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Controls on Bacterial Functional Trait Expression with Carbon and Nutrient Cycling
Consequences

DISSERTATION

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in Ecology and Evolutionary Biology

by

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DEDICATION

To

my parents, Robert and Ethel Ramin

in recognition of their lifetime of hard work
and sacrifice

Be faithful in small things because it is in them that your strength lies.

Mother Teresa

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ABSTRACT OF THE DISSERTATION

Controls on Bacterial Functional Trait Expression with Carbon and Nutrient Cycling
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By

Kelly I. Ramin

Doctor of Philosophy in Ecology and Evolutionary Biology

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Professor Steven D. Allison, Chair

Bacteria are key drivers of global biogeochemical cycling. By producing extracellular enzymes
they are able to turnover organic matter so that it can be assimilated for new biomass or energy
production. However, enzymes are metabolically expensive, resulting in decreased fitness with
their production. How bacteria allocate their resources determines not only the rate at which
carbon is cycled, but its fate in an ecosystem. Therefore, the central role that enzymes play in this
process makes them an important research target. Understanding the mechanisms that impact the
expression of extracellular enzymes is fundamental to predicting ecosystem carbon and nutrient
cycling. In this dissertation, we began with a literature review that explores the role that
microbial interactions play in soil carbon cycling dynamics through phenotypic plasticity and
evolutionary processes. Next, to determine the physiological limitation of extracellular enzyme
production and how bacteria allocate resources, we assessed the trade-offs between extracellular
enzyme production and growth rate relative to resources across several strains of bacteria.
Bacteria do trade off these traits, though selectively, and with a stronger effect in nutrient-poor
media. Finally, we examined the functional capacity in a river system to determine if enzyme

expression is determined by community structure, nutrients, or environmental parameters. Enzyme expression was most strongly determined by biofilm productivity, and had no relationship to alpha or beta diversity. While there is some evidence to suggest a phylogenetic signal of extracellular enzyme production, from the empirical results presented here, bacterial expression of extracellular enzymes, in both populations and communities, appears to be predominantly determined by nutrient parameters.

INTRODUCTION

With the genomics revolution arrived an explosion of research in microbial ecology. Previously, there had been limited acknowledgement within the field of biology of the central role that bacteria hold in all processes fundamental to life. Bacteria had been predominantly considered in their capacity as disease agents. The levels of diversity in the microbial world were unfathomable, as most bacteria derived from the natural environment were unable to be cultured and identification via microscopy was only successful at a very coarse taxonomic scale. As new technology emerged that enabled a more profound assessment of bacterial diversity, ubiquity, and functional potential, the scope of bacteria's role in our world has become realized (Stahl and Tiedje 2002). Bacteria form symbiotic relationships with humans, impacting several factors associated with our well-being, from our immune system to our mood (Rhee et al. 2009). They fix nitrogen for plants and help ward of herbivores (Sugio et al. 2018). Bacteria are involved in hyena social interactions, allowing them to mark their territory (Theis et al. 2013). Perhaps most importantly, though, bacteria drive biogeochemical cycles (Falkowski et al. 2008).

One of the key processes in biogeochemical cycling by bacteria is the degradation of dead plant and animal matter, with its subsequent conversion to new biomass or CO_2. To effect this process, bacteria produce extracellular enzymes that break down macromolecules so that the resulting monomers of this reaction can be assimilated for biomass or energy production. However, production of extracellular enzymes is metabolically expensive, leading to a reduction in fitness. Bacteria have evolved various adaptations that allow them to be prudently responsive to the surrounding milieu of conditions created through interactions with other organisms, available nutrients, and the physical habitat. This functional plasticity alters the rate and fate of

organic matter turnover. Therefore, it is critically important to study the determinants of bacterial enzyme expression.

There are many perspectives from which we can consider bacterial function. At a fundamental level, the expression of functional traits is dependent upon bacterial physiology and genetic potential. Multiple studies have analyzed the functional potential present within communities via metagenomics. In addition, studies have been performed that characterize population and community-level functional plasticity of carbon-cycling traits such as enzyme production relative to specific parameters, including pH, interacting species, and nutrients. The aim of this dissertation is to assess the impact of bacterial physiology, community structure, and nutrients on extracellular enzyme production.

In the first chapter, the role of ecological interactions combined with evolutionary processes is explored in relation to phenotypic expression of carbon-cycling traits. This review focuses on how the fate of carbon in the soil ecosystem is dictated by bacterial resource allocation patterns. The hypotheses that were addressed were: 1) changes in allocation patterns resulting from interactions will lead to both ecological and evolutionary consequences for carbon cycling, and 2) microbial interactions have important ramifications for community structure that feed into associated community functioning. The relative amount of carbon that is converted to forms that are stored in the soil versus what is respired into the atmosphere is influenced by population-level regulation of enzyme expression, biofilm production, and dormancy.   Evolution also plays a large part in resource allocation by altering functional potential of carbon-cycling genes through horizontal gene transfer and selective gene loss. Finally, allocation of resources is

influenced by interactions with other taxa in a community, with outcomes dependent upon resource and spatial niche overlap.

The second chapter investigates bacterial functional expression as constrained by physiology and life history strategy. The growth and enzyme production of thirty-eight strains of bacteria isolated from plant litter in a Mediterranean grassland ecosystem were measured to assess 1) if bacteria trade-off between production of extracellular enzymes and investment in growth across broad taxonomic groups, 2) whether there is a weaker tradeoff in these traits in high resource conditions, and 3) if the relative investment of enzyme activity to growth rate is phylogenetically conserved. The results suggest that for some strains, the ratio of extracellular enzyme activity to growth rate is conserved in high resource conditions. The tradeoff is stronger for some strains in low resource conditions, though some strains eliminated enzyme production all together. There was mixed evidence on whether the relative investment ratio of enzyme production and growth rate was phylogenetically conserved, dependent upon the statistical test performed. The expressed phenotypes were categorized according to their expression profiles to create life history strategy classifications that incorporate this trait.

Finally, the last chapter analyzes the bacterial community structure, and how resources affect community abundance and enzyme activity in a highly impacted river biofilm that flows through a Mediterranean chaparral ecosystem. The hypotheses tested were 1) biofilm community composition will remain stable across reaches due to stable dissolved oxygen levels within the system, 2) bacterial abundance will be driven by biofilm organic carbon and nutrients, and 3) extracellular enzyme activity will be determined by the stoichiometric needs of the biofilm

bacterial community. Bacterial abundance increased with biofilm carbon and nutrient availability. There were no significant changes in community structure spatially or seasonally, most likely due to maintenance of sufficient dissolved oxygen levels that supported biofilm diversity. The associated enzyme activity had no relationship to community structure. Organic nutrients in the biofilm were so high that heterotrophic bacteria were not limited by their stoichiometric demands. Instead, extracellular enzyme activity scaled with heterotrophic abundance, biofilm C and the detrital biomass ratio.

Together, this dissertation attempts to answer some of the important questions regarding extracellular enzymes. While there is some evidence to suggest a phylogenetic signal of extracellular enzyme production, from the empirical results presented here, bacterial expression of extracellular enzymes, in both populations and communities, appears to be predominantly determined by nutrient parameters.

CHAPTER 1

Carbon Cycle Implications of Soil Microbial Interactions

1.1 Introduction

Soil holds the largest store of carbon on Earth, estimated to be >2300 Pg C (Jobbágy and Jackson 2000). Flux rates of carbon from the soil exceed anthropogenic emissions by up to ten times yearly (Chapin et al. 2002). Owing to the scale of soil carbon inputs into the atmosphere, and major concerns over human disruption of the global carbon cycle, it is important to understand the drivers of the soil carbon flux. Because microbes are responsible for the degradation and transformation of organic matter, soil carbon cycling is dependent upon microbial metabolism (Falkowski et al. 2008). Yet microbial processes that govern the turnover of carbon in the soil are not fully understood (Prosser 2012).

Microbial processes have been difficult to study owing to the microscale at which they take place, the spatial and temporal fluctuation of conditions in the soil, and the incredible diversity of interacting organisms and abiotic parameters. With advancements in molecular tools, the diversity of the soil biota and its associated carbon cycling potential have become more resolved. Many stressors in the soil environment have been explored for their impact on carbon cycling. Yet less attention has focused on how microbial interactions influence the evolution and phenotypic expression of microbial traits that affect carbon cycling in the soil environment. This chapter will therefore discuss the impact of microbial interactions on traits involved with carbon cycling.

For the purposes of this chapter, interactions will be defined as processes driven by one microbe that have either positive or negative effects on survival or reproduction of one or more other microbes. We will focus on interactions that influence phenotypic expression and genotypic capacity of traits with consequences for carbon cycling. We propose that microbial interactions

act as pressures that result in changing the cellular allocation of resources underlying these

processes. These pressures alter fitness cost:benefit ratios and ultimately impact carbon cycling.

This chapter also aims to address how microbial interactions influence community structure.

Community structure may be important to carbon cycling if organisms show inter-taxa variation

in their capacity for carbon cycling and if the breakdown of carbon is limited by cellular

processes (Schimel and Schaeffer 2012). There is extensive evidence that changes in microbial

community structure have impacts on carbon turnover (Balser and Firestone 2005; Matulich and

Martiny 2014). More broadly, changes in diversity are often linked to altered functioning (Bell

et al. 2005; Tilman et al. 2001).   Interactions that alter diversity at the microsite, such as niche

partitioning, or prevention of competitive exclusion, such as non-transitive interaction networks

and negative frequency-dependent selection, therefore, will likely have effects on community

carbon cycling (Cordero and Datta 2016).
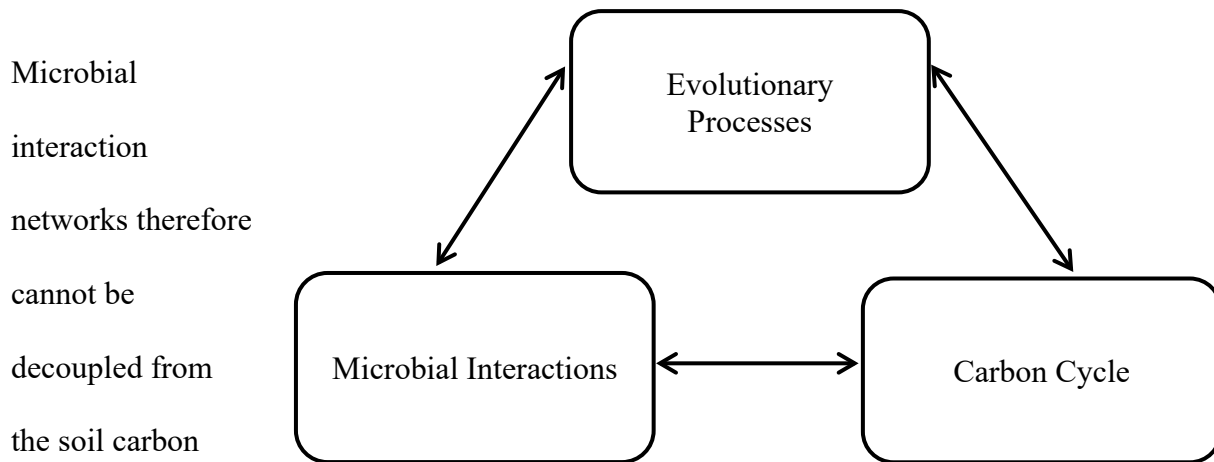
Microbial interaction networks therefore cannot be decoupled from the soil carbon cycle. The purpose of this

Figure 1.1. A conceptual diagram of the feedback between microbial interactions, evolutionary processes, and the carbon cycle.

chapter is to explore the implications of microbial interactions in soil carbon cycling (Fig. 1.1).

We hypothesize that changes in allocation patterns resulting from interactions will lead to both ecological and evolutionary consequences for carbon cycling. Furthermore, we hypothesize that microbial interactions have important ramifications for community structure that feed into associated community functioning. While these metabolic constraints on carbon transformation and shifts in allocation that change the fate of carbon may take place at the microsite, evidence suggests that microbial metabolic processes collectively scale up and contribute to carbon cycling at the ecosystem level (Brown et al. 2004; Elser 2006; Sinsabaugh et al. 2015). Therefore, the effect of microbial interactions on soil carbon flux potentially has relevance across multiple spatial and temporal scales, including the global scale over decades to centuries.

1.2 Allocation patterns

Microbial growth has been shown to drive soil organic matter (SOM) decomposition, indicating that metabolic mechanisms that impact growth rate have a large influence on soil carbon dynamics (Neill and Gignoux 2006). While growth rate is partly determined by rRNA copy number, or codon usage bias (Vieira-Silva and Rocha 2010; Stevenson and Schmidt 2004; Goldfarb et al. 2011), carbon use efficiency (CUE) is phenotypically variable and depends upon maintenance costs. As a metric, CUE defines the amount of growth achieved per unit of acquired carbon, and may be an important control on carbon sequestration in soil (Allison et al. 2010; Bradford and Crowther 2013). Maximum possible microbial CUE has been estimated at approximately 60% of acquired carbon being assimilated into biomass or ATP, but declines with growing maintenance costs (Schmidt and Konopka 2009). Maintenance costs vary with conditions, and may increase with temperature, nutrient limitation, starvation, physiological

stress, allocation to storage, extracellular products, and transporters (Lipson 2015; Matsumoto et al. 2013).

Microbes often face competition for limited resources in the soil environment. The investment in acquiring resources, part of cellular maintenance costs, generally lowers the overall metabolic efficiency of the cell (De Mattos and Neijssel 1997). The phenotypic response of microbes living in resource-limited conditions includes synthesis of enzymes that acquire limiting resources to maximize uptake rates, synthesis of enzymes targeting alternative forms of the limiting resources, a decrease in anabolism to match the uptake of the limiting resources, and use of storage polymers to compensate resource deficiencies (Harder and Dijkhuizen 1983; Schmidt and Konopka 2009).

Metabolic theory posits that thermodynamics define absolute constraints on the uptake, transformation and secretion of energy and matter, as well as the rates of these processes (Brown et al. 2004). These controls over energy and matter fluxes also dictate ecological interactions among organisms by defining a bacterium's ability to grow, produce molecules that impact surrounding bacteria, respond to declining resources, or counter chemical attacks. The cellular response to interactions may lead to a shift in allocation of resources that impacts the rate of carbon turnover and its ultimate fate in the soil environment. These metabolic interactions influence what percentage of acquired carbon is transformed and immediately released into the atmosphere, converted to biomass or extracellular products, or stored as recalcitrant compounds in the soil.

Many of the effector molecules associated with maintenance costs are proteins. Protein production requires the greatest amount of energy and resources of all microbial processes (Koch 1985). Even under optimal conditions, maximum growth rate is limited by macromolecular synthesis, energy production, and transport of molecules, all processes driven by proteins. Therefore, allocation of resources towards non-growth protein synthesis represents a decrease in fitness (Chubukov et al. 2014). This burden creates a strong selective pressure for microbes to reduce nonessential protein production.

In addition to the increase in resource-acquiring mechanisms, microbes in the soil alter their growth rates and production levels of other potentially costly molecules in response to interactions. Toxins attack predators and competitors for nutrients. Defense systems respond to interspecific assaults. Biofilm polymeric substances protect microbes against desiccation and antibiotics, while slowing diffusion of nutrients away from the producing cells. Siderophores chelate iron to make it bioavailable. Production of these may also represent a decrease in fitness for a microbe.

1.2.1 Interaction-mediated phenotypic plasticity

Phenotypic plasticity is beneficial in highly heterogeneous environments, allowing microbes to adjust their response to a range of conditions. This has the potential to ameliorate the severity of circumstances causing negative fitness effects for the microbe on a short-term scale. Phenotypic plasticity arguably carries costs with its maintenance, though. Evolutionary biologists have analyzed the costs and limits on phenotypic plasticity (DeWitt et al. 1998), as well as constraints on the evolution of plasticity. A loss of plasticity may be due to accumulation of mutations or

loss of genes if their products are unused or being produced by other community members (Murren et al. 2015). Multiple studies have found loss of core metabolic genes in obligate symbiotic, parasitic or commensal microbes. In contrast, some free-living microbes have streamlined their genomes by maintaining core functional genes while reducing the relative amount of intergenic spacer DNA and number of paralogous genes (Giovannoni et al. 2014; Solden et al. 2016). Microbes must balance their capacity for plasticity with the burden of DNA replication, immediate ecological and environmental pressures, and availability of genetic material through horizontal gene transfer (HGT).

1.2.1.1 Interaction agents in the soil environment

Interaction-induced phenotypic alterations are often initiated via direct contact, metabolic byproducts, or diffusible autoinducer molecules that interact with regulatory pathways, such as quorum signals, volatile organic compounds (VOCs), or even toxins (Davies et al. 2006; Decho et al. 2011; Effmert et al. 2012; Straight and Kolter 2009). Multiple studies have shown coordinated phenotypic responses to environmental or competitive stressors within and between populations (Challis and Hopwood 2003; Rigali et al. 2008). When this occurs, autoinducers are considered signals. In some cases, however, phenotypic responses are induced that are not part of an effort to enact a cooperative, coordinated response. For example, it is possible that some autoinducer producers may force metabolic changes in other microbes for their own benefit, which is termed coercion. Some microbes appear to have evolved the capacity for 'crosstalk,' or the ability to eavesdrop on heterospecific autoinducers in the surrounding environment. These autoinducers are known as cues. (Traxler and Kolter 2015; Netzker et al. 2015; Federle and Bassler 2003; Diggle et al. 2007).

Microbial interactions may act to alter the expression of various traits that have implications in carbon cycling, such as growth rate and production of extracellular products. The production of many exoproducts is temporally and spatially modulated through intercellular signals within and between populations (Diggle et al. 2007; Huang et al. 2013; Strickland et al. 2013), as may be differentiation and predatory behavior (Müller et al. 2014; Schuster et al. 2003; Straight et al. 2006). Autoinducers are also involved in efficiency sensing: detection of diffusion rates to optimize production amounts of extracellular products (Hense et al. 2007). The impact of autoinducers on fitness for an individual microbe in relation to its community, through both competition and cooperation, confers a level of importance that is reflected in the capacity for a wide diversity of genes for signals found in many microbes (Challis and Hopwood 2003; Krug et al., 2008; Schuster et al. 2003). Furthermore, as mediators of interactions that result in altered expression of functional traits, autoinducers are fundamental to ecosystem function (Seneviratne 2015; Zhuang, et al. 2013).

Autoinducer efficacy and persistence in the soil environment is affected by the size and adsorption properties of the autoinducer molecules, and may be altered by pH and the ratio of clay to organic material (Lv et al. 2013; Subbiah et al. 2011; Traxler and Kolter 2015). Mineral soil is comprised of approximately 50% air and water-filled pores, which are temporally and spatially dynamic (O'Donnell et al. 2007). This creates a high surface area within the soil matrix, on which many soil microbes form biofilms. Biofilms alter the autoinducer potential of a community through changes in diffusion rates, redox gradients and pH (Stewart 2003; Decho et al. 2011). Additionally, some microbes produce degrading enzymes, agonists, and antagonists of

autoinducer molecules (Wang and Leadbetter 2005; Xavier and Bassler 2005). Not only do these compounds serve to manipulate microbial interactions, but some of the degraded products may form new carbon and nutrient sources, and act as antimicrobial compounds or iron chelators (Leadbetter and Greenberg 2000; Kalia 2013).

Another direct mechanism that may force interspecific changes in microbial phenotype, and hence shifts in resource allocation, is contact-dependent inhibition (CDI) (Blanchard et al. 2014; Ruhe et al. 2013). This not only causes shifts in resource allocation and a decrease in growth for the CDI-producing cell, but also decreases in growth or death of the recipient. This mechanism requires close proximity for action, conditions that arise in soil microbial biofilms.

Finally, microbes may cause changes to neighboring cells' phenotypes through indirect agents. Metabolic byproducts can change the local abiotic conditions, such as pH, creating stressful conditions and altering metabolic efficiency of neighbors. Likewise, metabolic byproducts can alter efficiency as newly available resources that benefit neighbors through cross-feeding.

1.2.1.2 Soil biofilms

Biofilm formation is important to many soil microbes for survival. It offers protection against several soil environment stressors such as predation, desiccation, and toxin exposure (Matz and Kjelleberg 2005; Mah and O'Toole 2001; Roberson and Firestone 1992; Jefferson 2004). The prevalence of biofilm formation amongst bacteria, estimated to be at 99% of taxa, supplies evolutionary evidence of life in biofilm as an important adaptation. Though fungi, algae, protozoa, and yeast also grow in biofilms alongside bacteria, the primary focus in research of

biofilms has been on bacteria (Jass et al. 2002; Vu et al. 2009). Regardless of taxonomic identity, biofilms establish conditions that alter contact between microbes by immobilizing the biofilm cells next to each other, forming barriers to inhibit interactions, or altering diffusion rates of extracellular molecules.

The exact composition of biofilms varies widely, but contains polysaccharides, proteins, lipids, nucleic acids, and other biopolymers such as humic substances, along with the resident microbes. While some of the matrix can be easily degraded as a nutrient source, humic substances are resistant to degradation, contributing to long-term soil carbon stocks (Flemming and Wingender 2010). The combined, 3-dimensional matrix of molecules is broadly termed 'extracellular polymeric substances,' or EPS. Each species of bacteria produces a distinct set of polysaccharides and proteins for their respective EPS, which is integrated into multispecies biofilms (Vu et al. 2009). Biofilm matrix architecture varies widely based on EPS molecular structure and environmental conditions, with the different architectures impacting important physical parameters of microbial existence, such as diffusion gradients (Flemming and Wingender 2010). The dramatic change in phenotype that accompanies the transition to a sedentary lifestyle within a biofilm makes it difficult to isolate the changes in cellular efficiency or changes in allocation of resources due to production of EPS. However, initial colonization is marked by high production of metabolically expensive carbon compounds and proteins, so an immediate reduction in growth might be expected. In fact, a decline in growth has been observed in some cases (Burmolle et al. 2014; Mah and O'Toole 2001).

