analysis of ribosomal RNA (rRNA) is frequently employed to characterize the “active” fraction of microbes in environmental samples. However, a growing body of evidence indicates that the general use of rRNA as an indicator of metabolic state in microbial assemblages has serious limitations. This report highlights the complex and often contradictory relationships between rRNA, growth, and activity. Potential mechanisms for confounding rRNA patterns are discussed, including differences in life histories, life strategies, and non-growth activities. Ways in which rRNA data can be used for meaningful characterization of microbial assemblages are presented, along with questions to be addressed in future studies.



## Introduction

Microorganisms play essential roles in shaping and controlling virtually all ecosystems including the atmosphere, oceans, soils, and plant- and animal-associated biomes. Microbes exist in different metabolic states in these systems: *growing*, *active*, *dormant*, and recently *deceased* (Figure 1-1). These metabolic states correspond to different degrees of influence that a microbe can have on its environment. Therefore, to understand the relationships between microbial community patterns and ecosystem functions, it is important to accurately associate microbial identity with concurrent metabolic state. Simultaneous identification of microbes and their metabolic states has been a longstanding goal in microbial ecology and methods to achieve this have recently been accumulating in our molecular toolboxes.

Nucleic acid analysis has proven to be an effective avenue for characterizing the phylogenetic, taxonomic, and functional structure of microbial assemblages, but this approach has limitations when attempting to assess current metabolic state. Ribosomal RNA genes (rRNA genes) have frequently been used to identify microorganisms present in environmental samples regardless of metabolic state, while ribosomal RNA (rRNA) has been widely applied to identify the active fraction of microbes. In our literature survey, we found more than 100 studies that used rRNA for this purpose (e.g. (DeAngelis et al 2010, Duineveld et al 2001, Egert et al 2011, Gentile et al 2006, Jones and Lennon 2010, Mannisto et al 2012, Mills et al 2005, Muttray and Mohn 2000, Schippers et al 2005, Wüst et al 2011)). However, conflicting patterns between rRNA content and growth rate indicate that the assumption that rRNA is a reliable metric for growth or growth-related activity could be incorrect and in some cases grossly misleading. We recognize that virtually all molecular characterization methods are imperfect, but we believe that there is now enough information published regarding rRNA to warrant a critical reevaluation of the assumption that rRNA is directly and quantitatively linked to activity in microbial assemblages. Using rRNA to evaluate microbial assemblages requires that limitations and assumptions be clearly identified and understood. This article first explores critical limitations and potential causes of inconsistencies in rRNA/growth relationships. Second, it recommends applying rRNA abundance as an activity potential and proposes perspectives for future applications within this framework. The methodological aspects of rRNA extraction and characterization are continuously being examined and improved, and will not be included here. The present article will focus on bacteria, which have been extensively studied, but it is important to acknowledge that many of the limitations discussed likely affect rRNA measurements for other microbes including archaea, fungi, and algae.

## **Ribosomal RNA and its use in microbial ecology**

RNA exists in different forms within a cell, but the total RNA pool is mainly composed of rRNA (82-90%) (Neidhardt and Magasanik 1960, Neidhardt 1987, Tissieres and Watson 1958). As an integral structural component of ribosomes, rRNA is a fundamental constituent of all known microorganisms. rRNA is mostly ribosome-associated, but also exists as free rRNA in a cell (Lindahl 1975, Nomura et al 1984). Total RNA concentration is generally proportional to rRNA concentration and to the number of ribosomes in the cell, and has often been employed as a proxy for both (Bremer and Dennis 1996, Kerkhof and Ward 1993, Poulsen et al 1993). In pure culture experiments, counting cells can be used to determine the average concentration of ribosomes per cell. In mixed communities, other methods of normalization are usually necessary. Commonly, rRNA is normalized to the number of cells by using rRNA genes to calculate an rRNA:rRNA gene ratio (e.g. (Dortch et al 1983, Kemp et al 1993, Kerkhof and Ward 1993, Poulsen et al 1993)), since rRNA gene concentration per cell is generally assumed to be more stable than rRNA concentration; note however, that genome content per cell can vary significantly (Cooper and Helmstetter 1968, Schaechter et al 1958, Sukenik et al 2011), but this issue will not be addressed here.

Microbial ecology studies commonly use rRNA analyses to either quantify a population's growth rate in a mixed community (e.g. (Muttray et al 2001, Poulsen et al 1993)) or identify active populations within an assemblage (e.g. (Felske et al 1998, Mattila et al 2012)). Two principal lines of evidence are invoked to justify these applications. First, total RNA and rRNA content have been found to correlate well with microbial growth rate for a handful of microbes in pure culture, over a wide range of growth rates under balanced growth conditions (i.e. growing in an unchanging environment) (Bremer and Dennis 1996, Kemp et al 1993, Kerkhof and Ward 1993, Koch 1970, Neidhardt and Magasanik 1960, Poulsen et al 1993, Ramos et al 2000, Rosset et al 1966, Schaechter et al 1958, Wagner 1994). Second, decreased rRNA content is associated with decreased growth rate in some organisms growing under specific nutrient-limiting conditions (Davis et al 1986, Kramer and Singleton 1992, Mandelstam and Halvorson 1960, Tolker-Nielsen et al 1997). It is important to note that the relationship between rRNA concentration and growth rate is frequently coupled with an assumption that growth and activity are synonymous. Here we distinguish between growth and activity; while all growing organisms can be considered active, not all active organisms are measurably growing (Figure 1-1).

Experimental evidence has demonstrated numerous limitations of rRNA-based analyses, many of which have been addressed previously in molecular method reviews (e.g. (Molin and Givskov 1999)) or in brief statements in discussion sections of research articles (e.g. (Griffiths et al 2003)). However, these limitations are frequently overlooked or ignored in practice. Here we provide a

concise list of limitations (Box 1-1) and discuss relevant examples to assess what information rRNA data can actually provide.

**Box 1-1: Limitations of rRNA as Activity Metric**

1. **Concentration of rRNA and growth rate are not always simply correlated** (Binder and Liu 1998, Flårdh et al 1992, Kemp et al 1993, Kerkhof and Kemp 1999, Lepp and Schmidt 1998, Mandelstam and Halvorson 1960, McKillip et al 1998, Morgenroth et al 2000, Oda et al 2000, Schaechter et al 1958, Schmid et al 2001, Tolker-Nielsen et al 1997, Worden and Binder 2003).
2. **The relationship between the concentration of rRNA and growth rate can differ significantly among taxa** (Binnerup et al 2001, Kemp et al 1993, Mandelstam and Halvorson 1960, Oda et al 2000, Pang and Winkler 1994, Rosset et al 1966, Wade and Robinson 1965, Worden and Binder 2003).
3. **Dormant cells can contain high numbers of ribosomes** (Bishop and Doi 1966, Chaloupecky 1964, Chambon et al 1968, Filion et al 2009, Sukenik et al 2011).
4. **The relationship between non-growth activities and concentration of rRNA has not been investigated.**

**Critical analysis of rRNA as a metric for growth and activity**

*Concentration of rRNA and growth rate are not always simply correlated*

The first line of evidence that supports a predictable relationship between rRNA concentration and growth rate is based on single-strain studies under balanced growth conditions. However, even under these constrained conditions the correlation between growth rate and rRNA concentration is commonly not straightforward and in some cases can break down altogether. For example, the relationship between growth rate and rRNA content is not always linear or consistent across all growth rates measured. Under balanced growth conditions, *Synechococcus* and *Prochlorococcus* strains can have a three-phase relationship between growth and rRNA concentration: 1) rRNA concentration remains constant for a range of low growth rates, 2) rRNA concentration increases linearly with growth rate over an intermediate growth rate range, and 3) at higher growth rates the relationship reverses, with rRNA content decreasing as growth rate increases (Binder and Liu 1998, Worden and Binder 2003). Obviously, rRNA concentration will not be a robust proxy for growth rate, and assuming a positive linear correlation between rRNA concentration and growth rate may lead to false conclusions. Additionally, balanced growth conditions are unlikely in most environments. Little work has characterized how rRNA concentration varies with growth rate under more environmentally realistic non-

steady state conditions. In one study, three different patterns were observed for the relationship between rRNA concentration and growth rate for strains of Proteobacteria under non-steady state conditions (Kerkhof and Kemp 1999): 1) a direct linear relationship, 2) an indirect relationship where cell growth rate is consistently lagging behind rRNA concentration, or 3) no discernible relationship. For the strain with no obvious relationship (*Vibrio fischeri*), there were clearly periods during which growth rate decreased while rRNA content increased. The assumption of a positive correlation between rRNA concentration and growth rate would lead to the conclusion that growth rate had increased, when in reality it had decreased. Further evidence showing potential for misleading environmental interpretations includes the significant increase in cellular rRNA in *Aphanizomenon ovalisporum* cells as they change from a vegetative state to a dormant state (Sukenik et al 2011). These results indicate that for rRNA quantification to provide useful information about changes in growth rate, the relationship between rRNA and growth rate needs to be well characterized for the strains of interest under relevant/appropriate experimental conditions (i.e. balanced or non-steady state). The resistance of many, if not most, microorganisms to pure culture cultivation is a serious limitation to this approach.

A second line of evidence often cited as support for rRNA as an indicator of growth or activity arises from studies on RNA stability under different growth-limiting conditions (e.g. carbon or nutrient limitation). Several studies reported that exponentially growing cells subjected to starvation of a nutrient could degrade much of their rRNA in a relatively short time. However, the dynamics of cellular rRNA may be more strongly related to previous growth conditions. In one example, *Azotobacter agilis* was grown on different substrates and then starved for 72 hours (Sobek et al 1966). When originally grown on glucose, RNA did not decrease during the starvation period but O<sub>2</sub> consumption dramatically dropped, indicating that cell activity declined. In another example, *Rhodospseudomonas palustris* cells were grown at different growth rates and then carbon-starved (Oda et al 2000). The rRNA concentration of *R. palustris* cells grown at maximum growth rate decreased by approximately 50% within a week of starvation; however, cells that had lower growth rates before the starvation period were able to maintain close to pre-starvation rRNA concentration for more than a week of starvation. These results indicate that rRNA detectability can be influenced not only by current conditions, but also by recent history.

*The relationship between rRNA concentration and growth rate can differ significantly among taxa*

Relating rRNA concentration and growth rate becomes even more difficult when considering microbial assemblages. rRNA concentration may correlate well with growth rate in some strains of bacteria, but correlations can differ significantly between strains (Binnerup et al 2001, Kemp et al 1993, Pang and Winkler 1994,

Wade and Robinson 1965, Worden and Binder 2003). Hence, using rRNA to compare the relative growth rate of different taxa is unlikely to provide useful information. Further, even at the “species” level of bacteria, the relationship between rRNA and growth rate can differ significantly between subpopulations (Licht et al 1999, Rosset et al 1966).

#### *Dormant cells can contain high numbers of ribosomes*

Another major limitation is that dormant organisms are known to contain measurable amounts of rRNA (Chambon et al 1968) and in some cases can contain significantly more rRNA in the dormant state than in the vegetative state (Sukenik et al 2011). Detectability of rRNA in dormant cells can be more affected by methodology (due to changes in cell structure) than by low levels of rRNA (Filion et al 2009). The issue of dormant cells containing measurable quantities of rRNA can be especially problematic when applying rRNA data to identify active organisms in environments likely to contain many dormant organisms such as soil, deep subsurface, or the atmosphere. For example, Mediterranean grasslands are characterized by long hot dry summers, at the end of which there is almost no measurable soil microbial respiration. Yet, during this dry period, the amount of total extractable soil rRNA is equivalent to that found in the soil after the first wet-up event when respiration is dramatically stimulated (Placella et al 2012).

#### *The relationship between non-growth activities and concentration of rRNA has not been investigated*

A final point to consider is that the relationship between rRNA concentration and growth rate is frequently considered equivalent to that between rRNA and activity. However, many important microbial activities are not necessarily associated with growth, such as cell motility, osmoregulation, defense against oxidative stress, communication, exopolysaccharide production, or conjugation (van Bodegom 2007). To our knowledge, no published work has investigated the relationship between non-growth activities and rRNA concentration. It has been hypothesized that under certain conditions of stress, microbes can dramatically increase the portion of metabolism geared towards non-growth maintenance activities (Schimel et al 2007), indicating that under appropriate conditions non-growth activities could contribute significantly to ecosystem processes.

#### **Relationship between rRNA, growth and activity: physiological links**

The multiple levels of modulation and regulation of most cell functions may easily invalidate simple correlations between metabolic state and rRNA abundance. For

example, the relationship between microbial activity and measurable rRNA can be influenced by heterogeneity of cell physiology within a population (Licht et al 1999), changes in the ratio of non-growth metabolic activity to growth-specific activity, life history (Oda et al 2000), life strategy (Flärdh et al 1992, Lepp and Schmidt 1998, Mitchell et al 2009, Sukenik et al 2011), environmental heterogeneity, changing environmental conditions, and of course fundamental enzyme kinetics. Additionally, it is useful to recognize that the concentration of rRNA in a cell at a given point in time is the net result of rRNA synthesis (i.e. transcription) and degradation rates (Gausing 1977), each of which may be under distinct controls. All of these factors can affect the relationship between ribosome cycling and microbial activity at multiple levels (Figure 1-2) and should be considered when utilizing rRNA data for analysis of environmental samples.

### **rRNA analyses in community ecology**

In light of the limitations and relationships discussed, we suggest that it may be most useful to consider rRNA abundance as an indicator of potential protein synthesis activity. The distinction between actual activity and potential activity is critical when attempting to identify and characterize the dynamics of organisms that drive ecosystem functions (Figure 1-1). The concept of potential protein synthesis activity of microbial populations is analogous to that of potential enzyme activities. Enzyme activities measured *in vitro* will generally not represent actual rates *in vivo*, since many factors can prevent the enzymatic process *in vivo* from resembling that under the ideal conditions of the assay (Nannipieri et al 2002). Thus, process rates determined from enzyme potential activity assays are considered as potential process rates, not as actual rates. Although ribosomes are more complex than most enzymes, they do function as enzymes to mediate protein synthesis. Hence, the number of ribosomes present at a given time provides a control of the maximum protein synthesis activity for a population (Figure 1-2). Thus, instead of directly measuring protein synthesis, the number of ribosomes present is measured. However, since the rate at which ribosomes operate is highly regulated, the abundance of rRNA is indicative of a protein synthesis *potential*, not of a realized rate of protein synthesis. Note that unlike an enzyme activity assay, rRNA analysis enables simultaneous taxonomic and phylogenetic characterization in addition to quantification, providing an extremely valuable link between identity and protein synthesis potential. In addition, rRNA may offer greater sensitivity of detection compared to rRNA gene, since a cell generally contains thousands of ribosomes whereas rRNA genes are usually found in the single digits.

## Applications in microbial ecology: future directions

What does measuring 'protein synthesis potential' tell us about microbial populations? The connection between the number of ribosomes and the ability to synthesize proteins links the quantity of rRNA found in a population with its growth potential and acclimation potential (i.e. its potential to up-regulate or change currently expressed metabolic functions). However, there may be measurable rRNA present that is not associated with viable cells and cannot contribute to a population's acclimation potential or to ecosystem processes. We previously discussed how rRNA data will also reflect historical activity and conditions. It is furthermore possible for rRNA to represent potential future activity: for example, some microorganisms have a life strategy of increasing ribosome concentration as they enter a dormant state, apparently to give them a growth advantage when environmental conditions improve and they return to a vegetative state (Sukenik et al 2011). Similarly, non-dormant populations that maintain ribosome levels above current protein synthesis demands likely maintain the ability to rapidly shift metabolic functions to adapt to changing conditions, thereby becoming better competitors (Alton and Koch 1974, Flårdh et al 1992, Koch 1971).

Recognizing that rRNA concentration reflects past, current, and future activities and different life strategies restricts its usefulness as a metric of real-time activity, but provides the basis for generating and testing important hypotheses. For example, there is evidence that microbes are capable of recognizing (adapting to) repeated temporal patterns of changing environmental conditions and can consequently prepare for future environmental changes (Tagkopoulos et al 2008). Microbes in ecosystems that experience cyclic environmental conditions may develop an anticipatory life strategy, enduring one phase of the cycle while preparing for a more favorable phase that regularly follows. By accumulating or maintaining rRNA during periods of low metabolic activity, microbes may gain a competitive advantage during a favorable phase of the cycle. For example, over summer dry-down in Mediterranean soils, changes in the relative abundance of rRNA in the dominant bacterial taxa reflected contrasting drought-related bacterial strategies (Barnard et al 2012). During the dry period, there was almost no measurable microbial activity yet total extractable soil rRNA was found in quantities similar to those after the microbes become activated by the first wet-up event (Placella et al 2012), which could reflect an anticipatory strategy for the upcoming annual rainy season. Such anticipatory or predictive rRNA regulation has been documented in microbes that undergo regular diurnal cycles. In incubations with light and dark diurnal cycles, the rRNA content of *Synechococcus* sp. increased during dark periods compared to light periods; in contrast, growth occurred during the light periods and ceased during the dark periods (Lepp and Schmidt 1998). It is possible that these organisms built up rRNA at the end of the dark period in preparation for the anticipated light period. Similar results were found for a strain of *Prochlorococcus* in which expression of

ribosomal genes was higher during a dark cycle than during a light cycle (Zinser et al 2009). Further evidence for anticipatory behavior in bacteria is found in *E. coli*, which can manifest a Pavlovian-type response to a primary stimulus by preemptively modifying genetic expression for a secondary stimulus before it occurs (Mitchell et al 2009, Tagkopoulos et al 2008). If anticipatory life strategies are common in microbial populations experiencing repeated cyclic patterns, then can this information be usefully applied to predict future changes in ecosystem function?

In order to utilize rRNA data to characterize microbial assemblages, we need to better our understanding of how these data relate to environmental conditions and community interactions; this understanding could be furthered by several experimental approaches:

- a) Coupling direct measurements of metabolic activity to rRNA data.
- b) Explicit testing of the relationship between non-growth activities and rRNA concentrations.
- c) Measuring ribosome turnover (for both viable and non-viable cells) under different environmental conditions.
- d) Fine scale analysis of the heterogeneity of rRNA content at spatial scales meaningful for microbes.

## **Conclusion**

A number of pure-culture studies have shown a positive correlation between growth rate and rRNA concentration. This relationship makes intuitive and biological sense, since rRNA is a critical component of ribosomes and ribosomes are necessary to synthesize protein. However, the correlation between activity and rRNA in environmental samples is inconsistent due to differences in life histories, life strategies, and non-growth activities. Using rRNA analysis as a general indicator of the active microbes in environmental samples is not valid under many circumstances, and may in fact hinder progress in understanding metabolic states in microbial assemblages. Viewing rRNA measurements as indicators of protein synthesis potential provides microbial ecologists with a more robust framework within which to view rRNA measurements, facilitating a more prudent yet comprehensive understanding of the complex dynamics operating in microbial communities.



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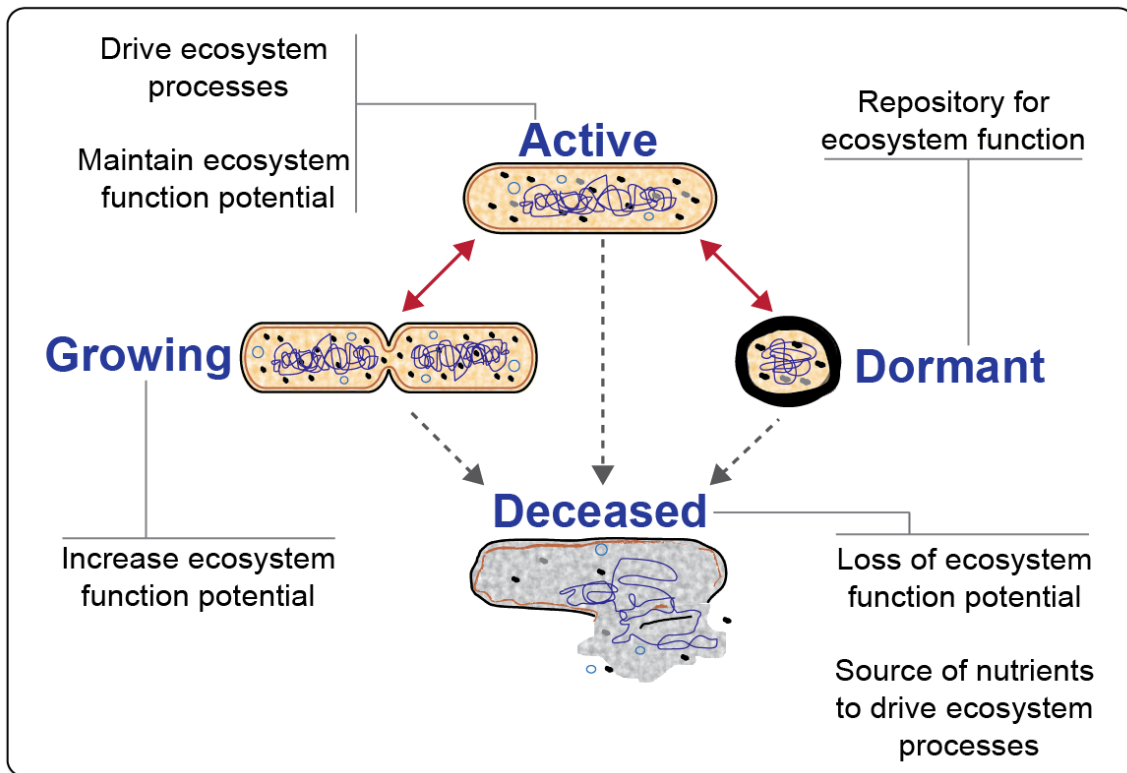
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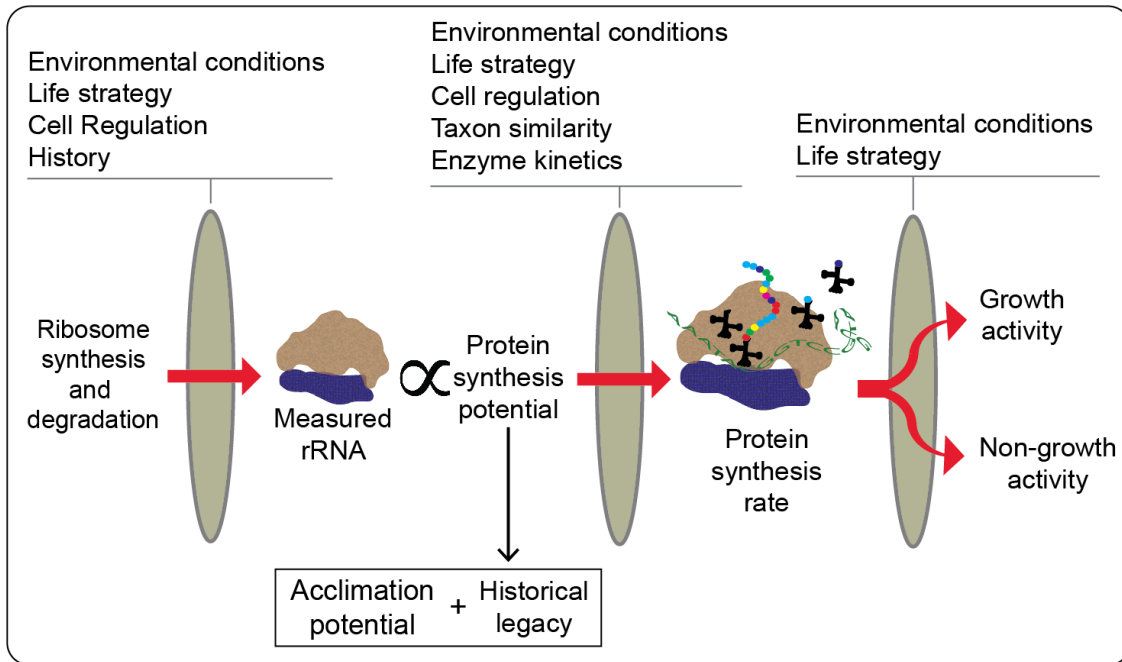
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**Figure 1-1.** Microorganism metabolic states and their contribution to ecosystem functioning. Viable microorganisms exist in one of three general metabolic states that are all subject to mortality. Definitions of terms: *Growing* – cells are actively dividing, *Active* – cells are measurably metabolizing (catabolic and/or anabolic processes) but are not necessarily dividing, *Dormant* – cells are not measurably dividing or metabolizing, *Deceased* – cells are not metabolically active or capable of becoming metabolically active in the future, but intact macromolecules may persist.





**Figure 1-2.** From ribosome cycling to microbial activity: Factors that affect ribosome quantities and their relationship to microbial activities.

# 2

## **Growth, Mortality, and Survival of Bacteria and Fungi Following Wet-up of Dry Soil Revealed by H<sub>2</sub><sup>18</sup>O Stable Isotope Probing**

### **Abstract**

The rapid stimulation of microbial activity that occurs when a dry soil is rewetted has been well documented and is of great interest due to implications of changing precipitation patterns on soil C dynamics. Many studies have characterized net changes in microbial populations, but gross population dynamics for bacteria and fungi following wet-up are not well understood. Here we applied DNA stable isotope probing with H<sub>2</sub><sup>18</sup>O coupled with quantitative PCR to characterize new growth, survival, and mortality for bacteria and fungi following the rewetting of a seasonally dried California annual grassland soil. Microbial activity, as determined by CO<sub>2</sub> production, increased significantly within 3 h of wet-up, yet new growth was not detected until 24 h, suggesting a pulse of non-growth activity immediately following wet-up likely due to resuscitation from dormancy and cellular response requirements associated with the rapid change in water potential. Net microbial abundance revealed little change throughout the 7 d post-wet incubation, but there was substantial turnover of both bacterial and fungal populations (49 and 52% respectively). New growth was linear between 24 and 168 h for both bacteria and fungi with average growth rates of  $2.3 \times 10^8$  bacterial 16S gene copies  $g_{dw}^{-1} h^{-1}$  and  $4.3 \times 10^7$  fungal ITS copies  $g_{dw}^{-1} h^{-1}$ . While bacteria and fungi differed in their mortality and survival characteristics during the 7-day incubation, mortality that occurred within the first 3 h was similar with 25 and 27% of the respective pre-wet cohorts disappearing. The high mortality that was evident at 3 h after wet up indicates that cell death, occurring either during the extreme dry down or during the rapid change in water-potential due to wet-up, generates a significant pool of available C that likely contributes to the large pulse in CO<sub>2</sub> associated with wet-up. Interestingly, the new growth and CO<sub>2</sub> production were tightly associated following wet-up. Bacterial and fungal populations underwent dynamic changes following wet-up yet the balance between mortality and growth resulted in relatively stable net population abundances even after such a profound and sudden change in environment.

