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Linking Microbial and Ecosystem Ecology Using Ecological Stoichiometry: A Synthesis of Conceptual and Empirical Approaches

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Abstract

Currently, one of the biggest challenges in microbial and ecosystem ecology is to develop conceptual models that organize the growing body of information on environmental microbiology into a clear mechanistic framework with a direct link to ecosystem processes. Doing so will enable development of testable hypotheses to better direct future research and increase understanding of key constraints on biogeochemical networks. Although the understanding of phenotypic and genotypic diversity of microorganisms in the environment is rapidly accumulating, how controls on microbial physiology ultimately affect biogeochemical fluxes remains poorly understood. We propose that insight into constraints on biogeochemical cycles can be achieved by a more rigorous evaluation of microbial community biomass composition within the context of ecological stoichiometry. Multiple recent studies have pointed to microbial biomass stoichiometry as an important determinant of when microorganisms retain or recycle mineral nutrients. We identify the relevant cellular components that most likely drive changes in microbial biomass stoichiometry by defining a conceptual model rooted in ecological stoichiometry. More importantly, we show how X-ray microanalysis (XRMA), nanoscale secondary ion mass spectroscopy (NanoSIMS), Raman microspectroscopy, and in situ hybridization techniques (for example, FISH) can be applied in concert to allow for direct empirical evaluation of the proposed conceptual framework. This approach links an important piece of the ecological literature, ecological stoichiometry, with the molecular front of the microbial revolution, in an attempt to provide new insight into how microbial physiology could constrain ecosystem processes.

Key words: biogeochemistry; ecological stoichiometry; community structure; ecosystem processes; homeostasis; nutrient ratios; Raman; NanoSIMS; XRMA.

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Introduction

Biogeochemical cycles are intimately linked with microbial communities from local to global spatial scales and on daily to geologic timescales in all ecosystems (Falkowski and others 2008). Our understanding of the composition and biology of environmental microorganisms is accumulating at an unprecedented rate due to the application of an ever-increasing suite of culture-free methods (Zak and others 2007). Whereas these advances have increased the understanding of microbial structure and function in the environment, microbially focused ecological theory (that is, synthetic frameworks within which to organize this wealth of information) has not kept pace with the accumulation of empirical results (Prosser and others 2007). Recently, theoretical approaches have yielded insight into how environmental microorganisms are distributed in time and space (Hughes Martiny and others 2006). Such theoretical or conceptual frameworks are required to direct hypothesis-driven research and to develop a clearer understanding of how microorganisms constrain ecosystem processes.

We argue that a more rigorous application of ecological stoichiometry to microbial ecology can provide novel insight into how microorganisms constrain ecosystem processes. Ecological stoichiometry is an increasingly broad body of work that evaluates how the relative quantity of specific elements constrains or facilitates the movement of organic and inorganic matter through an ecosystem (Sterner and Elser 2002). Using a stoichiometric approach to integrate biological and ecological systems across wide physical scales (for example, from microorganisms to ecosystems) has several inherent advantages. For example, elemental ratios are unitless and therefore allow tracking of the same response at scales where quantities differ greatly (for example, from single cells to landscapes). In addition, although rate processes may be heavily influenced by temperature and precipitation in some ecosystems, focusing on the relative changes in individual elements (for example, increasing litter N:C) corrects for temporal differences and allows for direct comparison of processes under a wide range of climatic conditions (Manzoni and others 2008). Also, using stoichiometry as an organizational framework places explicit focus on the law of conservation of mass, which allows for 'truthing' of empirical data by conducting mass balances. Unbalanced analyses can point to previously unconsidered sources or sinks of elements into a biogeochemical cycle. These points, coupled with the dominance both metabolically and often by mass of microorganisms in many ecosystems (Whitman and others 1998), emphasize the strength and importance of using ecological stoichiometry to link microbial and ecosystem processes in both terrestrial and aquatic ecosystems.