The transition from a planktonic lifestyle to a biofilm is accomplished through multiple changes in gene expression. Many of the differentially expressed genes associated with the transition from planktonic to biofilm life code for metabolic function and starvation responses (Booth et al. 2011; Donlan 2002; Jefferson 2004; Prigent-combaret et al. 1999; Sauer and Camper 2001; Stewart 2003). These changes in gene expression can be initiated by environmental cues, but have also been observed to be engendered through intercellular autoinducers (Parsek and Greenberg 2005; Jefferson 2004). For example, Lopez et al (2009) found that a diverse set of natural molecules that cause potassium leakage by temporarily creating membrane pores in *Bacillus subtilis* were responsible for inducing biofilm formation. These molecules are produced by other strains as well as *B. subtilis* itself. They proposed that a membrane receptor was likely able to detect lowered intracellular concentrations of potassium and initiate a transcriptional response leading to biofilm production.

Though the specific interacting molecules were not always determined, several other studies have shown either induction or an increase of biofilm formation in strains of bacteria grown together versus when grown in monocultures (Bleich et al. 2015; Burmolle et al. 2007; Shank et al. 2011), whereas other studies have found inhibition of biofilm production (Powers et al. 2015). Monoculture biofilm formation may be a cooperative mechanism (West et al. 2007); however, induction of biofilm production by heterospecific strains could also mean that biofilm formation is a defensive or coercive strategy.

Through the progressive stages of development of a biofilm, colonizers transform their created biofilm environment through cell autoinducers, waste products, and degradation of soil organic

matter (SOM) (Stewart 2003).   This transformation creates microenvironments that magnify

spatial and temporal heterogeneity within the biofilm due to restricted diffusion, leading to

changes in microbial phenotype relative to available resources and interacting organisms

(Stewart and Franklin 2008). Some microbial processes also have bistable switches that respond

to intercellular autoinducers that may affect the phenotypic heterogeneity displayed within a

mature biofilm (Chai et al. 2008; Dubnau and Losick 2006). These mechanisms that increase

heterogeneity may lead to an increase in community or population-level efficiency through

specialization in tasks and reduction of the unicellular burden of enzyme production, or a

reduction in the waste of resources through cross-feeding, and may act to alter soil carbon

turnover rates (Ackermann 2015; Bernstein et al. 2012; Folse and Allison 2012; Huang et al.

2013; Jefferson 2004).

The physical structure of EPS in the soil affects microbial processes and interactions by affecting

diffusion rates. As the amount of EPS accumulates, diffusion rates of oxygen, nutrients, and

waste products decrease, creating conditions that might decrease growth rates through nutrient

limitation, triggering of a stress response, and transition of metabolism to inherently less efficient

anaerobic respiration or fermentation (Stewart 2003; Mah and O'Toole 2001; Prigent-combaret

et al. 1999). Thus, it is possible that conditions generated through biofilm structural and

chemical differentiation created by indirect microbial interactions lead to lower metabolic

efficiency. Likewise, the stress response that has been noted in biofilms represents a shift toward

allocation of resources to maintenance (Schimel et al. 2007).

Alternatively, decreased diffusion associated with the EPS matrix may benefit microbes. Extracellular products that are available to and benefit all members of a community—or public goods—such as enzymes, quorum molecules, and siderophores, remain closer to the producing cell, increasing its return on investment (Burmolle et al. 2014; Flemming and Wingender 2010). Because restricted diffusion effectively lowers the productive need of these molecules, it may allow the producing cells to devote more of their resources towards growth, improving metabolic efficiency and biomass accumulation. One study showed that 63% of four-species biofilm-producing consortia synergistically increased biofilm production relative to strains grown independently in the lab (Ren et al. 2014). The highest-producing four-species consortia contained a dominant biofilm producer, *Xanthomonas retroflexus*, however, all of the interacting species in that group increased in both biofilm production and relative cell number compared to monoculture biofilms. Only two of the thirty-five combinations of 4-species consortia showed decreased biofilm production relative to monocultures.

1.2.1.3 Growth and dormancy

Interactions among microbes, whether positive or negative, and direct or indirect, have the potential to affect growth and soil carbon cycling. Exploitation competition between microbes is indirect and involves depletion of a common limiting resource, with the winner having a higher capacity for resource acquisition. Higher resource acquisition increases growth rate, effectively starving the loser of resources. An evolutionary focus on this strategy may only be successful when resources are available (Stevenson and Schmidt 2004; Goldfarb et al. 2011; Moorhead and Sinsabaugh 2006). Given the highly variable availability of resources, it is unsurprising that the soil environment hosts a wide diversity of microbial growth strategies, beyond the simple

dichotomy of copiotrophs and oligotrophs (Ernebjerg and Kishony 2012; Vieira-Silva and Rocha 2010). Yet the ability of microbes to maintain relatively high growth rates down to nanomolar or micromolar concentrations of substrate due to the maximization of uptake suggests a strong selective advantage for exploitative competition (Schmidt and Konopka 2009).

Indeed, some bacteria may have evolved measures to manipulate their growth rate as a competitive measure. By switching to a high-growth rate low-yield strategy, bacteria disproportionately acquire available resources even though their metabolic efficiency declines (Lipson 2015; Pfeiffer, et al. 2001). While this low-yield strategy might not immediately improve fitness, it functions to decrease fitness of competitors by reducing resources available for their growth. This strategy has the effect of increasing carbon turnover and flux, but is only beneficial under conditions with high rates of resource diffusion (Lipson 2015). Therefore, this mechanism would likely only occur at the surface of biofilms where high diffusion rates of oxygen and resources take place.

Additionally, interference competition, in which competitors directly and aggressively fight over resources, often supports exploitative efforts. Some microbes may respond to nutrient stress, which is associated with exploitative competition, by slowing growth and producing growth inhibitory antibiotics (Rigali et al. 2008; Cornforth and Foster 2013; Garbeva and de Boer 2009). This slowed growth may accompany an allocation towards cellular maintenance costs of antibiotic production, but it has also been proposed that the slowed growth is a preemptive protective measure against antibiotic attacks (Mah and O'Toole 2001). The reason why slowed growth imparts protection is unclear. However, because resistance to antibiotics may also carry

a fitness cost, the slowed growth could be associated with this shift in allocation away from growth and towards resistance (Andersson and Levin 1999; Andersson and Hughes 2010; Dykes and Hastings 1998). Garbeva et al (2011) found differential regulation of ribosomal protein and stress response genes along with induction of antibiotic production, suggesting that slowed growth is partly due to a cellular stress response. Slowed growth may also be caused by production of coercive molecules to suppress antibiotic production in a neighboring cell, or to trigger antibiotic production in a third cell that is forced into the role of bodyguard (Tyc et al. 2014; Abrudan et al. 2015; Galet et al. 2014). Given the fitness cost of production of some growth inhibitory molecules, it is surprising that one study found 33% of soil bacteria constitutively produce antibiotics, lending credence to the hypothesis that antibiotics may also serve as autoinducers (Tyc et al. 2014).

Dormancy or a reduced metabolic state will have indirect fitness consequences for a population by freeing up for their kin the resources that microbes otherwise would have consumed for themselves. (Ratcliff et al. 2013). These microbes may be the persister cells noted in biofilms, that are more inclined to switch into a dormant or reduced metabolic state (Stewart and Franklin 2008). In the soil environment, approximately 80% of all bacteria are in a dormant state (Lennon and Jones 2011). Though the reduced metabolic state is energetically prudent, the cost of going into this state is not zero. Multiple metabolic processes must first prepare for cellular shut down, including production of machinery to go into and out of dormancy, as well as resting structures (Lennon and Jones 2011). Ultimately, microbial interactions affect the rate at which neighboring microbes transition into a dormant state, either through exploitation or kin selection, thus altering soil carbon turnover rates.

1.3 Evolution of traits with carbon cycling consequences

Studies of social evolution are often performed using microbes due to their relative simplicity. Even though laboratory experiments often cannot specifically prove that the evolutionary response to selective pressures in the experiment is solely due to the interaction and therefore social, these experiments inform about potential mechanisms that may occur through interactions, and as such, are important to begin understanding how evolution impacts carbon cycling (Rainey et al. 2014). Social behaviors have fitness effects for both the actor and the recipient. Cooperative behaviors can be mutually beneficial, in which both the actor and recipient receive positive fitness results, or altruistic, in which the actor does not. Likewise, competitive behaviors are broken down into selfish, with the actor receiving a fitness benefit while the recipient is harmed, or spiteful, with both being harmed (Hamilton 1964). Natural selection acts on genetic variation, often a single, specific locus in microbes (Mitri and Foster 2013). For many social evolutionary mechanisms, relatedness is determined at one specific gene, such as for a public good or toxin.

Pressures that shift the cellular balance away from reproduction, such as those that occur through microbial interactions, act as selective forces that may have implications for carbon cycling. The higher the incurred cost to fitness and the longer it occurs, the more likely a change in allocation will lead to evolutionary changes. Presumably, costly traits such as production of extracellular goods, will be maintained if the benefit outweighs the fitness cost. Benefits to a producing cell may be direct, as is the case with enzymes that scavenge high energy resources, or indirect, such as a reduction in competition for resources.

Conversely, costly traits may be maintained if the cost of loss increases, as occurs with enforcement tactics carried on mobile genetic elements (MGEs).

1.3.1 Horizontal gene transfer

Many of the genes responsible for microbial interactions and carbon cycling are part of the accessory genome, which constitutes upwards of 90% of a bacterial taxon's pan-genome (Haq et al. 2014; Rankin et al. 2011; Touchon et al. 2009). The accessory genome- those genes contained within a microbe that are shared through HGT via mobile genetic elements (MGEs) such as transposons, bacteriophages, and plasmids- predominantly codes for secreted proteins, but can also encode metabolic traits and pathways (Falkowski et al. 2008; Nogueira et al. 2012; Ochman et al. 2000). The more complex pathways may be difficult to transfer, however, because of their multi-gene nature and incongruity with preexisting pathways (Schimel and Schaeffer 2012). This has likely led to the deeply conserved nature of these large metabolic units (Martiny et al. 2015).

Transmission of MGEs increases at higher cellular densities (McGinty et al. 2013; Sorensen et al. 2005). Biofilms promote HGT by creating a matrix for microbes to interact closely for conjugation, maintaining the naked DNA of lysed cells in proximity to the biofilm's residents for transformation, and even potentially facilitating viral infection for transduction (Donlan 2002; Flemming and Wingender 2010; Hausner and Wuertz 1999; Burmolle et al. 2014; Sorensen et al. 2005; Molin and Tolker-Nielsen 2003). Because of this, it is likely that plasmids and bacteriophages have incorporated genes that facilitate biofilm formation to ensure their own propagation (Jefferson 2004; Madsen et al. 2012). Therefore, the biofilm acts as a reservoir of

genetic information, allowing rapid adaptation to fluctuating conditions, and redefinition of an

ecological niche (Haq et al. 2014; Norman et al. 2009).

Because many of the genes carried on MGEs code for public goods that are secreted from the

cell, the potential loss of public goods by diffusion implicitly increases the cost of production to

the cell and likelihood of gene ejection. As is the case with whole organisms, MGEs success

depends upon propagation. To resolve a potential conflict of survival between the host and the

MGE, an evolutionary compromise has been observed in which the biosynthetic cost of secreted

and outer membrane proteins are often lower than those for purposes elsewhere in the cell,

improving the likelihood of the MGE maintenance within the cell (Nogueira et al. 2009; Smith

and Chapman 2010).

It is important to consider that MGEs have also evolved mechanisms of forced maintenance.

These mechanisms impact interactions between microbes as well as metabolic efficiency through

shifts in allocation of resources towards fabrication of MGE products. For example, addiction

complexes contain a toxin-antitoxin complex, with the antitoxin degrading more rapidly than the

toxin (Zhang 2012). Because the toxin remains in effect for a longer period than the antitoxin,

the cell loses immunity upon loss of the MGE, and fitness lowers to zero.

Through MGEs a picture emerges of how function and interactions feed into one another.

Microbes create biofilms that favor HGT, and MGEs contain traits that impact neighboring cells.

The toxin-antitoxin complexes force production of their products while killing local cells that

have not acquired the same complex. Depending on what other genes might be carried with

these complexes, this may also have a large impact on the production of public goods that are involved in carbon cycling or sequestration. Even without a toxin-antitoxin complex, the associated increase of relatedness involved with HGT creates a dynamic of kin selection that promotes production of public goods encoded on MGEs (McGinty et al. 2013). Despite this immediate and localized increase in relatedness, HGT is thought to contribute to the larger process of speciation (Boto 2010). In fact, genes associated with secreted proteins have been found to evolve at a relatively high rate (Nogueira et al. 2012), which may have more downstream effects on carbon cycling as discussed in the continuing sections.

1.3.2 Cheaters

Cheating is an evolutionary strategy that either eliminates the cost of production of a public good for the cheater while using the goods produced by others, or disproportionately increases access to a limiting resource for the cheating microbe. The success of any cheating strategy is density dependent, as a competitive strategy only has benefits insomuch that it is distinct amongst its competitors (Ross-Gillespie et al. 2007; West et al. 2007). It also depends upon diffusion rates, spatial structure, and available resources. Despite the population-level benefit of cooperative public good production, cheating is a strategy that commonly arises (Allison et al. 2014; Darch et al. 2012; Kim et al 2014). Multiple mechanisms exist to buffer populations against cheaters, including those associated with MGE maintenance, but the rapid generation time of microbes combined with the relatively high evolvability of genes for secreted products suggests that cheating mutations may occur often (Travisano and Velicer 2004; Diggle et al. 2007; Popat et al. 2015).

Cheating with EPS production in biofilms alters allocation of resources, metabolic efficiency, and growth through an increase in production of EPS. Cells at the surface of a biofilm experience higher resource and oxygen levels. Cheaters have arisen with an increased ability to produce biofilm compounds, effectively pushing themselves to the surface of the biofilm to acquire more of these resources while suffocating the wild type strain (Kim et al. 2014; Xavier and Foster 2007). This allocation to biofilm polymers, however, comes at the expense of reproduction as indicated by lower density of cheater cells compared to wild-type cells. Genetic analysis confirms that increased competitive ability was not achieved through faster growth, but through increased biofilm polymer production (Kim et al. 2014).

Because microbial growth is positively correlated with SOM degradation, this competitive interaction, resulting in a decreased growth rate, may represent slowed carbon turnover and lower relative biomass. Depending upon the molecular composition of the produced EPS, more resistant forms of soil carbon may be formed. However, the increased allocation of resources towards production of EPS may be associated with decreased metabolic efficiency and consequently a greater proportion of acquired carbon being respired.

When members of a population are producing the same public goods, it is evolutionarily expedient for an individual microbe to evolve a loss of production. Because the cheater is still being provided with communal public goods, the loss of function represents an increase in fitness and has a positive competitive effect against surrounding producers. An example of public goods commonly involved in cheating is siderophores. Because soil is often an aerobic environment, bioavailability of iron is limited. Siderophores chelate iron, an important element

Table 1.1. Potential effects of microbial interactions on soil carbon cycling.

Interaction type	Effect upon soil carbon storage	Potential mechanisms
Exploitation	-	Rate of SOM degradation increases with increasing growth of exploiting population
Decrease in CUE	-	Reduction in biomass accumulation and increasing amount of carbon released as CO_2
Toxin production	+/-	Metabolic production costs may decrease carbon storage but growth inhibition might increase it. Reduction in niche overlap may contribute to increased SOM degradation.
Signal degradation	+	The targeted population will be unable to function cohesively in SOM degradation
Coercion	+/-	Effects are dependent upon what action is being coerced
Dormancy	+	Reduces total SOM degradation if dormancy caused by stressors other than nutrient limitation
Cross-feeding	-	Rate of SOM degradation increases, but yield may decrease
Syntrophy	-	Streamlines metabolic processes and facilitates SOM degradation in anoxic environments
Siderophore cheating	+/-	May increase or decrease SOM degradation depending on the relative metabolic costs of siderophore production and growth rates of the cheater and producer
Enzyme cheating and Black Queens	+	Reduction of degradation of SOM by lowering total enzyme production
Biofilm cheating	+/-	Increased carbon allocation to EPS and humic substances increases storage though associated production costs may cause greater CO_2 flux
Soil pore formation	-	Facilitates access to SOM, oxygen, and water resulting in increased degradation

involved in many metabolic pathways. Many species of bacteria and fungi produce multiple

types of siderophores and their receptors, some that are more metabolically costly than others

(Dumas et al. 2013). It is common for cheaters to arise that pirate xenosiderophores, those

produced by other species. Cheating is done by expressing xenosiderophore receptors, that are

likely acquired through HGT (Cornelis and Bodilis 2009). There is often a concomitant

reduction in the production of endogenous siderophores, and a subsequent increase in fitness

(Galet et al. 2015; Miethke et al. 2013). Traxler et al (2012) found that siderophore piracy can be used to reduce growth of competitors, as well. In their experiment, *Amycolatopsis sp.* AA4 arrested development of *Streptomyces coelicolor* through manipulation of iron availability via siderophore production, then through piracy of *S. coelicolor* siderophores. The shifts in allocation of resources through siderophore cheating lead to changes in metabolic efficiency and growth rate, thereby altering the fate of carbon in the soil.

Extracellular enzymes are another public good that are subject to cheating. Enzyme cheating potentially has a high impact on soil carbon turnover because enzymes are the proximate agents by which microbes access SOM and begin degradation and subsequent mineralization of carbon. Some extracellular enzymes can also contribute to production of recalcitrant SOM in the soil (Burns et al. 2013), affecting the amount of carbon that is sequestered. As with siderophores and EPS, their production costs are relatively high, potentially resulting in reduced fitness through allocation of carbon and energy towards enzymes, and a lowered metabolic efficiency. This cost is a trade-off with the increase in fitness resulting from higher resource acquisition due to enzyme activity (Allison 2011).

Enzyme cheaters have been shown to benefit from high diffusion rates, especially as the cost of production rises (Allison 2005; Allison et al. 2014; Folse and Allison 2012). In biofilms, where a majority of microbes grow in the soil, loss of enzymes through diffusion will be partially mitigated by the EPS matrix. This decreases overall cheater success because enzymes remain localized to the producers, and because producers form patches that exclude cheaters (Allison

2005; Allison et al. 2014). Likewise, low nutrient concentrations allow producers to form insulated patches against cheaters (Mitri et al. 2011; Nadell et al. 2016; Nadell et al. 2010).

1.3.3 Black queen, cross-feeding, and syntrophy

With higher percentages of cheaters, a public good supply is reduced, causing a decline in both producers and cheaters. However, in nutrient-limited environments that impose slowed growth rates, such as mature biofilms, the relative number of cheaters in communities might be more likely to stabilize (Morris et al. 2012). The Black Queen Hypothesis stipulates that the stabilization of cheaters in a population of public good producers occurs when producers are forced to be helpers, individuals that collectively make a minimum required amount of a vital public good that can sustain both populations, thereby ensuring their own survival (Morris et al. 2012). This requires close proximity of the producers and cheaters. Furthermore, Morris (2015) suggests that, over time, cheating may arise in helpers for other public good traits that the first cheater still produces, creating an auxotrophy.

Stabilization of a mutual auxotrophy was shown in an experiment in which *Escherichia coli* strains were grown together that had null mutations for different amino acid production pathways (Pande et al. 2014). The cross-feeding mechanism created a division-of-labor fitness advantage over the ancestral strain that was stabilized through negative frequency-dependent selection for the pair of metabolic dependents. The benefit received by auxotrophic microbes is reflected in a recent analyses that shows the widespread nature of this strategy (Solden et al. 2016).
In the case of enzymes, decreasing production, either through cheating or as mutual auxotophy, may slow carbon turnover in the soil by decreasing the relative amount of active enzymes to

break down carbon substrates relative to the number of individuals in the community (Folse and Allison 2012; Oliveira et al. 2014). Alternatively, cheating may facilitate a transition to a more efficient community metabolism that frees cellular resources to be allocated towards growth. This may increase total carbon consumption in the community and the rate of carbon turnover (Pande et al. 2014).

Cross-feeding interactions have arisen through optimization of metabolic pathway length (Costa et al. 2006; Pfeiffer and Bonhoeffer 2004). Cross-feeding is pervasive in microbial communities, and is often associated with metabolic traits whose expression can be altered dependent upon the surrounding circumstances (Ponomarova and Patil 2015). Production of ATP in metabolic pathways involves a step-wise series of multiple enzymes. These enzymes are energetically expensive and resource intensive. By eliminating some of the ATP-generating steps, an overflow metabolism occurs, in which the resource is only partially oxidized and then secreted from the cell (De Mattos and Neijssel 1997). This increases the ATP production rate, though at the expense of yield (Pfeiffer and Bonhoeffer 2004). In microbes adapted to resource limitation, overflow metabolism occurs with excess carbon supplies. The partially oxidized compound is then used by another microbial community member.