## Introduction

It is well documented that when a dry soil is rewetted, a pulse of microbial activity, respiration, and mineralization results (Birch 1958, Bloem et al 1992, Kieft et al 1987, Lund and Goksoyr 1980, Rudaz et al 1991, Waksman and Starkey 1923), but the dynamics of microbial mortality and growth associated with such an event has received little attention. There has been a great deal of interest in wet-up stimulated microbial activity in the last decade due to the importance of trace gas fluxes resulting from such events and due to predicted shifts in rainfall patterns with potentially associated changes in soil C dynamics. In Mediterranean-type ecosystems, the CO<sub>2</sub> pulse associated with wet-up can be large enough to be measurable at the field scale (Xu et al 2004), with up to 10% of annual net ecosystem production released in the pulse following a single rain event (Lee et al 2004). This CO<sub>2</sub> pulse is associated with increased microbial activity and degradation of organic carbon and is especially important in arid and semiarid ecosystems such as Mediterranean environments, where soils can become extremely dry between infrequent rainfall events. Despite the observed rapid changes in microbial activity and associated soil C mineralization, little is known about the gross population dynamics that underscore these ecosystem characteristics.

Knowledge of population dynamics such as survivorship, fecundity, and mortality within a community is fundamental to understanding the relationship between community characteristics and ecosystem function and has long been studied for humans and other multicellular animals (Harcourt 1969, Malthus 1798, Pearl and Parker 1921). While population dynamics are important in microbial communities, several of these basic characteristics are generally not considered in environmental studies. This is likely due to difficulties associated with characterizing gross population dynamics such as new growth, mortality, and survivorship for microbes living in heterogeneous matrices such as soil. In terms of ribosomal gene or biomass abundance, microbial communities in soils appear to generally be fairly stable. While we know that the populations comprising a total community can change over time, it requires relatively sensitive molecular methods to follow the changes in community membership over periods of hours, days, or weeks. After a disturbance event such as the wet-up of dry soils, microbial activity can be stimulated but net changes in community composition can be very small (Fierer et al 2003, Placella et al 2012). Does the stability of gene copies and biomass over time indicate that cell division and cell mortality does not happen rapidly in soil? How dynamic are soil communities in terms of mortality and new growth? Few studies have moved beyond investigating net population or community dynamics; tracking changes in DNA or biomass does not allow for the identification or quantification of organisms that survived, died, or recently grew. The small number of studies that have directly measured bacterial and fungal growth rate in soils using uptake of labeled substrate reveal that growth rates can be fairly rapid (Bååth 1990, Bååth 1998, Harris and Paul

1994, Iovieno and Bååth 2008, Rousk and Bååth 2011). This suggests that concurrent growth, survival, and mortality all contribute significantly to microbial population and community stability, perhaps in a balanced manner so as to maintain microbial abundances.

Life tables are often utilized in macroecology to organize population information such as survivorship, growth, and mortality (Carey et al 2012, Harcourt 1969, Pearl and Parker 1921). In dynamic life tables, information will be provided for a cohort over time. Dynamic life tables have yet to be applied to microbial populations, but could provide useful information about microbial dynamics when a cohort can be defined as a population of microbes that share a common experience. For example, the rapid water potential change associated with the wet-up of dry soils can be considered as a definitive event (Kieft et al 1987, Schimel et al 2007), and all of the organisms that were present during the dry period and experienced the rapid change in soil water potential caused by the wet-up event can be considered as members of a cohort. If this cohort can be monitored for new growth, survival, and mortality after the wet-up event, it may provide a more detailed picture of the microbial dynamics and how these dynamics relate to the large associated CO<sub>2</sub> pulse. Using a combination of molecular and stable isotope techniques it is possible to investigate these dynamics for microbes within soil communities.

Direct identification of active microbes through the incorporation of stable isotopes is called stable isotope probing (SIP). This family of approaches is recognized as among the most powerful techniques in microbial ecology (Kuypers and Jorgensen 2007, Neufeld et al 2007b), and the recently-developed application of heavy water (H<sub>2</sub><sup>18</sup>O) in SIP experiments can provide insight into the population dynamics in soil microbial communities (Schwartz et al 2007). In heavy water SIP experiments, H<sub>2</sub><sup>18</sup>O is introduced to a system. All newly formed cells (and thus newly replicated DNA) incubated in heavy water becomes enriched in <sup>18</sup>O (Aanderud and Lennon 2011, Blazewicz and Schwartz 2011, Schwartz 2007). DNA that was present at the beginning of incubation and persisted throughout the incubation without replication will not become enriched in <sup>18</sup>O. Consequently, the differences in density of the natural abundance DNA and the <sup>18</sup>O-enriched DNA can be exploited to isolate and subsequently characterize DNA from actively growing organisms and from those that survived but did not reproduce throughout the incubation. Deceased organisms that were decomposed during the incubation can also be characterized by identifying organisms that were present at the beginning of an incubation, but not at the end (Adair and Schwartz 2011). Heavy water SIP provides an ideal approach for investigating microbial dynamics during the wet-up of dry soil since the heavy water addition simultaneously increases water potential and serves as the isotopically enriched substrate necessary for density separation.

In this investigation we applied  $\text{H}_2^{18}\text{O}$ -DNA-SIP coupled with quantitative PCR (qPCR) to study microbial population and community response to wet-up of soil after a 5-month characteristically dry Mediterranean-type summer. Specifically, we characterized net growth, new growth, survival, mortality, and turnover for the total soil microbial community and for bacterial and fungal populations from a California annual grassland soil. We then compared microbial community and population dynamics with  $\text{CO}_2$  pulse dynamics that result from the wet-up of soil.

## Methods

### *Soil Collection*

Soil samples were collected from Hopland Field Station in northern California (38° 59.5784' N, 123° 04.0469' W) at the end of the summer dry season during which there had been no measurable rain for five months. The site is classified as annual grassland with soils of the Laughlin series and Mediterranean climate. Additional site and soil characteristics are given in Table 2-1. Surface vegetation was removed and soil cores (10 cm x 10 cm) were collected every meter along five 8 m transects. Cores from each of transects were combined, homogenized in the field, and sealed in air-tight jars prior to returning to the laboratory to prevent alterations in water potential due to differences between field and laboratory relative humidity. Jars were stored at room temperature in the dark until the incubations were begun, one week after collection. Soil water content of field soil was determined by drying soil to constant weight at 105 °C.

### *Isotope Labeling Incubation*

Dry soils ( $2.000 \pm 0.002$  g) were incubated in sterile plastic aerobic culture tubes (17 x 100 mm) with 400  $\mu\text{l}$  heavy water (97 atom%  $\text{H}_2^{18}\text{O}$ , Cambridge Isotope Laboratories) or natural abundance water (double-distilled  $\text{H}_2\text{O}$ ) for specified times ( $T = 0, 3, 24, 72, 168$  h) in triplicate for a total of 27 samples. Soils were destructively sampled and immediately frozen in liquid nitrogen and then stored at -80 °C.

### *DNA Extraction and Density Gradient SIP*

DNA was extracted from the 27 soil samples using a modified hot phenol extraction (Sambrook and Russel 2001). Briefly, two replicate extractions were conducted for each sample and then replicate DNA extracts were combined. For each extraction soil (0.5 g  $\pm$  0.001g) was mixed with TE (420  $\mu\text{l}$  1X TE),  $\text{PO}_4^{3-}$  buffer (150  $\mu\text{l}$  0.2 M in 1 M NaCl), and phenol:chloroform:isoamyl alcohol (600  $\mu\text{l}$  25:24:1) in lysing matrix E tubes (MP Biomedicals) and bead beat (30s at 5.5

m/s). Tubes were incubated at 65 °C for 10 min and then spun at 10K RCF<sub>avg</sub> for 5 min. Supernatant was transferred to a clean tube. Soil was extracted a second time using TE (220 µl 1X TE), PO<sub>4</sub><sup>3-</sup> buffer (80 µl 0.2 M in 1 M NaCl), vortexed for 10 s, and spun at 10K RCF<sub>avg</sub> for 5 min. Supernatant was combined with the first extract supernatant. Two phenol removal steps were done by adding an equal volume of chloroform:isoamyl alcohol (24:1), centrifuged 10K RCF<sub>avg</sub> for 5 min, and supernatant was transferred to a new tube. RNAase (6.44 µl, 10 mg/ml) was added and incubated at 50 °C for 10 min. NH<sub>4</sub><sup>+</sup> (244 µl 10 M) was added, incubated at 4 °C for 2 h, and spun at 16K RCF<sub>avg</sub> for 15 min. Supernatant was transferred to a new tube, 670 µl isopropal was added, and tubes were spun at 20K RCF<sub>avg</sub> for 20 min. Supernatant was removed, DNA pellet was dried in a clean hood for 15 min, and 30 µl 1X TE was added. DNA was stored at -80 °C. Since soils were from the same site and homogenized, it was assumed that extraction efficiency was similar for all samples. To ensure similar extraction efficiency between the pre-wet and wetted samples, the initial extraction buffer volume was varied slightly to result in equal (water volume):(dry soil mass) ratios for all extractions. Extraction efficiency was likely less than 100% so the abundances per g soil may be underestimated, nevertheless patterns observed for changes through time should be robust.

To separate isotopically enriched DNA from unenriched DNA, each sample was subjected to a cesium chloride density gradient. Gradients were generated by combining 4.60 ml CsCl stock (1.885 g/ml), 0.999 ml gradient buffer (add details), 5.5 µg DNA in 50 µl 1 x TE for a final density of 1.735 g/ml. After mixing, approximately 5.2 ml of the solution was transferred to an ultracentrifuge tube (Beckman Coulter Quick-Seal, 13 mm x 51 mm) and heat-sealed. Tubes were spun in an Optima L-90K ultracentrifuge (Beckman Coulter) using a VTi65.2 rotor at 44,000 rpm (176,284 RCF<sub>avg</sub>) at 20 °C for 109 h with maximum acceleration and braking. This length of time was chosen because using these spin conditions, all DNA large enough to be detected with the downstream analyses (> 24 bp) is expected to have reached equilibrium in the density gradient under these conditions.

The entire content of each ultracentrifuge tube was separated into approximately 75 fractions immediately following centrifugation using a syringe pump to deliver light mineral oil at 0.25 ml/min through a 25G needle inserted through the top of the tube to displace the gradient solution. The centrifuge tube was mounted in a fraction recovery system (Beckman Coulter) with a side port needle inserted through the bottom to collect extruded fractions. Approximately 5 drops (~70 µl) were collected for each fraction. The density of each fraction was measured using an AR200 digital refractometer (Reichert) fitted with a prism covering to facilitate measurement from 5 µl volumes, as previously described (Buckley et al 2007). DNA in each fraction was purified and concentrated using glycogen/PEG precipitations followed by an ethanol washing as described previously (Neufeld et al 2007a). Each fraction was resuspended in 25 µl of 1X TE and stored at -80 °C.

All fractions were analyzed for total DNA content via picogreen fluorescence assay (Figure 2-1). The SIP fractions were binned into three groups based on density for each sample (Figure 2-2). These binned fractions were combined and DNA was precipitated and suspended in equal volumes. The combined fractions were analyzed for total DNA content with picogreen (Figure 2-3).

### *Quantitative PCR*

Total bacterial 16S rDNA and total fungal ITS copies were quantified for the combined fractions using primers 16S EUB338/EUB518 and 5.8S/ITS1f, respectively, as previously described (Fierer et al 2005). All qPCR reactions were conducted with a CFX96 Real-Time PCR Detection System (Bio-Rad, USA). All samples were analyzed in triplicate with 20  $\mu$ l reactions containing 10  $\mu$ l Sso Fast Evagreen Supermix (Bio-Rad), 1  $\mu$ l of forward and reverse primers (10  $\mu$ M) (Sigma-Aldrich), 7  $\mu$ l PCR grade MQ-water (MP Biomedicals), and 1  $\mu$ l of template DNA (6.67 ng/  $\mu$ l). Plasmid standards were prepared by cloning purified PCR products from soil DNA into pCR4-TOPO vector and transformed into *Escherichia coli*. Plasmids were then extracted, purified, and quantified. Inserts were verified by sequencing. Thermocycling parameters consisted of 3 min at 95  $^{\circ}$ C, followed by 40 cycles of 30 s at 95  $^{\circ}$ C, 30 s at 53  $^{\circ}$ C, and 10 s at 60  $^{\circ}$ C, at which time fluorescence was measured at the end of each 60  $^{\circ}$ C step. To ensure that amplified products were representative of the target genes, a melting curve was calculated from 55 to 95  $^{\circ}$ C with an increase of 0.5  $^{\circ}$ C every 5 s following completion of thermocycling. Purity and amplicon product size were also verified by analysis random samples on agarose gels to visualize. Average PCR efficiencies were 98% and 90% and average slopes were -3.38 and -3.67 for bacteria and fungi respectively, with all standard curves having  $R^2 \geq 0.99$ .

### *CO<sub>2</sub> Flux Quantification*

CO<sub>2</sub> production was measured in parallel incubations that were not destructively sampled. Soil (40.00  $\pm$  0.01 g) was sealed in 8 oz mason jars. Water was added by syringe through a septum in each lid and distributed into soil by shaking. For measurements of CO<sub>2</sub>, incubation headspace (2 mL) was collected with a gas tight syringe and injected directly into a Hewlet Packard HP6890 (Agilent Technologies Inc., Santa Clara, CA) gas chromatograph fitted with a Hayesep DB 100/120 (1/16" x 1.5 m) column which fed into a Hayesep DB 120/140 (1/16" x 2.0 m) column leading to a pulse discharge detector (PDD). The PDD was calibrated for CO<sub>2</sub> using a 5 point-standard curve, and a single standard was analyzed hourly thereafter to correct for instrumental drift.

## Data Analysis and Rate Calculations

The following calculations were made using the assumption that changes in DNA abundances are representative of changes in microbial abundance. Here we use the term *growth* to indicate net increase in population, *new growth* to indicate cells that have been produced since the beginning of the incubation, *survival* to mean cells that were present at the beginning of the incubation and at a given time point during the incubation, and *mortality* as cells that were present at the beginning of the incubation but not at a subsequent time during the incubation.

The proportion of new growth for each group (total community, total bacteria, and total fungi) was calculated at each time point using abundance data from the three bins (light, middle, heavy) for total DNA (Figure 2-3), total bacterial 16S rRNA genes (Figure 2-4), or total fungal ITS (Figure 2-5) with the following equation:

$$\text{New Growth (\%)} = \text{\%Heavy DNA}_{18-0} + \text{\%Middle DNA}_{18-0} - \text{\%Heavy DNA}_{16-0} - \text{\%Middle DNA}_{16-0} \quad (1)$$

The percentage of new growth at each time point was used to calculate the quantities of new growth as follows:

$$\text{New Growth (gene copies g}_{dw}^{-1}) = \frac{\text{New Growth (\%)}}{100} * (\text{Total Gene Copies g}_{dw}^{-1}) \quad (2)$$

The percentage of microbes that were present before wet-up (pre-wet cohort) at each time point was calculated with equation 3, and the calculated percentages were used to calculate quantities of pre-wet cohort members present with equation 4.

$$\text{Prewet cohort (\%)} = \text{\%Light DNA}_{18-0} + \text{\%Middle DNA}_{16-0} + \text{\%Heavy DNA}_{16-0} \quad (3)$$

$$\text{Prewet cohort (gene copies g}_{dw}^{-1}) = \frac{\text{Prewet Cohort (\%)}}{100} * \text{Total Gene Copies g}_{dw}^{-1} \quad (4)$$

New growth and mortality rates were calculated using equations 6 and 7. It is important to note that calculations for new growth rate assume that death of new cells is zero and rates are only calculated for the pre-wet cohort, since it is not possible with this approach to determine mortality of newly generated (and  $^{18}\text{O}$  labeled) cells. Thus, the calculated rates may underestimate actual rates.



$$\text{New Growth Rate (gene copies } g_{dw}^{-1} h^{-1}) = \frac{\text{New Growth}_{t_{n+1}} - \text{New Growth}_{t_n}}{t_{n+1} - t_n} \quad (5)$$

$$\text{Mortality Rate (gene copies } g_{dw}^{-1} h^{-1}) = \frac{\text{Prewet cohort}_{t_{n+1}} - \text{Prewet cohort}_{t_n}}{t_{n+1} - t_n} * (-1) \quad (6)$$

Turnover time can be defined as the time it takes for members of a population or community to replace themselves and can be estimated using equation 8.

$$\text{Turnover time (d)} = \frac{100\%}{\frac{(\% \text{ New Growth})_{t_n}}{t_n - t_0}} \quad (7)$$

### *Statistics*

ANOVA with post hoc Tukey's Honestly Significant Difference test (HSD) was used to compare DNA or gene abundance data over time. Helmert contrasts were used to determine if significant changes occurred in gene abundances over time. Correlation analyses were used to compare quantities of DNA or gene abundances with CO<sub>2</sub> produced. All statistical analyses were performed within R version 2.12.2.

### **Results**

Following the wetting of dry California Mediterranean grassland soil with H<sub>2</sub><sup>18</sup>O, DNA was produced that was enriched in <sup>18</sup>O, resulting in a higher buoyant density for the isotopically labeled DNA as compared to the DNA that was present prior to the wet-up and to DNA from H<sub>2</sub><sup>16</sup>O control incubations. This increase in density facilitated the separation of DNA along a cesium chloride density gradient (Figure 2-1, 2-3). Based on comparison between the quantities of labeled DNA in the middle fractions of the <sup>18</sup>O incubations versus the DNA in the same density fractions for the <sup>16</sup>O controls, new microbial growth was not detectable until 24 hours after the wet-up event (24 h, *p*=0.006, Figure 2-3). In contrast to new growth, significant CO<sub>2</sub> production was detected within 3 hours of the wet-up event (*p*=0.004, Figure 2-6). The CO<sub>2</sub> production rate decreased back to pre-wet rates by the end of the incubation, indicating that the pulse in activity stimulated by the wet-up had largely subsided within 168 h.

### *Net changes*

Throughout the 7-day incubation following wet-up, there were small net changes in total soil DNA abundance, with the abundance at 24 h marginally lower than the preceding time points (Figure 2-7a, Helmert Contrast,  $p=0.08$ ). However, the total DNA abundance rebounded by 72 h resulting in no difference in total microbial abundance at pre-wet as compared the end of incubation (Figure 2-7a, Tukey's HSD,  $p=0.99$ ). Similarly, there was an apparent decrease in total bacterial abundance following wet-up that was marginally significant (Figure 2-7b, Helmert Contrast,  $p=0.08$ ); but again the total bacterial abundance had rebounded by 168 h such that there was no net difference in bacterial abundance compared to pre-wet (Tukey's HSD,  $p=0.93$ ). Fungi followed a similar pattern with a small but significant net decrease detectable immediately following wet-up (Figure 2-7c, Helmert Contrast,  $p=0.03$ ) followed by a return to pre-wet abundances by the final time point (Tukey's HSD,  $p=0.24$ ).

### *New growth*

In spite of the small net changes, a significant amount of new growth did occur within the total soil community during the incubation period (Figure 2-7). New growth was mostly linear throughout the incubation for both bacteria and fungi (Figure 2-7b and 2-7c, bacteria:  $R^2=0.81$ ,  $p<0.0001$ ; fungi:  $R^2=0.80$ ,  $p<0.0001$ ). New growth rates were calculated for bacteria and fungi at 24, 72, and 168 h, when new gene copies were quantifiable (Tables 2-2, 2-3). Growth rates remained relatively consistent from 24 h through the remainder of the incubation for both bacteria and fungi with average growth rates of  $2.3 \times 10^8 \pm 5.2 \times 10^7$  bacterial 16S gene copies  $g_{dw}^{-1} h^{-1}$  and  $4.3 \times 10^7 \pm 9.9 \times 10^6$  fungal ITS copies  $g_{dw}^{-1} h^{-1}$ .

### *Mortality and survival*

While growth patterns were similar for bacteria and fungi, they differed in their mortality characteristics. For the pre-wet fungal cohort, death was only measurable between wet-up and 3h, while bacteria continued dying through 72 h (Figures 2-7b, 2-7c). However, the mortality that occurred by 3 h was proportionally similar for bacteria and fungi with  $25 \pm 10$  and  $27 \pm 7\%$  of each cohort becoming undetectable. After this initial die-off, fungi were better survivors and maintained 71% of their pre-wet cohort while bacteria only maintained 46% of their original population (Tables 2-2 and 2-3). Mortality rates were highest at the 3 h time point for bacteria, but mortality continued through 72 h.

### *Turnover*

The percentages of new bacterial and fungal growth at each time point were used to calculate turnover times (Tables 2-2 and 2-3). Bacterial and fungal rates of turnover were highest at 24 and 72 h respectively, and then decreased significantly by 168 h. Interestingly bacteria and fungi both had similar average turnover times of  $11.0 \pm 1.0$  and  $9.7 \pm 1.1$  days, respectively.

### *Soil respiration and growth*

Correlation analyses were performed between cumulative CO<sub>2</sub> produced and net abundance or cumulative new growth for the total soil community and for bacteria and fungi populations (Figure 2-8). Soil respiration did not correlate significantly with net abundance for any of these groups. However, respiration was significantly related to new growth (using either power or logarithmic models) for all three of these groups (Figure 2-8).

## **Discussion**

The rapid change in water potential caused by soil wet-up was followed by a significant change in microbial activity as indicated by an increase in CO<sub>2</sub> production. The change in soil respiration was easily detectable within 3 hours indicating that microbial activity was rapidly stimulated (Figure 2-6). However, new microbial growth was not measurable until 24 hours after wet-up (Figure 2-7a). This could indicate that in short-term response to wet-up, most microbial activity is non-growth associated; this could include activity supporting germination of dormant cells or other cell regulation activities including osmoregulation, cellular repair, defense against oxidative stress, or energy spilling reactions (Allen 1965, Halverson et al 2000, Levinson and Hyatt 1956, van Bodegom 2007). Cells that are transforming from a dormant to a vegetative state can increase respiration rates prior to initiating growth. For non-spore forming organisms, maintenance costs would likely increase while microbes adjust to the rapid change in water potential and the resulting change in nutrient availability. It is also possible that some of the CO<sub>2</sub> efflux detected within 3 hours is the result of extracellular oxidative metabolism (Maire et al 2012) in which carbon is mineralized by previously secreted exocellular enzymes that persist through the dry period and enzymes that were released from dead organisms and stabilized by soil particles. Similar short-term decoupling of growth and respiration was observed after wetting dried soil using leucine and thymidine incorporation approaches (Iovieno and Bååth 2008), supporting the hypothesis that this decoupling is a characteristic of microbial response to wet up.

Relatively small and highly variable changes occurred in net abundances for the total community, bacteria, and fungi, yet significant new growth and mortality was observed for all three of these groups (Figure 2-7). This suggests that the commonly observed pulse of microbial respiration upon wetting of dry soils is in part from activity of newly divided members of the community and not solely from the activity of wet-up survivors, as the net abundance data might suggest. New growth proceeded linearly for bacteria and fungi through the incubation period. Linear growth has also been observed in soil using tritiated thymidine and leucine (Bååth 1990, Harris and Paul 1994, Iovieno and Bååth 2008, Rousk and Bååth 2011). This non-exponential growth pattern could be a result of nutrient limitations, especially due to diffusional limitations associated with the soil matrix. It is also important to keep in mind that the bacterial and fungal populations are likely comprised of a large diversity of organisms with potentially different life strategies such as maximizing growth rates versus maximizing growth use efficiency (Fierer et al 2007). Our observations of linear growth for these populations as a whole could be underlain by growth of distinct groups of microbes with varying growth rates and strategies.

This major wet-up event is accompanied by rapidly detectable death for both bacteria and fungi with 25% of bacteria and 27% of fungi dying (or becoming undetectable) within 3 hours of wet-up (Tables 2-2 and 2-3). This death may be the result of cellular desiccation, oxidative stress, or nutrient deprivation during the dry-down of the soil or it may be a result of plasmolysis caused by the rapid change in water-potential during wet-up. Likely, all of these factors contribute to the initial pulse of mortality. There has been a long-standing debate regarding the source of carbon and nutrients that fuel the microbial wet-up response with proposed contributions from the release of previously occluded carbon due to physical disturbance of soil particles (Fierer and Schimel 2003), the release of microbial intracellular solutes (Fierer and Schimel 2003, Halverson et al 2000), or from dead microbial bodies (Kieft et al 1987). While the data do not directly inform about the sources of C utilized, the high percentage of mortality observed for bacteria and fungi suggest that a sizeable portion of the wet-up response could be driven by the degradation of dead microbial bodies. Assessing only the initial pulse of bacterial mortality resulting from the dry-down and rapid wet-up (through 3 h), we can estimate how much CO<sub>2</sub> could potentially come from the complete mineralization of these dead microbial bodies (avg =  $2.2 \times 10^{10}$  16S copies/g soil). Using the conservative assumptions that there are six 16S operon copies per bacterial cell (Klappenback et al 2000), that the mass of bacterial C is  $2 \times 10^{-14}$  g C/cell (Lee and Fuhrman 1987), and that microbial growth has a carbon use efficiency of 50%, we can estimate  $3.6 \times 10^{-5}$  g CO<sub>2</sub>-C/g soil of potential CO<sub>2</sub> produced from dead bacterial biomass. This is equivalent to the amount of CO<sub>2</sub> released during the initial pulse of CO<sub>2</sub> measured at 3 h ( $2.2 \times 10^5$  g CO<sub>2</sub>-C/g soil). These calculations are approximations and it is unlikely that all of the dead cell material will be consumed. However, based on these estimates,

we can conclude that a sizeable portion of the CO<sub>2</sub> pulse observed after wet-up could be derived from the mineralization of dead microbial bodies.

After the initial rapid die-off, fungi appear to stabilize and the remaining pre-wet cohort survived throughout the remainder of the incubation. Bacteria, on the other hand continue to lose members of the pre-wet cohort through 72 hours of the incubation. Why are fungi better survivors than bacteria? It is unlikely that this is due to better mechanisms for immediately surviving the dry-down or rapid change in water potential due to wet up, since both bacterial and fungal pre-wet cohorts decreased in similar proportions within the first 3 h of wet-up. Perhaps fungi are less susceptible to predation in these soils. Direct investigation of the role of predators throughout the different stages of the dry-down/wet-up cycle could provide insight into the observed trends in mortality for both bacteria and fungi.