Traditionally, stoichiometric analyses have been focused on trophic interactions between metazoans and autotrophs (for example, zooplankton and algae, Sterner and Elser 2002). More recently, the power of combining traditional food web models with stoichiometric theory has been proposed (Hall and others 2009). It has been long known that Bacteria and Archaea provide an essential link between dissolved organic matter and higher trophic levels (Azam and others 1983). However, food web models routinely exclude the microbial component or deal with it only in passing, usually due to inability to quantify many of the necessary parameters associated with the microbial component of the food web. In this mini-review we take a single trophic-level view of nutrient recycling, however, it is important to note that the methods and concepts presented here also allow for the integration, both conceptually and empirically, of microorganisms into stoichiometrically specific whole food web models. Specifically, we outline a conceptual framework and a suite of complementary empirical methods that allow for deconstruction of microbial community stoichiometry to mechanistically understand what determines community biomass stoichiometry and how microorganisms may constrain nutrient cycling (Figure 1). We show how microbial biomass stoichiometry is linked to retention or recycling of mineral nutrients and how individual phylogenetic units (for example, populations, clades, domains) combine to form community stoichiometry. We then discuss how macromolecular composition influences cellular stoichiometry and how to empirically link macromolecular composition, cellular stoichiometry, and microbial community structure. Connecting these components of microbial communities links phylogenetically specific microbial physiology to community biomass stoichiometry, bringing together a well-developed body of ecological theory with the molecular front of the microbial revolution.

The Relationship Between Microbial Biomass Stoichiometry and Nutrient Cycling

In his seminal paper, Redfield (1958) identified the similarity between the carbon (C) to nitrogen (N)