More complex cross-feeding patterns have been found in nature. These syntrophies, or "obligate mutualistic metabolisms," are broadly described as a service mutualism, in which one species provides a chemical resource in exchange for a benefit from its interacting partner, such as removal of a secreted waste product (Bull and Harcombe 2009; Harcombe 2010). Syntrophies are largely anaerobic processes that are beneficial to the participants because they shift the

metabolic reactions toward thermodynamic favorability for the producing cell while providing

resources for the recipient (McInerney et al. 2008; Morris et al. 2013). Biofilms can function to

keep interacting partners in close proximity (Little et al. 2008), ultimately increasing carbon

turnover in mature biofilms with anoxic regions.

1.4 Community structure

Diversity-function relationships generally show a positive, asymptotically saturating relationship

between species richness and ecosystem function (Bell et al. 2005; Langenheder et al. 2010;

Tilman et al. 2001; Tiunov and Scheu 2005). The saturation point is likely related to redundancy

of functional traits that are held within a community (Allison and Martiny 2008). Conversely,

there are contrasting results of a 'negative complementarity effect.' This decreasing function

with increasing diversity, has been hypothesized to be caused by competitive interactions

(Becker et al. 2012; Jousset et al. 2011; Szczepaniak et al 2015; Van der Wal et al. 2013).

These conflicting patterns with increasing species diversity indicate that a community's

composition may have an impact on its overall function.

Mouillot et al (2011) showed that functional diversity, rather than taxonomic diversity was more

predictive of ecosystem multifunctionality, with a few specialist species contributing

disproportionately to primary production and degradation. However, complex, multi-gene traits

are more deeply conserved than simple traits like the ability to utilize a simple carbon substrate

(Berlemont and Martiny 2013; Martiny et al. 2013; Martiny et al. 2015; Zimmerman et al.

2013). Despite the rampant nature of HGT, it appears as though even simple traits are not

distributed completely randomly on a phylogenetic tree as would be expected if these traits were inherited horizontally.

Microbial community composition has been shown to affect ecosystem function in multiple studies (Tiunov and Scheu 2005; Bell et al. 2005; Langenheder et al. 2010; Reed and Martiny 2007). A recent literature synthesis that investigated the relationship between altered community composition and related function found that 75% of the papers that explicitly tested for a link between community structure and processes found a statistically significant link (Bier et al. 2015). When examining the link between community and function, the available techniques used to evaluate who is present in the community often do not take into account the metabolic states of the individual members of the community, which may be altered by microbial interactions (Baldrian et al. 2012; Lennon and Jones 2011; Schimel and Schaeffer 2012).

Community composition only indirectly controls the turnover of soil carbon by altering the genetic potential of the community and the context within which microbes operate. Ultimately, it is microbial physiology that directly controls carbon turnover (Allison 2012). Schimel and Schaeffer (2012) proposed that in order for the community composition to impact soil carbon turnover rates, 1) the organisms must vary in the functional traits that they possess, and 2) that the biological reactions they facilitate must be either the rate-limiting or the fate-controlling step in carbon breakdown. The authors argue that the rate-limiting step is more likely due to abiotic soil constraints, and therefore, it is the fate-controlling step that likely shows a relationships between microbial community composition and function.

1.4.1 Community composition as determined by microbial interactions

Microbial interactions, along with available resources and conditions, determine the composition

of microbial communities. The resource competition theory posits that the species with lower

resource requirements will outcompete other species with higher requirements when they are

both limited by the same resource. However, species can coexist if they are either limited by

different resources, or if they have nearly identical resource requirements (Tilma, 1981). Soils

are spatially and temporally heterogeneous, though. Microsite variation alters the outcome of

many competitive interactions beyond the resource competition theory, as do the additional

competitive mechanisms such as toxin production (Cordero and Datta 2016; Hibbing et al.

2010). A strategy that works in one location may not be as effective in the neighboring location.

For example, in environments with low nutrient levels or low diffusion rates, the competitive

ability conferred by a rapid growth rate is diminished (Dechesne et al. 2008). Rapid depletion of

resources upon initial colonization of a substrate surface and increase of EPS will likely create

that scenario, increasing coexistence.

1.4.1.1 Spatially defined interactions

The proximity of microbes to each other is relevant to interactions and microbial processes.

Importantly, HGT shapes communities and their function, and increases with microbial density

and activity (van Elsas and Bailey 2002). However, in a soil simulation parameterized using

photos taken at the microscale in soil, Raynaud & Nunan (2014) determined that the average

distance between microbes in the soil is 12 μm, with distances decreasing and aggregation

increasing closer to the surface of the soil. In lower density bulk soil, the average number of

interacting species was 11±4 within 20 μm, whereas in the higher-density rhizosphere, it was closer to 284±30 species (Raynaud and Nunan 2014).

Results from modeled two-species interactions show that spatial separation may result from microbial interactions, with antagonism leading to self-segregation and mutualism to homogenization (Blanchard and Lu 2015). Separation allows microbes to coexist that might normally compete (Ettema and Wardle 2002; Dechesne et al. 2008). So, while competitive exclusion may occur on a very small scale, diversity is maintained through the larger soil ecosystem, with stability of some communities dependent upon spatial structure (Kim et al. 2008). Furthermore, as was previously discussed with biofilms, each microsite is changed through the interactions it hosts, contributing to temporal heterogeneity.

Non-transitive interaction networks have been studied to determine how diversity can be maintained despite antagonistic interactions using rock-paper-scissor dynamics. Spatial structure allows sensitive strains to survive close to toxin-producing strains through shielding by strains resistant to the toxin (Kerr et al. 2002; Narisawa et al. 2008). This dynamic functions in communities with one toxin producing strain, or in communities with diverse toxin producers, with diverse toxin production leading to increased ecological stability (Biernaskie et al. 2013; Kelsic et al. 2015; Prasad et al. 2011). These models, however, do not account for the effects of antagonism strength on microbial interactions. For example, synthesis of communities with varying strength of bacteriocin action suggests that potent bacteriocins led the producers to extinction by stimulating heightened attack responses from their opponents, whereas weak bacteriocins supported coexistence through mild responses (Majeed et al. 2013).

1.4.1.2 Inhibition and reduction of niche overlap

Similar to the effects of physical separation with the previous examples, non-transitive interaction networks are applicable to modulation of antagonism through multispecies interactions. Neighboring cells can decrease antibiotic production of a focal species' antagonist, eliminating negative fitness impacts on the focal species and allowing all three to coexist (Tyc et al.,2015). In this respect, the identity of interacting species plays a strong role in ecological processes. Abrudan et al. (2015) demonstrated that inhibitory interactions were reduced by induction of antibiotic production combined with suppression of antibiotic production in competing species, which allowed maintenance of diversity. Moreover, they found that interactions were environmentally mediated.

Species with high niche overlap are predicted to be more competitive with each other (Freilich et al. 2011). Often, this means that phylogenetically related species engage in stronger competition than do more phylogenetically distant species (Jousset et al., 2011). Sympatric *Streptomycete* species showed higher degrees of antibiotic inhibition and reciprocated production than with allopatric *Streptomycete* species, with niche overlap being positively correlated with antibiotic inhibition (Kinkel et al 2014; Vetsigian,et al., 2011). This result supports the hypothesis that antibiotics function to mediate community interactions in attempts to reduce niche overlap. Indeed, sub-lethal levels of antibiotics altered independent growth rates of several *Streptomyces* strains on distinct substrates, as well as their range of substrate use (Jauri et al. 2013). Niche overlap declined in 56% of the isolate-isolate-antibiotic combinations, suggesting that sub-lethal antibiotics acted as an "escape from competition" mechanism. Consequently, antibiotic production may by an instrument to initiate niche differentiation, leading to speciation. Even

monoculture biofilms undergo adaptive diversification to eliminate intraspecific competition and form synergistic communities through spatial partitioning and cross-feeding, which leads to higher productivity (Poltak and Cooper 2011).

1.4.1.3 Fungal interactions

Fungi act as ecosystem engineers, creating pores that form new habitats and mining new resources for other microbes. Soil pore structure has been observed to be non-random in nature, with a highly structured bacterial distribution (Young and Crawford 2004). Using this evidence, in an experimental manipulation, Crawford et al (2012) found that at scales below 53 um, fungal hyphae were highly correlated with soil pore organization. Additionally, increasing the fungal:bacterial ratio increased soil aggregate formation, indicating that soil community structure plays a role in aggregate stabilization and pore formation in soil. The pores are speculated to improve local conditions for the engineering species by opening up channels for oxygen exchange and increasing water flow potential. These effects increase nutrient exchange and bacterial colonization through increased connectivity, and potentially increased carbon turnover. When varying hydration conditions were modeled as a function of the pore matrix potential, microbial dispersal increased dramatically (Kim and Or 2015). In addition, fungi facilitate bacterial movement in conditions with low water potential by providing a highway for bacterial biofilm formation and motility (Pion et al., 2013). Highly mobile bacteria species stimulated migration by less-mobile species along fungal hyphae, with no obvious fitness decline (Warmink et al. 2011; Warmink and Van Elsas 2009).

Fungi have been noted to dominate the litter horizon in a forest ecosystem, with the fungal: bacterial ratio evening out in the organic horizon (Baldrian et al. 2012). Bacteria often benefit from fungi due to the fungal release of extracellular enzymes that create nutrient "hotspots" or metabolic intermediates from degrading recalcitrant carbon sources (Van der Wal et al 2013; Tolonen et al. 2014). Increases in bacterial biomass are correlated with increasing fungal biomass in soil microcosms (Šnajdr et al 2011). Bacteria have lower yield than do fungi, though, so changes in the fungal:bacterial ratio have implications for ecosystem CO_2 flux (Lipson et al. 2009). Additionally, the area around fungal hyphae has been postulated to have concentrated horizontal gene transfer (HGT), including HGT occurrences between bacteria and fungi (Zhang et al. 2014), which has the potential to increase carbon turnover through transfer of carbon-degrading traits.

1.4.2 Evolutionary feedbacks on carbon cycling

Though multiple pairwise evolution experiments have been performed in the laboratory, the relevance of these experiments for communities of interacting microbes is unclear (Johnson and Stinchcombe, 2007; Turcotte, et al., 2012). Various pressures imposed by interactions with multiple species simultaneously may result in microevolution of a population that cannot be accounted for in simple two-species experiments (Johnson and Stinchcombe, 2007). Even though it is possible to extrapolate the fundamental niche of an organism, ecological interactions alter the niche, resulting in an altered range of conditions that permit survival.

Diffuse evolution refers to evolution that is caused by one species' effect on the evolving species, but depends upon multiple other species within the environment. Diffuse coevolution

occurs when the selection is reciprocal (Strauss et al. 2005). Research on the interplay between

ecological interactions and evolutionary mechanisms is still in its early stages (Johnson and

Stinchcombe 2007). By impacting evolutionary rate and direction, diffuse coevolution alters

interactions between members of a community of microbes, which then feeds back on function,

further affecting community interactions (Fussmann et al. 2007; Lawrence et al. 2012; Schoener

2011).

The community context of diffuse evolution may indicate that whole communities evolve

together through direct or indirect mechanisms (Little et al 2008; Barraclough, 2015). Generally,

it has been seen that over time, antagonistic communities evolve to be less competitive, with this

effect increasing with increasing diversity (Fiegna et al. 2015). Competition was observed to

cause a decrease in resource use diversity, associated with a decrease in relative growth rate and

yield compared to the ancestral strain, though this effect saturated at higher species richness

(Fiegna et al. 2015). The decrease in growth rate and yield mirrors microbial adaptation to

resource limited conditions, with increased uptake machinery and enzyme production for

resource acquisition (Schmidt and Konopka 2009).

Further eco-evolutionary dynamics were highlighted in an experiment performed by Lawrence et

al. (2012) whereby evolution in a community of four strains of bacteria was observed over

several generations. The researchers found that the strains grown in a community evolved faster

than did those same strains in monoculture. Additionally, the interacting species evolved

resource use divergence, and cross-feeding on metabolic waste products, indicative of character

displacement and positive interactions. Three of the four community-evolved species did more

poorly than their ancestors when grown in monoculture, revealing some degree of coadaptation. When compared to the community of ancestors, the evolved community had smaller population sizes, but higher CO_2 flux rate, suggesting a decrease in community CUE. This experiment demonstrated that community interactions can increase evolutionary rates above selection caused by abiotic pressures alone. It also reveals that adaptations caused by community interactions may function to transform environmental conditions, strengthening selective pressures, and altering carbon cycling through evolution-induced metabolic shifts.

1.5 Conclusion

Through effects on physiology, public goods, and evolution, microbial interactions play a large role in soil carbon cycling. The rate of microbial metabolism controls uptake, transformation, and allocation of carbon (Brown et al. 2004). Because microbial interactions change phenotypic allocation of carbon and drive selection that alters metabolic traits, these interactions are tied to the carbon cycle. Furthermore, the dynamics of populations and communities are largely determined by the rate of metabolism and the metabolic products of the member organisms. Waste products, biofilm formation, and growth rate affect a microbe's neighboring cells. Finally, ecosystem processes of energy flux and biomass production are also determined by metabolism. Sinsabaugh et al. (2015) showed an allometric relationship between extracellular enzyme-substrate and production-biomass reactions, indicating that these are linked metabolic processes with relevance at the ecosystem scale.

Though some techniques have been developed to study microbes in the soil environment (O'Donnell et al. 2007), the ability to determine ecological and evolutionary processes at the

microscale is still limited. The determinants of soil community structure and all of its associated network interactions are yet uncertain, as is the ability to deduce its functional capacity relative to spatial and temporal conditions (Prosser 2012). Multi-omics only offer a snapshot of community structure and function, and contain multiple biases and computational bottlenecks with increasing sample sets (Hahn et al. 2016; Nesme et al. 2016; Widder et al., 2016).

Models provide an alternative approach to extrapolate microbial metabolic processes, interactions with the abiotic and biotic environment, and effects on soil carbon cycling and storage (Allison 2012; Allison et al. 2010; Kim and Or 2015; Liang et al. 2011; Widder et al. 2016). Metabolic reconstructions are able to predict cellular and community processes such as biomass yields, formed consortia, evolution, stress adaptations, and the impact of specific phylotypes (Carlson and Taffs 2010; Harcombe et al. 2014; Khandelwal et al. 2013; Oberhardt et al. 2009). Furthermore, metabolic models can be used to explore ecosystem-level processes (Klitgord and Segre 2011). Combining models with new microscale experiments could rapidly advance a predictive understanding of microbial interactions in soil. Such efforts are critical given the myriad mechanisms by which microbial interactions potentially influence the carbon cycle.

CHAPTER 2

Bacteria Trade-offs in Growth Rate and Extracellular Enzymes

2.1 Introduction

One of the primary goals in microbial ecology is to analyze the link between microbial traits and their phylogeny (Martiny et al 2015). This linkage is particularly relevant to biogeochemical cycling because microbes drive organic matter decomposition, the largest transfer of carbon from the biosphere to the atmosphere (Schlesinger and Andrews, 2000; Swift et al. 1979). Multiple studies have found that depends on community structure (Allison et al. 2013; Bier et al. 2015; Matulich and Martiny 2014). Grouping carbon and nutrient cycling traits into meaningful functional categories that are phylogenetically linked will facilitate analysis and predictability of community function in the future (Krause et al 2014).

Life history strategies are sets of traits that have evolved to optimize fitness according to specific external conditions. Multiple life history strategy classifications have been proposed, beginning with r- and K-strategists (Pianka, 1970), or similarly, copiotrophs and oligotrophs (Koc 2001). Copiotrophs are generalists with a high maximum growth rate (μmax), adapted to nutrient-rich environments, and are relatively large in size, have large genomes, many copies of the rRNA operon, and high uptake capacity. Oligotrophs, on the other hand, are specialists with a low μmax, and are adapted to nutrient-poor environments, are relatively small, with small genomes, low rRNA operon copy number, and high substrate affinity (Lauro et al. 2009). While commonly utilized in bacterial studies, this binary categorization is limited in its ability to describe general patterns of evolutionary strategies in response to changing environmental conditions.

Grime's Ruderal-Competitor-Stress tolerator shifts classification of organisms to a more holistic schema, incorporating several traits that are directly related to multifaceted environmental

conditions beyond resource profiles. This creates an improved representation of species'

behavior and potential function, and has been recently applied specifically to microbial species

(Grime 1977; Fierer 2017; Krause et al. 2014). The proposed Stress tolerators have a high

capacity to tolerate stress but are similar to oligotrophs in terms of growth rate and substrate

affinity. Competitors have low tolerance of stress and disturbance, and have traits associated

with a highly competitive environment, such as antibiotic and siderophore production. Finally,

the Ruderal strategy takes advantage of disturbed niches with rapid growth and spore formation.

While the Ruderal-Competitor-Stress tolerator strategies include many important traits related to

the ecological and functional capacity of bacteria, the traits defined according to these strategies

vary between different research groups, and do not consider one of the most critical traits to

carbon and nutrient turnover: extracellular enzyme production (Allison et al. 2011).

Extracellular enzymes are produced by microbes to degrade complex organic matter into useable

products that can be taken up across their membranes. In leaf litter, this trait is potentially

significant for the rate of organic matter degradation because microbes are not physically limited

by access to resources as they are in soil. Extracellular enzymes, in concept, can access most of

the surrounding substrate.

While extracellular enzymes produce useable resources for community members, they also carry

an energetic, material, and reproductive cost of production (Koch 1985). Because bacteria are

limited in the amount of resources they can acquire, they are forced to allocate these resources

towards their most salient needs (Carlson and Taffs 2010). These needs are defined by many

factors, such as available resources, interactions with other species, pH, and water potential, but

are controlled by larger metabolic needs associated with a life history strategy. How bacteria allocate resources potentially plays a pivotal role in the fate of carbon in an ecosystem (Schimel and Schaeffer 2012). Increasing enzyme production lowers carbon use efficiency, resulting in higher rates of respiration and decreased soil carbon storage (Six, et al., 2006). Still, it is unknown a) what trade-offs exist among bacterial growth and enzyme production, b) how these potential trade-offs fit into an overall strategy, c) how the trade-offs are influenced by resource availability, and d) if these patterns are phylogenetically conserved.

Though the genes for extracellular enzymes largely display patterns of shallow phylogenetic conservation, the expressed metabolic investment in total enzyme production relative to growth should reflect an overarching life history strategy that will likely have deeper patterns of conservation (Martiny et al 2013; Martiny et al., 2015; Zimmerman et al. 2013). Multiple factors associated with growth rate (genome size, rRNA operon number, and abundance of genes encoding for periplasmic membrane) make it a more deeply conserved trait and also malleable in response to external conditions (Lauro et al. 2009; Matsumoto et al. 2013). Individual strains of bacteria may trade off growth and resource acquisition temporally within distinct phases of their growth curve, as resources decrease relative to population density (Muthusamy et al. 2017). Given this metabolically-controlled tradeoff in individual strains, it follows that a relationship could also emerge across multiple strains.

Across broad taxonomic groups, we hypothesize that bacteria will display strategies falling along a tradeoff between production of extracellular enzymes and investment in growth. Further, the ratio of investment in extracellular enzymes relative to growth will increase in low resource

conditions. We predict that there will be a weaker tradeoff in these traits in high resource conditions because bacteria will be less limited by available resources and able to invest more in all cellular processes. Finally, we propose that the investment in extracellular enzyme production relative to growth rate will be phylogenetically conserved due to deep evolutionary constraints on resource allocation.

To test these hypotheses, we analyzed extracellular enzyme activity, extracellular protein production, and growth rate with two different resource types in 38 strains of bacteria isolated from plant litter in a Mediterranean grassland ecosystem.

2.2 Methods

2.2.1 Strains used

The 38 strains of bacteria used in this study were originally isolated from plant litter in a Mediterranean grassland ecosystem in Southern California for previous studies (Mouginot et al. 2014; Matulich and Martiny,2014; Potts et al. 2012). There are 13 representatives from the Phylum Proteobacteria, 23 from Actinobacteria, and 2 from Bacteroidetes. These strains were chosen from among the originally isolated strains because their 16S rRNA sequences had already been obtained in the aforementioned studies, and because these strains could be revived from frozen glycerol stock cultures.

2.2.2 Growth and storage conditions

All strains were initially stored at -80°C in glycerol stocks. These stocks were used to streak onto luria broth (LB) agar plates. Once colonies formed, one colony from each strain was chosen to

inoculate a corresponding flask of liquid LB, which, upon reaching exponential growth, was then used to inoculate further flasks to produce growth curves and supernatant for enzyme and protein assays. All flasks were incubated at 28°C on a shaking platform.

Growth curves were performed in triplicate, with time intervals between measurements dependent upon rate of growth for the individual strain. For enzyme and protein assays, each strain was grown in quintuplicate for 14 hours until collection. At the point of collection, the optical density (OD_{600}) was measured, the cultures were centrifuged and the supernatant was collected and frozen at -20°C until further processing. To do these analyses, it was crucial to take measurements of enzyme activity and protein production during active growth to determine the overall investment relative to growth. We chose 14 hours as the assay time because this time fell before the fastest-growing strains reached stationary phase. Therefore, these results represent the amount of enzyme and protein that each strain could produce in 14 hours.

LB was chosen because these strains were shown previously to grow in this medium, and it represents nutrient-rich conditions, likely supporting the maximum growth rate possible for many of these strains. Plant litter broth (PB) was chosen because it more closely represents the native resources of the environment from which the bacteria were originally isolated, and because it is relatively nutrient poor. In preliminary growth assays, PB was found to support growth rates typically associated with nutrient poor conditions, that is, all strains had a relative instantaneous specific growth rate (μ) less than 1/20 of "maximum" μ (Konopka 2000).