Turnover for bacteria and fungi was fairly rapid following wet-up resulting in 49 and 52% new DNA for each population at the end of incubation (Tables 2-2 and 2-3). Bacteria and fungi had proportionally similar turnover rates with average turnover times of  $11.0 \pm 1.0$  and  $9.7 \pm 1.1$  d, respectively. While several studies using thymidine incorporation into DNA have estimated similar turnover rates for bacteria with a range between 1-33 d in different soils (Bååth 1990, Bååth 1992, Bååth 1998, Uhlířová and Šantrůčková 2003), the few investigations of fungal turnover have estimated much longer turnover times for fungi ranging between 130-170 d (Bååth 2001, Rousk and Bååth 2007). One possible reason for this disparity is that previous studies employed the <sup>14</sup>C-acetate into ergosterol approach. This approach should be specific in that labeled ergosterol is indicative of fungal and not bacterial growth. However, many bacteria are able to consume acetate, which could result in a decrease in the <sup>14</sup>C-acetate pool available for fungi by an unknown quantity; thus, potentially leading to an overestimation for turnover time if the original pool size is used to calculate rates. Another issue is that ergosterol not associated with viable fungi has been shown to be persistent in soil (Mille-Lindblom et al 2004). Slow degradation of ergosterol from dead fungi would result in increased estimates for turnover time. An alternative explanation could be that the heavy water SIP approach targets DNA and not specifically new fungal cells. Since fungi are multinucleated, it is possible that DNA is produced without new cell growth, which could result in underestimating turnover times. Regardless of the potential to underestimate fungal cell turnover, the heavy water approach provides robust evidence for the turnover of fungal DNA and therefore provides insight into fungal dynamics following wet-up.

Since an increase in CO<sub>2</sub> production is generally observed following the wetting of dry soils, it is interesting to compare this ecosystem characteristic with different microbial community and population characteristics. Changes in net growth correlated poorly with CO<sub>2</sub> production, but gross growth (new growth) was highly correlated with cumulative CO<sub>2</sub> production for the total soil community and

for bacterial and fungal populations (Figure 2-8b,d,f). Such correlations again highlight the importance of considering gross population dynamics when investigating ecosystem functions. It is important to note that we compared cumulative new growth and CO<sub>2</sub> by correlation analysis and not by regression analysis. These analyses were presented in order to illustrate the degree of association between the variables and not to illustrate causation with CO<sub>2</sub> described as a dependent variable of new growth. Conversely, it is interesting that cumulative new growth is tightly associated with total CO<sub>2</sub> production. The non-linear relationships suggest that disproportional changes occurred in new growth compared to CO<sub>2</sub> production during the incubation and could be due to factors such as changes in non-growth activities or carbon use efficiency. As demonstrated here and by others (Iovieno and Bååth 2008), non-growth activities were higher than growth activities immediately following wet-up. Such changes in non-growth:growth ratios could result in the non-linear associations observed between CO<sub>2</sub> and new growth. Similarly, as available C diminishes throughout the incubation, organisms with higher carbon use efficiency may become more active and therefore produce proportionally more new growth per carbon mineralized.

### *Conclusion*

Our study shows that bacterial and fungal populations underwent dynamic changes following the re-wetting of a seasonally dried grassland soil, yet net abundances were resilient to the sudden environmental change. A pulse of non-growth activity appears to immediately follow wet-up followed by linear growth for both bacteria and fungi. Mortality dynamics imply that dead microbial bodies provide a large pool of available C and nutrients, thus offering insight into possible sources fueling the CO<sub>2</sub> pulse following wet-up. Our results reveal that a vibrant assemblage of growing and dying organisms may comprise a seemingly static microbial community following a change in the environment. Understanding the underlying gross population dynamics should lead to stronger linkages between microbial population information with ecosystem characteristics.

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**Table 2-1.** Site and Soil Characteristics for Hopland Field Station.

Sampling date	9/18/2008
Previous rain event	4/23/2008
MAP (mm)	940 <sup>1</sup>
MAT (°C)	15 <sup>1</sup>
Soil Moisture (%)	1.0
Soil Water Potential (MPa)	-38 <sup>2</sup>
pH	5.0 <sup>2</sup>
Sand/Silt/Clay (%)	60/27/13 <sup>2</sup>
Total Carbon (%)	2.1 <sup>2</sup>
C:N Ratio	13 <sup>2</sup>

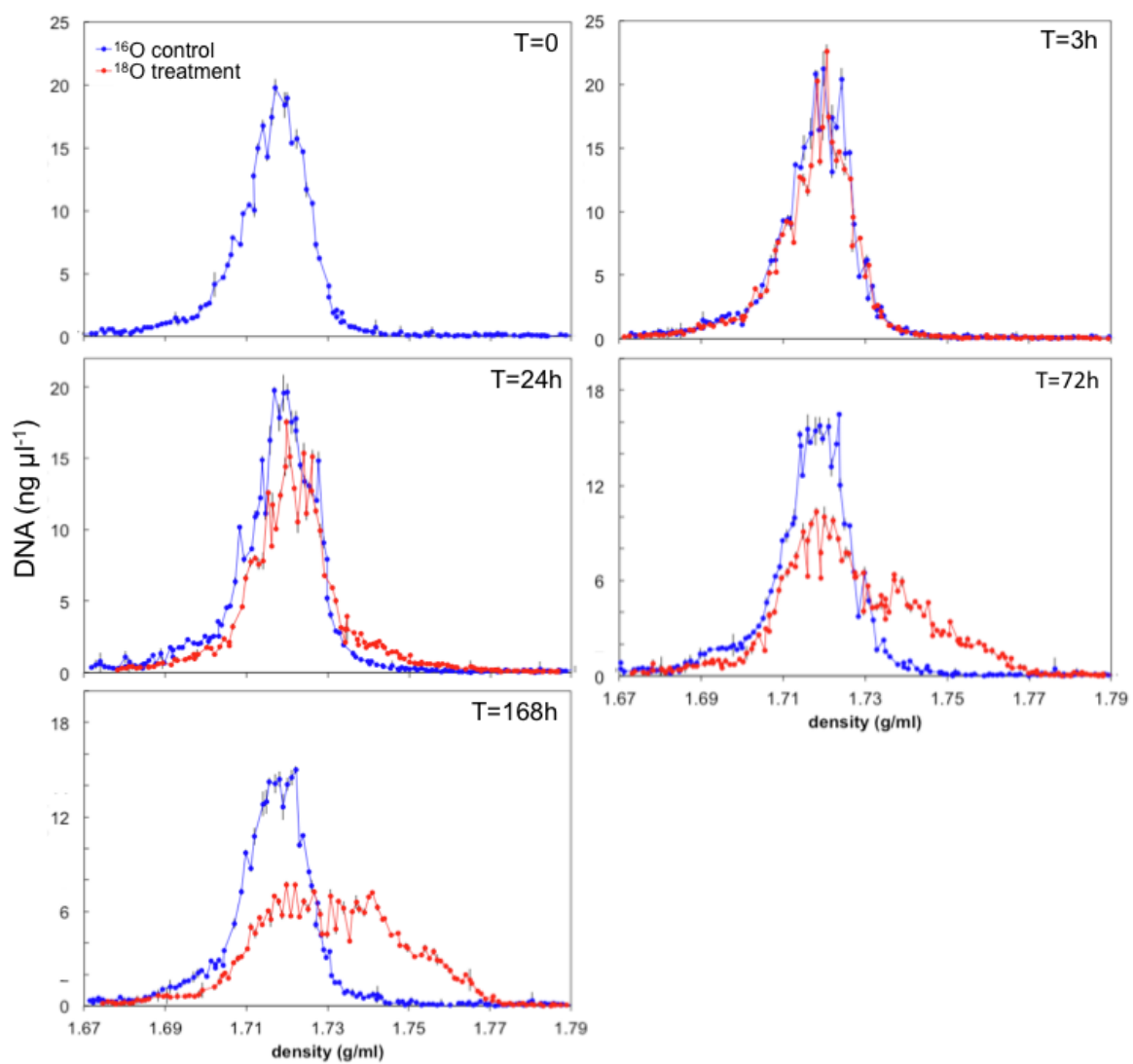
<sup>1</sup>(Waldrop and Firestone 2006), <sup>2</sup>(Placella et al 2012)

**Table 2-2.** Dynamic life table for bacteria following a wet-up event. Values in parentheses are standard error of the mean (n=3).

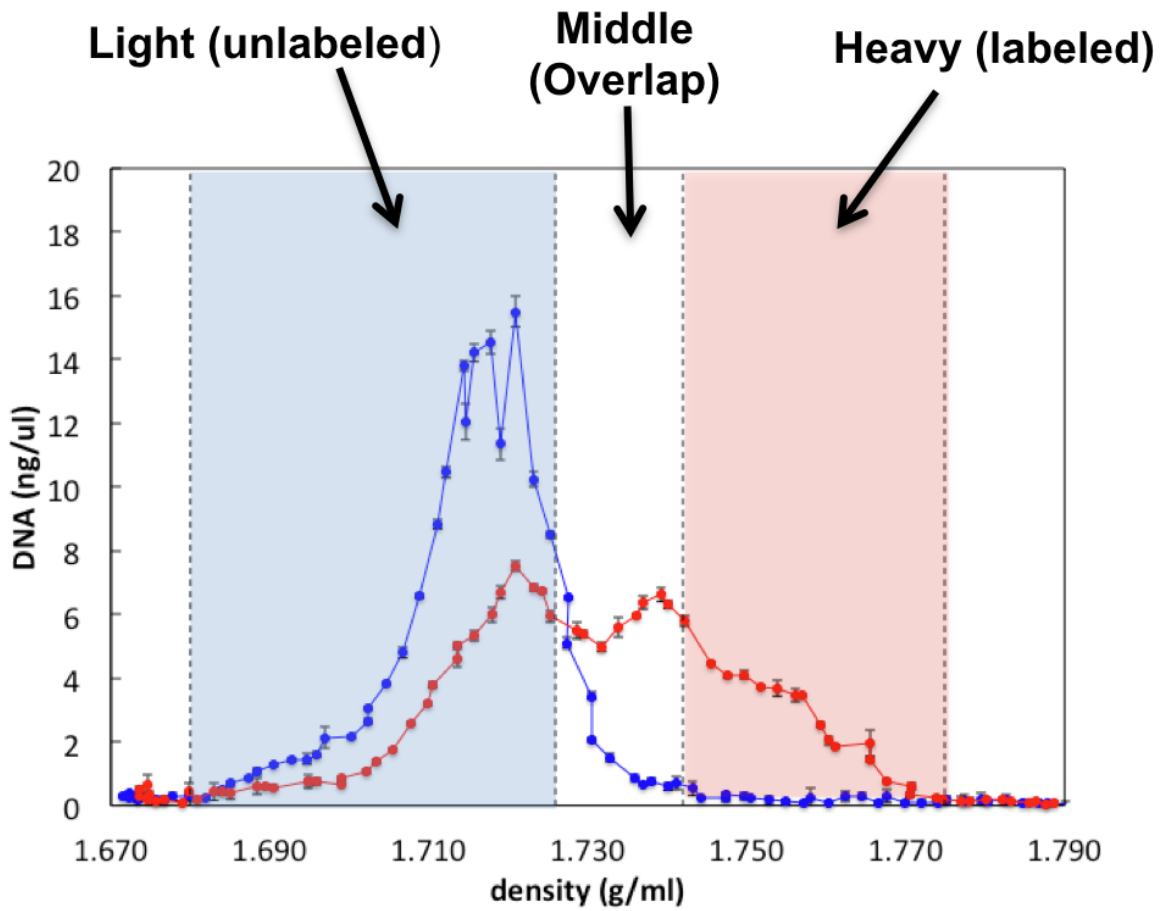
Time (h)	Total Bacteria Detected (16S gene copies/g soil)	Proportion of pre-wet cohort surviving	New Growth as a portion of pre-wet abundance	Rate of New Growth (16S gene copies/g soil/h)	Mortality rate (16S gene copies/g soil/h)	Turnover time (d)
0	$7.8 \times 10^{10}$ ( $1.2 \times 10^{10}$ )	1	n/a	n/a	n/a	n/a
3	$5.7 \times 10^{10}$ ( $2.1 \times 10^9$ )	0.75 (0.10)	n/a	n/a	$7.3 \times 10^9$ ( $3.9 \times 10^9$ )	n/a
24	$6.0 \times 10^{10}$ ( $6.6 \times 10^9$ )	0.72 (0.13)	0.11 (0.01)	$2.7 \times 10^8$ ( $3.1 \times 10^7$ )	$1.3 \times 10^8$ ( $4.0 \times 10^8$ )	9.5 (1.0)
72	$5.1 \times 10^{10}$ ( $1.1 \times 10^{10}$ )	0.42 (0.03)	0.34 (0.03)	$2.4 \times 10^8$ ( $1.4 \times 10^8$ )	$4.3 \times 10^8$ ( $1.9 \times 10^8$ )	9.0 (0.7)
168	$6.9 \times 10^{10}$ ( $7.0 \times 10^9$ )	0.46 (0.05)	0.49 (0.04)	$1.7 \times 10^8$ ( $1.0 \times 10^8$ )	$-1.5 \times 10^7$ ( $4.8 \times 10^7$ )	14.5 (1.2)

**Table 2-3.** Dynamic life table for fungi following a wet-up event. Values in parentheses are standard error of the mean (n=3).

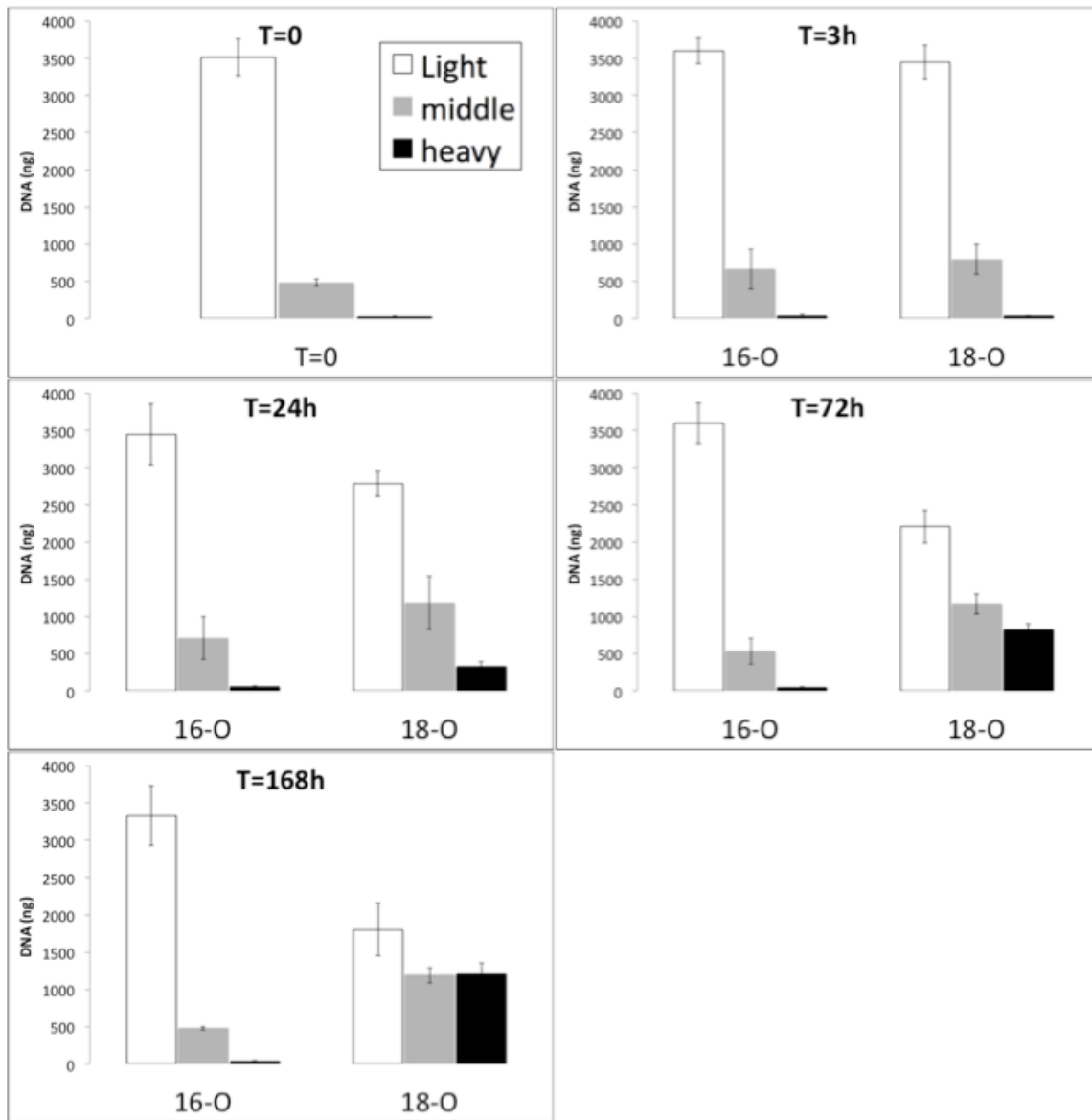
Time (h)	Total Fungi Detected (ITS copies/g soil)	Proportion of pre-wet cohort surviving	New Growth as a portion of pre-wet abundance	Rate of New Growth (ITS copies/g soil/h)	Mortality rate (ITS copies/g soil/h)	Turnover time (d)
0	$8.2 \times 10^9$ ( $1.2 \times 10^9$ )	1	n/a	n/a	n/a	n/a
3	$5.9 \times 10^9$ ( $2.7 \times 10^8$ )	0.73 (0.07)	n/a	n/a	$7.8 \times 10^8$ ( $3.2 \times 10^8$ )	n/a
24	$6.8 \times 10^9$ ( $6.3 \times 10^8$ )	0.75 (0.15)	0.13 (0.02)	$4.0 \times 10^7$ ( $7.6 \times 10^6$ )	$-2.8 \times 10^6$ ( $3.7 \times 10^7$ )	8.2 (1.7)
72	$9.1 \times 10^9$ ( $1.9 \times 10^9$ )	0.64 (0.11)	0.42 (0.05)	$6.6 \times 10^7$ ( $2.2 \times 10^7$ )	$1.7 \times 10^7$ ( $2.9 \times 10^7$ )	7.3 (0.9)
168	$1.2 \times 10^{10}$ ( $1.0 \times 10^9$ )	0.71 (0.07)	0.52 (0.03)	$2.2 \times 10^7$ ( $1.3 \times 10^7$ )	$-6.2 \times 10^6$ ( $4.1 \times 10^6$ )	13.6 (1.0)



**Figure 2-1.**  $^{18}\text{O}$  incorporation into DNA over time.

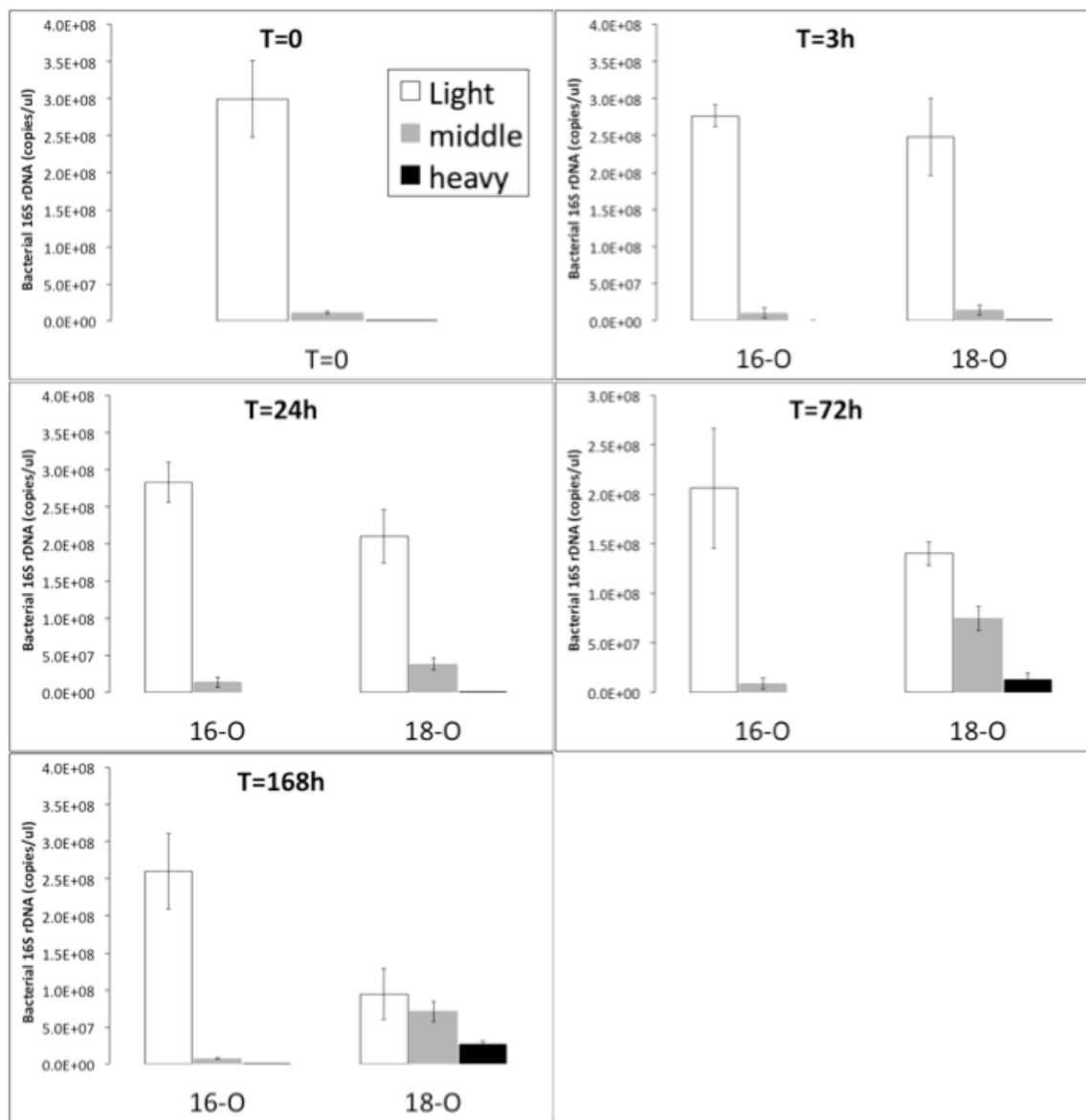


**Figure 2-2.** SIP fractions were binned into three groups based on density for each sample (light, middle, and heavy), and binned fractions were then combined for each sample.