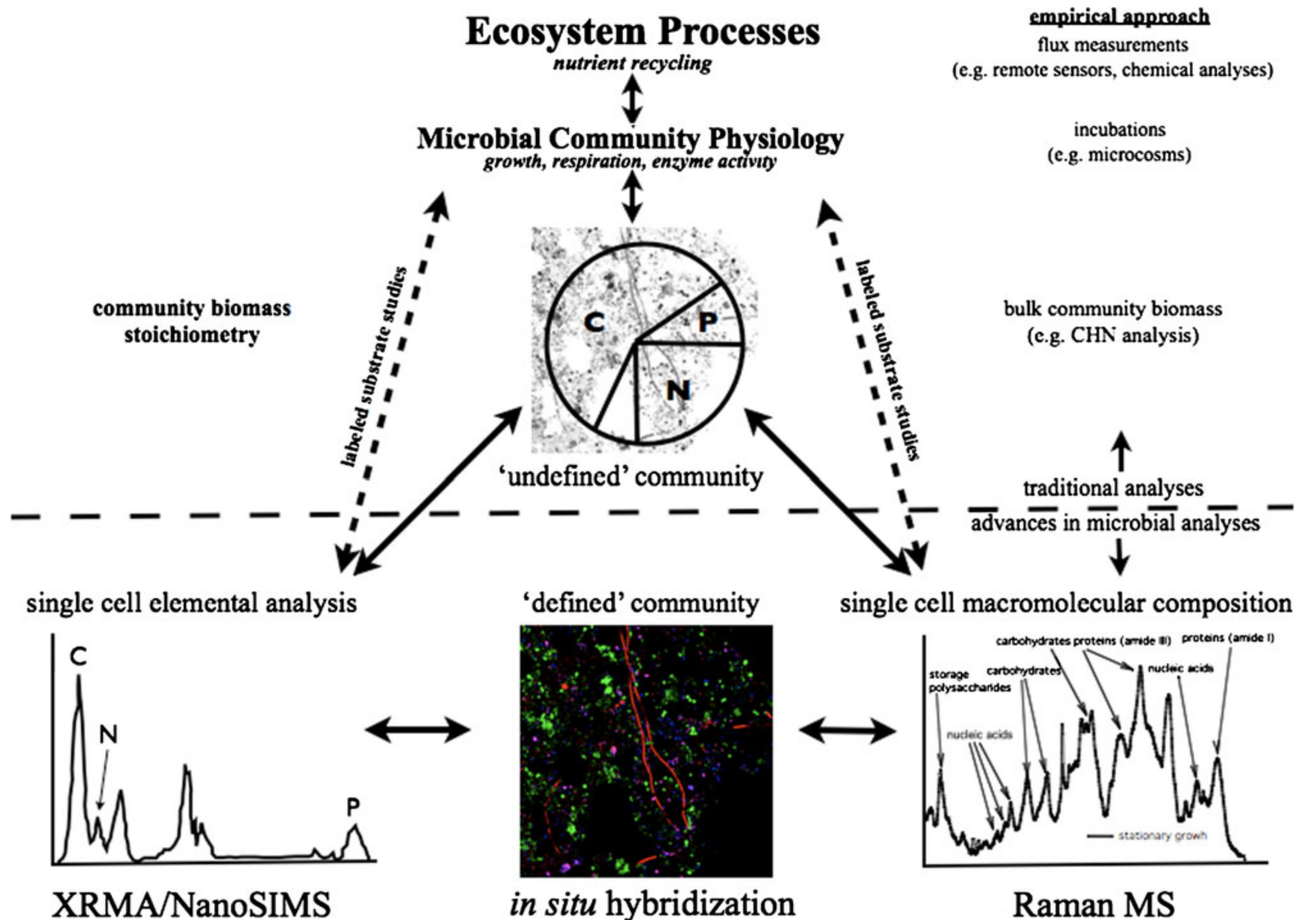

Figure 1. Conceptual framework linking microbial ecology to ecosystem processes using ecological stoichiometry. Listed are broad categorical concepts with explicit examples in smaller font (for example, ecosystem processes—nutrient recycling). Small font on the right of the figure indicates how each parameter is empirically evaluated. Until recently microbial analyses were limited to those above the dotted line. Recently developed methods (below *the line*) can now be used to test hypotheses on the cause of variation in community biomass stoichiometry. Each novel method is listed below a figure of representative output (with the exception of NanoSIMS), with what it evaluates listed above the image. Arrows link concepts/methods that can be coupled empirically (for example, macromolecular composition and community structure using Raman-FISH).

to phosphorus (P) ratio in marine algae and that of the dissolved resource pool. Although this work is well known, the key conclusion, that the biomass stoichiometry of marine algae regulates inorganic resource pool stoichiometry, is often overlooked. Since the publication of Redfield's work, the metabolic dominance of marine ecosystems by Bacteria and Archaea has come to light (Strom 2008) suggesting that additional insight into marine nutrient cycles may be achieved by extending stoichiometric analyses to heterotrophic microorganisms.

This idea is supported by the recent recognition of the relationship between microbial biomass stoichiometry and nutrient recycling across a wide range of freshwater and terrestrial ecosystems. Using data compiled across continental-scale watersheds, Taylor and Townsend (2010) noted a consistent inverse and non-linear relationship between nitrate (NO_3^-) and dissolved organic carbon (DOC) levels. They concluded that the inflection point (that is, where NO_3^- accumulates at low DOC levels) was strongly related to the biomass N:C of the microbial community (Taylor and Townsend 2010). Similar meta-analyses that focused on litter decomposition also pointed to microbial biomass stoichiometry as an important driver in elemental cycles. For instance, an impressively large data set (60 globally distributed locations, 55 litter types, 2,800 observations) on terrestrial litter decomposition showed that microbial community function switched between immobilization and mineralization of N when the stoichiometry of the litter

approached the stoichiometry of the microbial biomass (Manzoni and others 2008). This result is consistent with an earlier study that also suggested, although in a less mechanistic manner, that decomposer physiology was an important constraint on N-release during litter decomposition (Parton and others 2007).

A similar relationship between microbial biomass stoichiometry and the proportion of nutrients consumed or recycled to the environment has also been observed in freshwater ecosystems, both lentic and lotic. In an analysis of 10 stream ecosystems from three biomes, N-uptake increased with increasing N:C of different compartments of organic matter, both biotic and abiotic (Dodds and others 2004). Although this study contained no compartments composed exclusively of microbes, differences in N:C of these pools, many with associated microbial biomass (for example, epilithon), were inversely related to N-uptake. Compartmental N:C ratio alone could predict approximately 40% of the variation in N-retention for a given reach of stream (Dodds and others 2004).