PB was made using grass litter from the site where the strains were originally isolated. Litter

was dried, ground, and added to deionized water. After 6 hours of heated stirring, the litter was

removed using centrifugation and filtered, then autoclaved for sterilization. All results in this

study in PB were produced using the same litter batch to ensure consistent nutrient conditions.

2.2.3 Growth curves

Spectrophotometer measurements (OD_{600}) were taken until the cultures appeared to be no longer

increasing in biomass, using 96 well plates and a Biotek Synergy2 plate reader.

The Grofit package in R was used to determine the μ and the maximum attainable biomass (A) of

each growing population in both broths (Kahm et al. 2010). Grofit uses logistic, Gompertz,

modified Gompertz, and Richards models to fit the data. The parameters of the best-fit model

that was returned in the results for each strain was used for further analysis. If a model could not

be significantly fitted to the growth curve, the corresponding strain was omitted from the study.

Additionally, some strains displayed a diaxic growth pattern. In these instances, μ was calculated

only using the portion of the curve corresponding to the initial growth phase.

2.2.4 Enzyme and protein assays

Once the supernatants from all strains in this study had been collected, they were frozen at -20°C

and later thawed for fluorometric enzyme and Lowry assays. Protein assays were carried out to

complement the results of extracellular enzyme production as an additional metric of

extracellular product.

Hydrolytic enzymes were assayed using the methods in (German et al. 2011), and include assays for the C-targeting enzymes α-glucosidase, β-glucosidase, cellobiohydrolase, β-xylosidase, and N-acetyl-glucosidase, P-targeting acid phosphatase, and N-targeting leucine aminopeptidase. Enzyme activities were calculated based on the mean linear change in fluorescence readings taken every 30 minutes for two hours. Each reading is the calculated nmol product released g substrate^{-1} OD^{-1}). All final enzyme and protein values are biomass-specific (per unit OD). For statistical analyses, values were log+1 -transformed.

2.2.5 Phylogeny

The 16S rRNA sequences were retrieved from the GenBank at NCBI database. The accesssion numbers are: KF733300-01, -03 to -09, -11 to -20, -22, -23, -25, -27 to -31, -34 to -40, and KF881974, -76, -77, -80, -81, and -84. We used SILVA to verify taxonomic identification against the EMBL, LTP, RDP, SILVA, and GenBank databases, however, the only complete set of taxonomic identification was retrieved from GenBank, with the sequence identity from GenBank being supported by the other databases. We aligned our sequences with a phylogeny-aware algorithm in webPRANK (https://www.ebi.ac.uk/goldman-srv/webprank/) (Löytynoja and Goldman 2010).

Using these aligned sequences, we generated a majority consensus tree with 100 bootstrap replications using a transition/transversion ratio=2, a constant base rate variation among sites, and empirical base frequencies in the PHYLIP v. 3.695 software package (Felsenstein 2005). This tree was then visualized with iTOL v.4.0.3 (https://itol.embl.de/) (Letunic and Bork 2016).

2.2.6 Statistical analysis,

Because non-normal distribution of data was found within each trait using the Shapiro-Wilk

normality test, we used the Wilcoxon Rank Sum Test to examine the differences in measured

traits between the two broths. Spearman rank correlations were performed to test for

relationships between traits. The Kruskal-Wallace test was used to analyze the differences in

traits among taxonomic groups (Phylum and Class), as well the strategies of Resource

acquisition-Growth-Maintenance that emerged from the LB data.

Analysis of the phylogenetic signal of traits was done using the phylosignal v. 1.1 package in R

(Keck, et al., 2016), with the results including Bloomberg's K and Pagels λ. To understand how

our proposed strategies of Resource acquisition-Growth-Maintenance relate to phylogeny, the

strategies were compared to Phylum and Class using the categorical Pearson's chi-squared test.

2.3 Results

2.3.1   Growth parameter

LB supported a higher μ in all 38 strains of bacteria, with a range from 0.02 to 0.66 OD hr^{-1}. In

PB, the range for μ was significantly lower, with values from 7.6×10^{-5} to 0.02 hr^{-1} (Table 2.1).

The maximum biomass reached, A, ranged in LB from OD_{600} 0.40 to 5.70, while it was only 0.03

to

0.14 in PB. We found that μ and A are significantly correlated in both LB and PB (Spearman

ρ=0.86, p<0.001; and ρ =0.85, p<0.001, respectively).

However only 14 of the 38 strains grew in PB, with representatives growing in PB from all three

phyla (Proteobacteria, Actinobacteria, and Bacteroidetes) in our study. Five of the 23

Table 2.1: Range of values of enzyme activity, growth, and protein production in both broths, as well as Wilcoxon Rank Sum p-values of these values between broths.

		LB		PB		Wilcoxon p-value	
		No. of strains with detectable activity	Range of expression	No. of strains with detectable activity	Range of expression	All strains	Non-growing strains removed
Enzyme activity (nmol g^{-1} OD^{-1}hr^{-1})	AG	17	$0 - 6.0 \times 10^5$	2	$0 - 3.9 \times 10^4$	<0.001	0.04
	AP	6	$0 - 9.5 \times 10^3$	1	$0 - 4.8 \times 10^2$	0.043	0.38
	BG	15	$0 - 1.7 \times 10^2$	2	$0 - 1.0 \times 10^3$	0.002	0.25
	BX	3	$0 - 4.7 \times 10^3$	0	0	0.08	0.30
	CBH	4	$0 - 6.4 \times 10^2$	0	0	0.043	0.22
	LAP	32	$0 - 5.1 \times 10^5$	6	$0 - 5.9 \times 10^4$	<0.001	0.03
	NAG	9	$0 - 2.2 \times 10^4$	1	$0 - 2.8 \times 10^2$	0.006	0.16
	Total	34	$0 - 1.2 \times 10^6$	8	$0 - 9.8 \times 10^4$	<0.001	0.008
	Total C	23	$0 - 6.2 \times 10^5$	3	$0 - 3.9 \times 10^4$	<0.001	0.02
Growth hr^{-1}	μ	38	$0.02 - 0.66$	14	$7.6 \times 10^{-5} -$	<0.001	<0.001
	A		$0.40 - 5.70$		$0.03 - 0.14$	<0.001	<0.001
Protein		37	$0 - 455.7$	14	$0 - 1039.1$	<0.001	0.09

Actinobacteria members in this study grew in PB, 1 of the 3 Bacteroidetes, and 8 of the 14

Proteobacteria. Of Proteobacteria, only Class Gammaproteobacteria actively grew in PB, while

the single member of Bacteroidetes that grew in PB is in the Class Flavobacteriia. The only Class

of Actinobacteria in this study was Actinobacteria.

2.3.2 Extracellular enzyme activity

Total enzyme activity in LB ranged from 0 to 1.2×10^6 nmol $g^{-1}OD^{-1}hr^{-1}$, with 32 of the 38 strains

producing some enzyme (Table 2.1). However, in PB, total activity was 0 to 9.8×10^4 nmol

$g^{-1}OD^{-1}hr^{-1}$, with only 8 of the 14 strains showing any enzyme activity. The remaining 6 strains

that grew in PB that had no detectable activity were Strains 122, 160, 17

(Gammaproteobacteria), and Strains 212, 115, 145 (Actinobacteria). Strain 122 also had no

activity in LB, but all other strains that had no detected activity in LB did not grow in PB (Figure

2.2).

No strains increased their C enzyme activity in PB relative to the levels in LB, but a few

increased their N enzyme activity, including Strains 117, 124, 114, 9 (Gammaproteobacteria),

and 41 (Flavobacteriia). Strains 117, 124, 114, and 9 all had no detected C enzyme activity in

either broths. More strains produced at least some N enzymes in both broths, but not necessarily

C or P enzymes. Conversely, P enzyme activity showed the lowest levels of production in both

broths. Only Strain 117 produced any P enzyme in PB, increasing its amount of production

relative to LB.

2.3.3 Extracellular protein

In LB, 35 of the 38 strains produced detectable protein, up to 455.7 µg $ml^{-1}OD$. All 14 strains

growing in PB produced protein, ranging up to 1039.1 µg/ml*OD. We found that protein

production and the log of enzyme activity had a significant relationship in PB, but not in LB

(Spearman ρ =-0.59, $p<0.001$ in PB; ρ =-0.11, p=0.5 in LB). All strains that had no detectable

amounts of protein in LB did not appear to grow in PB, nor did those with the highest amount of

produced protein in LB. All Actinobacteria and the single Bacteroidetes that grew in PB

increased their protein production in PB relative to LB, however, 5 of the 8

Gammaproteobacteria showed a reduction of protein in PB.

2.3.4 Relationships between traits

To test the first hypothesis, that extracellular enzyme production and growth rate trade off, we performed Spearman rank correlations, expecting a negative relationship. Overall, there was no significant relationship between extracellular enzyme production and μ in either broths. Yet when we removed the
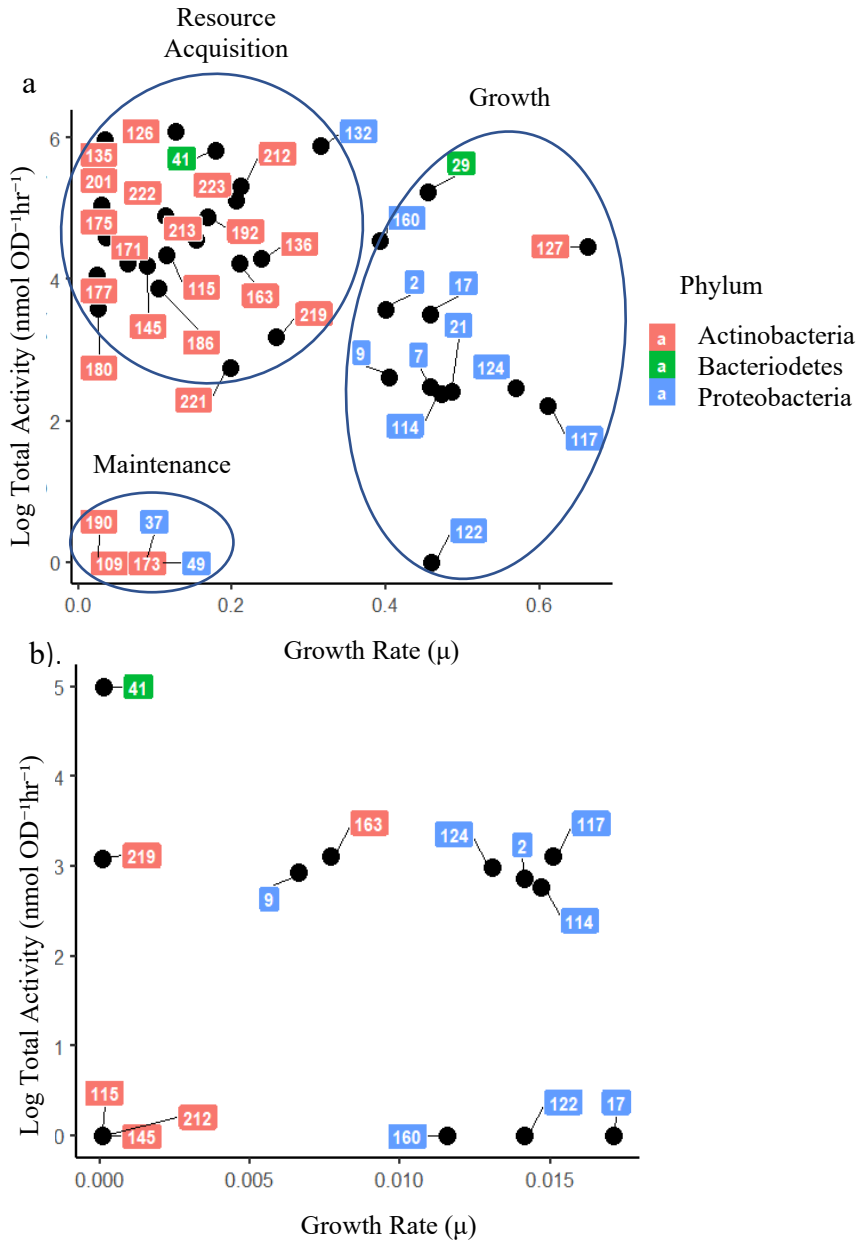
Figure 2.1. Graph of the growth rate, μ, and the log of total enzyme activity in LB (a) and PB (b). Strategies of resource acquisition, maintenance, and growth are defined by circles in

strains with low growth rate and no enzyme production, we found a correlation between the log

of extracellular enzyme production and growth rate in LB, but not in PB (Spearman ρ=-0.48, p=0.004 in LB) (Figure 2.1).

Similarly, when we tested the relationships between growth and C or N enzymes specifically, there were no significant relationships unless we removed the low-producing strains. Only in LB, μ correlated with the log of both total C and N enzyme (Spearman ρ=-0.44, p=0.001 and ρ=-0.61, p<0.001). P enzyme relationships could not be tested because the number of individual strains producing P enzymes was too low. Growth and protein showed no relationship in either broths.

We also analyzed how the growth medium affected the log of Total Activity:μ ratio using a Wilcoxon rank sum test. This ratio was significantly different between the two broths when considering only the 14 strains that grew in PB (p-value=0.004). The median value of the log of Total Activity:Mu was 15.98 in LB, and 195.53 in PB. This difference partially supports our second hypothesis that bacteria will increase their relative investment in extracellular enzyme production and decrease their investment in growth under low resource conditions. All strains had less absolute total activity in PB than in LB. Eight strains increased their investment in extracellular enzymes relative to growth in PB, while, unexpectedly, 6 strains eliminated extracellular enzyme investment completely. All strains dramatically decreased their growth in PB with reductions from 96.3% to 100% relative to LB. The strength of the tradeoff in low resource environment is mixed in PB. Some strains increased their Total Activity relative to μ, but some strains reduced it to zero or very low values (Figure 2.1b).
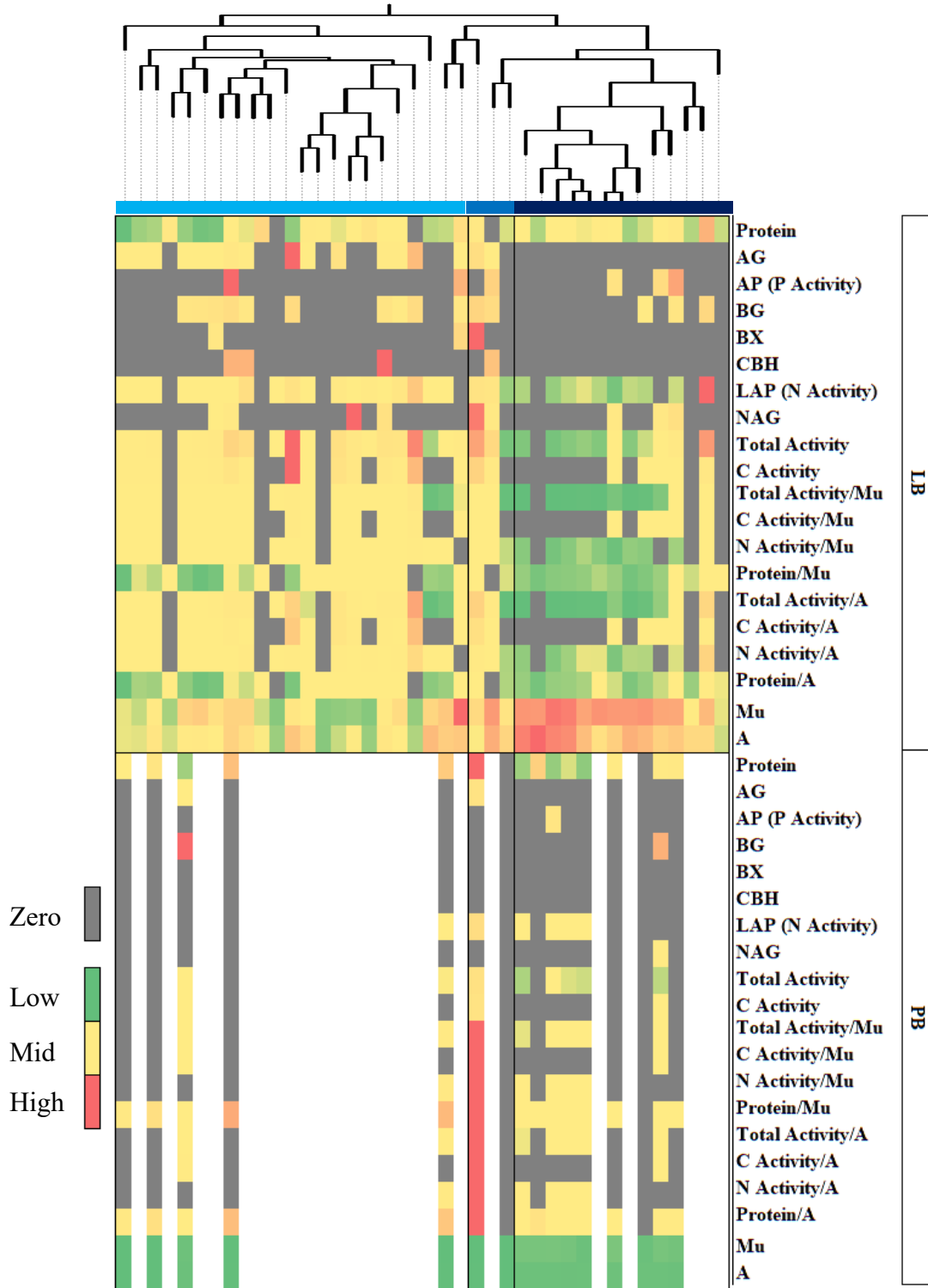
Figure 2.2: A majority consensus tree with 100 bootstrap replications and a corresponding heatmap of the values of measured traits in both LB and PB. Phyla are Actinobacteria (light blue), Bacteroidetes (medium blue), and Proteobacteria (dark blue). The color coding indicates the relative expression levels of the measured traits associated with each strain, in both broths.

2.3.5 Phylogenetic signal of traits

We used Blomberg's and Pagel's tests to analyze the phylogenetic conservation of our measured traits (Table 2.2) (Blomberg et al.,2003; Freckleton et al. 2002). According to Blomberg's test, the two growth parameters, μ and A, were more similar between relatives than would occur under Brownian motion evolution in PB only, but extracellular enzyme activity and protein production had no phylogenetic signal in either broth. Pagel's test found that λ approached or surpassed 1 for multiple traits, including the ratio of Total Activity:μ, μ, and A in both broths, indicating that these traits correlate with phylogeny in a manner dependent upon shared evolutionary history.

2.4 Discussion

In reference to our first hypothesis, we found that some bacteria exhibit strategies that align with a tradeoff between extracellular enzyme production and investment in growth under high resource conditions. However, this pattern was not observed in low resource conditions. There was also some evidence to support our second hypothesis, that the ratio of investment in extracellular enzymes relative to growth will increase in low resource conditions, though this was not observed in all strains. Some strains instead eliminated extracellular enzyme production completely. There were mixed findings for our final hypothesis that the extracellular enzyme production relative to growth rate will be phylogenetically conserved. Blomberg's test did not support conservation of the ratio of Total activity:μ, but Pagel's did.

Our results show that our hypotheses are supported, but with multiple exceptions. To interpret these patterns, it is helpful to categorize bacteria into strategies that incorporate an explanation

for the varied response present in our data. Adding enzyme production into a life history strategy confounds previous life history strategy classifications, though, so we have proposed new strategies based on our findings.

There is a significant relationship between extracellular enzyme production and growth rate across multiple species of bacteria in high resource media when non-enzyme producing strains are removed. The pattern that emerged through the data corresponding to LB display three distinct bacterial strategies that are suggestive of an evolutionary tradeoff. We defined a Growth strategy, which displayed high growth rates ($\mu \geq 0.35$), with a median log Total Activity of 2.54 nmol/g*OD, and median A of 3.22 OD (Figure 2.3). Our Resource Acquisition strategy is heavily invested in extracellular enzyme production, with growth rates from the low- to mid-range ($0 < \mu \leq 0.35$), median log Total Activity of 4.55 nmol g^{-1}OD^{-1}hr^{-1}, and median A nearly half of the Growth strategy at 1.65 OD. Finally, our Maintenance strategy is characterized by low growth rate ($\mu < 0.10$), no extracellular enzyme production, and median A of 1.14 OD. It is possible that the Maintenance strategy is reflective of an unknown resource

Resource Acquisition
$0 < \mu \leq 0.35$
Median A=1.65 OD
Median Total Activity=4.55
nmol g^{-1}OD^{-1}hr^{-1}

Growth
$\mu > 0.35$
Median A=3.22 OD
Median Total Activity=2.54
nmol g^{-1}OD^{-1}hr^{-1}

Maintenance
$\mu < 0.10$
Median A= 1.14 OD
Median Total Activity=0

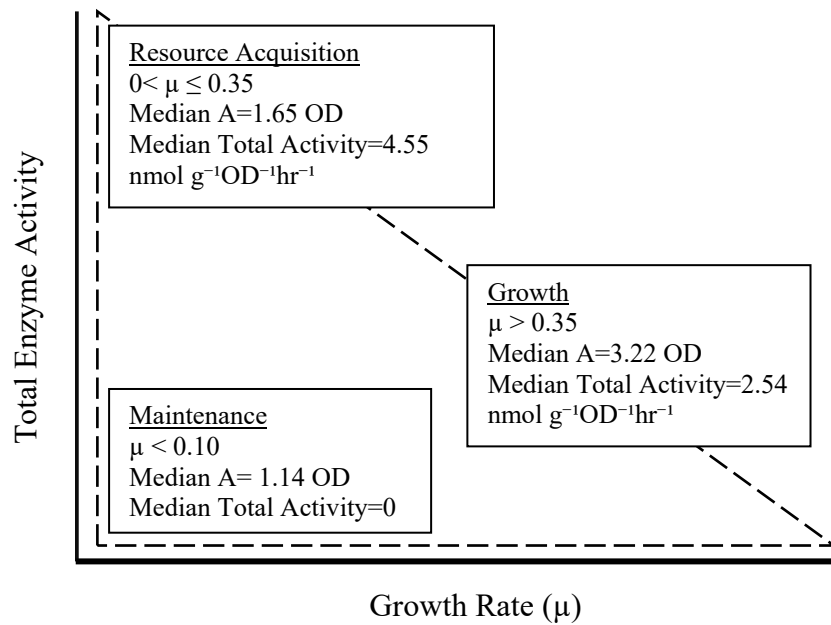Total Enzyme Activity

Growth Rate (μ)

Figure 2.3: A schematic representation of the tradeoff between extracellular enzyme production and growth rate, along with the corresponding strategies.

limitation affecting the strains characterized in this group, however, it is also a possibility that this is reflective of a distinct strategy. Expression of μ, A, and total enzyme activity were significantly different between these three groups in LB, as well as expression of μ and A in PB (Table 2.3). No maintenance strategists grew in PB.