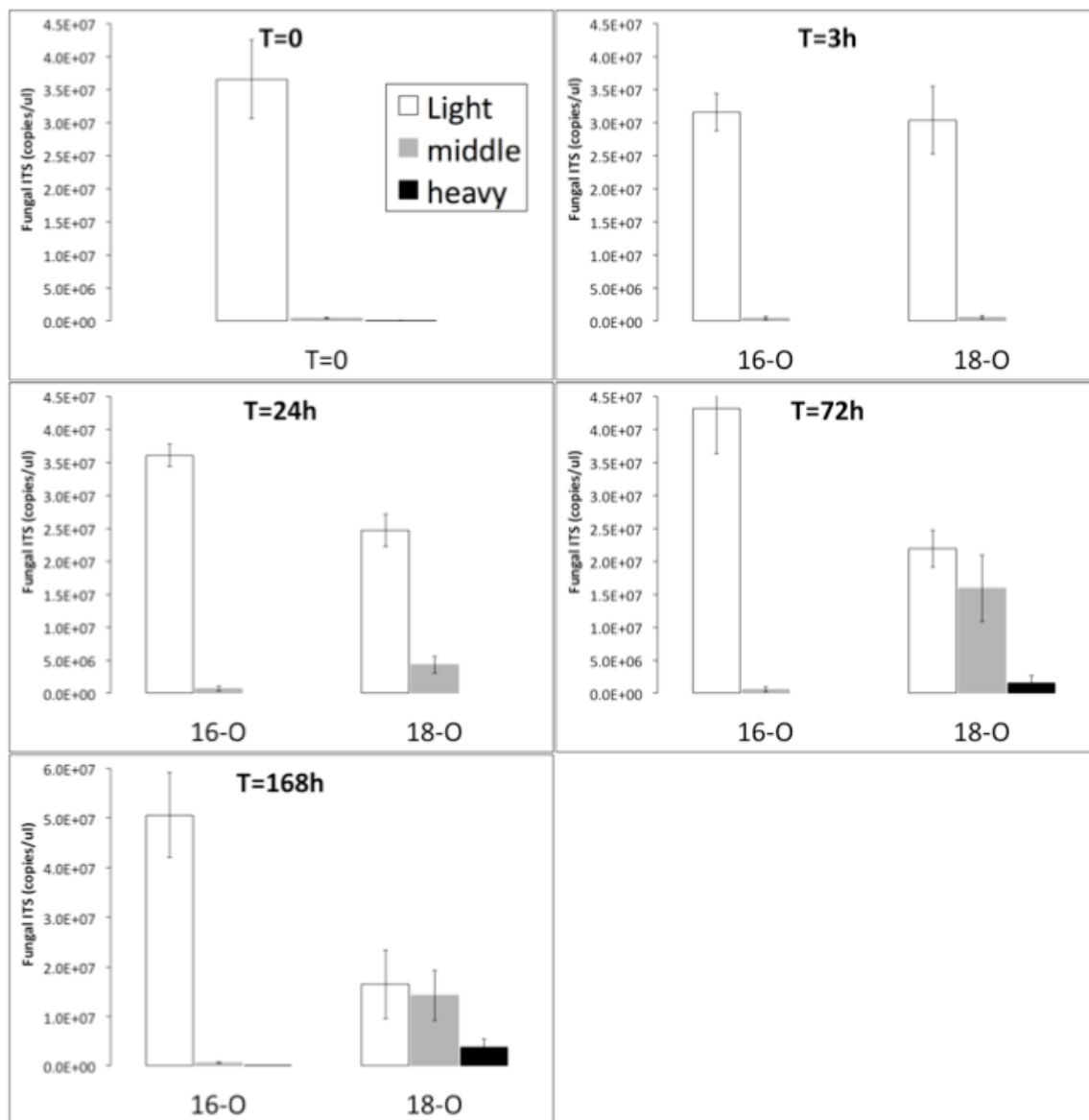


**Figure 2-3.** Total DNA abundance in combined SIP fractions measured by picogreen.

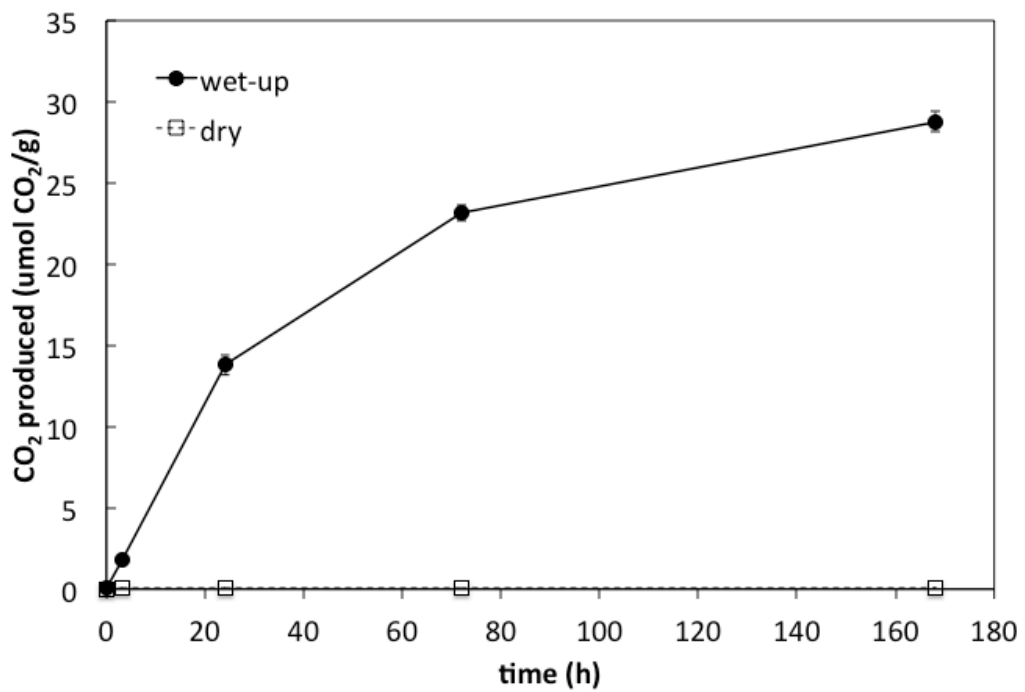




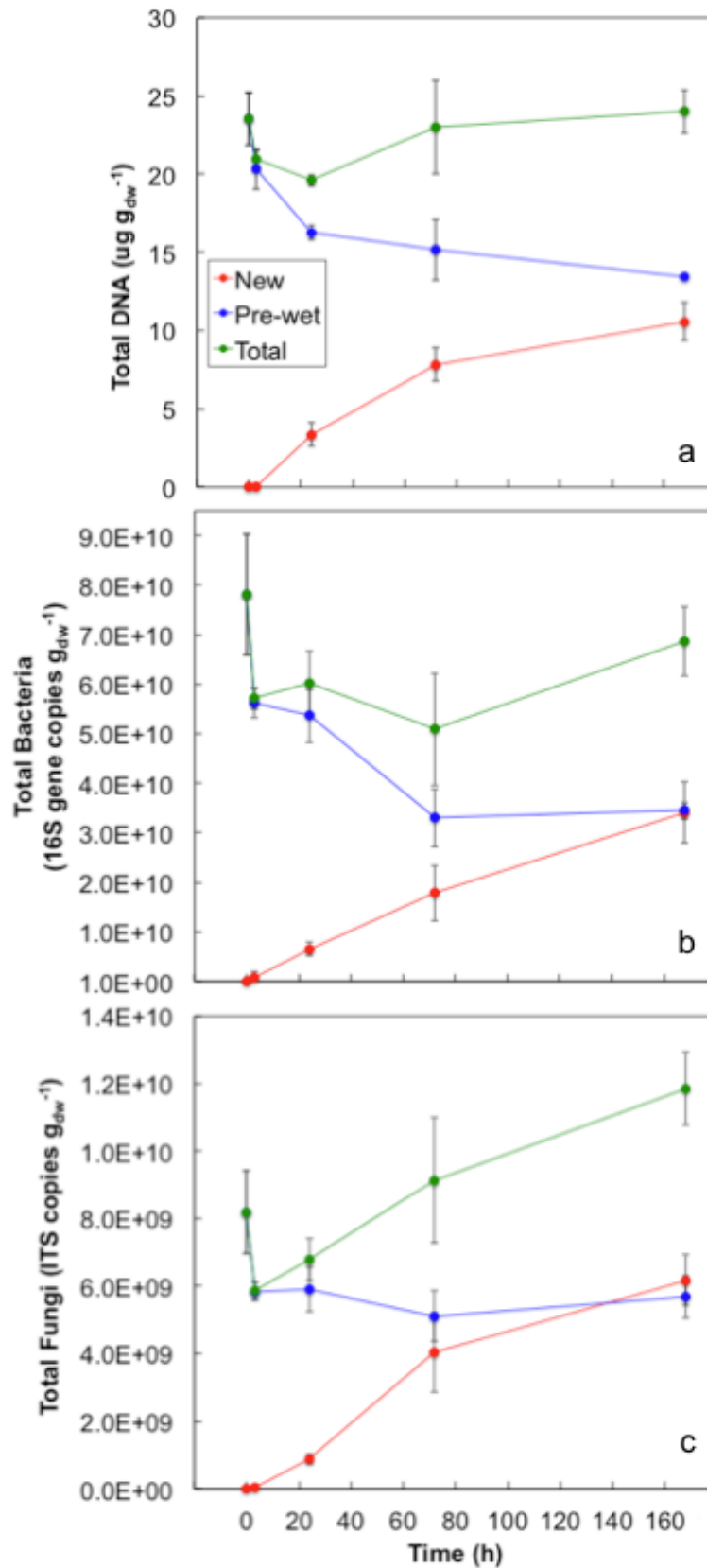
**Figure 2-4.** Total bacterial 16S rRNA gene abundance in combined SIP fractions.



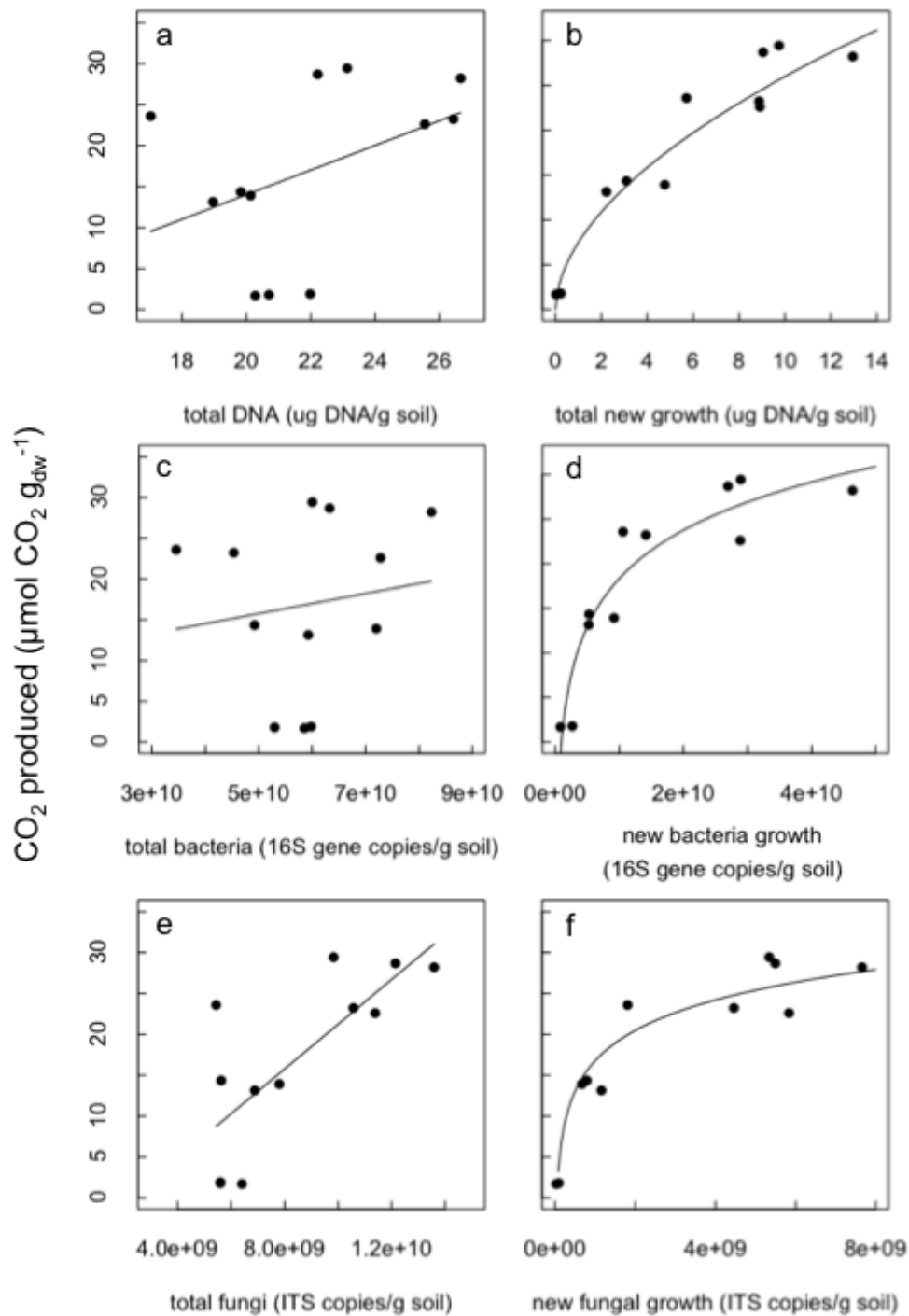
**Figure 2-5.** Total fungal ITS abundance in combined SIP fractions.



**Figure 2-6.** Cumulative CO<sub>2</sub> production for soils that were wet-up and for dry controls.



**Figure 2-7.** Net growth, new growth, and survival dynamics for a) the total soil community, b) bacteria, and c) fungi. Green = total measured, blue = present before wet-up, red = new growth. Error bars represent standard error (n=3).



**Figure 2-8.** Correlation analysis for cumulative  $\text{CO}_2$  produced versus: a) total extracted DNA ( $R^2 = 0.18$ ,  $p=0.17$ ), b) new DNA produced (power model,  $R^2 = 0.93$ ,  $p<0.0001$ ), c) total bacteria ( $R^2 = 0.022$ ,  $p=0.64$ ), d) new bacterial growth (logarithmic model,  $R^2 = 0.88$ ,  $p<0.0001$ ), e) total fungi ( $R^2 = 0.57$ ,  $p=0.005$ ), and f) new fungal growth (logarithmic model,  $R^2 = 0.91$ ,  $p<0.0001$ ).

# 3

## Sequential Growth of Bacteria Following the Rewetting of a Seasonally Dried Grassland Soil

### Abstract

Microbial activity is stimulated by the rewetting of a dry soil, resulting in a pulse of carbon mineralization and nutrient availability. This phenomenon is of particular interest because predicted changes in precipitation patterns in Mediterranean ecosystems could profoundly change soil C dynamics and nutrient availability. While there has been much interest in the response of indigenous communities to wet-up of dry soil, to date no work has identified the microorganisms in semi arid soils that grow in response to soil wet-up. We used heavy water ( $\text{H}_2^{18}\text{O}$ ) DNA stable isotope probing coupled with high throughput sequencing of bacterial 16S rRNA genes to characterize taxonomic and phylogenetic composition of bacteria following the rewetting of a seasonally dried California annual grassland soil. Bacterial growth was detected at all time points throughout the incubation (3, 24, 72, 168 h), with patterns of sequential growth observable at the phylum and order levels. Of the 25 phyla detected in the pre-wet community, members of the Firmicutes Bacillales order were the only detectable early responders with approximately 5% increase in relative abundance from growth during the first 3 h after wet up. The second group of growing bacteria detected at 24 h included only Betaproteobacteria and Bacteroidetes. Members of the Burkholderiales order in the Betaproteobacteria phylum were the dominant growers during this period showing a 21% increase in relative abundance. The highest richness of growing bacteria was detected during the third time-period (between 24-72 h), with significant changes in relative abundance due to growth detected for 11 phyla. Nonmetric multidimensional ordination of community composition data through time shows a somewhat cyclical pattern for phylogenetic composition of

growing bacteria with composition at 3 hours differing slightly from the pre-wet community, differing greatly at 24 h, and then becoming progressively more similar to the pre-wet community at 72 and 168 h. This suggests a degree of community resilience in response to this abrupt environmental change; composition of new growth first diverges from the original community composition but eventually results in a partial return to the original composition. However, some net compositional changes were observed following wet-up. Actinobacteria were the most dominant pre-wet phylum, but Proteobacteria became the most dominant phylum by 168 h. This change in composition was likely driven by new growth since Proteobacteria were found to increase in relative abundance across most time periods following wet-up, unlike Actinobacteria where comparatively small increases in growth were only observed during the latter two time points. Sequential growth patterns found at the phylum and order level suggest that an ecologically coherent response was observable at a high taxonomic level with members of certain groups employing different life strategies in response to rewetting of a dried soil.

## Introduction

Following a long dry period, the first rain event causes a rapid water potential change in soils that catalyzes changes in microbial activity, function, and biomass (Kieft et al 1987). Microorganisms are essential controllers of many crucial soil functions such as nutrient cycling and plant productivity; thus it is important to understand factors that contribute to microbial community compositional changes or compositional stability. Fundamental factors controlling community composition include evolution, immigration, emigration, growth, and mortality. Over short periods of time, evolution can be down weighted as a factor affecting changes in composition, and in “closed” system *in vitro* incubations, immigration and emigration are regulated. Under such conditions, growth and mortality become the principal controllers of composition. Soil community composition can alter in response to environmental changes, but it often requires relatively sensitive molecular methods to follow the changes in community membership over short time periods such as hours or even days. After a punctuated environmental change such as the wet-up of dry soils, microbial activity can be significantly stimulated but net changes in community composition can appear to be very small (Barnard et al 2012, Fierer et al 2003, Placella et al 2012).

Compositional stability following a change in environmental conditions could be the result of one of two mechanisms (Griffiths and Philippot 2012, Pimm 1984). The community could be resistant to change where little mortality or growth occurs and composition remains consistent. Alternatively, the community could be resilient where community composition changes temporarily due to selective new growth and/or mortality, but eventually growth and mortality balance and the community returns to the original composition (Botton et al 2006). It is likely that compositional stability following a seasonal ecosystem change is the consequence of both resistance and resilience to change. Given the prominent role that bacteria play in controlling biogeochemical cycling, production of atmospherically reactive trace gases, and plant productivity, knowledge of factors and mechanisms that control community compositional stability are of fundamental importance.

Soil bacterial communities are generally comprised of a large diversity of organisms likely employing different life strategies. For example, the abundance of some soil bacteria were found to be related to soil C availability with certain bacteria preferring high C availability (copiotrophs) and others preferring low C availability (oligotrophs) (Fierer et al 2007, Koch 2001, Poindexter 1981). This differential preference can be related to growth rate strategies where copiotrophs maximize growth rates while oligotrophs maximize growth use efficiency. Alternatively, growth strategies could be connected to soil moisture preferences with some taxa growing better in the dry season and others growing better when soil moisture is higher (Cruz-Martínez et al 2009, Zvyagintsev et al 2007). Most



studies investigating growth strategies in soils have only characterized net changes in community composition, but net increases in relative abundance are not necessarily indicative of growth since relative changes are driven by mortality as well. Using stable isotope labeling techniques it is possible to characterize the organisms that are definitively growing in soil.

Stable isotope probing (SIP) with heavy water ( $\text{H}_2^{18}\text{O}$ ) can be used to identify and characterize the growing microorganisms in soil (Aanderud and Lennon 2011, Schwartz 2007). In heavy water SIP experiments,  $\text{H}_2^{18}\text{O}$  is added to a sample and incubated. All newly formed cells (and newly replicated DNA) incubated in heavy water will become enriched in  $^{18}\text{O}$  (Aanderud and Lennon 2011, Blazewicz and Schwartz 2011, Schwartz 2007). DNA that was present at the beginning of incubation and persisted throughout the incubation without replication will not become enriched in  $^{18}\text{O}$ . Consequently, the differences in density of the natural abundance DNA and the  $^{18}\text{O}$ -enriched DNA can be exploited (by density gradient centrifugation) to isolate and subsequently characterize DNA from actively growing organisms and from organisms that survived but did not reproduce throughout the incubation.

Using a combination of heavy water DNA-stable isotope probing coupled with high throughput 16S rRNA gene sequencing, growing bacteria were identified following the wet-up of California grassland soil after a seasonally dry summer. Specifically, we examined the taxonomic and phylogenetic composition of the growing bacteria at different time points after an *in vitro* wet-up event. We hypothesized that after an abrupt change in soil water potential due to wetting of dry soil, new growth would follow a sequential response pattern due to differences in growth strategies.

## Methods

### *Soil Collection*

Soil samples were collected from Hopland Field Station in northern California ( $38^\circ 59.5784'$  N,  $123^\circ 04.0469'$  W) at the end of the summer dry season during which there had been no measurable rain for five months. The site is classified as annual grassland with soils of the Laughlin series and Mediterranean climate. Additional site and soil characteristics are given in Table 2-1. Surface vegetation was removed and soil cores (10 cm x 10 cm) were collected every meter along five 8 m transects. Cores from each of transects were combined, homogenized in the field, and sealed in air-tight jars prior to returning to the laboratory to prevent increase in water potential due to the higher humidity in Berkeley, CA. Jars were stored at room temperature in the dark until the incubations were begun, one week after collection. Soil water content of field soil was determined by drying soil to constant weight at  $105^\circ\text{C}$ .

### *Isotope Labeling Incubation*

Triplicate samples of dry soils ( $2.000 \pm 0.002$  g) were incubated in sterile plastic aerobic culture tubes (17 x 100 mm) with 400  $\mu$ l heavy water (97 atom%  $\text{H}_2^{18}\text{O}$ , Cambridge Isotope Laboratories) or natural abundance water (double-distilled  $\text{H}_2\text{O}$ ) for different lengths of time ( $T = 0, 3, 24, 72, 168$  h) for a total of 27 samples. Soils were destructively sampled and immediately frozen in liquid nitrogen and then stored at  $-80$  °C.

### *C Mineralization Quantification*

$\text{CO}_2$  production was measured on parallel incubations that were not destructively sampled. Soil ( $40.00 \pm 0.01$  g) was sealed in sealed 8 oz mason jars. Water was added by syringe through a septum in each lid and distributed into soil by shaking. For measurements of  $\text{CO}_2$ , incubation headspace (2 mL) was collected with a gas tight syringe and injected directly into a Hewlett Packard HP6890 (Agilent Technologies Inc., Santa Clara, CA) gas chromatograph fitted with a Hayesep DB 100/120 (1/16" x 1.5 m) column which fed into a Hayesep DB 120/140 (1/16" x 2.0 m) column leading to a pulse discharge detector (PDD). The PDD was calibrated for  $\text{CO}_2$  using a 5 point-standard curve, and a single standard was analyzed hourly thereafter to correct for instrumental drift.

### *DNA Extraction and Density Gradient SIP*

DNA was extracted from the 27 soil samples using a modified hot phenol extraction (Sambrook and Russel 2001). Briefly, two replicate extractions were conducted for each sample and then replicate DNA extracts were combined. For each extraction soil (0.5 g  $\pm$  0.001g) was mixed with TE (420  $\mu$ l 1X TE),  $\text{PO}_4^{3-}$  buffer (150  $\mu$ l 0.2 M in 1 M NaCl), and phenol:chloroform:isoamyl alcohol (600  $\mu$ l 25:24:1) in lysing matrix E tubes (MP Biomedicals) and bead beat (30s at 5.5 m/s). Tubes were incubated at 65 °C for 10 min and then spun at 10K  $\text{RCF}_{\text{avg}}$  for 5 min. Supernatant was transferred to a clean tube. Soil was extracted a second time using TE (220  $\mu$ l 1X TE),  $\text{PO}_4^{3-}$  buffer (80  $\mu$ l 0.2 M in 1 M NaCl), vortexed for 10 s, and spun at 10K  $\text{RCF}_{\text{avg}}$  for 5 min. Supernatant was combined with the first extract supernatant. Two phenol removal steps were done by adding an equal volume of chloroform:isoamyl alcohol (24:1), centrifuged 10K  $\text{RCF}_{\text{avg}}$  for 5 min, and supernatant was transferred to a new tube. RNAase (6.44  $\mu$ l, 10 mg/ml) was added and incubated at 50 °C for 10 min.  $\text{NH}_4^+$  (244  $\mu$ l 10 M) was added, incubated at 4 °C for 2 h, and spun at 16K  $\text{RCF}_{\text{avg}}$  for 15 min. Supernatant was transferred to a new tube, 670  $\mu$ l isopropal was added, and tubes were spun at 20K  $\text{RCF}_{\text{avg}}$  for 20 min. Supernatant was removed, DNA pellet was dried in a clean hood for 15 min, and 30  $\mu$ l 1X TE was added. DNA was stored at  $-80$  °C. Since soils were from the same site and homogenized, it was assumed that

extraction efficiency was similar for all samples. To ensure similar extraction efficiency between the pre-wet and wetted samples, the initial extraction buffer volume was varied slightly to result in equal (water volume):(dry soil mass) ratios for all extractions. Extraction efficiency was likely less than 100% so the abundances per g soil may be underestimated, nevertheless patterns observed for changes through time should be robust.

To separate labeled DNA from unlabeled DNA, each sample was subjected to a cesium chloride density gradient. Gradients were generated by combining 4.60 ml CsCl stock (1.885 g/ml), 0.999 ml gradient buffer (add details), 5.5 µg DNA in 50 µl 1 x TE for a final density of 1.735 g/ml. After mixing, approximately 5.2 ml of the solution was transferred to an ultracentrifuge tube (Beckman Coulter Quick-Seal, 13 mm x 51 mm) and heat-sealed. Tubes were spun in an Optima L-90K ultracentrifuge (Beckman Coulter) using a VTi65.2 rotor at 44,000 rpm (176,284 RCF<sub>avg</sub>) at 20 °C for 109 h with maximum acceleration and braking. This length of time was chosen because using these spin conditions, all DNA large enough to be detected with the downstream analyses (> 24 bp) is expected to have reached equilibrium in the density gradient under selected spin conditions.

The entire content of each ultracentrifuge tube was separated into approximately 75 fractions immediately following centrifugation using a syringe pump delivering light mineral oil at 0.25 ml/min through a 25 g needle inserted through the top of the tube to displace the gradient solution through the centrifuge tube mounted in a fraction recovery system (Beckman Coulter) with the side port needle inserted through the bottom for the outlet. Approximately 5 drops (~70 µl) were collected for each fraction. The density of each fraction was measured using an AR200 digital refractometer (Reichert) fitted with a prism covering to facilitate measurement from 5 µl volumes, as previously described (Buckley et al 2007). DNA in each fraction was purified and concentrated using glycogen/PEG precipitations followed by an ethanol washing as described previously (Neufeld et al 2007a). Each fraction was resuspended in 25 µl 1 x TE and stored at -80 °C.

All fractions were analyzed for total DNA content via picogreen fluorescence assay. The SIP fractions were binned into three groups based on density for each sample (Light = 1.680-1.7259 g/ml, Middle = 1.726-1.7419 g/ml, Heavy = 1.7420-1.775 g/ml). The binned fractions were combined and the DNA was precipitated and suspended in equal volumes. The condensed fractions were analyzed for total DNA content with picogreen.

### *Quantitative PCR*

Total bacterial 16S rRNA gene copies were quantified for the condensed fractions using bacterial 16S qPCR (EUB338/EUB518) as previously described (Fierer et al 2005). All qPCR reactions were conducted with a CFX96 Real-Time

PCR Detection System (Bio-Rad, USA). All samples were analyzed in triplicate with 20  $\mu$ l reactions containing 10  $\mu$ l Sso Fast Evagreen Supermix (Bio-Rad), 1  $\mu$ l of forward and reverse primers (10  $\mu$ M) (Sigma-Aldrich), 7  $\mu$ l PCR grade MQ-water (MP Biomedicals), and 1  $\mu$ l of template DNA (6.67 ng/  $\mu$ l). Plasmid standards were prepared by inserting a copy of purified PCR product from soil DNA into *Escherichia coli*. The *E. coli* was then cultured and the plasmids were extracted, purified, and quantified. The plasmid was verified to contain appropriate inserts by sequencing. Thermocycling parameters consisted of 3 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 53 °C, and 10 s at 60 °C, at which time fluorescence was measured. Following qPCR analysis, a melting curve was conducted from 55 to 95 °C with an increase of 0.5 °C every 5 s to ensure that results were representative of the target gene. Purity and correct targeting of amplicons were also checked by running random samples on an agarose gel to visualize qPCR products. Average PCR efficiency was 98% and average slope was -3.38, with all standard curves having  $R^2 \geq 0.99$ .

### Sequencing

DNA samples were normalized to 10 ng per reaction prior to PCR. Samples were amplified using the PCR primer pair of F515/R806 targeting the V4 region of the 16S rRNA (Caporaso et al 2011). The forward and reverse primers were modified to contain an Illumina adapter region for sequencing on the Illumina GAIIx platform. The reverse PCR primer is barcoded with a 12-base error-correcting Golay code to facilitate multiplexing. PCR reactions were performed using Takara Hot Start Ex Taq polymerase (Takara, Madison, WI), with the following thermocycling parameters: initial denaturation at 98°C for 2 minutes followed by 30 cycles of 98°C for 20 seconds, 30 seconds of annealing at 50°C and extension at 72°C for 45 seconds. Final product extension was at 72 °C for 10 minutes. Reaction primer dimers were removed from the PCR products via SPRI bead purification according to the manufacturer's protocol (AMPure XP, Beckman Coulter Genomics, Danvers, MA) before being checked for quality and average size distribution on a Bioanalyzer 2100 using a DNA 7500 chip (Agilent Technologies, Santa Clara, CA). Cleaned amplicons were quantified using Quant-It Picogreen dsDNA reagent in 10 mM Tris buffer (pH 8.0) (Life Technologies, Grand Island, NY). A pooled sample for sequencing was created by combining equimolar ratios of amplicons from the individual samples.