The relationship between nutrient recycling and microbial biomass stoichiometry can be especially insightful when the drivers of microbial stoichiometry are independent of the resource stoichiometry. For example, in a survey of temperate lakes (n = 47), after considering several predictor variables (chlorophyll a, total dissolved nitrogen, Secchi depth, soluble reactive phosphorus, and DOC), latitude (interpreted as a proxy for mean annual temperature) and in situ temperature were the only significant predictors of bacterial biomass P:C (Hall and others 2009). Here, by only focusing on these two significant predictor variables, we were able to extend this analysis to include 46 additional lakes from the same study, for a total of 93 lakes. The entire data set used here includes lakes from a wide trophic gradient (chlorophyll a 0.32–91, median 4.2 μg l^{-1}) in the Upper Mid-West (South Dakota, Minnesota, Iowa, and Michigan) as described by Hall and others (2009). We found latitude was again a significant predictor of bacterial biomass P:C (by atom), explaining 25% of the variance of bacterial biomass P:C (P:C = −5.8 + 0.0009 * latitude, R^2 = 0.25, $P < 0.0001$). More importantly, although total dissolved phosphorus (TDP) was significantly and positively correlated with biomass P:C (Figure 2A), SRP was significantly and negatively correlated with biomass P:C (Figure 2B). The inverse relationship between SRP and biomass P:C suggests that microbial biomass P:C was, in part, driving the availability of reactive P in these temperate lake ecosystems (Figure 2B). This result is consistent with the relationship between microbial biomass stoichiometry and nutrient recycling from the soil, litter, and stream studies outlined above.

There is further evidence for the relationship between microbial biomass stoichiometry and nutrient recycling from experimental studies at both the community and population level. For instance, bacteria from a Canadian shield lake

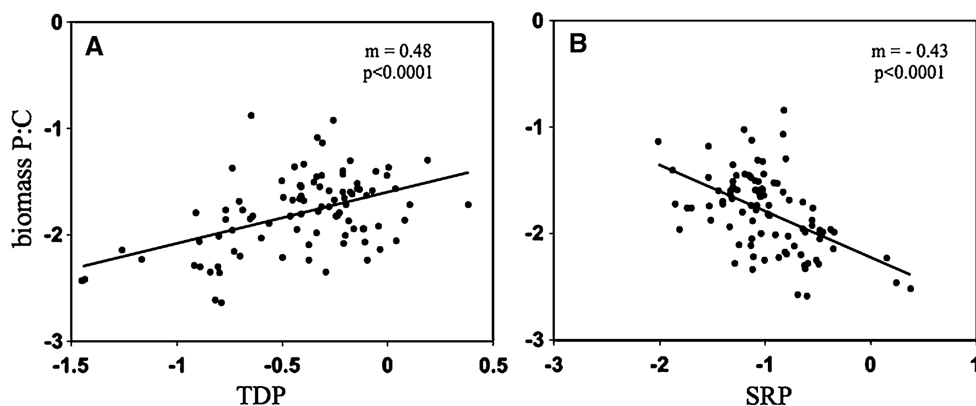

Figure 2. Plots from a multiple regression analysis of the individual effect of total dissolved phosphorus (TDP) and soluble reactive phosphorus (SRP) against biomass P:C of pelagic bacteria (defined here as <1 μm seston, full model: R^2 = 0.27, F = 16.48, $P < 0.0001$). Each plot shows the effect of the variation of each variable on the response after the effect of the other variable has been accounted for. Both TDP and SRP (μmol l^{-1}) were significantly and positively correlated for the 93 lakes used in this analysis (TDP = 0.17 + 0.58 * SRP, R^2 = 0.52, $P < 0.0001$). A subset of this data (n = 47) and a complete detail of the study was presented in Hall and others (2009). Each variable was log (base 10) transformed to meet the assumptions of linear regression. m = slope of each parameter from the MLR analysis.

showed an inverse correlation between N-flux and biomass N:P (Elser and others 1995). Specifically, when bacterial biomass N:P (by atoms) was greater than about 25, no N was recycled to the environment although below a biomass N:P of 25 there was a positive N-flux. Similarly, a chemostat study using a monoculture of *Pseudomonas fluorescens* also demonstrated recycling of N below a biomass N:P of 25 but not above (Chrzanowski and Kyle 1996).