All Gammaproteobacteria grouped exclusively in the Growth strategy, with the rest of the Proteobacteria phyla spread across the other two strategies. Bacteroidetes was assigned to either Growth or Resource Acquisition, and Actinobacteria had representatives in all 3 strategies. Because we could not test the phylogenetic conservation of strategy, we used a Pearson's chi squared test to analyze whether our strategy categorization was correlated with Phylum and Class. We found that it was significantly correlated with both (with Phylum H=22.838, df=4, p-value<0.001, and with Class H=39.973, df=4, p-value<0.001). Some evidence suggests that life history strategy is conserved at the Phylum level, specifically the copiotroph-oligotroph spectrum (Fierer et al 2007; Philippot et al., 2010). Growth rate is a trait particularly associated with life history strategy definitions, and we found that growth rate is a trait that is phylogenetically conserved in both media.

Growth and Resource Acquisition strategies appear to lie on a spectrum of expression of extracellular enzyme activity and growth, apart from the Maintenance strategy. Under reduced resources, the altered expression of Growth and Resource Acquisition strategies is likely part of a sliding-scale response.   While μ is more closely aligned with strategy groupings, extracellular enzyme activity is more variable than μ dependent upon resource conditions. There are several solutions for a bacterium that might allow for decreased production or elimination of

extracellular enzymes. Some bacteria could be preferentially switching to membrane-tethered enzymes to reduce the loss of their investment, or upregulating transport machinery (Traving et al. 2015). Enzymes that are attached to the membrane would not be measured in our assays due to separation of the supernatant from the cells. Likewise, there may be a decrease in cell size, which increases the cell surface:volume ratio, which concentrates the capacity for resource uptake relative to size (Harder and Dijkhuizen 1983; Koch 1985). Ultimately, if the cost of enzyme production is greater than the resource benefits received as a result of production, bacteria should adjust growth rate according to other remaining resources to optimize fitness, and avoid investment in costly enzyme production (Dekel and Alon 2005).

Overall, the Activity:μ ratio was dramatically higher in PB than in LB. Several studies have found that a transition from exponential growth phase to stationary phase and starvation response coincides with a decrease in production of growth-related proteins and an increase in resource acquisition proteins, including enzymes and receptors (Ferenci1999; Harder and Dijkhuizen, 1983; Kooijman et al. 1991; Matsumoto et al. 2013; Muthusamy et al. 2017; Navarro Llorens et al. 2010; De Mattos and Neijssel 1997). Because of the low growth rates associated with PB, it is reasonable to assume that the metabolic response to this media may be similar to what occurs with a starvation response.

We found the relative investment in resource acquisition and growth was distinct between strategies. In LB, the median values of the Activity:μ ratio were 5.93 for the Growth strategy, 0 for Maintenance, and 37.11 for Resource Acquisition. In PB, though, the median value was 195.53 for the Growth strategy and 202.09 for the Resource Acquisition strategy. Both Growth

and Resource acquisition strategies had member strains that eliminated all extracellular enzyme activity in PB. Strains with high Activity:μ ratios, members within the Resource acquisition strategy, had lower relative biomass when compared to Growth strategists. The Resource acquisition strategy also had much higher median protein production in PB than did the Growth strategy (168.42 μg g^{-1}OD^{-1}hr^{-1} Resource acquisition and 17.20 μg g^{-1}OD^{-1}hr^{-1} Growth).

It appears that the relationship between protein production, μ, and biomass production A is critical for distinguishing strategies in PB. There may be other extracellular enzymes being produced that we did not measure, especially because total enzyme activity and protein production were not related in either broth. There could also be other types of proteins being produced, such as signaling molecules or siderophores, which we did not analyze.

Aside from μ and A, the Protein:A and Protein:μ ratios were the only traits that were significantly different between the Resource acquisition and Growth strategies in PB. The Resource acquisition strategy again displayed higher ratios than the Growth strategy. Similar to the case with extracellular enzyme activity, this difference may be attributed to a lower carbon use efficiency caused by energetically expensive protein production. For example, Strain 122, a Pseudomonad with a Growth strategy, produced no detectable enzyme in either broth, and had the highest biomass reached in LB and one of the highest in PB. Strain 41, a member of Bacteroidetes and a Resource acquisition strategist, stood out for its high investment in extracellular enzyme activity and protein in both broths, but reached a relatively low biomass, particularly in PB. A sister species within the same genus as this strain possesses many genes for

peptidases and polysaccharide-degrading enzymes, including hemicelluloses and chitinase, and produces high amounts of protein for use in motility (McBride et al. 2009).

Based on our data, we can make limited comparisons to previously defined life history strategies for microbes. Growth and metabolic output, as protein, extracellular enzyme activity, or biomass production, are the primary traits relevant to existing life history frameworks. We can also infer catabolic diversity based upon whether the strains are capable of growth in both media. Our Growth strategy is similar to copiotrophy primarily because it is characterized by a high μ. Additionally, though copiotrophs are poor competitors in low resource environments, they can maintain some growth because they are classified as resource generalists with higher catabolic diversity (Freilich et al. 2009). Eight of our 12 Growth strategists were capable of growth in both media, a higher fraction than any of our other strategies, lending support to this idea.

It is difficult to fit our Resource Acquisition strategists onto the copiotroph-oligotroph spectrum. Though they do have a lower median growth rate than the Growth strategists, which overlaps with the Maintenance strategists, they have high metabolic output in the form of extracellular enzymes, opposing the characterization of oligotrophs as having low metabolic capabilities.

Our Maintenance strategists are similar to oligotrophs owing to their slow growth rate, low extracellular enzyme activity, and possible resource specialization (Freilich et al. 2009). None of these strategists grew in PB, which contradicts the understanding of oligotrophs as capable of growth in low resource environments, particularly because PB was made with litter from the plots from which these bacteria were isolated. It is possible that the resources that these Maintenance strategists require might not have effectively leached into the broth. Also, our

Maintenance strategists had the highest median protein production in LB, at 20.01 µg ml^{-1}OD^{-1}, indicating higher metabolic output than might be anticipated from an oligotroph.

The Stress tolerator-Ruderal-Competitor strategies might be useful in describing our results, though the traits associated with each strategy are further from consensus than the oligotroph-copiotroph spectrum. Our maintenance strategy would most closely resemble the stress tolerators of this schema, which are likely auxotrophic, requiring metabolic byproducts from other taxa to survive (Fierer 2017). Nutrient limited conditions have been associated with streamlining, a process in which bacteria jettison unnecessary genes to reduce metabolic and reproductive inefficiencies. This results in strains with high resource specialization that are interdependent with other taxa (Giovannoni et al. 2014). Contrary to this mechanism, there is evidence that reduction in nutritional competence is an indirect effect of adaptation to a high stress environment, predicated on alterations to cellular signaling pathways (Ferenci and Spira 2007). Either way, adaptations to stressful environments may explain why the Maintenance strategists in our study could not grow as monocultures in PB. Similar to our Maintenance strategists, Stress tolerators are also defined by slow growth. An inverse relationship between stress tolerance and growth rate is widely recognized, potentially due to the antagonistic quality between the cellular growth and stress pathways (López-Maury et al. 2008).

Aligning our Resource acquisition and Growth strategies with Grime's framework is more problematic. There is not a clear pattern of trait assignment to either of these strategies in previously defined work, though it could be argued that both our Resource acquisition and Growth strategies have high catabolic diversity, though in different forms. Resource acquisition

strategists produced a relatively high amount of free extracellular enzymes to utilize the surrounding resources, while the Growth strategists were clearly able to utilize diverse resource types based on their ability to grow in both media. This may be due to diverse uptake machinery or membrane-bound enzymes. There is also conflicting evidence on the relationship between growth rate and substrate use diversity. In soil, this relationship is positive, while in freshwater aquatic systems, the trend is negative (Kurm et al. 2017; Livermore et al. 2014).

A previous study showed that while closely-related taxa shared similar substrate-use profiles and growth rates, their catabolic diversity was not phylogenetically conserved (Dolan et al. 2017). Because our strategies are highly correlated with phylogeny, we infer that catabolic diversity is not a specific trait related solely to either Resource acquisition or Growth strategies.

Relative growth rate is likely determined by total incoming resources and growth efficiency associated with life history strategy, while previous evidence suggests that enzyme expression levels can be influenced by many factors. Substrate abundance has a strong influence on extracellular enzyme activity (German et al. 2011; Vetter et al. 1998; Chatterjee et al. 2005; Sachia et al. 2015; Allison et al. 2014). Additionally, optimal strategies for extracellular enzyme production depend on diffusion rates, whether a bacterium is solo or in a colony, if it is mobile, and if cheaters are present (Allison et al. 2014, 2011; Traving et al. 2015). Enzyme expression has even been regulated by the presence of seemingly unrelated substrates, such as amino acids repressing the expression of a lipase (Eggert et al. 2003). There are likely many complicated regulatory mechanisms that have evolved to maintain stoichiometric balance (You et al. 2013). These factors may have affected trait measurements in PB.

As bacteria live a majority of their existence under nutrient limitation, adaptations to this state are critically important and influenced by strategy. There is a high reproductive and competitive cost to low resource adaptation because of the growth lag and growth machinery required when resources are reintroduced (Geisel et al. 2011). In highly variable environments, Growth strategists may be most fit if they double down on growth investment in the face of resource limitation. However, for Resource acquisition or Maintenance strategists, it may improve fitness to convert to maintenance physiology or dormancy. We found that none of the Maintenance strategists, and none of the Resource acquisition strategists with $\mu<0.085$ were able to grow in PB. Strains that did not grow in PB either sustained OD over extended periods of time, or showed declining OD in PB (Figure 2.4). This pattern may suggest either a transition to dormancy, maintenance physiology, or death in the strains that would not grow in PB (Kempes et al. 2017; Lennon and Jones 2011). Consistent with our results, Actinobacteria is a Phylum characterized by many species with diverse metabolisms and morphologies, and a high prevalence of sporulation upon nutrient limitation (Barka et al. 2016). Only 5 of our 23 representatives from Actinobacteria actively grew in PB, the lowest fraction of all three phyla in this study.

2.5 Conclusion

The fate of carbon in an ecosystem is potentially determined by how microbes allocate their resources. Adding in the dimension of resource acquisition as part of a distinct microbial strategy, and linking that to phylogeny, is important to furthering our understanding of community function. More physiological studies are needed to understand how traits are expressed relative to environmental conditions and interacting community members. Using a

more complete trait-based framework to define microbial life history strategies may facilitate the integration of environmental genomics to community and ecosystem models, and even conservation efforts (Vandenkoornhuyse et al. 2010; Krause et al. 2014; Parker 2010).

Table 2.2: Blomberg's and Pagel's phylogenetic signal of traits across 38 strains of bacteria

| | | Blomberg's | | Pagel's | |
		K	p	λ	p
LB	Protein	0.21	0.32	7.09e-05	1
	AG	0.54	**0.02***	0.49	**0.004***
	AP	0.25	0.24	0.04	0.86
	BG	0.33	0.16	0.71	0.07
	BX	0.20	0.41	7.09e-05	1
	CBH	0.21	0.43	7.09e-05	1
	LAP	0.21	0.59	7.09e-05	1
	NAG	0.30	0.20	0.06	0.87
	Total Activity	0.21	0.61	7.09e-05	1
	C Activity	0.32	0.24	0.23	0.12
	Total Activity:Mu	0.34	0.12	1.07	**0.001***
	C Activity:Mu	0.31	0.15	0.26	0.19
	N Activity:Mu	0.30	0.15	1.07	**0.001***
	Protein:Mu	0.22	0.31	7.09e-05	1
	Total Activity:A	0.29	0.25	0.30	0.16
	C Activity:A	0.40	0.07	0.32	**0.04***
	N Activity:A	0.26	0.29	0.27	0.27
	Protein:A	0.21	0.41	7.09e-05	1
	Mu	0.90	**0.002***	0.78	**0.001***
	A	0.74	**0.03***	0.76	**0.001***
PB	Protein	0.33	0.2	7.33e-05	1
	AG	0.27	0.58	7.33e-05	1
	AP	0.25	0.80	7.33e-05	1
	BG	0.23	0.93	0.99	1
	LAP	0.48	0.16	0.35	0.31
	NAG	0.26	0.64	7.33e-05	1
	Total Activity	0.40	0.28	0.36	0.37
	C Activity	0.27	0.66	7.33e-05	1
	Total Activity:Mu	0.41	0.197	1.11	**0.001***
	C Activity:Mu	0.29	0.196	7.33e-05	1
	N Activity:Mu	0.41	0.191	1.11	**0.001***
	Protein:Mu	0.37	0.154	1.11	**0.001***
	Total Activity:A	0.43	0.157	1.09	0.12709
	C Activity:A	0.29	0.352	7.33e-05	1
	N Activity:A	0.43	0.156	1.09	0.097099
	Protein:A	0.34	0.173	7.33e-05	1
	Mu	1.57	**0.001***	1.11	**0.001***
	A	1.47	**0.002***	1.11	**0.001***

Table 2.3: Kruskal-Wallace values of traits in both broths relative to the strategy groups, Phylum and Class. The values in PB reflect only the strains that grew in PB.

|		Strategy			Phylum			Class		
		H	p	df	H	p	df	H	p	df
LB	Protein	2.77	0.2501	2	0.75	0.69	2	1.25	0.87	4
	AG	15.43	**<0.001***	2	14.56	**<0.001***	2	14.56	**0.006***	4
	AP	7.90	**0.02***	2	2.62	0.27	2	4.00	0.41	4
	BG	6.08	**0.05***	2	8.21	**0.02***	2	10.39	**0.03***	4
	BX	0.49	0.7811	2	6.44	**0.04***	2	6.44	0.17	4
	CBH	1.04	0.5943	2	4.28	0.12	2	4.28	0.37	4
	LAP	24.68	**<0.001***	2	10.90	**0.004***	2	12.85	**0.01***	4
	NAG	2.01	0.3656	2	7.11	**0.03***	2	8.24	0.08	4
	Total Activity	20.15	**<0.001***	2	10.27	**0.006***	2	11.83	**0.02***	4
	C Activity `	15.07	**<0.001***	2	9.82	**0.007***	2	11.22	**0.02***	4
	Total Activity:Mu	29.31	**<0.001***	2	13.59	**0.001***	2	14.04	**0.007**	4
	C Activity:Mu	17.44	**<0.001***	2	9.65	**<0.001***	2	9.95	**0.04***	4
	N Activity:Mu	28.89	**<0.001***	2	12.20	**0.002***	2	12.67	**0.01**	4
	Protein:Mu	7.60	**0.02***	2	1.21	0.55	2	4.74	0.32	4
	Total Activity:A	26.45	**<0.001***	2	11.14	**0.004***	2	11.88	**0.02***	4
	C Activity:A	17.44	**<0.001***	2	9.76	**0.008***	2	10.27	**0.04***	4
	N Activity:A	27.79	**<0.001***	2	10.00	**0.007***	2	11.15	**0.03***	4
	Protein:A	3.56	0.1687	2	0.12	0.94	2	1.16	0.8843	4
	Mu	25.41	**<0.001***	2	14.49	**<0.001***	2	19.83	**<0.001***	4
	A	19.78	**<0.001***	2	11.46	**0.003***	2	16.48	**0.002***	4
PB	Protein	3.27	0.07	2	4.46	0.11	2	4.46	0.11	2
	AG	2.87	0.09	2	7.8	**0.02***	2	7.8	**0.02***	2
	AP	0.75	0.39	2	0.75	0.69	2	0.75	0.69	2
	BG	0.10	0.75	2	0.39	0.82	2	0.39	0.82	2
	LAP	0	1	2	3.55	0.17	2	3.55	0.17	2
	NAG	0.75	0.39	2	0.75	0.69	2	0.75	0.69	2
	Total Activity	0.22	0.64	2	2.82	0.24	2	2.82	0.24	2
	C Activity `	1.16	0.28	2	5.18	0.08	2	5.18	0.08	2
	Total Activity:Mu	0.22	0.64	2	2.82	0.24	2	2.82	0.24	2
	C Activity:Mu	1.16	0.28	2	5.18	0.08	2	5.18	0.08	2
	N Activity:Mu	0	1	2	3.55	0.1692	2	3.5531	0.17	2
	Protein:Mu	6.02	**0.01***	2	6.78	**0.03***	2	6.78	**0.03***	2
	Total Activity:A	0.37	0.54	2	2.82	0.24	2	2.82	0.24	2
	C Activity:A	1.16	0.28	2	5.18	0.08	2	5.18	0.08	2
	N Activity:A	0	1	2	3.55	0.17	2	3.55	0.1692	2
	Protein:A	5.4	**0.02***	2	6.24	**0.04***	2	6.24	**0.04***	2
	Mu	8.82	**0.003***	2	8.94	**0.01***	2	8.94	**0.01***	2
	A	8.82	**0.003***	2	8.94	**0.01***	2	8.94	**0.01***	2