The pooled 16S rRNA gene library was diluted to 2nM. Following NaOH denaturation, the libraries were applied to a v4 TruSeq Paired End GA flow cell/cluster kit (Illumina Inc., San Diego, CA) at 5pM. For clustering sequencing of read 1, index read, and read 2, custom sequencing primers (Integrated DNA Technologies, Coralville, Iowa) were used at a final concentration of 500nM to spike into the primers from the cluster kit. Sequencing on the GAIIx (TruSeq SBS kit v5 reagents) was done according to manufacturer's instructions.

### *Quality Filtering of Sequence Reads, OTU Picking, and Taxonomy Assignment*

Sequences were demultiplexed then trimmed using the program Trimmomatic, where bases were trimmed using the sliding window method and sequences shorter than 75bp were removed from the dataset. The window size was set at 4 base pairs, and the average Sanger quality (Q) value threshold was set at 20 (Lohse et al 2012). The forward reads were discarded due to poor quality and only the reverse reads used for community analysis.

Sequences were clustered using UCLUST (Edgar 2010) and classified using the open-reference OTU-picking algorithm from QIIME (version 1.5-dev) at the 97% similarity level (Caporaso et al 2010b). The RDP classifier (Wang et al 2007) was trained using the Greengenes 99% similarity 16S gene rRNA database (October 2012) trimmed to the reverse section of the V4 region (*E. coli* positions 2489-4090) (Desantis et al 2006). Sequences were aligned using Pynast (Caporaso et al 2010a). Sequences were checked for chimeras using UCHIME (Edgar et al 2011), but no chimeras were found in the dataset. Pynast failures, singletons, and sequences present in only one sample were removed from the dataset. After this analysis, 11,386,219 sequences remained in the dataset.

Sequences were rarefied to 32,762 sequences for alpha and beta diversity analyses. The community distance matrix was calculated using the unifrac distance measure (Lozupone and Knight 2005).

### *Data Analysis and Statistics*

Here we use the term *growth* to indicate DNA that has been produced since the beginning of the incubation, *survival* to mean DNA that was present at the beginning of the incubation and at a given time point during the incubation, and *mortality* as DNA that was present at the beginning of the incubation but not at a subsequent time during the incubation.

Relative abundances were summarized at the order taxonomic level using QIIME. Increases in relative abundance for growing taxa were determined by first comparing <sup>18</sup>O middle density fraction samples at each time point with <sup>18</sup>O middle density fraction samples for the preceding time point using two-tailed t-tests with Benjamini-Hochberg correction. Taxa that had  $p < 0.05$  and increased in relative abundance were considered potential growers. The same analysis was performed for corresponding samples and time points using the <sup>16</sup>O control data. The potential growers determined from <sup>18</sup>O middle density fractions were then compared to taxa that were shown to increase in relative abundance in the <sup>16</sup>O control using two-tailed t-tests with Benjamini-Hochberg correction. Taxa that were shown to increase significantly ( $p < 0.05$ ) more in <sup>18</sup>O middle density fractions as compared to <sup>16</sup>O controls were considered as actively growing. All

taxa in heavy density fractions from  $^{18}\text{O}$  treatments were treated as growing since 16S rRNA gene abundance was below qPCR detection limits in the heavy density fraction for  $^{16}\text{O}$  controls. Increases in relative abundances from growth for  $^{18}\text{O}$  treatment heavy fractions at 72 h were determined by identifying taxa that had relative abundance greater than zero, and at 168 h by comparing 168 h abundances with 72 h abundances (two-tailed t-tests with Benjamini-Hochberg correction). The calculated increases in relative abundance were combined for middle and heavy fractions at 72 and 168 h by first weighting the relative abundance of each sample based on the quantity of DNA in the original density fraction, and then adding the weighted abundances.

Taxa that significantly changed between time points were mapped onto a maximum likelihood tree. One representative sequence for each taxonomic order in the dataset was selected, and the full-length Greengenes sequence associated with that OTU was used to create the tree. The tree was calculated using FastTree (version 2.1.3) using the generalized time-reversible (GTR) model and gamma settings (Price et al 2010). The tree was then visualized using the Interactive Tree of Life (Letunic and Bork 2011).

Nonmetric multidimensional scaling (NMDS) ordinations were created using the metaMDS function in the vegan package (Oksanen et al 2011), with unifrac distance matrixes as input and Bray-Curtis dissimilarity indices. The 95% confidence intervals were calculated using the ordihull function in vegan with standard deviation of points. Community differences between time points for each density group were evaluated with Analysis of Similarity (ANOSIM) using the vegan package (Oksanen et al 2011). All statistical analyses were performed within R version 2.12.2.

## Results

### *Density gradient ultracentrifugation and qPCR*

Following the wetting of dry California grassland soil with  $\text{H}_2^{18}\text{O}$ , DNA was produced that was enriched in  $^{18}\text{O}$ , resulting in a higher buoyant density for the isotopically labeled DNA as compared to the DNA that was present prior to the wet-up and to DNA from  $\text{H}_2^{16}\text{O}$  control incubations. This increase in density facilitated the separation of DNA along a cesium chloride density gradient. 16S rRNA gene abundances were quantified via qPCR for the different density fractions (light, middle, and heavy), and the quantity of  $^{18}\text{O}$ -enriched 16S rRNA genes present in the middle and heavy fractions increased through time (Figure 3-1). Light fractions were considered to contain genes from pre-wet bacteria that survived the wet-up. The middle fractions were analyzed as an overlap region potentially containing genes from both pre-wet and growing organisms, while heavy fractions were analyzed as containing genes exclusively from growing

organisms. Based on comparison between the quantities of labeled rRNA genes in the middle fractions of the  $^{18}\text{O}$  incubations versus the genes in the same density fractions for the  $^{16}\text{O}$  controls, new microbial growth was not measurable until 24 hours after the wet-up event (t-test,  $p=0.008$ ).

### *Sequencing*

Bacterial 16S rRNA genes were sequenced for all times and density fractions (light, middle, heavy) for  $^{18}\text{O}$  samples and  $^{16}\text{O}$  controls except for samples that did not contain significant gene abundance (All  $^{16}\text{O}$  heavy fractions and 3h and 24h  $^{18}\text{O}$  heavy fractions). Illumina sequencing resulted in  $11.4 \times 10^6$  sequences following quality control curation. Rarefaction of all samples to 32762 sequences resulted in 71388 total OTU detected (<97% similarity) with 3873 different OTU from 27 different phyla present with greater than 0.01% relative abundance.

### *Composition of growing bacteria*

Organisms that were actively growing were identified by presence in heavy density fractions or by greater presence in  $^{18}\text{O}$  middle density fractions as compared to  $^{16}\text{O}$  controls. Changes in relative abundance due to growth were assessed through time by combining weighted relative abundances for growing organisms in middle and heavy density fractions for a single time point and then comparing abundances at adjacent time points at the order level of taxonomic classification. A significant increase was found for bacteria within a single order within the Firmicutes phylum (Bacillales) by 3 h, indicating that rapid growth of some bacteria did occur following wet-up (Figure 3-2a, Table 3-1). After 24 h there was significant growth within two groups, the Betaproteobacteria and Bacteroidetes. However, the Betaproteobacteria were by far the dominant growers during this period. Of the growing Betaproteobacteria, members of the Burkholderiales order grew the most during this period with a 21% increase in relative abundance. By 72 h there was growth in 11 phyla with 6 phyla increasing by more than 1% relative abundance. During this period, the Proteobacteria were again the dominant growers with a 28% increase in relative abundance for the entire phylum. Betaproteobacteria and Alphaproteobacteria comprised the majority of this growth with 15% and 10% increases, respectively. Again, the members of the Burkholderiales order were the dominant order of growers in the Proteobacteria phylum with an increase in relative abundance of 12.7%. Within the Alphaproteobacteria, the members of the Sphingomonadales order increased the most (8%). Actinobacteria grew rapidly as well with a 5% increase in relative abundance. Following this period, there was a decrease in the number of growing taxa that increased significantly between 72-168 h with only members of the Acidobacteria and Actinobacteria phyla increasing by more than 1% relative abundance. To assess cumulative changes in relative abundance due to growth

for the length of the entire incubation, abundances for growing taxa at 168 h were compared with pre-wet abundances. Alphaproteobacteria and Betaproteobacteria increased in relative abundance the most (Figure 3-2b). Growth was found in 15 phyla with multiple taxa actively growing in most of these phyla (Table 3-2). For cumulative growth, the 3 orders that grew most dominantly were Sphingomonadales (Alphaproteobacteria), Burkholderiales (Betaproteobacteria), and Actinomycetales (Actinobacteria) with 15%, 13%, and 4% increases in relative abundance, respectively.

A phylogenetic tree was created using all of the orders detected to grow at each time point. Rings that surround the tree highlight the actively growing organisms that increased in relative abundance between each time point (Figure 3-3). Within this incubation period, growth was not detected for many of the pre-wet taxa, and the detected growth was distributed throughout the phylogenetic tree.

### *Composition of SIP fractions*

NMDS ordinations indicate that  $^{18}\text{O}$  light density fractions remained similar throughout the incubation, while the composition of  $^{18}\text{O}$  middle and heavy density fractions changed significantly (Figure 3-5). A small separation was observed between pre-wet light density fractions and the post wet  $^{18}\text{O}$  light density fractions (Figure 3-5a). However, the light post-wet  $^{18}\text{O}$  samples clustered together through time (Figure 3-5a) and remained clustered with  $^{16}\text{O}$  controls through time (Figure 3-5d). This indicates that after a small initial change that occurred during the first 3 h after wetup, the phylogenetic composition of the pre-wet DNA remained fairly consistent throughout the incubation since light fractions should primarily contain unlabeled DNA. There was a small shift in composition of the  $^{18}\text{O}$  middle density fractions between pre-wet and 3 h, but the bulk of compositional changes were observed after 24 h (Figure 3-5b). The 24, 72, and 168 h  $^{18}\text{O}$  middle density fractions separated significantly from the  $^{16}\text{O}$  middle controls (Figure 3-5e), indicating that differences in composition were driven by the production of  $^{18}\text{O}$ -enriched DNA from growing bacteria. When all of the  $^{18}\text{O}$  density fractions through time are ordinated together, a general pattern emerged where samples that contain predominantly pre-wet DNA remained relatively clustered, but samples that contain new growth changed in a somewhat cyclical pattern through time (Figure 3-6).

### *Net community composition*

The pre-wet soil bacterial community included bacteria from 25 different phyla and was dominated by Actinobacteria and Proteobacteria, which comprised approximately 42% and 23% of the total community, respectively (Figure 3-7). The number of phyla present in greater than 1% relative abundance were similar



at the end of the incubation (168 h), but changes in relative abundance were observed. For example, Actinobacteria decreased significantly to 26% ( $p=0.0001$ ) while Proteobacteria increased to 41% ( $p=0.0003$ ), as shown in Figure 3-7.

## Discussion

The rewetting of dry California grassland soil was followed by dynamic growth of bacteria (Figure 3-1). New growth was not readily apparent in qPCR 16S gene abundances in the middle and heavy fractions until 24 h, but sequence data indicate that some new growth occurred within 3 h. Interestingly, this rapid new growth was only observed for bacteria within the Bacillales order of the Firmicutes phylum, and resulted in close to a 5% increase in relative abundance for this phylum (Figure 3-2a, Table 3-1). It is important to note that this increase in relative abundance is found in the  $^{18}\text{O}$  samples as compared to the  $^{16}\text{O}$  controls indicating that this increase is conclusively from new growth and not an artifact caused by the decrease in abundance for other taxa. Many bacteria within the Bacillales order are spore formers, and therefore it is possible that many of these organisms were present in the pre-wet soil in such a protected state. As spores they would be protected during both the dry down and the rapid change in water potential associated with wet up. Once the water potential equilibrated, they would potentially be able to quickly germinate and begin growing. Spores can germinate on the order of minutes (Hashimoto et al 1969, Levinson and Hyatt 1956). After the initial burst of rapid growth, Firmicutes were not found to increase in relative abundance for the remainder of the incubation. This suggests that a coherent life strategy response to wet-up is observable at the phylum and orders levels where this group becomes active and replicate quickly at the first signs of a suitable and fertile environment but then replication slows or ceases once conditions became more nutrient limited. Perhaps the Bacillales play a role analogous to ephemeral plants where they spend most of their lives in a dormant state, but when conditions are correct they germinate quickly and then return to dormancy after a short growth phase. Such a life strategy would poise them to be among the first taxa to take advantage of the resource pulse that accompanies the wet-up of dry soil.

During the second time period (3-24 h), taxa within two different phyla were identified as growing. Members of the Proteobacteria (Betaproteobacteria) and Bacteroidetes increased in relative abundance. The Burkholderiales (Betaproteobacteria) were by far the dominant growers during this period with an increase of 21% in relative abundance by 24 h (Figure 3-2a, Table 3-1). Betaproteobacteria and Bacteroidetes have been shown to exhibit copiotrophic attributes with relative abundances increasing with soil C availability (Fierer et al 2007). In this experiment, the dominant growth between 3-24 h after rewetting supports the concept of a copiotrophic life strategy for members of these phyla

since available soil C can increase dramatically following the rewetting of dry soil (Fierer et al 2003, Kieft et al 1987). In support of C availability being highest during the first 24 h, net C mineralization can provide a rough estimate of available soil C (Robertson and Paul 2000), and the highest proportion of total C mineralization was found in the first 24 h (Figure 3-4). An alternative explanation could be that the phyla growing during this period follow a 'mesic' life strategy and grow fastest when soil moisture is higher. Both Bacteroidetes and Betaproteobacteria have been shown contain a high percentage of subfamilies that correlate positively with soil moisture (Cruz-Martinez et al 2012).

During the third incubation period (24-72 h) the phyla that increased in relative abundance were much more diverse in terms of measured richness with bacteria from 47 different orders in 11 phyla (Figure 3-2a, Table 3-1). Dominant phyla for the tertiary responders included Actinobacteria and Proteobacteria (Alpha, Beta, and Delta). With a similar experimental approach applied to a temperate grassland soil, Alpha, Beta, and Delta Proteobacteria were also found to be dominant growers following rewetting of dried soil in a 72 h incubation (Aanderud and Lennon 2011). This suggests that organisms in these two classes show similar life strategies even in vastly different climates. Members of rare phyla that were grouped together in the below 1% bin also grew significantly during this period.

Between 72 and 168 h, the number of taxa growing was reduced to 26 orders in 10 phyla. Of these orders, members of the Acidobacteria and Actinobacteria phyla were the dominant growers. In contrast to the copiotrophic life strategy of Betaproteobacteria and Bacteroidetes, Acidobacteria have been shown to have oligotrophic tendencies with higher abundances found in soils with low resource availability (Fierer et al 2007). Our data support that members of the Acidobacteria phylum follow an oligotrophic life strategy, since the majority of growth within this phylum was observed during this final incubation period when available C had significantly decreased (Figure 3-4). Additionally, growing members of the below 1% continued to increase in relative abundance during this period indicating that many of the rare phyla may also employ an oligotrophic growth strategy.

Results indicate that growth after wet-up followed a sequential pattern. During the first 24 h, growth was dominated exclusively by members of three phyla (Firmicutes, Betaproteobacteria, and Bacteroidetes) that could be considered copiotrophic. The Firmicutes were the first detected to grow, perhaps because they are Gram-positive spore formers and are able to recover quicker from the rapid change in water potential than the Gram-negative Betaproteobacteria and Bacteroidetes (Halverson et al 2000). Taxa that had a slower response to wet-up and did not grow until later time periods when C availability had been reduced such as Acidobacteria and Actinobacteria phyla could be considered oligotrophs. This leaves the groups growing during the middle time period (24-72 h). This

period included most of the richness for organisms identified as growing, and these organisms were found in many different phyla (Figures 3-2a, 3-3). The estimated C availability during this time period was in between the rich flush available during the first time period and the highly reduced pool by the final time period. This moderate C availability and the evidence that most of the organisms grew temporally between the copiotrophs and the oligotrophs suggest that this large group did not fit simply into the copiotrophic/oligotrophic categorization.

Ordination of the light and middle density fractions through time shows that the pre-wet (0 h) groups differ slightly from the 3 h light and middle density fractions in the presence of  $^{18}\text{O}$ -water (Figures 3-5a,b). The difference observed in the middle fraction may be partially driven by the growth of the Firmicutes taxa, but this is not the case for the difference in the light fraction. Likely, much of this change was due to mortality, which was shown to be significant with 25% of pre-wet DNA degraded during this time period (discussed in Chapter 2). After this initial change in composition, the remainder of the light fraction samples grouped together through time, indicating that the composition of the pre-wet population persisted through the rest of the incubation (Figure 3-5a). From 3h to 72h, mortality consumed an additional 29% of the starting community (Chapter 2), yet this mortality could not be seen to change the composition of the pre-wet community. This apparent resistance to compositional change could be due to a combination of several factors including: a) mostly rare taxa dying and thus not driving changes in composition, b) large variability in the organisms that die thus preventing any change in composition from being detected, c) proportional or uniform death throughout the groups of organisms that drive the sample composition, or from d) recycling of cell material by related taxa that are likely to be in close proximity resulting in equal but opposite contribution from mortality and growth on compositional changes.

Ordination of all  $^{18}\text{O}$  samples through time shows a somewhat cyclical pattern for phylogenetic composition of growing bacteria with composition at 3 hours differing slightly from the pre-wet community, differing greatly at 24 h, and then becoming progressively more similar to the pre-wet community at 72 and 168 h (Figure 3-6). This suggests a degree of community resilience to the punctuated environmental change, where composition of new growth first diverges from the original community composition but eventually results in a partial return to the original composition. The changing composition of new growth through time supports that there is a sequential growth response following wet-up.

While the composition of the growing community did begin to move back towards the pre-wet community at 72 and 168 h, net compositional changes were observed at the phylum level by the end of the incubation as compared to the pre-wet community (Figure 3-7). Actinobacteria decreased by 16% relative abundance between pre-wet and 168 h, while Proteobacteria increased by 18%. Proteobacteria were found to grow in abundance for much of the incubation likely

driving this net change in taxonomic composition. Actinobacteria were shown to grow during the later time points (72 and 168h), but increased much less in relative abundance as compared to the Proteobacteria (Figure 3-2). Members of the Actinobacteria phylum can be drought-resistant and actually grow under challenging dry conditions (Goodfellow and Williams 1983) (Zvyagintsev et al 2007). It is likely that the high abundance of Actinobacteria observed in the pre-wet samples is from growth that occurred during the spring dry down period and/or the summer dry season. This could be suggestive of a seasonal change in dominance with Proteobacteria dominating during the wet season and Actinobacteria dominating during the dry summer months. In a study of seasonal responses for bacteria in a CA grassland soil, Proteobacteria were shown to increase in abundance during the spring when soils remained moist, while Actinobacteria were suppressed during this period (Cruz-Martínez et al 2009). Further support is found in a study of community dynamics during the dry down of CA grassland soils, Actinobacteria were found to increase strongly in potential activity and abundance during dry-down (Barnard et al 2012).

It is interesting to note that the sequential growth patterns observed were found at the phyla and order level. Studying bacteria at higher levels of taxonomy than species can integrate and reduce the immense diversity commonly found in soil (Curtis and Sloan 2005, Gans 2005), perhaps leading to a more practical approach for understanding ecosystem function and fitness. Many high taxonomic levels of bacteria, even up to the phylum level, have shown evidence of ecological coherence where members of specific groups share characteristic life strategies that are distinct from other groups (Fierer et al 2007, Philippot et al 2009, Philippot et al 2010, Placella et al 2012). Due to the extensive diversity of bacteria, ecological coherence will likely not be universal within a group and may not be observed at the same level of taxonomy for all bacterial lineages, but as demonstrated here, this approach has potential to identify general unifying traits or life strategies that provide insight into connections between community composition and ecosystem functioning.

In conclusion, bacterial growth response to a punctuated environmental change followed a sequential growth pattern with different life strategies dominating during different time-periods. Importantly, these patterns were observable at a high taxonomic level suggesting ecological coherence in growth response at the phylum and order level. New growth that occurred between 3 h and 7 days became sequentially more similar to the pre-wet community through time; thus bacterial community response shows a high degree of resilience in response to the wet-up event. Net changes that did occur over the week following rewetting point towards seasonal patterns in community composition.

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**Table 3-1.** Growing taxa with significant increase in relative abundance between the different time points. Orders that increased by more than 1% are highlighted in gray.

Phylum	Class	Order	Increase in Relative Abundance (%)	Standard Deviation (%)
<b>time period 0 - 3 h</b>				
Firmicutes	Bacilli	Bacillales	4.59	0.832
<b>time period 3 - 24 h</b>				
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	0.28	0.134
Proteobacteria	Betaproteobacteria	Burkholderiales	21.14	0.917
Proteobacteria	Betaproteobacteria		1.84	0.292
<b>time period 24 - 72 h</b>				
Acidobacteria	Acidobacteria-6	CCU21	0.02	0.005
	Acidobacteria-6	iii1-15	0.92	0.190
	Acidobacteria-6		0.01	0.000
	Chloracidobacteria		0.00	0.002
	Other		0.02	0.008
Actinobacteria	Acidimicrobiia	Acidimicrobiales	0.06	0.012
	Actinobacteria	Actinomycetales	4.21	0.833
	Actinobacteria		0.01	0.002
	Thermoleophilia	Gaiellales	0.07	0.040
	Thermoleophilia	Solirubrobacterales	0.62	0.158
	Thermoleophilia		0.01	0.003
	Other		0.08	0.011
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	0.42	0.114
	Other		0.03	0.002
Chloroflexi	Ellin6529		0.00	0.002
Firmicutes			0.00	0.001
Gemmatimonadetes	Gemm-1		0.01	0.004
	Gemm-3		0.43	0.052
	Gemmatimonadetes		0.01	0.001
	Gemmatimonadetes	Gemmatimonadales	0.57	0.168
	Gemmatimonadetes	N1423WL	0.07	0.004
	Gemmatimonadetes		0.56	0.138
	Other		0.04	0.005
Other			1.05	0.089
	Other	Other	0.01	0.001

Table 3-1 continued.

Planctomycetes	Phycisphaerae		0.05	0.019
Proteobacteria	Alphaproteobacteria	Caulobacterales	0.75	0.042
	Alphaproteobacteria	Ellin329	0.18	0.024
	Alphaproteobacteria	Rhizobiales	1.41	0.228
	Alphaproteobacteria	Sphingomonadales	7.68	1.802
	Alphaproteobacteria		0.37	0.032
	Betaproteobacteria	A21b	0.01	0.001
	Betaproteobacteria	Burkholderiales	12.70	4.060
	Betaproteobacteria	Ellin6067	0.62	0.168
	Betaproteobacteria	MND1	0.02	0.009
	Betaproteobacteria	SC-I-84	0.04	0.014
	Betaproteobacteria		1.90	1.140
	Deltaproteobacteria	Bdellovibrionales	0.40	0.070
	Deltaproteobacteria	MIZ46	0.04	0.016
	Deltaproteobacteria	Myxococcales	0.62	0.147
	Deltaproteobacteria		0.21	0.040
Gammaaproteobacteria		0.25	0.034	
	Other		0.44	0.041
TM7	SC3		0.01	0.001
Verrucomicrobia	Spartobacteria	Chthoniobacterales	0.38	0.070
	Opitutae	Opitutaes	0.02	0.003
	Other		0.01	0.001

## time period 72 - 168 h

Acidobacteria	Acidobacteria-6	iii1-15	1.22	0.188
	Chloracidobacteria		0.19	0.027
	iii1-8	DS-18	0.03	0.005
	Solibacteres	Solibacterales	0.07	0.018
	Other		0.08	0.021
Actinobacteria	Acidimicrobiia	Acidimicrobiales	0.17	0.013
	Thermoleophilia	Gaiellales	0.76	0.113
	Thermoleophilia	Solirubrobacterales	1.37	0.560
	Thermoleophilia		0.03	0.006
Armatimonadetes	Armatimonadia	Armatimonadales	0.01	0.003
Bacteroidetes			0.04	0.007
Chloroflexi	Bljii12	AKYG885	0.04	0.013
	Bljii12	B07	0.02	0.009
	Chloroflexi	Roseiflexales	0.02	0.006
	Ellin6529		0.27	0.041
Gemmatimonadetes	Gemm-1		0.22	0.016

Table 3-1 continued.

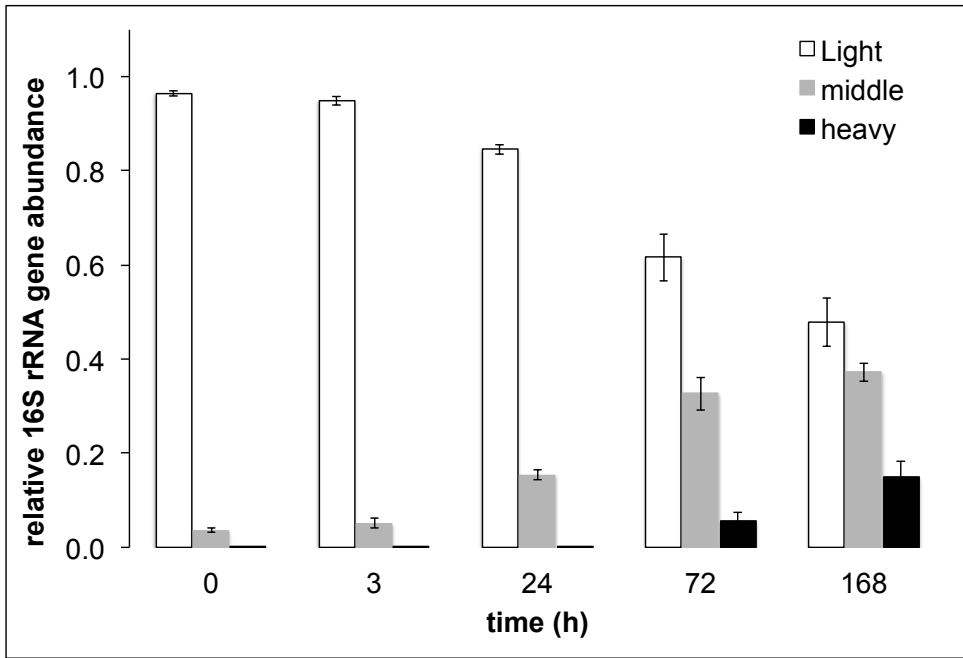
Other			0.75	0.059
Planctomycetes	Phycisphaerae		0.19	0.069
	Planctomycetia	Gemmatales	0.62	0.246
	Other		0.05	0.009
Proteobacteria	Alphaproteobacteria	Rhodospirillales	0.37	0.134
	Alphaproteobacteria		0.31	0.001
	Other		0.18	0.026
Verrucomicrobia	[Pedosphaerae]	[Pedosphaerales]	0.30	0.063
	Opitutae	Opitiales	0.05	0.014
	Other		0.01	0.004

**Table 3-2.** Growing taxa with significant increase in relative abundance between pre-wet and 168 h. Orders that increased by more than 1% are highlighted in gray.