Taken together, these observational and experimental studies, from an exceptionally broad range of environments, suggest that there is an intimate link between microbial biomass stoichiometry and nutrient recycling. In addition, the results from the lake survey demonstrate how bacterial biomass stoichiometry may be driven, or at least constrained, by factors other than resource stoichiometry (for example, temperature) and therefore allows for predictive linkages between environmental variables and the role of microbes in nutrient recycling. Mechanistically defining the controls and constraints on microbial biomass stoichiometry should help predict under which environmental conditions the microbial biomass pool is likely to act as a sink or source of mineral nutrients.

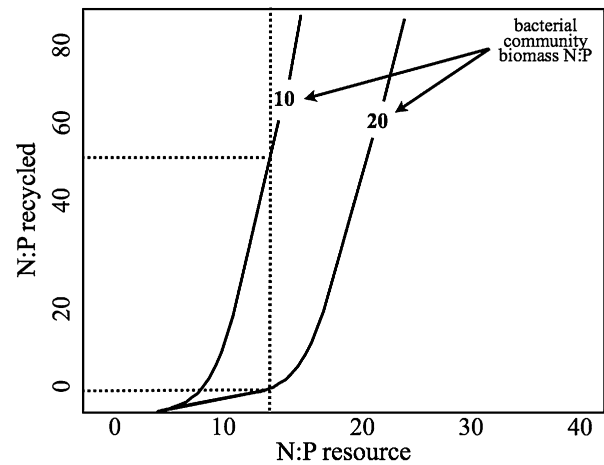

Figure 3. The relationship between the N:P stoichiometry of the recycled pool and resource pool for two microbial communities with different (10 vs. 20) biomass N:P. *The dotted line* that connects each axis shows the difference in relative N recycling for each community consuming a resource pool with a resource N:P of 15. Makino and Cotner (2004) showed that lake bacterial communities growing on the same media in continuous culture but at different growth rates had biomass N:P that ranged from 11 to 31 (by atom). Thus independent of media, bacterial community biomass N:P can vary by at least as much as the examples shown here. (Adapted from Figure 6.2 in Sterner and Elser 2002).

Theoretical Basis for Biomass Stoichiometry and Nutrient Cycling

The relationship between biomass X:C (where X can be N or P) and the amount of resource recycled to the environment has a well-developed theoretical framework with broad empirical support (Elser and Urabe 1999; Sterner 1990). Using a model system of a metazoan feeding on algae, Sterner (1990) showed that for a given resource N:P, consumers with higher biomass N:P recycled less N relative to P (Figure 3). Assuming homeostatic biomass (that is, some level of resistance to change of consumer biomass stoichiometry in response to changing resource stoichiometry) of the consumer (or in this case microbial community) when the resource N:P is less than the consumer N:P, the consumer will retain more N relative to P. Conversely, when the resource N:P is greater than the consumer N:P, the consumer recycles more N relative to P. This leads to substantially different recycling of N relative to P when communities with distinct biomass N:P ratios are exposed to an intermediate N:P resource (Figure 3). Although the presence of a 'consumer driven nutrient recycling' (CDNR) mechanism was established using pelagic herbivore—autotroph interactions, recent studies have shown a similar mechanism in microorganisms consuming both dissolved and particulate organic matter. A theoretical analysis demonstrated that through a CDNR-like recycling mechanism, terrestrial microorganisms can be more important than herbivores in determining the nutrient that limits autotrophic growth (Cherif and Loreau 2009). Due to the low amount of primary production that is consumed by herbivores in forest ecosystems, the role of a microbially driven recycling mechanism was hypothesized to be especially pronounced and promote P limitation due to relatively high biomass P in microbial decomposers (Cherif and Loreau 2009). Shifts in the limiting nutrient caused by differential retention and recycling by microorganisms due to differences in biomass stoichiometry can result in shifts in autotrophic community composition driving changes in ecosystem processes (for example, C acquisition) (Cherif and Loreau 2009). This mechanism was demonstrated empirically where the inclusion of a bacterial community switched nutrient limitation in an algal culture from N to P limitation (Danger and others 2007).