a)
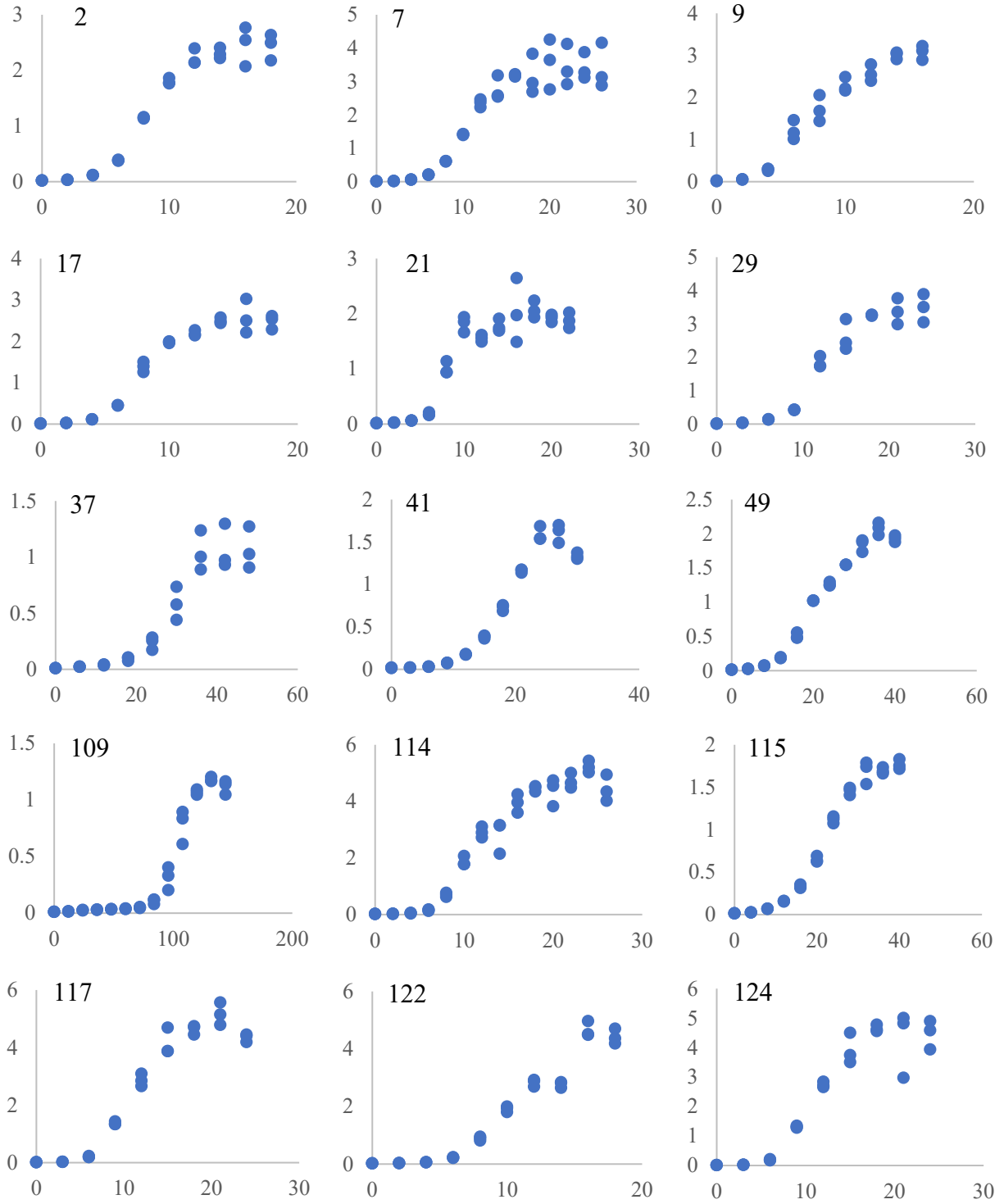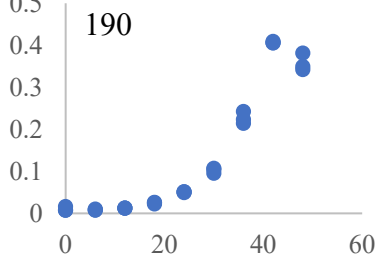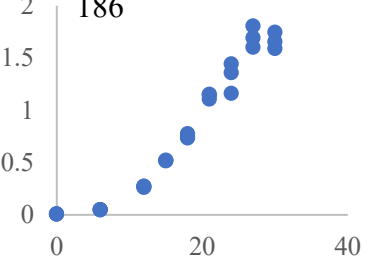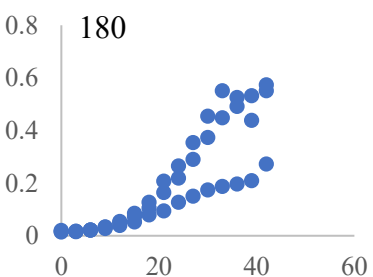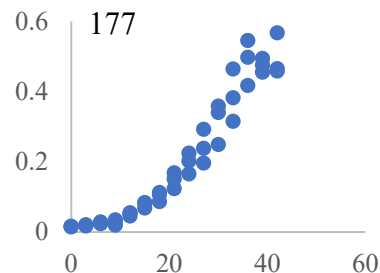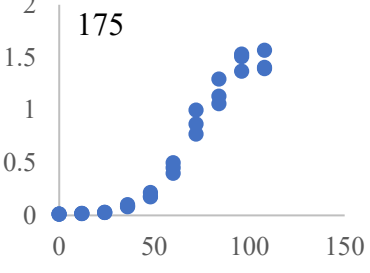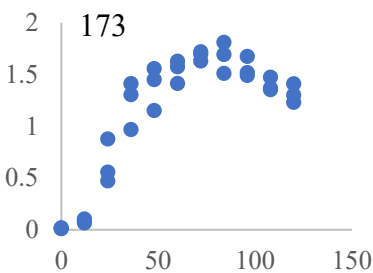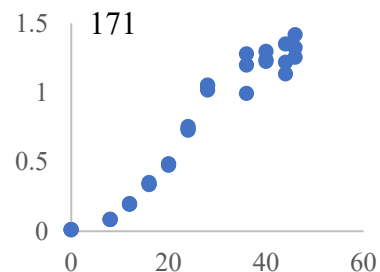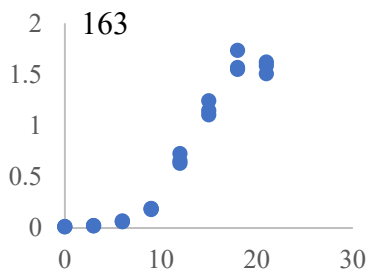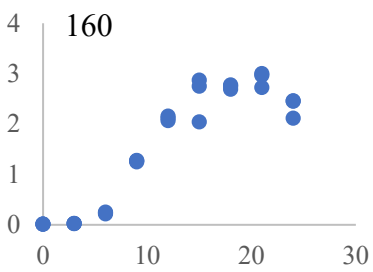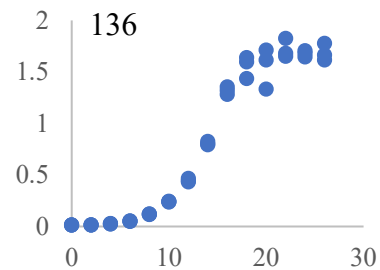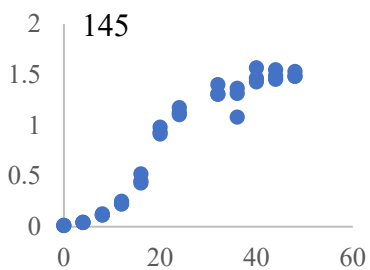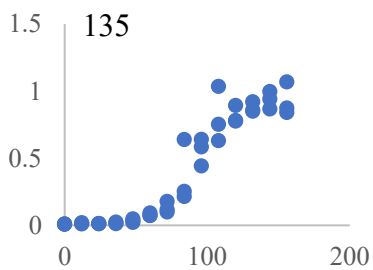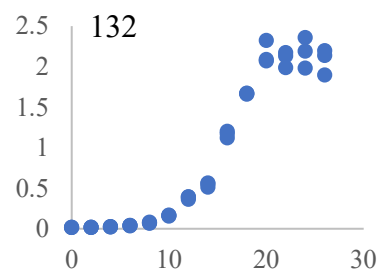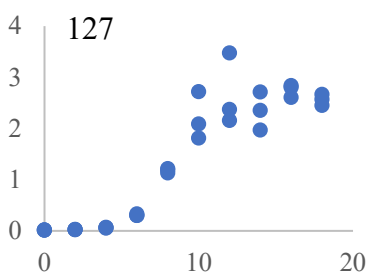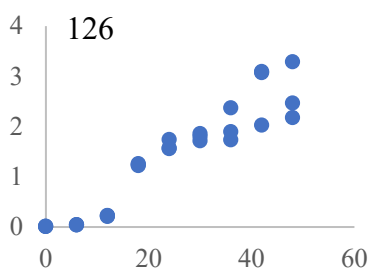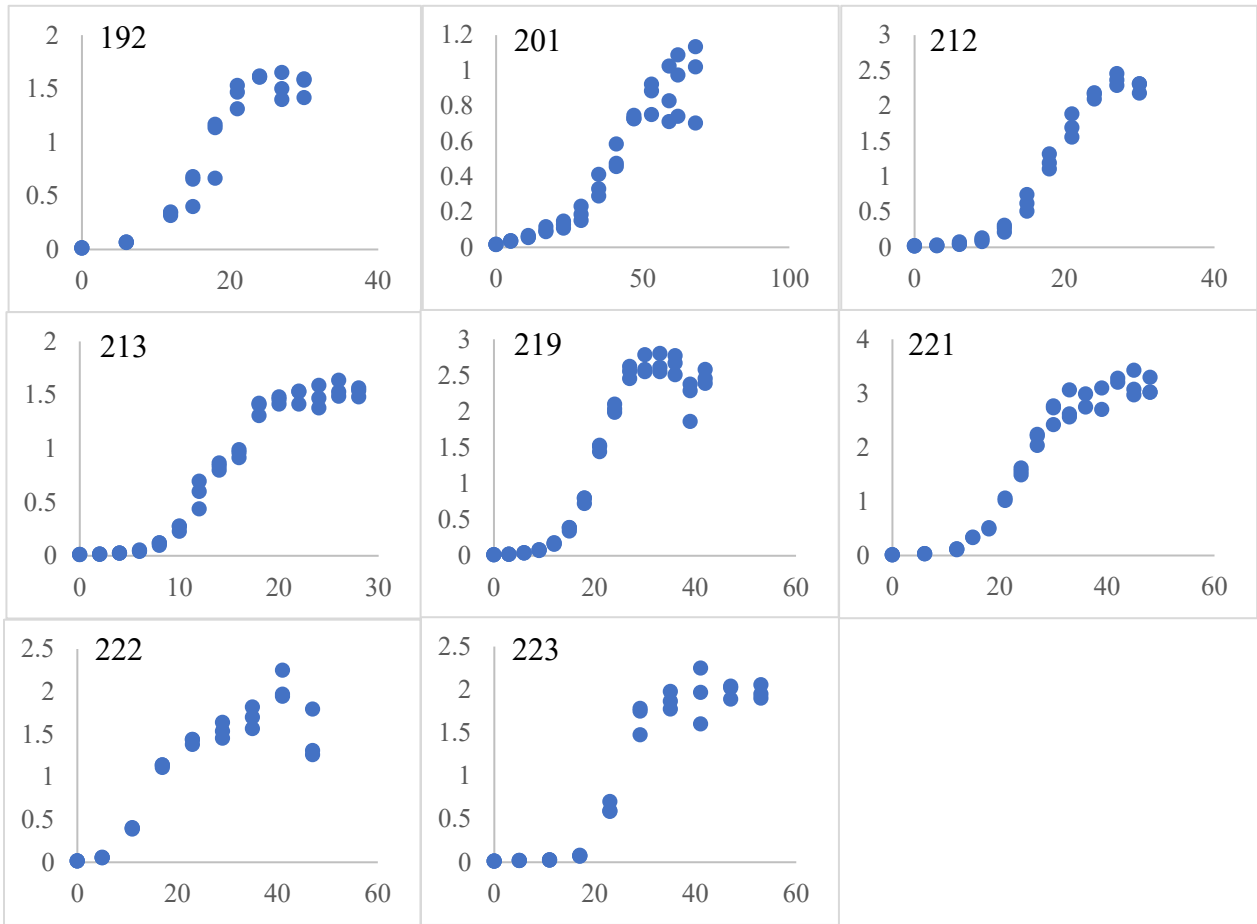
b)
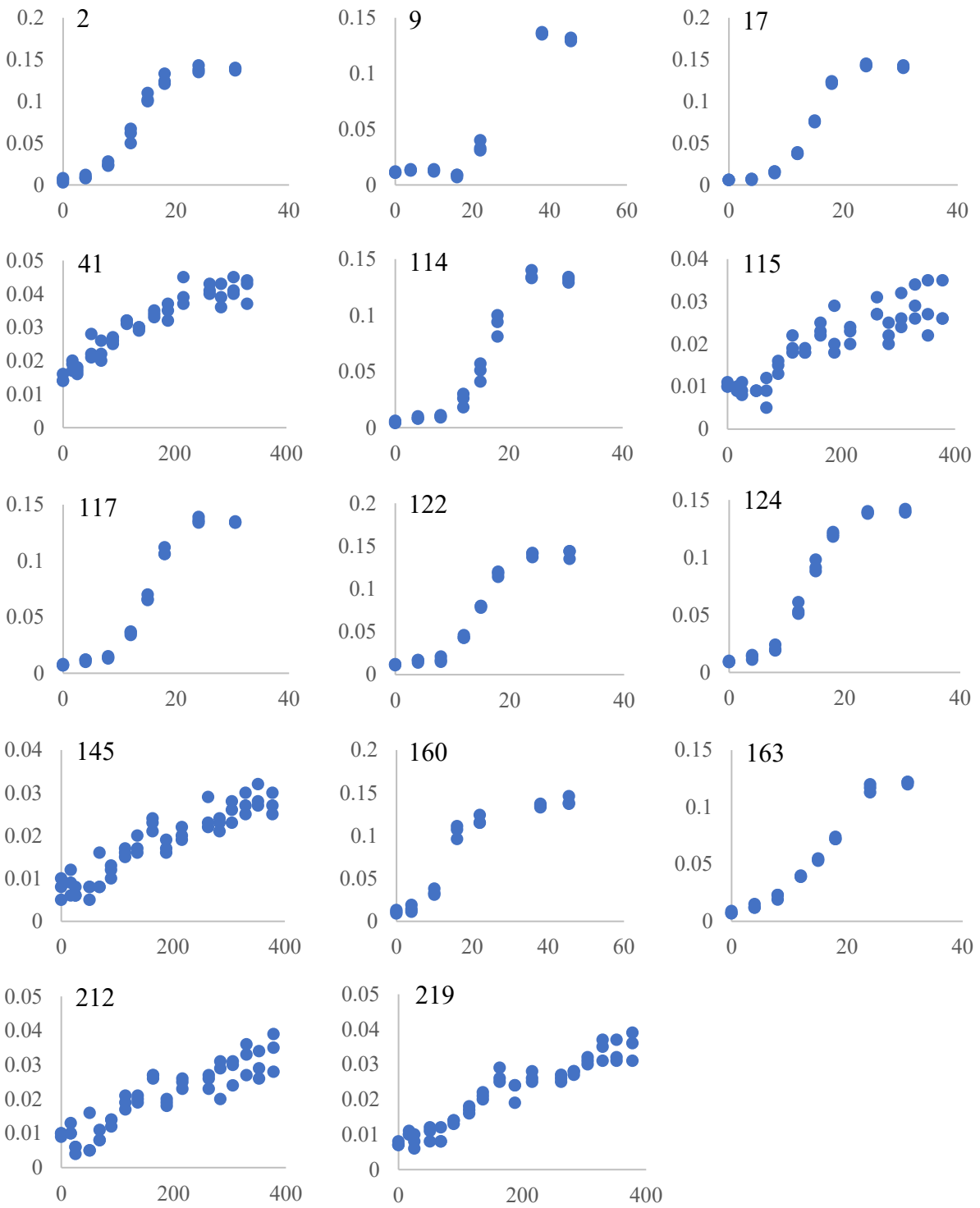

Figure 2.4: Growth curves of all strains in both a) LB and b) PB.

CHAPTER 3

Bacterial Community Structure and Function in an Impaired Mediterranean River Biofilm

3.1 Introduction

Global freshwater biodiversity is threatened due to pollution, habitat degradation, species invasion, flow modification, and overexploitation (Dudgeon et al. 2006). Freshwater ecosystems are estimated to offer 12,512 international $/ha/yr in services (2011 values) (de Groot et al. 2012; Costanza et al. 2014). Much of the services offered by rivers occur within biofilms, dynamic communities of algae, bacteria, protozoa, and fungi. The biofilm forms the base of the aquatic food web and filters out various types of toxins. Through the extent of the river system, the biofilm community also acts as a conduit of biogeochemical cycling, continuously turning over nutrients and organic matter through primary production, nutrient assimilation, and organic matter degradation.

The capacity of the biofilm to perform these functions depends upon many factors. Function in a biofilm is the collective expression of traits, or phenotypic characteristics, that are implicated in processes such as organic matter turnover. Central to function is the biofilm microbial community composition, which determines functional potential. Some traits are ubiquitous, decreasing the likelihood that alterations in community structure will have an impact on biofilm function. However, functions that are more specialized, or that are part of multigenic complexes, are more likely to be altered when changes in community structure occur (Martiny et al. 2015). Chemical (pH, salinity, nutrients, dissolved oxygen (DO)), physical (flow, substrate, light availability), and biological (community interactions, grazing) factors all have been shown to impact community structure.

Furthermore, expression of functional traits associated with the biofilm microbial community shift according to many pressures. Resource availability, for example, may alter the expression of traits associated with resource acquisition, affecting biogeochemical cycling within the biofilm. Heterotrophic bacteria have been shown to produce extracellular enzymes in response to the presence of corresponding substrates (German et al., 2011). Conversely, bacteria may also produce enzymes to target limiting resources if stoichiometric needs are not being met.

Under high nutrient, eutrophic conditions, increasing nutrient inputs into an aquatic system can result in declining function and overall health. The release from nutrient limitation allows algae biomass to grow until it reaches limitation by another resource. If light becomes the limiting resource, algae below the layer with access to light die, leading to heterotrophic degradation of the resulting detrital biomass, and depletion of oxygen through this increase in bacterial metabolic processes. Bacterial metabolic processes, as well as respiration from large mats of algae during the night, can lead to hypoxic conditions which may harm higher trophic levels. This decrease in oxygen may also cause dramatic shifts of the biofilm heterotrophic bacterial community structure (Spietz et al. 2015). Depending upon the function under consideration, this shift may lead to an alteration in system function, and a decline in its health. However, if dissolved oxygen levels remain high enough to support metabolism and prevent dramatic changes in community composition, function may be retained in a nutrient polluted system despite large algal blooms.

For this study, we analyzed the upper main stem of the Santa Margarita River (SMR), situated in a coastal and inland chaparral Mediterranean ecosystem. SMR hosts several state- and federally-

listed threatened and endangered species, such as the Southern California Steelhead Trout and the Least Tern. The watershed associated with this river encompasses 750 mi² in San Bernardino and San Diego Counties in Southern California. SMR and its tributaries flow through agricultural and urban areas, resulting in hydromodifications and nutrient pollution. It is characterized by nuisance algal blooms and has several reaches that have been put on the 2010 Clean Water Act section 303(d) list of impaired water bodies for nitrogen (N), phosphorus (P), or eutrophication. Though this river has been part of a multi-year intensive research project tied to its listed status, biofilm heterotrophic bacterial community structure and function have not been explicitly analyzed in this system.

Typical Mediterranean river ecosystems experience unique conditions associated with highly fluctuating seasonal flow that impact both their ecology and biogeochemical cycling (Romani et al. 2013). These conditions are likely to become more extreme when combined with climate change, the effects of which are predicted to be strong in Southern California (Cook et al. 2015). Climate change will also magnify the problem of eutrophication by increasing surface water temperatures, a mechanism associated with an increase in algal abundance (Rabalais et al. 2009). However, due to a water rights issue in downstream segments of SMR, flow is currently supplemented in the upper reaches of SMR, altering the natural flow patterns. The increased flow in SMR likely has several beneficial effects, such as dilution of nutrients, mitigation of algal biomass accumulation, and attenuation of daily DO fluctuations.

Our primary objective is to assess biofilm function in SMR, considering its characteristics of eutrophic conditions and modified flow regime. Large changes in heterotrophic community

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structure may be indicative of changes in function. However, for our first hypothesis, we predict that bacterial community composition in the upper reach of SMR will remain stable across the reaches sampled due to stable dissolved oxygen (DO). The increase of flow in SMR created by the supplemental water should aerate the system, stabilizing community composition across reaches. Partly because of the stabilization of the bacterial community, we expect continued biogeochemical cycling within the biofilm. Therefore, for our second hypothesis, we propose that biofilm bacterial abundance will be directly driven by organic carbon and nutrients within the biofilm. In spring and summer, SMR should have lower allochthonous carbon inputs into the system, making bacteria predominantly dependent upon autochthonous carbon sources. Additionally, the thick biofilm that should develop will limit diffusion of water column resources into the deeper layers for access by bacteria (Sabater et al 2002). Finally, we hypothesize that extracellular enzyme activity will be determined by the stoichiometric needs of the biofilm bacterial community (Artigas et al,2015). Because of the high cost of extracellular enzyme production, bacteria will allocate resources to enzyme production predominantly to fulfill stoichiometric demand.

3.2 Methods

3.2.1 Study system

All reaches sampled in this study maintained continuous water flow throughout the year, though the discharge levels varied. Because SMR is located in a Mediterranean climate (33.40 to 33.47 latitude, -117.14 to -117.25 longitude), there was a strong seasonal component to water flow, with higher discharge occurring during the cooler months, October 1st-March 31st. The winter preceding sampling was characterized by a higher-than-average rainfall amount for the region.

2016-2017 experienced 172% of the normal average rainfall in San Diego County of 9.9 inches (Sutula and Shultz 2018). The river was reported at the 75th percentile of discharge levels over a 30-year period (Sutula and Shultz 2018) All reaches contained sand and gravel but varied with respect to the amount of bedrock and cobble.

3.2.2 Biofilm collection

There were four sampling reaches that were each sampled four times between March and the end of July in 2017 (Figure 3.1, Table 3.1), including Fallbrook Sump (FB), Rainbow (RB), Municipal Water District Crossing (MWD), and the Gorge (GG). Due to the threat of storms in April, field work did not take place in this month.

Figure 3.1. Santa Margarita River, a) located in southern California, and the four sampling reaches for this study: b) the Gorge, c) MWD Crossing, d) Rainbow, and e) Fallbrook Sump.

Sampling was carried out using a modified version of the California Surface Water Ambient Monitoring Program (SWAMP) bioassessment and physical habitat procedures. Each reach was 500m long, with two sites of focused sampling of 150m length within each reach. Each site was divided into eleven main transects, with three biofilm samples taken across each transect, at 25, 50, and 75% width measures of the stream. Samples from each transect were pooled into three

separate collection buckets then prepared for analysis. This resulted in 6 separate buckets

sampled from each reach per day.

Table 3.1. The four sampling reaches in Santa Margarita River, dates sampled, and general reach characteristics. Values in the canopy cover column are mean values for the duration of the study with their standard deviation. This is a unitless measurement, with larger numbers indicative of more coverage.