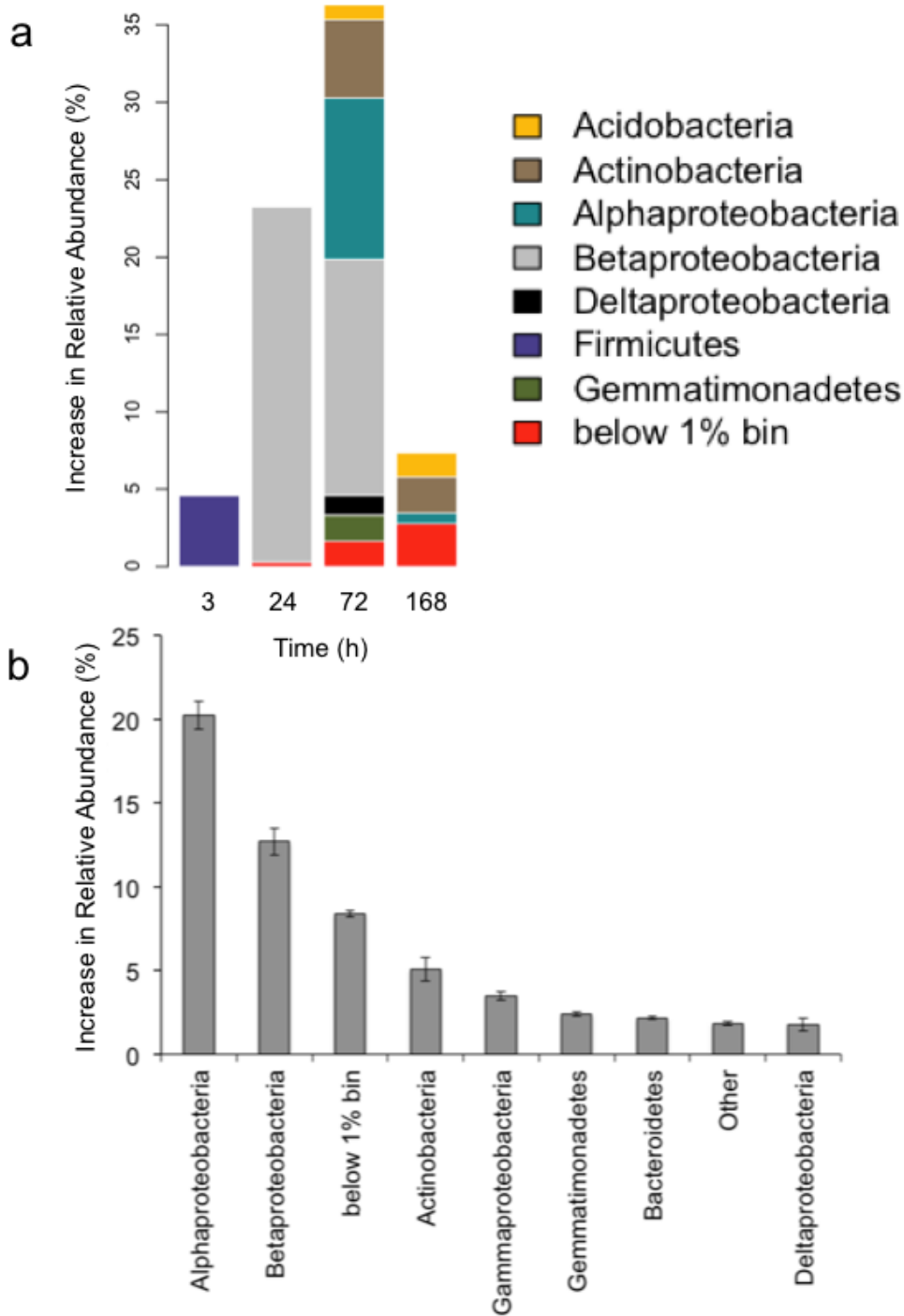
Phylum	Class	Order	Increase in Relative Abundance (%)	Standard Deviation (%)
Acidobacteria	Acidobacteria-6	iii1-15	0.64	0.063
	Acidobacteria-6		0.04	0.006
	Acidobacteria	Acidobacteriales	0.62	0.079
	Chloracidobacteria		0.14	0.021
	iii1-8	DS-18	0.03	0.001
	PAUC37f		0.00	0.002
	Solibacteres	Solibacterales	0.09	0.011
	Other		0.08	0.003
Actinobacteria	Actinobacteria	Actinomycetales	4.05	0.718
	Thermoleophilia	Solirubrobacterales	1.02	0.086
	Acidimicrobiia	Acidimicrobiales	0.17	0.013
	Actinobacteria		0.01	0.002
	MB-A2-108	0319-7L14	0.01	0.002
	Thermoleophilia	Gaiellales	0.23	0.017
	Thermoleophilia		0.02	0.002
	Other		0.14	0.007
Armatimonadetes	Fimbriimonadetes	Fimbriimonadales	0.02	0.007
	Armatimonadia	Armatimonadales	0.01	0.002
Bacteroidetes	Sphingobacteriia	Sphingobacteriales	2.15	0.092
	Other		0.08	0.006
Chlorobi	OPB56		0.01	0.002
Chloroflexi	Ellin6529		0.09	0.015
	Other		0.01	0.004
Cyanobacteria	S15B-MN24		0.01	0.002
Elusimicrobia	Elusimicrobia	Iib	0.02	0.003
Firmicutes	Bacilli	Bacillales	0.03	0.002
Gemmatimonadetes	Gemmatimonadetes	Gemmatimonadales	1.23	0.065
	Gemmatimonadetes		1.16	0.106
	Gemm-1		0.04	0.014
	Gemm-3		0.39	0.054
	Gemm-5		0.06	0.027
	Gemmatimonadetes	Ellin5290	0.01	0.001
	Other		0.06	0.022
	Other		1.84	0.110
Planctomycetes	Phycisphaerae		0.30	0.020
	Planctomycetia	Gemmatales	0.16	0.035
	Other		0.02	0.004

Table 3-2 continued.

Proteobacteria	Alphaproteobacteria	Caulobacterales	1.21	0.037
	Alphaproteobacteria	Rhizobiales	3.56	0.148
	Alphaproteobacteria	Sphingomonadales	15.47	0.832
	Betaproteobacteria	Burkholderiales	12.69	0.807
	Deltaproteobacteria	Myxococcales	1.78	0.378
	Gammaproteobacteria	Xanthomonadales	3.49	0.285
	Alphaproteobacteria	Ellin329	0.34	0.051
	Alphaproteobacteria	Rhodospirillales	0.42	0.012
	Alphaproteobacteria		0.76	0.020
	Betaproteobacteria		0.01	0.003
	Betaproteobacteria	Ellin6067	0.80	0.039
	Betaproteobacteria	MND1	0.16	0.015
	Betaproteobacteria	Rhodocyclales	0.00	0.002
	Betaproteobacteria	SC-I-84	0.16	0.008
	Deltaproteobacteria	Bdellovibrionales	0.22	0.019
	Deltaproteobacteria	MIZ46	0.14	0.021
	Deltaproteobacteria		0.32	0.047
	Gammaproteobacteria		0.23	0.006
	Other		0.60	0.037
TM7	SC3		0.04	0.005
Unclassified	Other	Other	0.01	0.002
Verrucomicrobia	Pedosphaerae	Pedosphaerales	0.24	0.058
	Spartobacteria	Chthoniobacterales	0.34	0.078
	Opitutae	Opitales	0.07	0.016
	Other		0.01	0.003

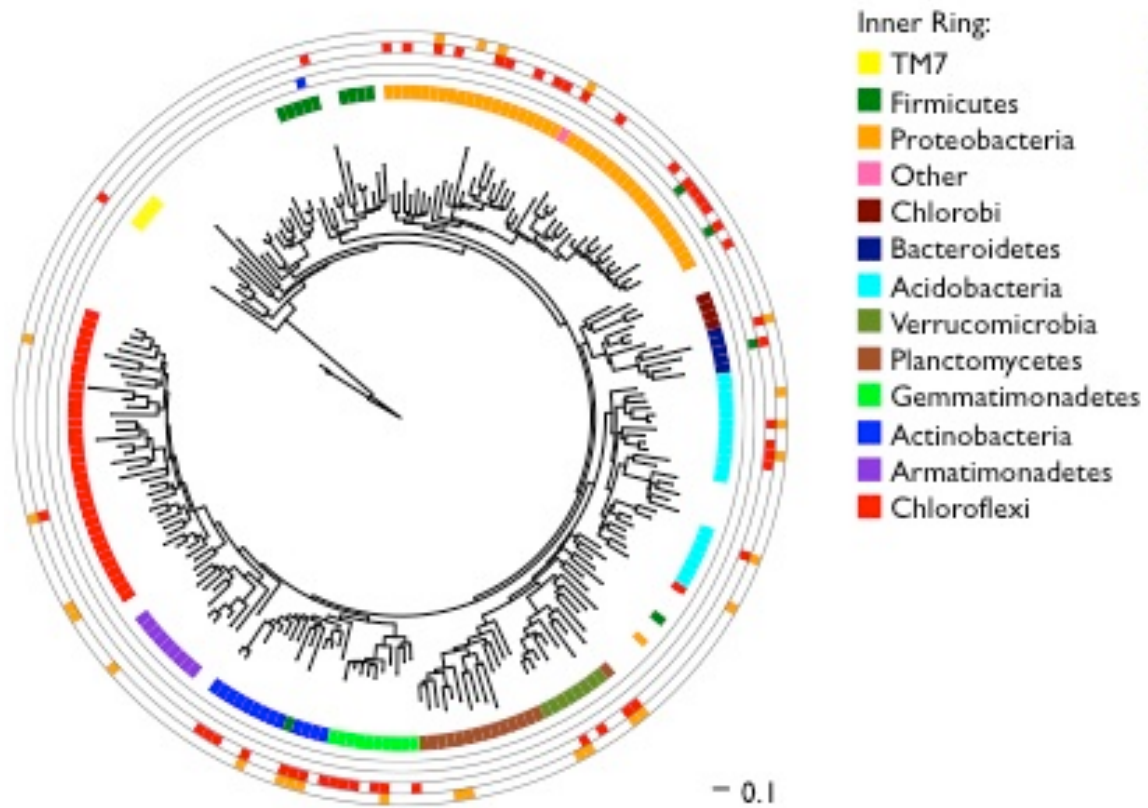


**Figure 3-1.** Bacterial 16S rRNA gene abundance, as measured by qPCR, in the condensed SIP fractions for pre-wet and  $^{18}\text{O}$  treatment. The proportions of gene abundance in  $^{16}\text{O}$  controls did not change through time and were not distinguishable from the pre-wet samples (data not shown), indicating that a shift in density was not observed.

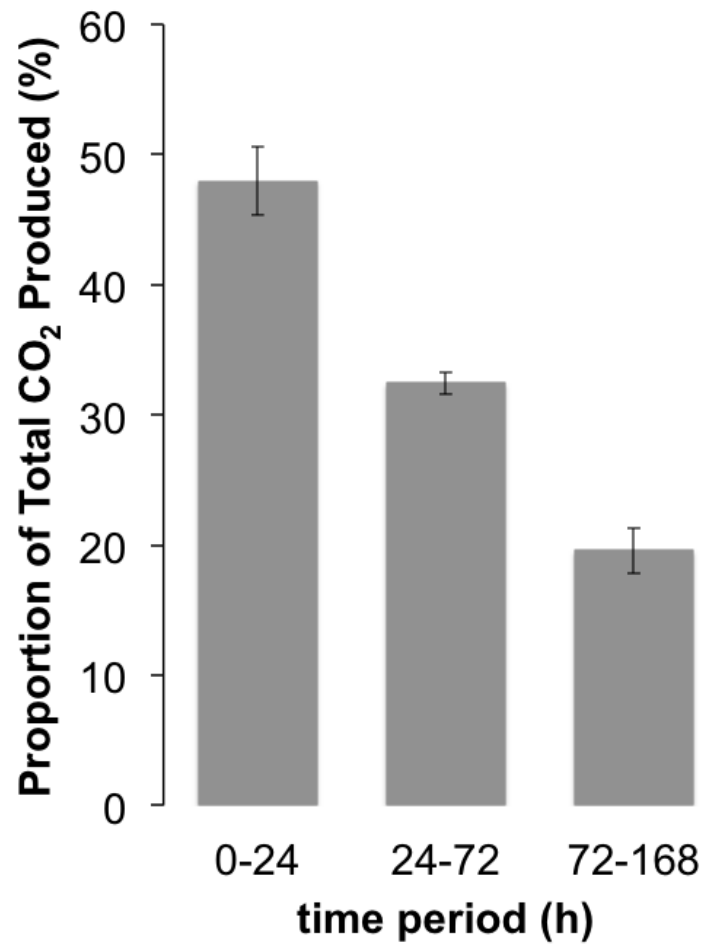


**Figure 3-2.** Growing bacteria that increased in relative abundance through time. a) The increase in relative abundance as compared to previous time point. b) The increase in relative abundance at 168 h as compared to pre-wet. Comparisons were made using 2-tailed t-test with a Benjamini Hochberg correction. Only taxa with >0.01% relative abundance were considered for comparisons and  $p < 0.05$  denoted a significant change. Data from middle and heavy fractions were combined at 72h and 96h.

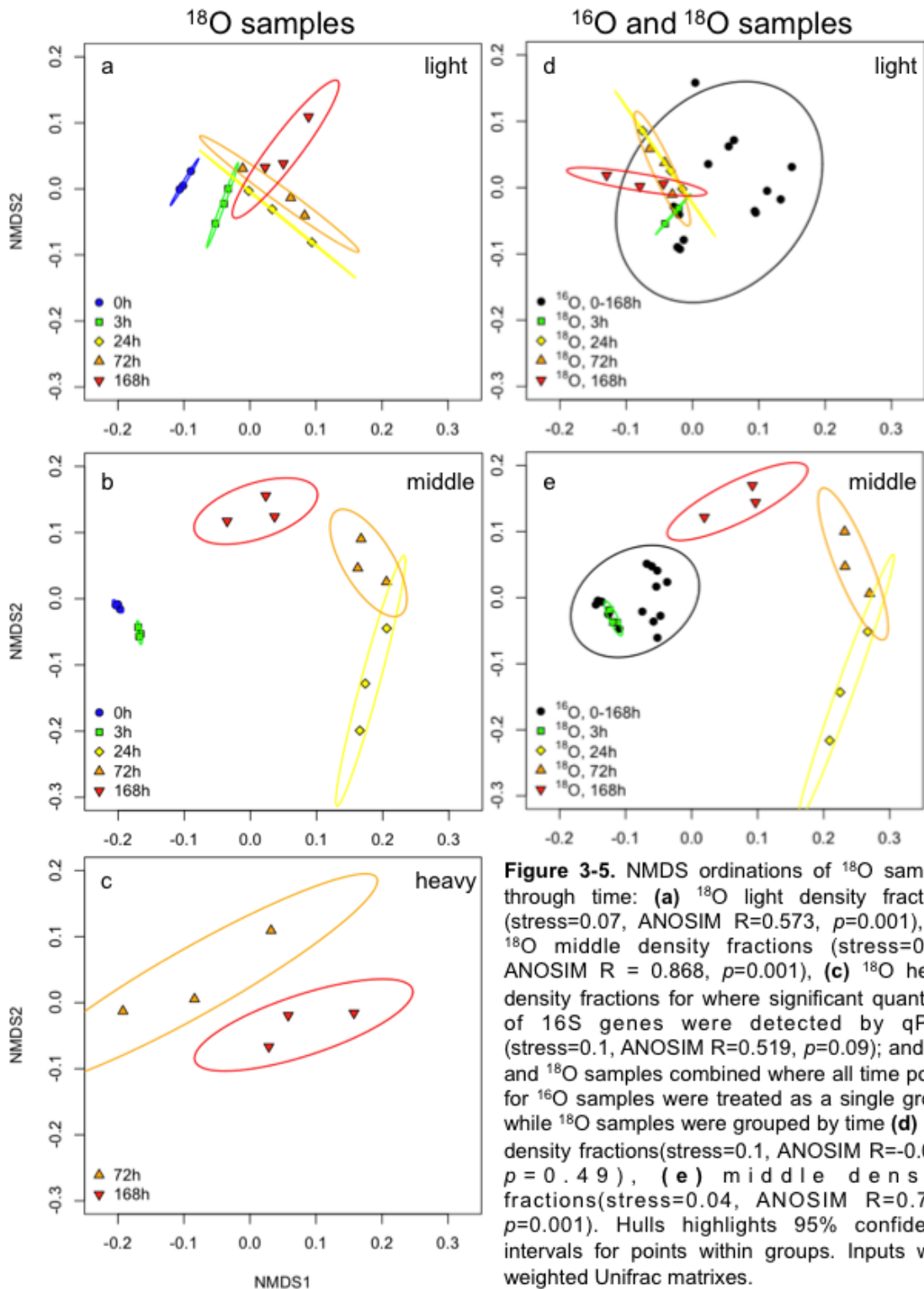




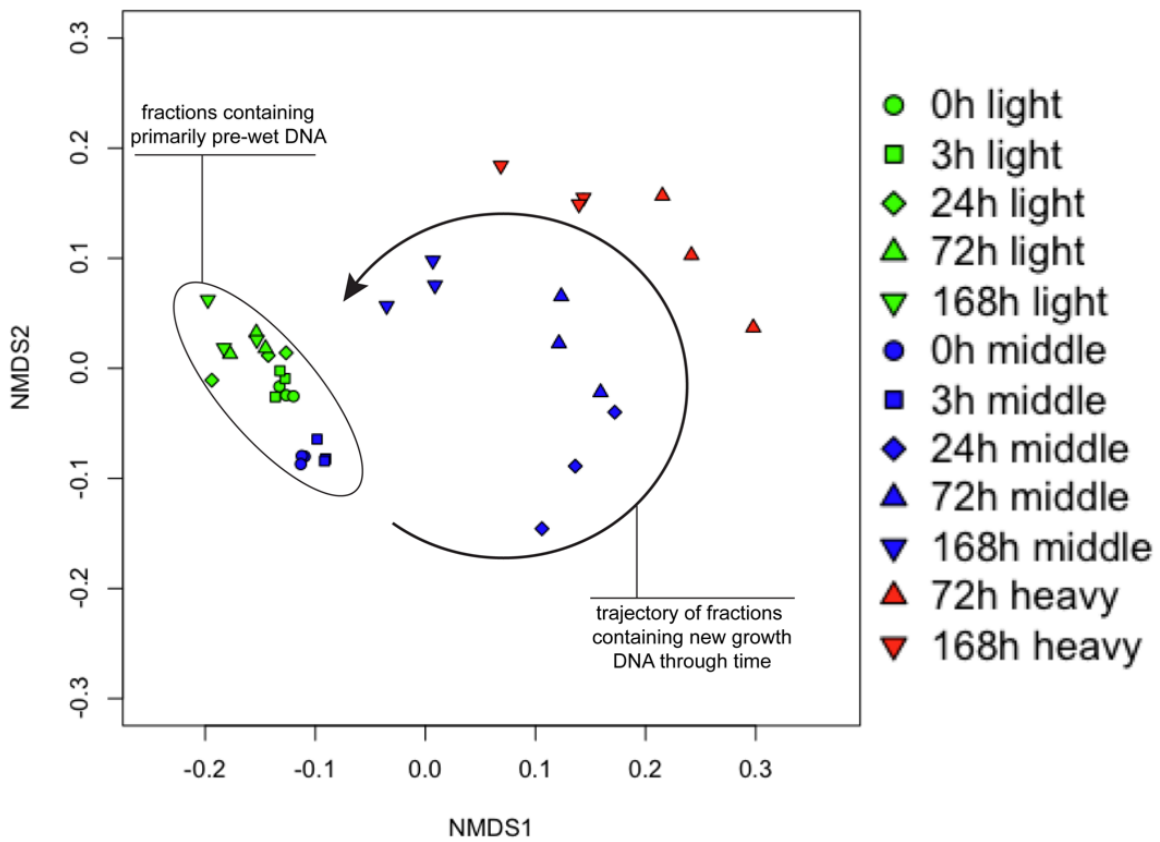
**Figure 3-3.** Phylogenetic tree showing the taxa that increased significantly in relative abundance at each time as compared to the previous time point. From the inner ring to the outer ring, rings show a) phyla, and the taxa that grew from b) 0 – 3 h, c) 3 – 24 h, d) 24 – 72 h, e) 72 – 168 h.



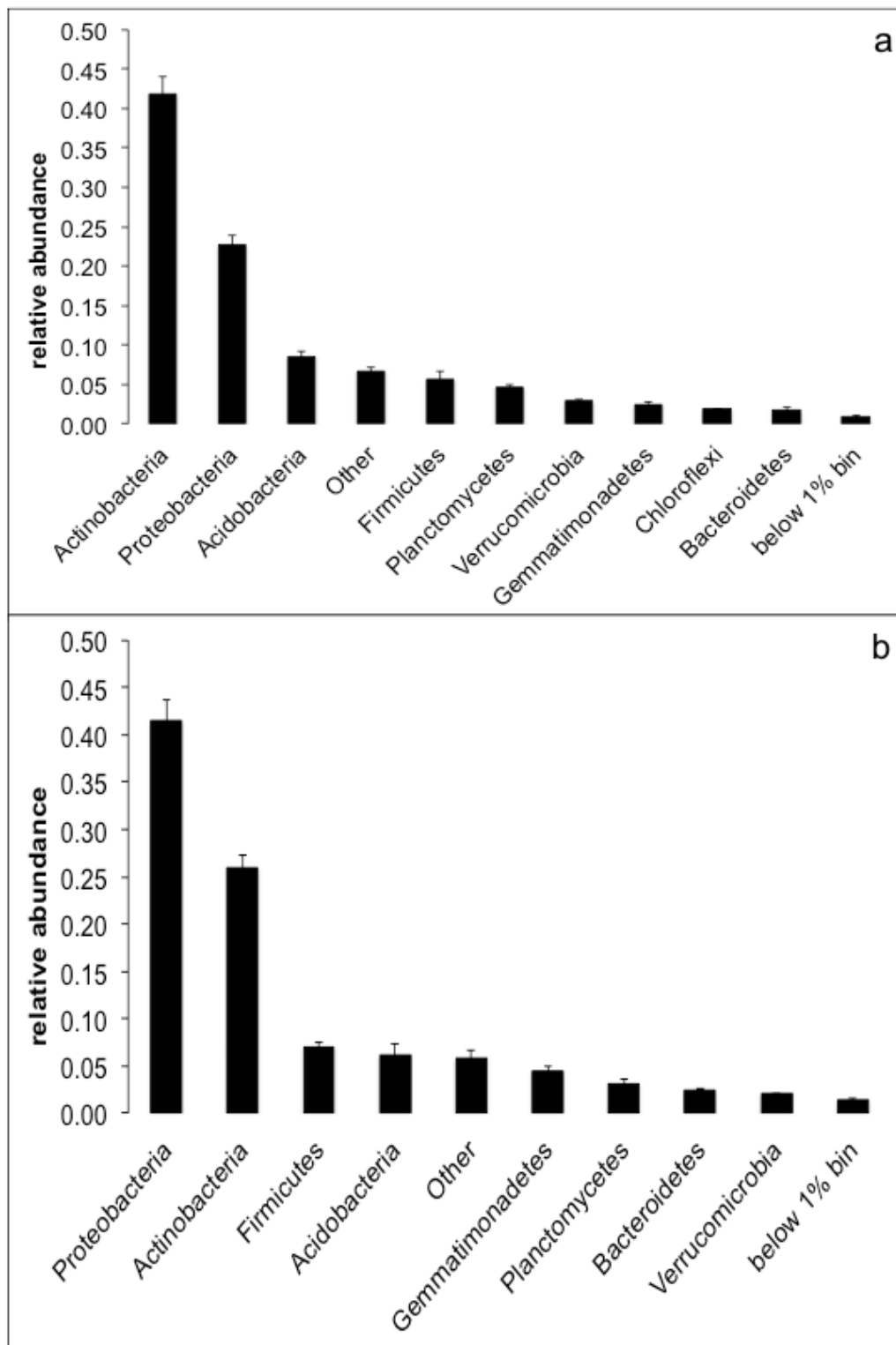
**Figure 3-4.** The proportion of cumulative CO<sub>2</sub> produced during different time periods compared to the total CO<sub>2</sub> produced during the entire incubation. Error bars show standard deviation (n=3).



**Figure 3-5.** NMDS ordinations of  $^{18}\text{O}$  samples through time: **(a)**  $^{18}\text{O}$  light density fractions (stress=0.07, ANOSIM  $R=0.573$ ,  $p=0.001$ ), **(b)**  $^{18}\text{O}$  middle density fractions (stress=0.02, ANOSIM  $R = 0.868$ ,  $p=0.001$ ), **(c)**  $^{18}\text{O}$  heavy density fractions for where significant quantities of 16S genes were detected by qPCR (stress=0.1, ANOSIM  $R=0.519$ ,  $p=0.09$ ); and  $^{16}\text{O}$  and  $^{18}\text{O}$  samples combined where all time points for  $^{16}\text{O}$  samples were treated as a single group, while  $^{18}\text{O}$  samples were grouped by time **(d)** light density fractions(stress=0.1, ANOSIM  $R=0.005$ ,  $p = 0.49$ ), **(e)** middle density fractions(stress=0.04, ANOSIM  $R=0.762$ ,  $p=0.001$ ). Hulls highlights 95% confidence intervals for points within groups. Inputs were weighted Unifrac matrixes.



**Figure 3-6.** NMDS ordination of all  $^{18}\text{O}$  samples density fractions through time (stress=0.09, ANOSIM  $R=0.847$ ,  $p<0.001$ ).



**Figure 3-7.** Relative abundance of dominant bacterial phyla in (a) pre-wet samples and (b) at the end of the incubation (168 h).