Reach	Location (latitude, longitude)	Sampling Dates	Primary Substrate Type	Canopy cover
Fallbrook Sump (FB)	33.403861, -117.251214	3/14/17, 5/02/17, 6/06/17, 7/11/17	Sand and gravel	9.06±1.09
Rainbow (RB)	33.406051, -117.219396	3/16/17, 5/04/17, 6/08/17, 7/13/17	Gravel and cobbles	8.70±1.90
MWD Crossing (MWD)	33.455589, -117.171385	3/28/17, 5/18/17, 6/13/17, 7/18/17	Bedrock	4.28±2.30
Gorge (GG)	33.472561, -117.144391	3/30/17, 5/24/17, 6/15/17, 7/20/17	Sand and bedrock	3.52±1.88

Methods of biofilm collection varied depending upon substrate type. For bedrock, rock, or

wood, a circular guide defined the area of collection in which the biofilm was scraped off. With

sand and gravel substratum, a plastic coring device was used to press into the sand or gravel,

with a spatula slid underneath to remove the sample from the stream bottom. Previous years of

bioassessment in SMR resulted in low-biased estimates of benthic C, N, P, ash-free dry mass

(AFDM), and chlorophyll a (chl-a) in areas with large filamentous algal mats due to difficulty of

collection. The protocol was therefore modified to include additional representative samples of

these mats that were then combined with the biofilm collection. All samples were transported in

the dark and on ice to the laboratory for processing and then analyzed for AFDM, chl-a, benthic

organic C, N, and P, and heterotrophic bacterial community structure. For AFDM, chl-a, benthic

C, N, and P, the samples were filtered and stored for 28 days at -20° C. The protocols and

Table 3.2. All of the analytes included in this study with the associated protocols used for analysis and processing laboratory.

Analyte	Matrix	Analytical Method/SOP	Location of Analysis
Ammonium	Water column	EPA 350.1	Chesapeake Bay Laboratory
Nitrate + nitrite		EPA 353.2	
Orthophosphate		EPA 365.1	
TDN		USGS I-4650-03/	
		USGS I-2650-03, EPA	
TDP		USGS I-4650-03/	
		USGS I-2650-03, EPA	
DOC		EPA 415.1	
Dissolved oxygen		SM 45000-G	In-field measurements
Temperature		SM 2550-B	
pH by meter		SM 4500-H+B	
Chlorophyll a	Water column & biofilm	EPA 445.0	SCCWRP
AFDM		SM 10300 C	

location of analyses are listed in Table 2. Reported values for biofilm AFDM, chl-a, C, N, and P are normalized to the area collected.

3.2.3 Chemical and habitat assessment

Canopy cover measurements and channel substrate classification were taken at transects established in between the main transects used for bioassessment, resulting in 10 per site. This was done on one site only per reach per sampling day. These measurements were performed in

accordance with those in the SWAMP algae sampling protocol for Physical Habitat data (Fetscher et al., 2009). For data analysis, the data collected from these 10 transects was averaged at each site on each sampling day.

Surface water samples were collected at the upper and lower transects of each site during each sampling day. Samples were transported on ice and in the dark to the lab, where they were filtered and frozen at -20°C in preparation for later analyses. These samples were used to determine water column concentration of ammonium (NH4), nitrite + nitrate (NN), orthophosphate (PO4), total dissolved nitrogen (TDN), total dissolved phosphate (TDP), and dissolved organic carbon (DOC) (Table 2). The data from May 2 (FB) and May 4th, 2017 (RB) have been omitted due to low quality.

Velocity was measured once per reach, at one of the places that water was collected. A Marsh-McBirney velocity meter was used to measure velocity as laid out in the SWAMP PHAB protocol (Ode 2007). Velocity measurements that were taken across the width of the river were averaged for reported values. Velocity measurements from March 14th at FB and June 13th at MWD are missing due to low quality of data.

Sondes were deployed downstream from the lower site within each reach to measure temperature, dissolved oxygen concentration (DO), and pH continuously between 24 hours to 30 days. Measurements were taken using a YSI 6600 meter. The median DO, median respiration, and diel DO variance were calculated over a 24 hour period tied to a sampling event and used for further analyses. The median DO is the median during a 24 hour period, in which measurements

were recorded every 15 minutes. The median respiration is calculated as the maximum value of the median DO minus the 24 hour minimum, over a 24 hour period of measurements taken every 15 minutes. Finally, the diel DO variance is one half of the DO range. Because of constraints imposed by cost and field conditions, sonde measurements were omitted from two sampling dates: FB on June 6th and RB on June 8th, 2017.

We compared environmental variables in SMR to statewide medians from 2008-2011 in Fetscher et al. (2014), as well as the California Stream Condition Index (CSCI) scores. This is a metric that incorporates several measurements, focusing on either macroinvertebrates or algae, to represent the health of a river (Rehn et al 2015). The scores presented here are from the California Surface Water Ambient Monitoring Program from 2008 to 2016 (https://www.waterboards.ca.gov/water_issues/programs/swamp/bioassessment/csci_scores_map .html).

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3.2.4 DNA extraction and amplification

During the initial processing of samples on the day of collection, each sample was filtered and stored in the Mo Bio PowerBead solution of guanidine thiocyanate at -80°C. The MoBio Power Soil DNA Isolation kit was then used to extract DNA, which was sent to Laragen (Culver City, CA) for amplification and sequencing with Illumina MiSeq. We targeted the V4 region of the 16S rRNA gene using the Earth Microbiome 515F-806R primer pair for paired-end sequencing (Caporaso et al. 2010).

3.2.5 Bioinformatics

The DNA sequences were processed using QIIME2 v. 2018.4 (https://qiime2.org, Caporaso et al. 2010). Sequences were denoised, and the chimeras removed, using DADA2 (Callahan et al., 2016), and trimmed at 230 bp. Sequence alignment was performed using the MAFFT alignment software v7 (Katoh and Standle 2016), followed by filtering of the highly variable positions to create a maximum likelihood rooted tree with FastTree 2 (Price et al. 2010). Sequences were classified using a QIIME2 SILVA reference (Pruesse et al. 2012), then filtered to remove chloroplasts and mitochondria from the community sequences. We grouped together the samples of each site on a given sampling date, resulting in two community data samples for each day

3.2.6 Bacterial abundance

Prior to this study, test samples were analyzed to optimize the protocol for flow cytometry. Sonication to remove bacteria that were bound to algae, as well as filtration to remove algae from the samples were assessed but found to have no impact or to dramatically reduce cell count numbers, respectively. Therefore, we used a method of diluting samples with 0.9% NaCl when preparing samples for FLOW cytometry, then adding glutaraldehyde at a final concentration of 0.5% to fix the cells. The samples were then stored at 4° C. Cells were stained with SYBRGreen I (Promega) prior to being run through the FLOW cytometer.

3.2.7 Enzyme assays

Hydrolytic enzyme activity for alpha-glucosidase (AG), beta-glucosidase (BG), beta-xylosidase (BX), cellobiohydrolase (CBH), N-acetyl-glucosidase (NAG), acid phosphatase (AP), and leucine-aminopeptidase (LAP) was measured for each sample collected using a modified version

of the protocol established in German et al. (2011). Fluorometric substrates and standards for all of the samples were prepared in maleate buffer prior to starting the sampling season, and frozen at -20° C until used. After prepping the biofilm samples for DNA extraction and biofilm nutrient analysis, the remaining samples were transported on ice to the laboratory at UCI. Samples were briefly and vigorously shaken to ensure even distribution of bacteria prior to plating for the assays.

Enzyme assays were also performed on water collected from the same location. The activity present in water was used to correct the biofilm activity calculations due to water that was collected with the samples during the sampling event. The enzyme activity is expressed as nmol of substrate cleaved per m² of area sampled per hour. The final values used are the mean of the three samples collected at each site, for a total of two data points per reach.

3.2.8 Statistical analyses

We tested for differences in functional, water column, and physical variables using a Kruskal-Wallace test, grouping samples by reach and month of sampling. Pearson correlations and linear regressions were performed to determine potential relationships between the measured variables.

The VEGAN package in R was used to perform PERMANOVA and Mantel analyses (Oksanen et al. 2013). All analyses used Bray Curtis distances. The Mantel test was used to assess the relationship between beta diversity and the continuous variables in this study, including the results for functional assays, water column data, and environmental measurements. PERMANOVA was used to test for a distinction in beta diversity dependent upon categorical variables. Independent variables included reach and month of sampling.

Alpha diversity was calculated using both the Shannon index and Faith's phylogenetic diversity (PD).

3.3 Results

3.3.1   Environmental variables

When comparing statewide values from 2008-2011 to SMR values from April to June, 2017, SMR had higher median total phosphorus (TP) and total nitrogen (TN) (Table 3.3). Both biofilm chl-a, a measurement of algal abundance, and ash-free dry mass (AFDM) exceed state values by several orders of magnitude. The CSCI scores in SMR, however, where in line with the statewide values.

Within SMR, biofilm C, N, P, and C:N, C:P, N:P, and C:chl-a ratios varied significantly by both reach and month, but chl-a was only significantly different between months (Figure 3.3, Table 3.5). Additionally, median DO, median respiration, diel DO variance, and temperature were distinct between reach and month (Figure 3.2). Velocity, however, did not vary spatially or temporally.

Median DO declined consistently throughout the season at all reaches apart from GG (Figure 3.2). Median respiration and diel DO variance peaked in May, and temperature steadily increased from March to July at all reaches except GG.

Table 3.3: A comparison of California state and Santa Margarita River (SMR) values for nutrients, algal biomass, and the index scores of system function (CSCI).

Indicator	State		SMR	
	Range of Measured Values (N)	Estimated Median (CI)	Range of Measured Values	Estimated Median (CI)
TP (mg/L)	0.002-4.5 (536)	0.028 (0.024-0.031)	0.01-0.12	0.04 (0.037-0.073)
TN (mg/L)	0.01-26 (538)	0.131 (0.111-0.156)	0.53-5.11	1.09 (0.962-1.180)
Chl-a (mg/m²)	0.22-1504 (536)	8 (6-12)	231.63-13,125.2	2,040.65 (1,598.99-2,493.10)
AFDM (g/m²)	0.07-489 (525)	7 (6-8)	12.81-1,023.42	119.28 (54.527-163.163)
CSCI (macroinvertebrates)	0.11-1.35 (2566)	0.865 (0.855-0.873)	0.63-0.99	0.84 (0.722-0.96)

3.3.2 Taxonomic diversity

Over 50% of the sequence reads from SMR were from the phylum Proteobacteria, with the second most dominant phylum being Bacteroidetes (Figure 3.3a). On average, the communities were 36.58 ±10.27% Alphaproteobacteria, 18.99±5.74% Gammaproteobacteria, 11.87±3.45% Betaproteobacteria, 7.47±3.36% Deltaproteobacteria, 4.96±2.26% Sphingobacteriia, 4.04±2.43% unidentified bacteria, and 2.97±% Flavobacteria. There were 5,188 unique sequences among all of the reaches.

The highest Shannon diversity was at FB on July 11th (H'=8.75), and the lowest at GG on March 30th (H'=6.41). When using Faith's PD, we found alternatively that the lowest alpha diversity arises at GG on July 20th (PD=18.99), but that the highest alpha diversity is found at the same reach and date as the Shannon index (PD=55.2). Shannon diversity and Faith PD are significantly correlated (r=0.96, p<0.001). Both shannon diversity and faith's PD are significantly correlated with bacterial abundance (r=0.28, p=0.006; r=0.26, p=0.01). Similar to abundance, according to a Kruskal-Wallace test, Shannon and Faith PD were not significantly
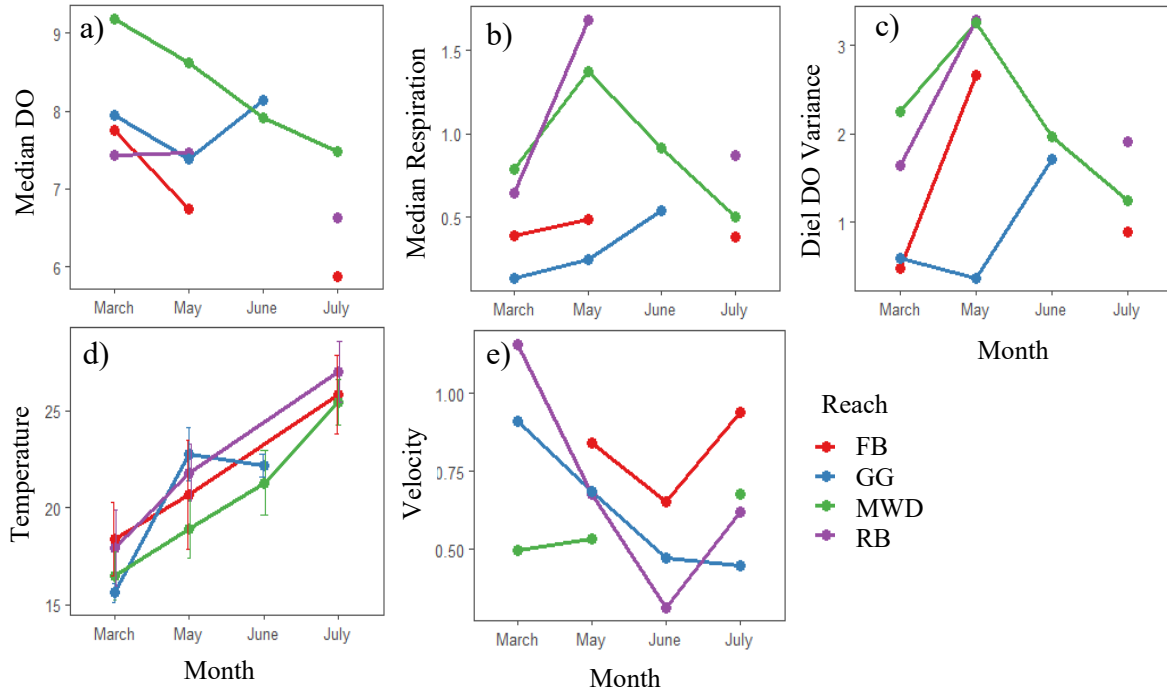
Figure 3.2: a) Median dissolved oxygen (DO), b) median respiration, c) diel DO variability, d) temperature, and d) velocity by reach, across time. The points on a-c are the mean of values for 2 days, and on d, the mean and standard deviation of temperature measurements taken every 15 minutes over 24 hours. e) shows the points of the mean of values taken in one sampling day.

distinct across reach or month. Shannon and Faith's PD are both correlated with the mean daily temperature (r=0.51, p<0.001; r=0.45, p<0.001) and median DO (r=-0.41, p<0.001; r=-0.38, p<0.001), but not median respiration or diel DO variance.

A PERMANOVA showed that there were no spatial effects on community composition. Month also had no significant impact on the differences in community composition between reaches. Bray Curtis distance across all reaches was not significantly associated with any of the biofilm, water column nutrients, or physical habitat measurements.
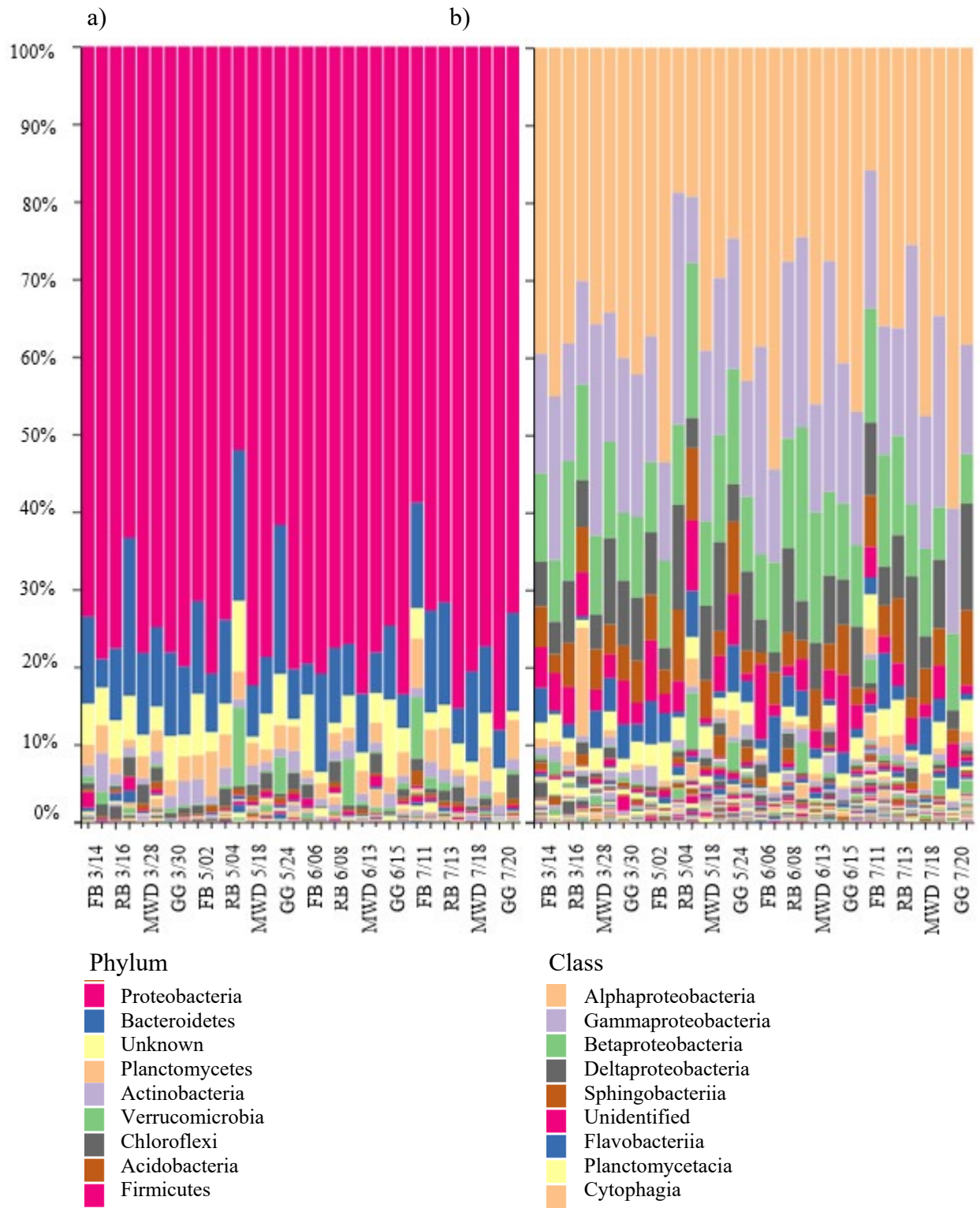
Figure 3.3. Taxonomic bar graph of all samples listed by date on the x-axis and grouped by phylum (a) or class (b) with relative frequency on the y-axis. Only the most abundant groups are shown in the legend.

3.3.2 Abundance

Bacterial abundance was not significantly different by reach or month, although March showed the lowest abundance, with a mean and standard deviation of $4.02 \times 10^{10} \pm 3.1 \times 10^{10}$ cells/m²
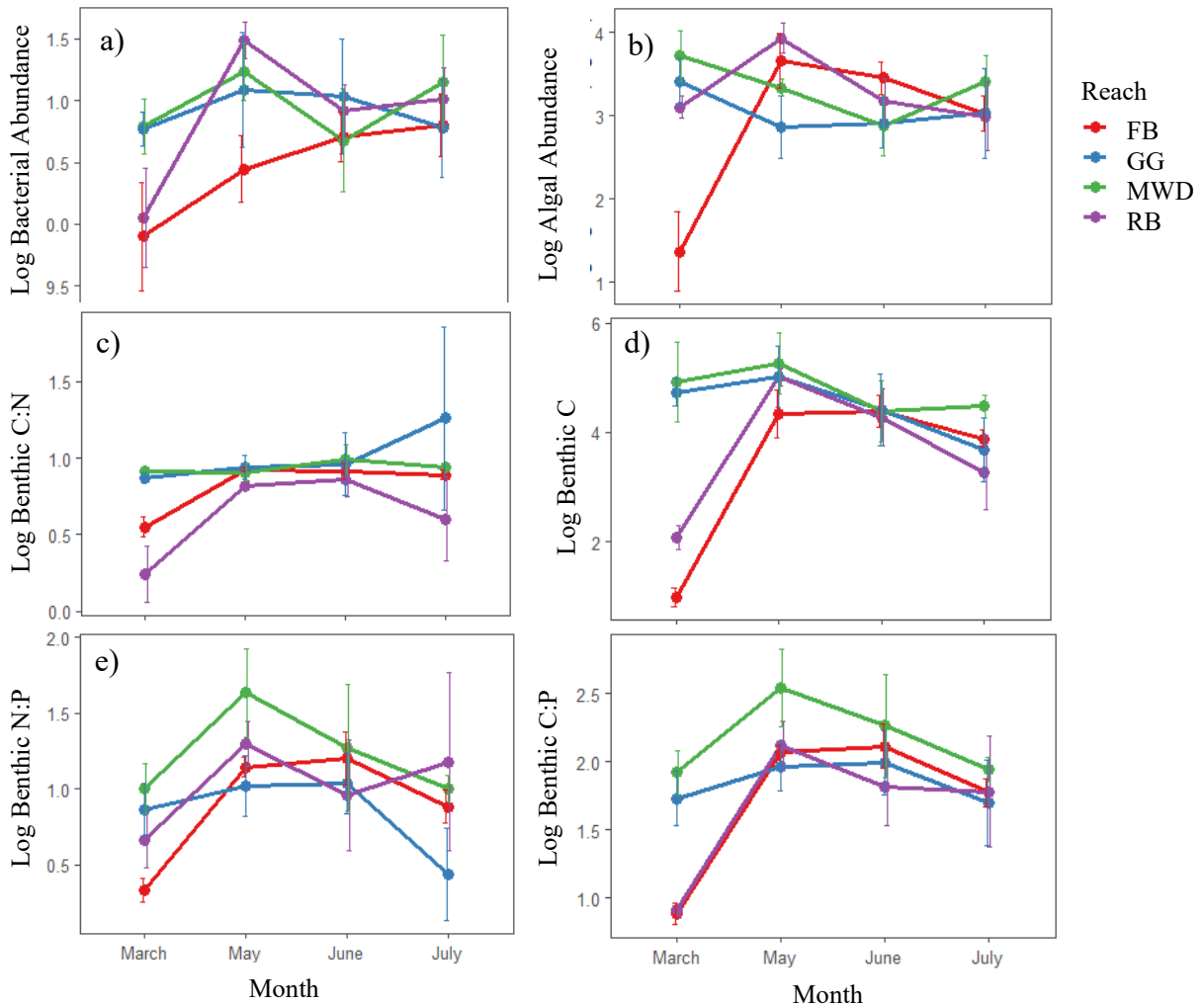
Figure 3.4: Log-transformed values of a) bacterial abundance (count m⁻²), b) algal abundance (mg m⁻²), c) benthic C:N, d) benthic carbon (mg m⁻²), e) benthic N:P, f) and benthic C:P by reach across time.

across all reaches (Figure 3.4a). May showed the highest abundance with a mean and standard deviation of $1.8 \times 10^{11} \pm 1.4 \times 10^{11}$ cells/m². Biofilm algal abundance, measured as mg/m² of chl-a, was distinct between month, but not reach. Bacterial abundance did weakly correlate with biofilm chl-a (r=0.25, p=0.03), but not with the C:chl-a ratio.

Across all reaches, bacterial abundance was positively correlated with the daily mean temperature (r=0.26, p=0.02), median respiration (r=0.44, p<0.001), and diel DO variance (r=0.29, p=0.01), but had no relationship to median DO or velocity.

For the water column nutrients, bacterial abundance was negatively correlated with TN (r=-0.55, p=0.00 4) and NN (r=-0.55, p=0.004), and positively with TP (r=0.41, p= 0.04), DOP (r=0.55, p=0.003), PO4 (r=0.41, p=0.04)

3.3.4 Enzyme Function

Activity for all enzymes differed significantly by reach, and all except BX differed by month (Table 3.5). We found that all enzymes had a strong, positive relationship with biofilm C, C:chl-a ratio, and bacterial abundance (Figure 3.5, Table 3.4). None of the enzymes correlated with
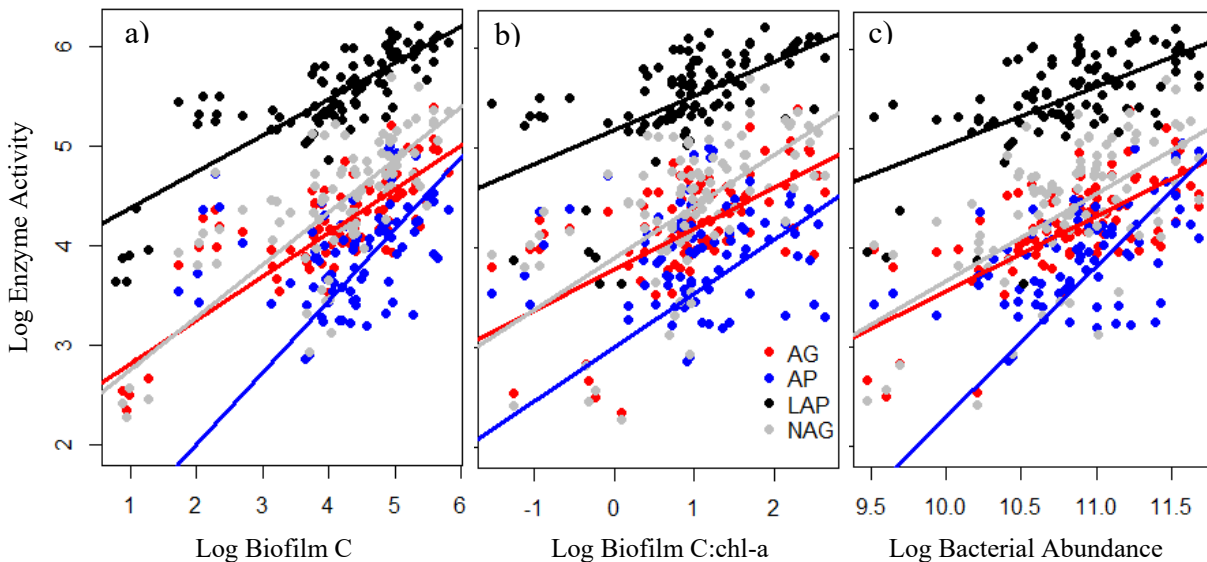

Figure 3.5: Linear regression of the log of enzyme activity (nmol m^{-2}hr^{-1}) and the log of a) biofilm C (mg m^{-2}), b) C:chl-a, and c) bacterial abundance (count m^{-2}). Lines are colored according to enzyme class.

Table 3.4: Results of linear regression using the log of biofilm C:chl-a, C (mg m⁻²), or bacterial abundance (count m⁻²) and the log of enzyme activity (nmol m⁻²hr⁻¹).

	Biofilm C:chl-a				Biofilm C				Bacterial Abundance			
	Adj R²	St. Error	F	p	Adj R²	St. Error	F	p	Adj R²	St. Error	F	p
AG	0.28	0.58	37.58	<0.001	0.57	0.45	127.9	<0.001	0.31	0.55	42.06	<0.001
AP	0.12	1.26	13.58	<0.001	0.4	1.05	63.31	<0.001	0.32	1.08	44.29	<0.001
BG	0.2	0.67	25.48	<0.001	0.4	0.59	63.99	<0.001	0.24	0.64	31.01	<0.001
BX	0.16	0.6	18.99	<0.001	0.46	0.48	81.62	<0.001	0.3	0.53	40.17	<0.001
CBH	0.18	0.64	21.37	<0.001	0.43	0.53	73.39	<0.001	0.27	0.59	36.26	<0.001
LAP	0.34	0.42	49.87	<0.001	0.69	0.29	208.4	<0.001	0.35	0.39	52.16	<0.001
NAG	0.31	0.69	42.73	<0.001	0.57	0.54	124.4	<0.001	0.28	0.68	37.49	<0.001

3.3.5 Community structure and function

Using a supervised learning regressor, we found that community structure was not predictive of any of the enzyme activities. Additionally, Mantel tests showed that enzyme activity was not correlated with Bray Curtis distances between communities, nor either of the alpha diversity metrics tested.

3.4 Discussion

3.4.1 Hydrological and biological characteristics of SMR

SMR is a unique ecosystem. Though it exists within a Mediterranean climate, the supplemental water being added to the river alters flow patterns, creating a deviation from the expectations typical of similar systems. The added water maintained relatively consistent flow velocities across space and time within the studied reaches of the river. While the water column N levels are only approximately one order of magnitude higher than the statewide value, and the water column P levels are similar to the statewide value, the median value of live algal biomass accumulation within the SMR biofilm is three orders of magnitude higher than the statewide median. Simply considering SMR from the perspective of algal abundance, a decline in system health might have been expected given the high values. However, the CSCI measurements of

macroinvertebrates, an indicator of river health, suggest that SMR is maintaining some system functions in line with the statewide median values.

3.4.2 Community composition

Our first hypothesis regarding stability of bacterial community composition was supported. In SMR, the heterotrophic bacteria community composition did not vary significantly between reaches, nor temporally within our experimental period. DO, maintained by flow, appears to be the driving factor for this stability of structure. Though several nutrient and physical parameters varied by reach, all reaches had a DO minimum above 5 mg O_2 L^{-1}, which is approximately the concentration threshold of DO at which microbial community shifts begin to take place (Spietz et al. 2015)

Further, DO concentrations appear to have no impact on bacterial abundance, as there is no relationship between abundance and median DO. The lack of relationship may also be reflective of DO concentration exceeding a threshold. We did find that Shannon diversity declined as median DO increased (r=-0.41, p=0.040). However, there is probably not a causal relationship between DO and Shannon diversity. Shannon diversity correlates positively with abundance, so changes in DO are likely driven by respiration from bacterial biomass rather than DO affecting diversity directly.

In other studies of eutrophication, it has also been noted that diversity increases with increasing nutrients (Sawall et al. 2012). In our study, there was no observable relationship between water

column or biofilm nutrients and alpha diversity of bacteria communities. Shannon diversity remained relatively stable throughout the season, with sampling events approximating the levels of diversity found in upper reaches of other river systems (Ruiz-González et al. 2015).

3.4.3 Bacterial and algal abundance

We found support for our second hypothesis, that bacterial abundance would be driven by organic compounds within the biofilm. Bacterial abundance increased with biofilm C, N, and P, as well as with the C:chl-a ratio. Bacterial abundance had the strongest relationship with biofilm C, however abundance increased most per unit of C relative to chl-a. C:chl-a is a proxy estimate of the detrital algal biomass relative to the live algae. Bacterial abundance is therefore probably driven more by detrital biomass than photosynthates secreted by live algae.

Bacterial abundance was negatively correlated with N measurements in the water column, but positively with P measurements. This is likely reflective of the algal assimilation of N, drawing down N in the water column. As algae increased in biomass, it provided more resources for bacteria within the biofilm, allowing more bacterial growth. This would result in the appearance of a negative relationship between bacterial abundance and water column N.

Bacteria produce exopolymeric substances (EPS), a structural matrix that attaches bacteria to surfaces, alters diffusion rates, and offers protection from toxins, among other functions (Flemming et al. 2016). When formed in close proximity to algae, biofilms may create an opportunity for increased access to algal-derived compounds above water column carbon and

nutrients. In addition, water column nutrients were likely secondary to organic biofilm carbon and nutrients due to limited diffusion into the biofilm.

Despite the high level of biomass accumulation, the biofilm C:N and C:P ratios were relatively low, suggesting luxury uptake of nutrients into storage tissue (Portielje and Lijklema 1994; Nambiar and Bokil 1981). Narrow C:nutrient ratios also suggest that algal biofilm growth is not nutrient-limited, but instead may be limited by another factor. Multiple aspects of the physical habitat impact the development of algal biomass, such as available light, flow, temperature, and river substrate (Artigas et al 2013; Munn,et al., 2010).

High flow rates may also restrict additional algal growth that could occur given the excess nutrients, especially at reaches with substrate dominated by sand and sediment. In reaches characterized by bedrock or large boulders, filamentous algae anchors to these substrates, preventing excess biomass from being carried downstream. However, in reaches predominantly characterized by sand or sediment, algae lack an anchor point that would allow large accumulations of biomass. This mechanism is illustrated by the drop in algal biomass after the May timepoint, caused by scouring from a large storm that increased flow, and is primarily apparent in the two reaches dominated by sand and sediment, RB and FB. Though GG and MWD are characterized by bedrock and boulders, they both contain some sand and sediment substrate patches that are included in the sampling protocol, and these patches would have been similarly affected by the increased flow.

Additionally, light availability is an important resource that impacts algal abundance (Frankforter et al 2010). Formation of large mats of algae at the water surface block sunlight to algae below. This shading may have played a large role in limiting biomass at MWD, where thick mats of filamentous algae began to form prior to our sampling dates and throughout our field season. Water turbidity also blocks light, inhibiting photosynthesis. In these instances, the C:N and C:P ratios might be driven down as a slowed photosynthetic rate would decrease carbon assimilation relative to available nutrients (Hessen et al. 2002).

3.4.5   Enzyme Function

We hypothesized that extracellular enzyme activity is determined by the stoichiometric needs of the biofilm bacterial community. However, our data suggests that increases in extracellular enzyme activity coincide with increases in bacterial abundance rather than stoichiometric nutrient demand. The low C:N and C:P ratios of the biofilm in SMR indicate that bacteria are not nutrient limited. If we consider the N requirement as an example, aquatic bacteria could easily maintain a biomass C:N ratio as low as 4 by consuming biofilm material with a C:N of 10 if they have carbon use efficiencies of 40% or lower (Godwin and Cotner 2015). In our study, average C:N ratio of the biofilm rarely exceeded 10, meaning there is sufficient biofilm N available to maintain bacterial stoichiometric requirements. Therefore, bacteria likely have enough resources available to invest heavily in enzyme production. (Jones & Lock 1989).

When assessing extracellular enzyme activity relative to biofilm C:chl-a or biofilm C, we found that activity scales more strongly with C:chl-a. While the actively photosynthesizing portions of the biofilm may be secreting photosynthates that bacteria can consume, as mats thicken, more of

the biofilm algae will become detrital when there is no longer access to light in the deeper layers. The measured bacterial extracellular enzymes primarily target the carbon compounds that are found within the cell wall of algae, which become available in the detrital layers of the biofilm (Arora et al. 2012).

Additionally, we found similar patterns of dominant enzymes being produced in SMR as in other systems. There is a strong seasonal component to enzyme activity in river biofilms, as the primary carbon sources shift from autochthonous carbon in the spring and early summer to allochthonous carbon in the fall (Romani et al. 2013). Thus, biofilm enzyme activity is probably responding to available resources rather than stoichiometric demands.

3.4.6 Conclusion

While nutrient pollution often leads to eutrophic conditions in aquatic systems, the physical habitat within a river can dramatically alter functional outcomes. In SMR, flow appears to mitigate algal accumulation as well as the associated swings in DO concentration that often drive the negative impacts of eutrophication. With no large transformations of the heterotrophic community caused by a switch to anaerobic conditions, and maintenance of a relatively high diversity, biofilm heterotrophic function is maintained in a river marked by nutrient pollution.

Table 3.5: Kruskal-Wallace values of all measured function, biofilm, and physical environment variables by reach and month.

		Reach		Month	
		H	P	H	p
Enzymes	Abundance (cell/m²)	4.59	0.20	5.96	0.11
	Shannon	1.38	0.71	6.34	0.10
	Faith's PD	1.52	0.68	4.11	0.25
	AG (nmol/m²*hr)	27.08	**<0.001**	15.08	**0.002**
	AP (nmol/m²*hr)	16.55	**<0.001**	12.38	**0.006**
	BG (nmol/m²*hr)	18.05	**<0.001**	17.13	**<0.001**
	BX (nmol/m²*hr)	19.31	**<0.001**	7.46	0.06
	CBH (nmol/m²*hr)	11.16	**0.01**	8.31	**0.04**
	LAP (nmol/m²*hr)	25.78	**<0.001**	19.73	**<0.001**
	NAG (nmol/m²*hr)	27.32	**<0.001**	23.65	**<0.001**
Biofilm	P (mg/m²)	18.73	**<0.001**	14.43	**0.002**
	C (mg/m²)	20.00	**<0.001**	26.62	**<0.001**
	N (mg/m²)	15.58	**0.001**	27.68	**<0.001**
	C:N	27.88	**<0.001**	15.89	**0.001**
	C:P	11.46	**0.009**	39.90	**<0.001**
	N:P	11.44	**0.01**	32.31	**<0.001**
	chl-a (mg/m²)	4.91	0.18	8.35	**0.04**
	AFDM (mg/m²)	8.39	**0.04**	35.68	**<0.001**
	C:chl-a	28.44	**<0.001**	19.39	**<0.001**
Physical environment	Median DO (mg/L)	11.95	**0.007**	29.65	**<0.001**
	Median Respiration (mg/L)	20.38	**<0.001**	24.17	**<0.001**
	Diel DO Variance (mg/L)	9.56	**0.02**	22.91	**<0.001**
	Mean Temp (°C)	8.15	**0.04**	72.66	**<0.001**
	Velocity (ft²/s)	2.08	0.56	3.96	0.27
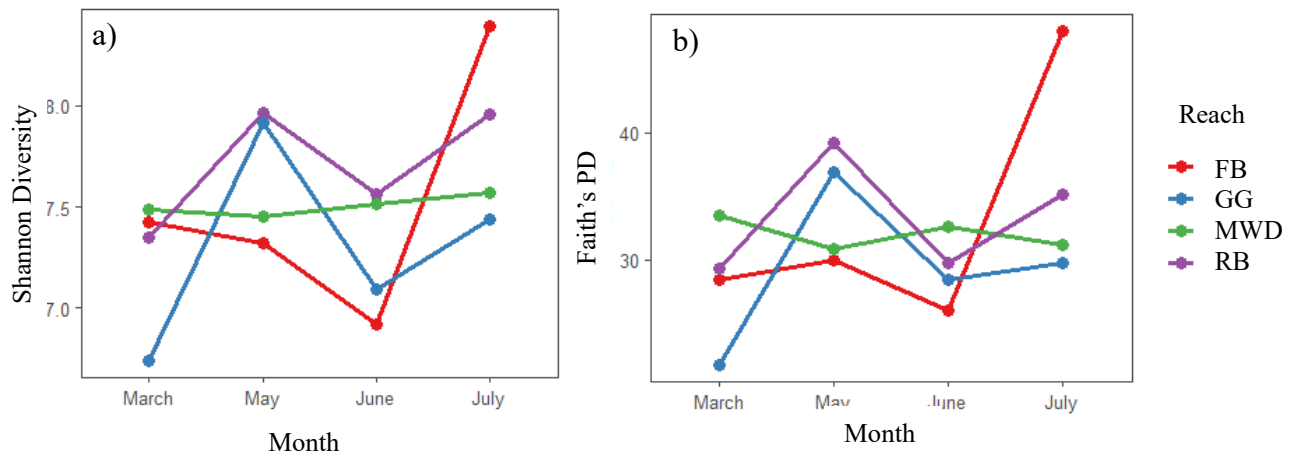
Figure 3.6: Alpha diversity plots of a) shannon diversity, and b) Faith's phylogenetic diversity across time by reach.
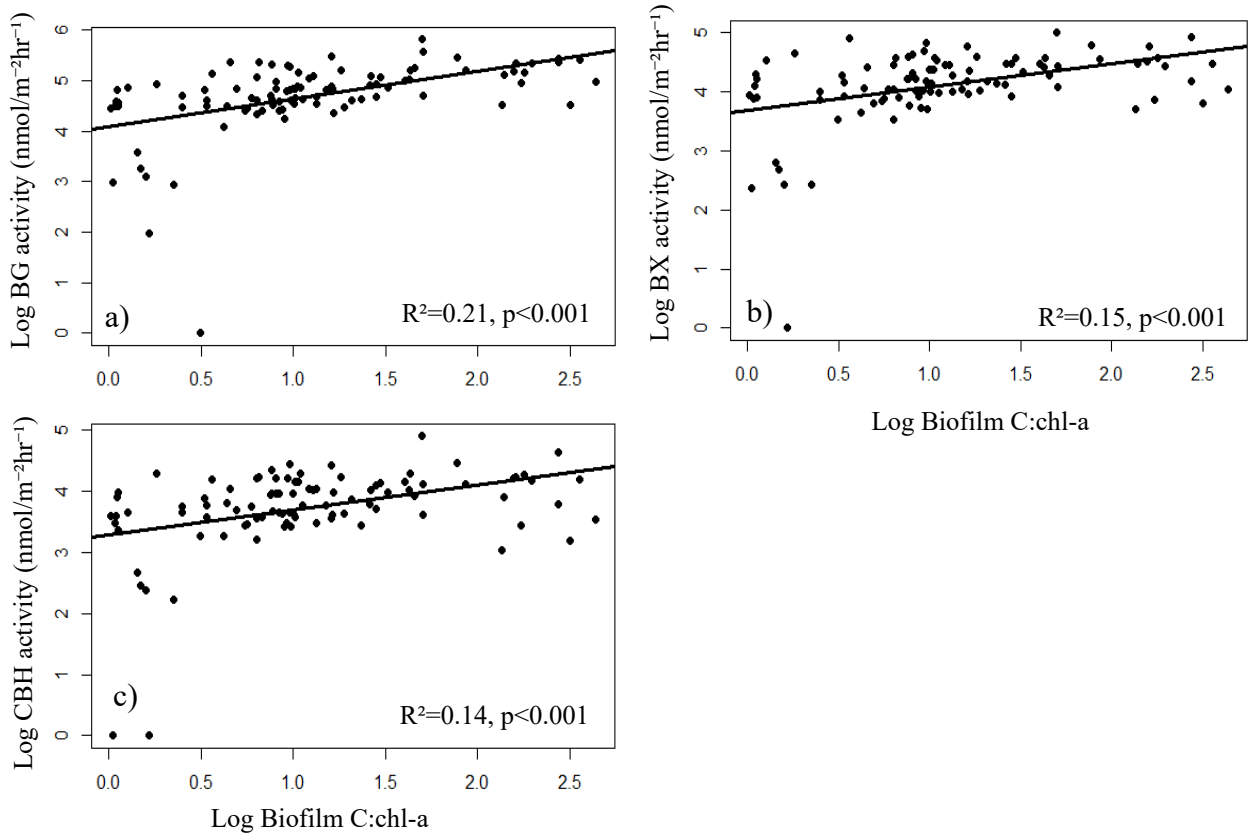
Figure 3.7: Linear regression plots of a) BG, b) BX, and c) CBH versus biofilm C:chl-a

Table 3.6 Pearson correlation values for enzyme activity (nmol activity/m²*hr) and water column chemical variables. *<0.05, **<0.01, ***<0.001

	Median respiration	Diel DO variance	Median DO	TN	NN	DOP	DON
AG	0.33**	0.39***		-0.53**	-0.5**	0.41*	
AP	0.39***	0.50***					
BG	0.31**	0.38***	0.22*				
BX	0.31**	0.39***					
CBH	0.30**	0.38***					
LAP	0.28**	0.34**	0.24*	-0.47*	-0.45*	0.41*	-0.38*
NAG	0.38***	0.45***	0.36**	-0.51**	-0.48*	0.41*	

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