# 4

## Anaerobic Oxidation of Methane in Tropical and Boreal Soils: Ecological Significance in Terrestrial Methane Cycling

### Abstract

Anaerobic oxidation of methane (AOM) is a considerable sink for the greenhouse gas methane (CH<sub>4</sub>) in marine systems, but the importance of this process in terrestrial systems is less clear. Lowland boreal soils and wet tropical soils are two hotspots for CH<sub>4</sub> cycling, yet AOM has been essentially uncharacterized in these systems. We investigated AOM in soils from sites in Alaska and Puerto Rico. Isotope tracers were utilized *in vitro* to enable the simultaneous quantification of CH<sub>4</sub> production and consumption without use of biological inhibitors. Boreal peat soil and tropical mineral soil oxidized small but significant quantities of CH<sub>4</sub> to CO<sub>2</sub> under anoxic conditions ( $p < 0.001$ ). Potential AOM rates were  $21 \pm 2 \text{ nmol g}_{\text{dw}}^{-1} \text{ d}^{-1}$  and  $2.9 \pm 0.5 \text{ nmol g}_{\text{dw}}^{-1} \text{ d}^{-1}$  for the boreal and tropical soils, respectively. The addition of terminal electron acceptors (NO<sub>3</sub><sup>-</sup>, Fe(III), and SO<sub>4</sub><sup>2-</sup>) inhibited AOM and methanogenesis in both soils. In all incubations, CH<sub>4</sub> production occurred simultaneously with AOM, and CH<sub>4</sub> production rates were always greater than AOM rates. There was a strong correlation between the quantity of CH<sub>4</sub> produced and the amount of CH<sub>4</sub> oxidized under anoxic conditions (Alaska:  $r = 0.875$ ,  $p < 0.0001$ ; Puerto Rico:  $r = 0.817$ ,  $p < 0.0001$ ). CH<sub>4</sub> oxidation under anoxic conditions was biological and likely mediated by methanogenic archaea. While only a small percentage of the total CH<sub>4</sub> produced in these soils was oxidized under anoxic conditions (0.3% and 0.8% for Alaskan and Puerto Rican Soils), this process is important to understand since it could play a measurable role in controlling net CH<sub>4</sub> flux.

## Introduction

Microorganisms that consume methane ( $\text{CH}_4$ ) play an important role in controlling climate forcing, since  $\text{CH}_4$  is approximately 26 times more effective than carbon dioxide ( $\text{CO}_2$ ) in retaining heat in the atmosphere (Lelieveld et al 1993), and the concentration of atmospheric  $\text{CH}_4$  is currently increasing (Rigby et al 2008). Although many studies have investigated soil  $\text{CH}_4$  consumption, they have focused on aerobic  $\text{CH}_4$  consumption, overlooking the potentially important role of anaerobic  $\text{CH}_4$  consumption (anaerobic oxidation of methane; AOM). AOM is an important process in marine environments and has been demonstrated in freshwater sediment (Knittel et al 2005, Pancost et al 2000, Raghoebarsing et al 2006), but the occurrence and importance of this process in terrestrial  $\text{CH}_4$ -cycling hotspots such as wet tropical and boreal soils has not been well investigated.

$\text{CH}_4$  was believed to be biologically inert under anoxic conditions until the 1970's (Strous and Jetten 2004). It is now clear that  $\text{CH}_4$  is directly oxidized under anoxic conditions by microorganisms, and this process plays an important role in controlling oceanic  $\text{CH}_4$  efflux, with an estimated 300-380 Tg  $\text{CH}_4 \text{ yr}^{-1}$  oxidized via AOM in marine sediments (Hinrichs and Boetius 2002, Reeburgh 2007). In the absence of oxygen, microorganisms can oxidize  $\text{CH}_4$  with or without the involvement of exogenous electron acceptors. Methanogenic archaea can oxidize trace amounts of  $\text{CH}_4$  without the use of exogenous electron acceptors, while simultaneously producing  $\text{CH}_4$  (Harder 1997, Moran et al 2005, Zehnder and Brock 1979). Alternatively,  $\text{CH}_4$  oxidation can be coupled to the reduction of various electron acceptors such as sulfate, ferric iron, nitrate, and nitrite (Beal et al 2009, Boetius et al 2000, Ettwig et al 2008, Hinrichs et al 1999, Raghoebarsing et al 2006). The common occurrence and metabolic versatility of AOM in marine systems indicates that further knowledge about this process in other systems could be valuable for  $\text{CH}_4$  cycling models.

Current models for  $\text{CH}_4$  cycling in soils posit that  $\text{CH}_4$  is produced by methanogenic archaea in anoxic regions of the soil, and  $\text{CH}_4$  is consumed by methanotrophic bacteria in oxic regions. The net atmospheric  $\text{CH}_4$  flux is simply a balance of these two processes. However, the widespread incidence of AOM in marine and freshwater systems suggests that AOM could be a previously unidentified sink in wet soils where anoxic conditions are commonly found. Interestingly, chemicals that inhibit  $\text{CH}_4$  production have been found to also inhibit AOM in culture and environmental samples (Krüger et al 2003, Nauhaus et al 2005, Zehnder and Brock 1979).  $\text{CH}_4$  oxidation measurements often utilize  $\text{CH}_4$  production inhibitors to allow for "accurate" quantification of oxidation rates (von Fischer and Hedin 2002), and the concurrent inhibition of both  $\text{CH}_4$  production and AOM by these inhibitors could explain why AOM has not yet been identified as an important process in wet soils. Factors that influence AOM processes include availability of  $\text{CH}_4$ , frequency of anoxic conditions, sources of

bioavailable exogenous terminal electron acceptors, and depth of suitable soil profile (affecting the influence of AOM on an areal basis). All of these factors suggest that AOM could be an important, unrecognized CH<sub>4</sub> sink in wet boreal and tropical soils.

Boreal and tropical forests play key roles in global carbon and CH<sub>4</sub> cycling. Boreal and subarctic peat soils are estimated to store one third of the total global soil carbon pool (Gorham 1991), and produce over 10% of all CH<sub>4</sub> emissions (Houweling et al 1999). Due to the slow diffusion of oxygen in water and the water saturated conditions characteristic of peatlands, anoxic conditions are typical throughout much of the soil profile. *Smemo and Yavitt (2007)* found evidence for AOM in peatlands from the contiguous United States and Sweden and suggested that this process could be an important controller of CH<sub>4</sub> release from peat soils (*Smemo and Yavitt 2011*). Wet tropical forests have somewhat different factors driving the CH<sub>4</sub> cycle; they contain approximately 50% of Earth's live biomass and facilitate around 25% of the global gross primary productivity (*Hanson et al 2008*). Wet tropical soils can become anoxic rapidly and frequently due to high moisture content and microbial activity (*Silver et al 1999*). These soils can produce and consume CH<sub>4</sub> simultaneously (*Teh et al 2005*), and are globally significant sources of atmospheric CH<sub>4</sub>, accounting for more than 17% of all CH<sub>4</sub> emissions (*Houweling et al 1999*). Indirect evidence has suggested AOM might occur in wet tropical soils (*Pett-Ridge 2005*).

The Luquillo Experimental Forest (LEF) in Puerto Rico and the Bonanza Creek experimental forest (BNZ) in central Alaska are tropical and boreal long-term ecological research sites (LTER) with extensive biogeochemical and climate data (*Pett-Ridge et al 2006*, *Silver et al 1999*, *Teh et al 2005*, *Teh et al 2006*, *Templer et al 2008*, *Turetsky et al 2008*). We chose to investigate AOM in LEF and BNZ soils because CH<sub>4</sub> cycling is dynamic in both soils [*Teh et al., 2005*; *Turetsky et al., 2008*], high rates of Fe(III) respiration have been found in LEF (*Chacon et al 2006*, *Dubinsky et al 2010*, *Peretyazhko and Sposito 2005*), and high rates of denitrification have been found in BNZ (*Petersen et al 2012*). Our initial working hypotheses were 1) AOM is coupled to Fe(III) reduction in the LEF tropical soils and 2) AOM is coupled to NO<sub>3</sub><sup>-</sup> reduction in the BNZ boreal soils. Additionally, nitrate and nitrite are found in LEF soils along with microbes capable of conducting denitrification (*Pett-Ridge et al 2006*), and deposition of sulfate from ocean water could provide a relatively constant source for this electron acceptor (*Asbury et al 1994*). AOM in LEF and BNZ soils could potentially be coupled to one or multiple terminal electron accepting processes. Given the dynamic CH<sub>4</sub> cycling and diverse anaerobic respiration capacity found in LEF and BNZ, they are ideal model systems to conduct preliminary investigations of the importance of AOM in tropical and boreal soils.

In this study, we conducted laboratory experiments with soils collected from Puerto Rico and Alaska to determine: 1) whether AOM occurs in tropical and



boreal soils, 2) potential rates of AOM in these systems, and 3) how the process is affected by the addition of different terminal electron acceptors. We used a stable isotope tracer approach to characterize CH<sub>4</sub> oxidation in order to circumvent using biological inhibitors to determine gross oxidation rates.

## Methods

### *Study Sites*

Boreal soils were collected from an area classified as continental boreal in the Bonanza Creek LTER in interior Alaska, USA (64.8°N, 147.9°W). The collection site is located in the Tanana River floodplain and is characterized as a rich fen. Vegetation is predominantly a diverse ground layer mixture of *Sphagnum*, brown moss, *Equisetum*, and *Carex* species. Tropical soils were collected from Luquillo Experimental Forest LTER in northeastern Puerto Rico (18.3°N, 65.8°S). The tropical site is characterized as a lower montane wet tropical forest and vegetation is dominated by *Cyrilla racemiflora* L. Additional site and soil characteristics for both sites are given in Table 4-1.

### *Sample collection*

In Alaska, soil cores were taken every 10 m along a 40 m transect for a total of 5 cores with each core representing a replicate. Cores were collected using a 2" diameter corer, and the living vegetation top layer was removed from each core. Cores were immediately sealed in sterile plastic bags to minimize exposure to atmospheric O<sub>2</sub>. Cores were approximately 15 cm deep (measured from below the vegetative layer), and the core tops were approximately equal with the level of the water table surface. Groundwater that filled in the space created via core removal was collected. Cores and groundwater were kept on ice and transported to UC Berkeley where they were stored at 4 °C until incubation experiments were initiated (approximately 1 week later).

In Puerto Rico, soil cores were collected using a spade, and cores were taken every 10 m along a 30 m transect for a total of 4 cores with each core representing a replicate. Living plants and intact litter was removed prior to core collection, and cores were approximately 10 cm deep. Cores were immediately sealed in sterile plastic bags to minimize exposure to atmospheric O<sub>2</sub>. Cores were transported, in a room temperature cooler to minimize temperature changes, to UC Berkeley where they were stored at room temperature until incubation experiments were initiated (approximately 3 days later).

### *Soil characteristics*

Soil water content was determined by drying soil to constant weight at 105 °C. Loss on ignition was measured on oven-dried soil after 6 h at 550 °C. Soil pH was determined using a soil pH electrode on a 1:1 soil/water slurry.

### *Incubation setup*

To determine the potential for AOM in boreal and tropical soils, anoxic laboratory incubations were conducted using Alaskan and Puerto Rican soils. A difficulty with accurately quantifying AOM rates in environments that also produce CH<sub>4</sub> is that chemicals such as bromoethane sulfonate or methyl fluoride that inhibit CH<sub>4</sub> production have also been found to inhibit AOM (Krüger et al 2003, Nauhaus et al 2005). Therefore, oxidation rates were determined using isotopic tracers in an approach similar to that developed by Moran et al. (2005). For both Alaskan and Puerto Rican samples, homogenized soils (10 g) were loaded into 100 ml serum bottles, and bottles were sealed with gas impermeable blue butyl rubber septa (Bellco, Vineland, NJ, USA). Visible roots and leaves were removed from Puerto Rican soil prior to loading. Anoxic conditions were created using a standard alternating vacuum and pressure approach (e.g. (Balch and Wolfe 1976, Zehnder and Brock 1980)). Briefly, bottle headspaces were evacuated by vacuuming to -51 kPa and then over-pressured with N<sub>2</sub> to 40 kPa, and this process was repeated 10 times. On final filling, headspace was only filled to 13 kPa with N<sub>2</sub>. The headspaces were then flushed with N<sub>2</sub>/CO<sub>2</sub> (98/2%) at 135 kPa for 5 minutes through the septa using 23-gauge needles as input and exhaust ports. This final flushing was done to set the starting concentration of CO<sub>2</sub> to 2% to facilitate isotope ratio analyses at early time points and to minimize recycling of <sup>13</sup>CO<sub>2</sub> back into <sup>13</sup>CH<sub>4</sub>. Headspace pressure was equilibrated to 10 kPa using a N<sub>2</sub> flushed syringe. Sterile anoxic groundwater (40 ml) was added to Alaskan soil incubation bottles, and sterile anoxic de-ionized water (10 ml) was added to Puerto Rican soil incubation bottles using a N<sub>2</sub> flushed syringe. Different volumes of water were used for the different soils because the soil bulk densities varied greatly between sites and different volumes were needed to minimally saturate the soils. Prior to additions, oxygen was removed from Alaskan groundwater and de-ionized water, using standard anaerobic culturing methods (e.g. (Balch et al 1979, Hungate 1950)), by bringing each to a boil while stirring under a steady stream of N<sub>2</sub> and then quickly cooling in ice water while under a continuous flow of N<sub>2</sub>; water bottles were sealed with blue butyl rubber septa and autoclaved at 121 °C for 20 min. High concentrations of CH<sub>4</sub> were added to incubation bottles to ensure that CH<sub>4</sub> was not a limiting factor for CH<sub>4</sub> oxidation. Labeled CH<sub>4</sub> (6 ml 99 atom% <sup>13</sup>C-CH<sub>4</sub>, Sigma Aldrich - Isotec, CAS 6532-48-5) was added to <sup>13</sup>C-incubations to allow for quantification of the oxidation of <sup>13</sup>CH<sub>4</sub> to <sup>13</sup>CO<sub>2</sub> over time. Natural abundance CH<sub>4</sub> (6 ml, Scotty analyzed Gas, Lot# 833605L) was added to parallel controls. All anoxic incubations were pre-incubated for 24 hours

before T=0 headspace samples were taken in order to allow any remaining oxygen to be consumed. Oxic incubations were set up in a similar manner except headspaces were not flushed with O<sub>2</sub>-free gas and incubation water did not have O<sub>2</sub> removed. Sodium 2-bromoethane sulfonate (BES), a specific inhibitor of methyl-coenzyme M reductase (Chidthaisong and Conrad 2000, Zehnder and Brock 1979), was added (10 mM) to additional anoxic Puerto Rican soil incubations to test for effects on CH<sub>4</sub> production and AOM. BES treatments were not conducted with Alaskan soil incubations because this treatment was added to the experimental design after the Alaskan incubations and analyses were already completed. Heat killed controls were autoclaved twice, 24 h apart, at 121 °C for 20 min. All treatments were incubated in the dark at 25°C.

Headspace samples (2 ml) were collected at multiple time points from incubation bottles through the septa, via needle and syringe, and transferred to 10 ml serum bottles sealed with thick black butyl rubber stoppers (Geo-Microbial Technologies, Inc.). All transfers, additions, and headspace samplings were done using gas tight syringes fitted with stopcocks that were first degassed with N<sub>2</sub> to avoid O<sub>2</sub> contamination. For all work, N<sub>2</sub> and N<sub>2</sub>/CO<sub>2</sub> were first passed through hot copper fillings (~350 °C) to remove traces of O<sub>2</sub> (e.g. (Balch et al 1979, Macy et al 1972)), and the copper fillings were reduced daily using H<sub>2</sub>.

### *Electron Acceptor Addition Experiments*

To determine the impact of different electron acceptors on AOM rates, anoxic soil samples were amended with NO<sub>3</sub><sup>-</sup>, Fe(III), and SO<sub>4</sub><sup>2-</sup>. NO<sub>3</sub><sup>-</sup> was added as NaNO<sub>3</sub> (5 mM), SO<sub>4</sub><sup>2-</sup> was added as Na<sub>2</sub>SO<sub>4</sub> (5 mM), and Fe(III) was added as soluble Fe(III) nitrilotriacetic acid (NTA) (10 mM) or poorly crystalline Fe(OH)<sub>3</sub> (10 mM). Fe(III)NTA was synthesized by combining equal quantities of Na<sub>2</sub>NTA and FeCl<sub>3</sub>•6H<sub>2</sub>O. While stirring, pH was brought to 6.9 using NaOH. Fe(OH)<sub>3</sub> was synthesized as previously described by slowly neutralizing a solution of FeCl<sub>3</sub>•6H<sub>2</sub>O with NaOH, and the minerals were repeatedly washed with de-ionized water to remove chloride ions (Lovley and Phillips 1986). Electron acceptor stock solutions were made anoxic by sparging with 2 streams (one at the bottom of the solution and one in the headspace just above the solution) of O<sub>2</sub>-free N<sub>2</sub> at 135 kPa for 20 min; bottles were sealed with blue butyl rubber septa and NO<sub>3</sub><sup>-</sup> and SO<sub>4</sub><sup>2-</sup> solutions were autoclaved at 121 °C for 20 min. Either <sup>13</sup>CH<sub>4</sub> or natural abundance CH<sub>4</sub> was added to soil incubation bottles as described above.

### *Gas and Isotope Analysis*

CH<sub>4</sub> and CO<sub>2</sub> concentrations were measured using a Hewlett Packard HP6890 (Agilent Technologies Inc., Santa Clara, CA) gas chromatograph fitted with a

Hayesep DB 100/120 (1/16" x 1.5 m) column which fed into a Hayesep DB 120/140 (1/16" x 2.0 m) column leading to a pulse discharge detector (PDD) for CO<sub>2</sub> quantification or into a Restek GS-Q plot (0.32 mm x 30 m) column leading to a flame ionization detector (FID) for CH<sub>4</sub> analysis. Carbon dioxide isotope analysis was conducted using a Europa 20-20 continuous flow isotope ratio mass spectrometer, IRMS (PDZ Europa, Cheshire, UK). Headspace sample CO<sub>2</sub> was dried by passing through a water trap (magnesium perchlorate), pre-concentrated by liquid N<sub>2</sub> cold trapping, and separated from contaminating gasses with two sequential chromatography columns (1.5 m x 1 mm Hayesep DB column, 30 m x 0.53 mm Restek GS-Q plot column, 0 °C) before entering the IRMS. The isotopic composition of C in CO<sub>2</sub> was determined in per mil values relative to the Vienna Pee Dee Belemnite (PDB) standard.

### Data Analysis and Rate calculations

The quantity of CH<sub>4</sub> oxidized at each time point was calculated using the following equation (Moran et al 2005),

$$n_{oxCH_4} = (F_{T_{CO_2}} n_{T_{CO_2}} - F_{T_{CO_2}^*} n_{T_{CO_2}^*}) \frac{n_{i_{CH_4}} + n_{b_{CH_4}}}{F_{i_{CH_4}} n_{i_{CH_4}} + F_{b_{CH_4}} n_{b_{CH_4}}}$$

where  $n_{oxCH_4}$  is moles of CH<sub>4</sub> oxidized,  $F_{T_{CO_2}}$  is the fractional abundance of carbon isotopes in total CO<sub>2</sub> from <sup>13</sup>CH<sub>4</sub> incubations,  $n_{T_{CO_2}}$  is total moles of CO<sub>2</sub> in headspace of <sup>13</sup>CH<sub>4</sub> incubations,  $F_{T_{CO_2}^*}$  is fractional abundance of carbon isotopes in total CO<sub>2</sub> from <sup>12</sup>CH<sub>4</sub> control incubations,  $n_{T_{CO_2}^*}$  is total moles of CO<sub>2</sub> in headspace of <sup>12</sup>CH<sub>4</sub> control incubations,  $F_{i_{CH_4}}$  is fractional abundance of carbon isotopes in initial CH<sub>4</sub>,  $n_{i_{CH_4}}$  is initial moles of CH<sub>4</sub> in headspace,  $F_{b_{CH_4}}$  is fractional abundance of carbon isotopes in biogenic CH<sub>4</sub>, and  $n_{b_{CH_4}}$  is biogenic moles of CH<sub>4</sub> in headspace.  $F_{T_{CO_2}}$  and  $F_{T_{CO_2}^*}$  values were determined via IRMS, and  $n_{T_{CO_2}}$ ,  $n_{T_{CO_2}^*}$ ,  $n_{i_{CH_4}}$  values were determined via GC-PDD/FID as described above. Values for  $n_{b_{CH_4}}$  were calculated as the difference between total moles of CH<sub>4</sub> at a given time point and  $n_{i_{CH_4}}$ . We assumed biogenic CH<sub>4</sub> produced during the incubation had a  $F_{b_{CH_4}} = 0.0106$  ( $\delta^{13}C = -50\text{‰}$ ). Biogenic CH<sub>4</sub> can be produced with a range  $\delta^{13}C$  values, but because the incubation systems were strongly labeled, realistic variations in  $F_{b_{CH_4}}$  would result in negligible changes to the results.  $F_{i_{CH_4}}$  was 0.99, since all initial CH<sub>4</sub> was from a know source. Since the CH<sub>4</sub> was strongly labeled, fractionation in the CH<sub>4</sub> oxidation process will not significantly affect measurements. Calculations assume CH<sub>4</sub> is oxidized to CO<sub>2</sub>.

AOM rates were calculated by difference in CH<sub>4</sub> oxidized using the first time point where the quantity of CH<sub>4</sub> oxidized was significant (ANOVA with Tukey's Honestly Significantly Difference test,  $p < 0.01$ ) and the immediately preceding time point (times 10 d and 15 d for Alaska and 45 d and 60 d for Puerto Rico). Aerobic CH<sub>4</sub> oxidation rates were calculated by difference in total CH<sub>4</sub> headspace concentration in the headspace at the beginning and end of the incubation period (Alaska = 5 d, Puerto Rico = 15 d). We used a two-point calculation because we were only concerned with average rates over a period comparable to the AOM rate calculations for each site.

### *Statistics*

In anoxic incubations,  $\delta^{13}\text{C-CO}_2$  values for <sup>13</sup>CH<sub>4</sub> and <sup>12</sup>CH<sub>4</sub> treatments were compared using ANOVA tests. In electron acceptor treatments, a post hoc Tukey's analysis was used to determine the earliest time points where significant CH<sub>4</sub> oxidation was observed. Correlation analyses were used to compare quantities of CH<sub>4</sub> consumed and produced. All statistical analyses were performed with R version 2.12.2.

### **Results**

Soils collected from a boreal peatland (BNZ-LTER, Alaska) and a tropical forest (LEF-LTER, Puerto Rico) exhibited the ability to oxidize CH<sub>4</sub> when incubated under anoxic conditions (Figure 4-1). Headspace CO<sub>2</sub> became significantly more <sup>13</sup>C enriched in incubations of Alaskan and Puerto Rican soils treated with <sup>13</sup>CH<sub>4</sub> as compared to controls (ANOVA of  $\delta^{13}\text{C-CO}_2$  for <sup>13</sup>CH<sub>4</sub> incubations versus <sup>12</sup>CH<sub>4</sub> controls; Alaska T = 71 d,  $p < 0.001$ , and Puerto Rico T = 77 d,  $p = 0.001$ ), indicating that CH<sub>4</sub> was indeed oxidized to CO<sub>2</sub>. Enrichment in <sup>13</sup>C-CO<sub>2</sub> was not observed in heat-killed soils incubated with <sup>13</sup>CH<sub>4</sub>.

To identify terminal electron acceptors coupled to AOM, soils were amended with NO<sub>3</sub><sup>-</sup>, Fe(III), or SO<sub>4</sub><sup>2-</sup>. However, none of the electron acceptors stimulated CH<sub>4</sub> oxidation as compared to the control treatment (Figure 4-2). In fact, all added terminal electron acceptors inhibited AOM either temporarily or throughout the entire incubation. The earliest time point where AOM was significant was in control treatments for both soils (Alaska T = 15; Puerto Rico T = 60 d, ANOVA with Tukey's HSD test,  $p < 0.05$ ). Total AOM was significantly greater in Alaskan control soils as compared to electron acceptor treatments (Alaska T=71 d,  $p < 0.001$ ). For Puerto Rican incubations, the mean total CH<sub>4</sub> oxidation was also greatest in the control treatment, but it was not significantly greater due to high variance in measurements (Figure 4-2). In both soils, the addition of poorly crystalline iron (Fe(OH)<sub>3</sub>) had a similar impact on AOM as the addition of soluble iron (Fe(III)NTA), data not shown.

The stable isotope tracer approach employed to quantify CH<sub>4</sub> oxidation, also facilitated the simultaneous quantification of CH<sub>4</sub> production. Net oxidation of CH<sub>4</sub> was not observed under anoxic conditions for either soil regardless of treatment. CH<sub>4</sub> was oxidized only when CH<sub>4</sub> was simultaneously being produced (Figure 4-2). For both soils, there was a strong correlation between the quantity of CH<sub>4</sub> that was oxidized and the CH<sub>4</sub> that was produced for a given time point, regardless of electron acceptor treatment (Figure 4-3); Pearson's product-moment correlation values ( $r$ ) were 0.875 ( $p < 0.001$ ) and 0.817 ( $p < 0.001$ ) for Alaska and Puerto Rico, respectively. Approximately, 0.3% and 0.8% of the total CH<sub>4</sub> produced was concurrently oxidized in boreal and tropical soils, respectively. Interestingly, the methanogenesis inhibitor, bromoethane sulfonate (BES), inhibited both CH<sub>4</sub> oxidation and CH<sub>4</sub> production under anoxic conditions in Puerto Rican soil (Table 4-2).

In order to put AOM rates into ecological perspective as a controller of CH<sub>4</sub> efflux to the atmosphere, CH<sub>4</sub> oxidation was also quantified under oxic conditions. Both Puerto Rican and Alaskan soils oxidized net quantities of CH<sub>4</sub> under oxic conditions. Potential aerobic CH<sub>4</sub> oxidation rates were two orders of magnitude greater than potential AOM rates (Table 4-2).

## Discussion

Isotopic evidence indicates that both tropical (LEF-LTER, Puerto Rico) and boreal soils (BNZ-LTER, Alaska) have the ability to oxidize CH<sub>4</sub> under anoxic conditions. In laboratory incubations, headspace <sup>13</sup>CO<sub>2</sub> became significantly more enriched in live soil incubations with <sup>13</sup>CH<sub>4</sub> added as compared to the <sup>12</sup>CH<sub>4</sub> live controls (Figure 4-1), verifying that the majority of <sup>13</sup>CO<sub>2</sub> enrichment in the <sup>13</sup>CH<sub>4</sub> incubations was from <sup>13</sup>CH<sub>4</sub> oxidation. <sup>13</sup>CO<sub>2</sub> was not enriched in <sup>13</sup>CH<sub>4</sub> heat-killed controls. These results demonstrate that AOM does occur in Alaskan boreal and Puerto Rican tropical soils and it is biologically mediated. Due to the low rates and temporal variability of this process, such demonstration required the precision of *in vitro* isotope tracer experiments to be successful. To the best of our knowledge, this is the first reported evidence indicating this process can occur in these two distinct terrestrial systems.

In both tropical and boreal soils, AOM was only observed when CH<sub>4</sub> production was also occurring (Figure 4-2), and there is a strong correlation between rates of the two processes (Figure 4-3) indicating that the oxidation of CH<sub>4</sub> under anoxic conditions is related to methanogenic activity and not to nitrate, ferric iron, or sulfate reduction. In order to gain energy from AOM, the oxidation of CH<sub>4</sub> must be coupled to the reduction of an electron acceptor. However, methanogens are able to oxidize CH<sub>4</sub> without the addition of exogenous electron acceptors (Harder 1997, Moran et al 2005, Moran et al 2007, Zehnder and Brock 1979). In our

anoxic incubations, approximately 0.3% and 0.8% of CH<sub>4</sub> produced was oxidized by Alaskan and Puerto Rican soils, respectively. These proportions of CH<sub>4</sub> oxidized to CH<sub>4</sub> produced are consistent with proportions observed for pure cultures of methanogens. *Zehnder and Brock* [1979] observed that up to 0.32% of produced CH<sub>4</sub> was oxidized to CO<sub>2</sub> by methanogenic archaea, and Moran et al. [2005] found a pure culture methanogen to oxidize 0.36% of CH<sub>4</sub> produced. Results indicate that the AOM observed in Puerto Rican and Alaskan soils is likely mediated by methanogens. This conclusion is supported for the Puerto Rican soils by the simultaneous inhibition of CH<sub>4</sub> production and oxidation by the addition of BES, a specific inhibitor of methyl-coenzyme M reductase (Chidthaisong and Conrad 2000). Concurrent inhibition of CH<sub>4</sub> production and CH<sub>4</sub> oxidation by BES was also observed in pure cultures of methanogens by *Zehnder and Brock* [1979].

Further evidence for the link between AOM and methanogenesis can be seen in the electron acceptor treatment data. The addition of terminal electron acceptors delayed the observable AOM in all treatments, and in one case (SO<sub>4</sub><sup>-2</sup> in Alaska) inhibited AOM for the entire incubation (Figure 4-2). After some time, many of the electron acceptor incubations began to conduct AOM. Similarly to AOM, methanogenesis was delayed by the addition of electron acceptors (Figure 4-2). The only apparent observed uncoupling of CH<sub>4</sub> production and AOM was in the Alaskan SO<sub>4</sub><sup>-2</sup> treatment. In this case, AOM was completely inhibited throughout the incubation, but CH<sub>4</sub> production was only partially suppressed. *Zehnder and Brock* [1980] observed a similar pattern where Na<sub>2</sub>SO<sub>4</sub> addition to lake sediment inhibited AOM to a greater extent than methanogenesis. It is likely that the SO<sub>4</sub><sup>-2</sup> initially inhibited AOM and methanogenesis through redox inhibition (i.e. substrate competition), however in the Alaskan soils, sulfide poisoning probably became a factor as sulfate reduction proceeded. This suggests that environmental factors can significantly affect the relative impact of AOM on net CH<sub>4</sub> emissions. Puerto Rican soils differ from the Alaskan soils in that they contain much bioavailable iron, which can be reduced to ferrous iron under anoxic conditions (Dubinsky et al 2010), and ferrous iron is known to react with sulfide forming a precipitant and effectively minimizing its inhibition on these microbial processes. This could explain why CH<sub>4</sub> production and AOM both proceeded by the final time point in SO<sub>4</sub><sup>-2</sup> treated Puerto Rican incubations.

The conclusion that CH<sub>4</sub> transformation in this system is likely mediated by methanogens raises the question of whether this transformation should be considered an oxidation reaction or simply the result of isotope exchange. Under anoxic conditions, it is well known that consumption of C<sub>1</sub> compounds is not necessarily associated with its use as an energy source for growth (Zeikus 1983). This non-fortuitous consumption is the result of exchange reactions in which the end product from an energy producing reaction is actively transformed back into the initial substrate through reverse enzymatic processes. Therefore, the movement of <sup>13</sup>C from CH<sub>4</sub> to CO<sub>2</sub> could be the result of “exchange

reactions". In our experiment, the transformation of CH<sub>4</sub> to CO<sub>2</sub> only proceeded when CH<sub>4</sub> was being produced, and therefore it is likely that our observed enrichment in <sup>13</sup>C-CO<sub>2</sub> was from biologically mediated isotope exchange that was catalyzed by active methanogenic enzymes. This said, we believe it to be useful to refer to this exchange reaction as oxidation of CH<sub>4</sub> or AOM when discussing ecosystem processes.

Reverse methanogenesis involves the movement of carbon from the CH<sub>4</sub> pool to the CO<sub>2</sub> pool, and enzymes mediate this transformation. Since the carbon is in fact being transformed from a -4 oxidation state in CH<sub>4</sub> to a +4 state in CO<sub>2</sub>, this transformation is a biologically mediated oxidation of CH<sub>4</sub>. This transformation is distinct from a spontaneous abiotic isotope exchange reaction that can occur rapidly without the aid of a biological catalyst.

Knowledge of gross reactions (e.g. forward and reverse reactions) is important in order to gain a comprehensive understanding of the combination of reactions that affect net ecosystem measurements. It is not necessary for a process to be fortuitous for the mediating microorganisms in order for it to be an influential ecosystem process. If CH<sub>4</sub> production and consumption are mediated by the same enzymes, it may be quite useful to consider the reverse reaction as a gross oxidation reaction distinct from the forward gross production reaction; this is particularly true since there appear to be factors that impact the forward (CH<sub>4</sub> production) and reverse (CH<sub>4</sub> oxidation) reactions differentially. For example, evidence in our experiment and others indicates that the reverse reaction is more sensitive to sulfate addition than the forward reaction (Figure 4-2 and (Zehnder and Brock 1980)). Another example can be found in the differential sensitivity of the forward and reverse reactions to BES addition in environmental samples (Zehnder and Brock 1980).

Finally, it is important to acknowledge that we do not know unequivocally that the observed consumption of CH<sub>4</sub> was simply the result of reverse reactions. While evidence in our system strongly indicates that the observed oxidation of CH<sub>4</sub> is a reverse reaction mediated by methanogens, the heterogeneity in soil leaves the possibility open that a separate and distinct oxidation of CH<sub>4</sub> was occurring and was coupled to the reduction of an unidentified electron acceptor. For example, AOM can be coupled with other inorganic electron acceptors such as manganese [Beal *et al.*, 2009], and it is thermodynamically possible for AOM to be coupled to the reduction of some organic molecules such as fumarate or methanol [Thauer and Shima, 2008].

Few studies have examined AOM in soil systems, so there is little relevant data to compare and contrast with our own. To our knowledge, only indirect evidence has been published for AOM in mineral soils. Several studies have indicated that AOM may occur in flooded rice paddy soils (Miura *et al* 1992, Murase and Kimura 1994a, Murase and Kimura 1994b, Murase and Kimura 1994c), but the



experimental designs were not necessarily strictly anoxic and AOM in these soils has yet to be verified with a direct approach. However, AOM has been directly observed in peat soils. Similarly to our study, *Smemo and Yavitt (2007)* conducted *in vitro* incubations with peat soils from locations in the United States and Sweden and concluded that this process could help to reduce CH<sub>4</sub> emissions in their sites. They observed average rates of AOM in soil from a fen in New York of 1469 nmol CH<sub>4</sub> g<sub>dw</sub><sup>-1</sup> d<sup>-1</sup>, which is much greater than the average rates we observed in Alaskan peat soils (Table 4-2). They also observed that rate of AOM was dependent on CH<sub>4</sub> concentration, indicating that the ratio of CH<sub>4</sub> oxidized to produced is dependent on substrate concentration in addition to other environmental factors. Similarly to our study, *Smemo and Yavitt (2007)* treated soils with different electron acceptors (NO<sub>3</sub><sup>-</sup>, Fe(III), or SO<sub>4</sub><sup>2-</sup>), but observed no stimulation of AOM and therefore were unable to identify a terminal electron acceptor coupled to the process. Further investigations are needed to explain the difference in AOM rates between studies and to characterize the ubiquity of AOM in peat and mineral soils.

Despite the slow AOM rates found in marine systems, this process consumes a substantial quantity of CH<sub>4</sub>, equivalent to 60-75% of the total global annual flux into the atmosphere (Hinrichs and Boetius 2002, Reeburgh 2007). The potential AOM rates measured in both Puerto Rican and Alaskan soils are slow as compared to the aerobic CH<sub>4</sub> rates in these soils (Table 4-2), but it is important to note that AOM rates in our soils do fall into the range of AOM rates measured in marine sediments (Knittel and Boetius 2009). The AOM measured in our soils is likely to be mechanistically different than AOM found in marine systems with the AOM in marine systems representing a net sink for methane, however it can still be insightful to compare gross rates of methane consumption in our soils with those found in marine systems. Acknowledging that CH<sub>4</sub> oxidation rates measured in this experiment should only be considered potential rates due to the experimental design, we limit comparisons to aquatic experiments that were similar in design where incubations were conducted *in vitro* with slurried sediments at constant temperatures. Sediment from a marine gas hydrate area coupled AOM to sulfate reduction with an *in vitro* AOM rate of approximately 1.5 μmol CH<sub>4</sub> g<sub>dw</sub><sup>-1</sup> d<sup>-1</sup> (Nauhaus et al 2002), which is two to three orders of magnitude greater than potential AOM rates in boreal and tropical soils, respectively. In a survey of different marine sediments, AOM rates as high as 8 μmol CH<sub>4</sub> g<sub>dw</sub><sup>-1</sup> d<sup>-1</sup> were found in sediments that were also from gas hydrate areas, but lower rates (0.01 – 0.05 μmol CH<sub>4</sub> g<sub>dw</sub><sup>-1</sup> d<sup>-1</sup>) that were more analogous to our soil rates were measured in lower carbon sediments from shallow intertidal zones (Kruger et al 2005). Not surprisingly, *Nauhaus et al. [2002]* and *Kruger et al. [2005]* found AOM rates were sensitive to both temperature and CH<sub>4</sub> partial pressure, thus it would be valuable for future experiments to characterize how AOM in boreal and tropical soils are affected by these environmental variables.

Aerobic CH<sub>4</sub> oxidation in dry forest and grassland soils is an important sink for atmospheric CH<sub>4</sub>, responsible for approximately 5 – 10% of the total global CH<sub>4</sub> sink (Cicerone and Oremland 1988, Crutzen 1991). In these dominantly oxic soil systems, methanotrophic bacteria are responsible for CH<sub>4</sub> consumption, and are often operating at fairly low rates. However, since this slow oxidation is prevalent over a large land area, it consumes significant quantities of CH<sub>4</sub>. Although conditions and oxidation mechanisms differ markedly in dry soils as compared to our study sites, it is interesting to note that aerobic dry soil CH<sub>4</sub> oxidation rates can be in the same order of magnitude as our measured AOM rates (Bull et al 2000). Although slow, AOM in boreal and tropical soils also has the potential to be found in a sizeable land area since humid tropical soils and boreal and subarctic peatlands cover approximately 7% ( $1.1 \times 10^9$  ha) and 2% ( $3.46 \times 10^8$  ha) of Earth's total land area, respectively (Achard 2002, Vitt 2006). The considerable depth of many peatlands also increases the potential area for AOM, since peatlands can be found with peat depths up to 8 m and have an average depth of 2.9 m (Martini et al 2006). Since only a small portion of the CH<sub>4</sub> produced is oxidized anaerobically in our soils, AOM appears to play a relatively minor role in controlling CH<sub>4</sub> efflux at the ecosystem level. However, if this process is ubiquitous in wet tropical and peatland soils, it could play a non-trivial role in CH<sub>4</sub> cycling at the global level. Future studies should investigate how cosmopolitan AOM is within wet tropical and boreal soils, investigate how rates change with depth, and integrate AOM rates for peatlands at the landscape scale.

### *Conclusion*

This experiment provides an important first glimpse into the capacity for CH<sub>4</sub> to be oxidized under anoxic conditions in wet tropical soil and boreal peat soil. Three lines of evidence indicate that anaerobic oxidation of CH<sub>4</sub> is likely mediated by methanogenic archaea in both soils: temporal trends of CH<sub>4</sub> production and oxidation, correlation analyses, and the ratio of CH<sub>4</sub> oxidation to production. Although anaerobic oxidation rates were much slower than aerobic CH<sub>4</sub> oxidation rates in both tested soil systems, it is important to recognize that this process could play a measurable role in controlling net CH<sub>4</sub> flux. Clearly, the total amount of CH<sub>4</sub> oxidized by this process will be a function of land area, but the importance of AOM in reducing net CH<sub>4</sub> flux from these soils will be dependent on environmental factors that affect the proportion of CH<sub>4</sub> oxidized relative to CH<sub>4</sub> produced. The *in situ* rates of AOM could be significantly different than rates calculated in this study, but we conclusively determined that soils from diverse and significant study sites had the potential to perform this process. Much is still unknown about AOM in these soils and the need for additional investigation is compelling.

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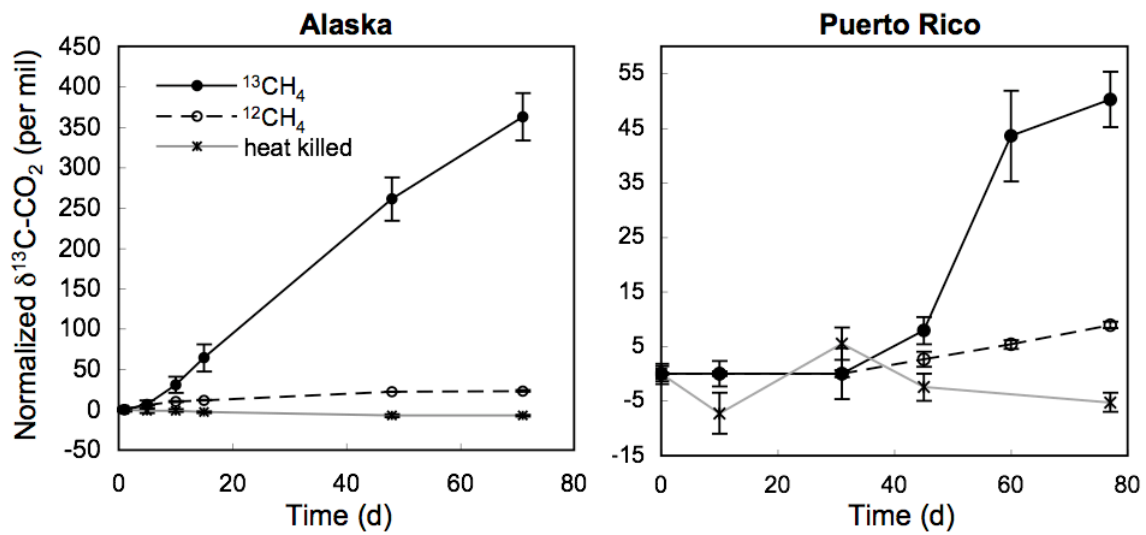
**Table 4-1.** Site and Soil Characteristics for BNZ and LEF. Standard error in parentheses (n = 5).

	Alaska	Puerto Rico
Ecosystem	Boreal	Subtropical
MAP (mm)	269 <sup>a</sup>	4500 <sup>b</sup>
MAT (°C)	-2.9 <sup>a</sup>	20 <sup>b</sup>
Soil moisture (%)	1100 (74)	111 (4)
Soil Organic Matter (%LOI)	81 (5)	19.5 (0.7)
pH	5.1 (0.2)	4.8 (0.1)

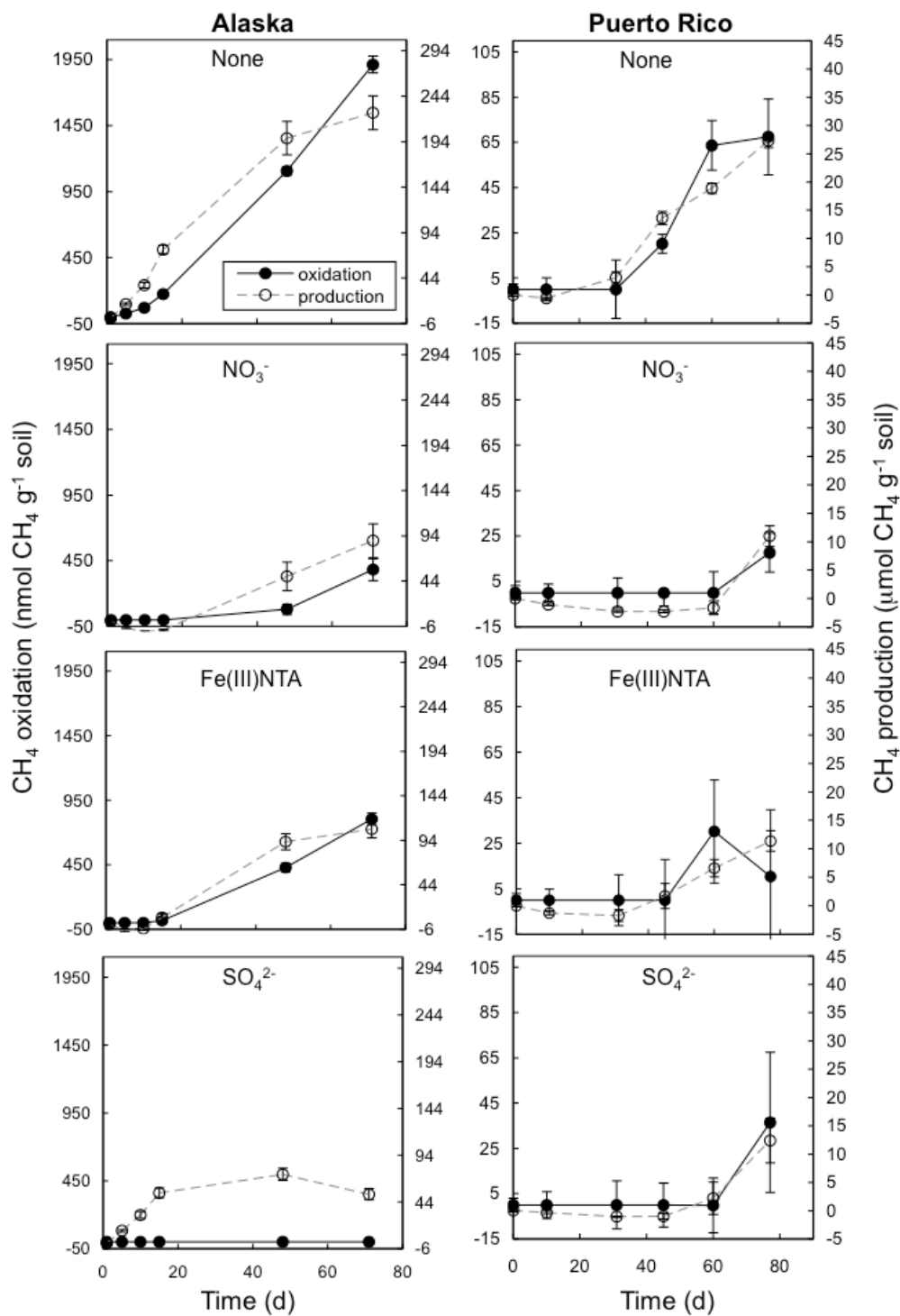
<sup>a</sup>(Turetsky et al 2008), <sup>b</sup>(Silver et al 1999)

**Table 4-2.** Rates of CH<sub>4</sub> oxidation and production. Standard error in parentheses.

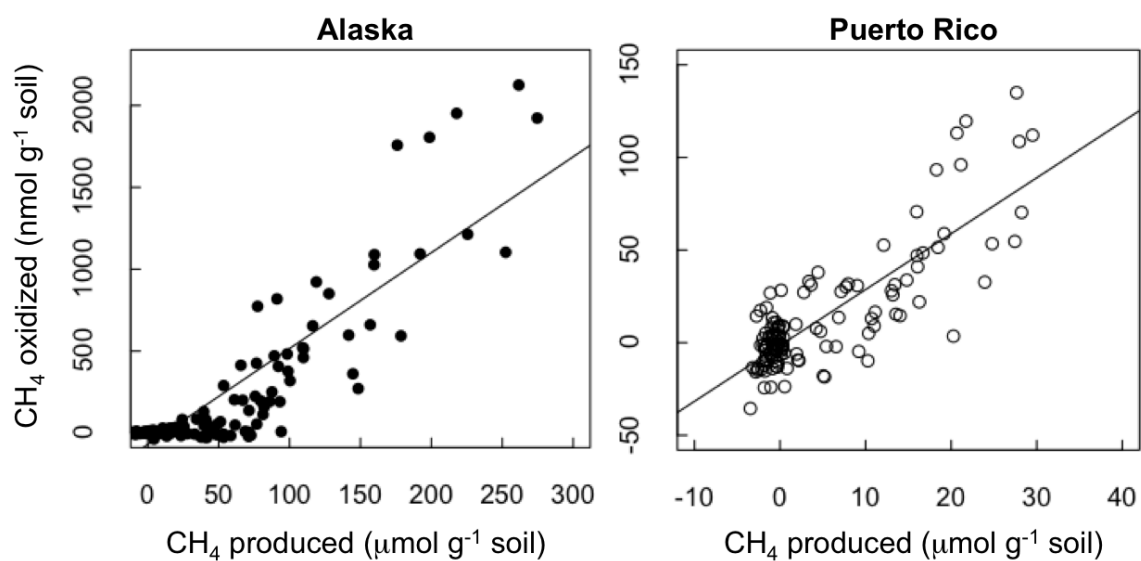
Treatment	CH <sub>4</sub> Oxidized (nmol g <sub>dw</sub> <sup>-1</sup> d <sup>-1</sup> )	CH <sub>4</sub> produced (nmol g <sub>dw</sub> <sup>-1</sup> d <sup>-1</sup> )
Alaska		
Oxic	1255 (58)	n/a
Anoxic	21 (2)	7858 (423)
Puerto Rico		
Oxic	533 (83)	n/a
Anoxic	2.9 (0.5)	351 (69)
Anoxic + BES	0 (15)	13 (70)



**Figure 4-1.** Changes in  $^{13}\text{C-CO}_2$  over time in anoxic incubations for Alaskan and Puerto Rican soils. Error bars indicate standard error (Alaska;  $n = 5$ , Puerto Rico;  $n = 4$ ). Values were normalized to T=0 for all treatments.



**Figure 4-2.** CH<sub>4</sub> oxidation and production over time in different electron acceptor treatments for Alaskan and Puerto Rican soils. Error bars indicate standard error (Alaska;  $n = 5$ , Puerto Rico;  $n = 4$ ). Values were normalized to  $T=0$  for all treatments.



**Figure 4-3.** Relationship between the quantities of CH<sub>4</sub> oxidized versus produced for a given sample under anoxic conditions. Graphs include all data for all measured time points and electron acceptor additions. Linear trendlines are shown for both data sets (Alaska;  $r = 0.875$ ,  $p < 0.001$ , Puerto Rico;  $r = 0.817$ ,  $p < 0.001$ ).

# 5

## Epilogue

Until recently, microorganisms have unequivocally dominated land and ocean biogeochemical dynamics in addition to production and consumption of greenhouse gasses. However, human activity is now also a major driver of greenhouse gas production, nutrient mobilization, and environmental change. Microbes continue to play essential roles in Earth's functions, but for the first time in history, human activities are driving changes in the environment that are affecting microbial activities. Unfortunately, we are greatly lacking the necessary knowledge and theory needed to effectively predict changes in microbial activities and integrate such information into biogeochemical and climatic models.

Rapidly evolving technology has resulted in the commonplace application of culture-independent molecular analyses to investigate microbial life in soils. This shift has resulted in an overwhelming quantity of data being collected. The acquisition of molecular data from microbial communities is outpacing the development of necessary theoretical framework for useful application. Fortunately, the discovery of general groups of molecular targets has likely reached an apex with several groups of target molecules of high interest for microbial ecology: phospholipid fatty acids, DNA, RNA, and proteins. Analysis of these groups has been successfully applied to provide useful information about microbes in the environment such as community composition, diversity, and potential activity, but many fundamental questions remain unanswered. What are identifiable features of molecular data that can capture soil community dynamics in a manner such as to inform predictive models? Can we apply molecular data to estimate gross process rates that are difficult to measure directly, and can this approach be applied to accurately predict realized net process rates?

Ideally, information obtained from molecular analysis of soil samples would inform about ecosystem functions and this information would be scalable in time and space. With this in mind, Chapter 1 highlights the importance of clearly defining the characteristics of target molecules to allow for meaningful characterization of microbial assemblages. As outlined for rRNA, it is likely that mRNA, DNA, and proteins all integrate information about past, present, and future activities and/or potentials. But how can we tease apart this information? For each group of macromolecules, it is important to carefully identify generalizable characteristics in order to increase understanding of the meaningful window of temporal information reflected in sample measurements.

Improving our knowledge of features such as stability and turnover for each of these groups in soil will certainly provide important clues towards this goal.

Chapters 2, 3, and 4 highlight the importance of understanding the gross changes in both community and process data. Chapters 2 and 3 focus on bacterial community and population dynamics, demonstrating that even though net changes in community abundance and composition may be fairly stable, this stability may in fact be the product of rapid gross changes, and these changes can be tightly linked with ecosystem processes. Chapter 4 focuses on microbial process measurements and demonstrates that controlling processes may not be apparent from net process measurements alone.

Increasing our understanding of the underlying gross dynamics for both population and process measurements should lead to stronger linkages between microbial community information and ecosystem characteristics. Information about gross dynamics for RNA, DNA, and proteins in soils under different conditions will provide critical information regarding timing of production, production rates, stability, and turnover for each of these groups. This knowledge will expand our view of microbial life in soil and will drive soil microbial ecology one step closer towards robust predictive capabilities.