The suite of studies discussed above, from the ecosystem (Taylor and Townsend 2010; Manzoni and others 2008; Hall and others 2009; Dodds and others 2004), community (Danger and others 2007; Elser and others 1995) and organismal level (Chrzanowski and Kyle 1996; Danger and others 2007) suggest that CDNR is applicable to microorganisms from many if not all environments. However, this interpretation as well as the CDNR mechanism itself hinge on the assumption that biomass of the microbial consumer is homeostatic. Although it is clear that microorganisms are not strictly homeostatic (Makino and Cotner 2003), multiple studies suggest some level of biomass homeostasis is present in microorganisms at multiple phylogenetic levels (Cleveland and Liptzin 2007; Danger and others 2008; Makino and Cotner 2004).

The Homeostasis of Microbial Biomass

The few studies that have directly evaluated the variation in bacterial biomass stoichiometry in response to changing resource stoichiometry have found different levels of homeostasis for different elemental ratios (for example, N:C vs. P:C) and for different levels of ecological organization, for example, population versus community (Chrzanowski and Kyle 1996; Goldman and others 1987; Makino and others 2003; Tezuka 1990). Microbial community biomass N:C both in the environment (Cleveland and Liptzin 2007) and in enrichment culture (Goldman and others 1987) appears to be relatively homeostatic, especially when considered in the context of the wide range of N:C of the potential resource pool (Cleveland and Liptzin 2007). Microbial biomass P:C, however, is considerably more variable and individual strains (that is, populations) appear to demonstrate a higher level of homeostasis than communities (Danger and others 2008; Makino and others 2003). The latter result has led to the hypothesis that community homeostasis might be a function of biological diversity, with more diverse communities having a lower level of stoichiometric homeostasis (Makino and others 2003). In such a scenario, changes in the community biomass P:C are due to shifts in the relative abundance of the more homeostatic constituent populations. Taxon-specific differences in biomass stoichiometry would suggest that biomass stoichiometry (or level of plasticity in biomass stoichiometry) is, to some extent, a genotypic trait or, more likely, that natural selection is acting on one or multiple genotypic trait(s) whose combined effect result in a constrained cellular stoichiometry.

Such a relationship can be elucidated by noting that variance in cellular stoichiometry is ultimately due to shifts in the relative abundance of constituent macromolecules (Hall and others 2010b), and the elemental composition of those macromolecules (Baudouin-Cornu and others 2001), which are tightly coupled to cellular physiology and life history (Vrede and others 2004). Viewing microbial community stoichiometry from this perspective links phylogenetically specific physiology and life history with biomass stoichiometry, and therefore community composition with community biomass stoichiometry.

Deconstructing Microbial Community Stoichiometry

Although community stoichiometry is a composite of population-level stoichiometry, population stoichiometry is directly attributable to the cellular stoichiometry of members of each population. Similarly, the stoichiometry of an individual cell is the composite of the relative abundance of its constituent macromolecules, each of which has a relatively fixed stoichiometric signal (Figure 4; Elser and others 1996). Changes in biomass stoichiometry, driven by changes in macromolecular

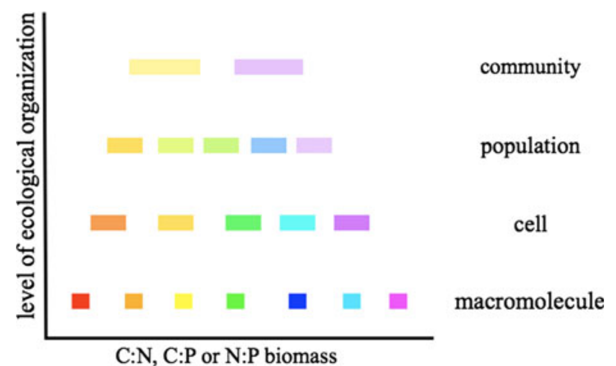

Figure 4. A conceptual schematic of the level of stoichiometric constraint at different levels of ecological organization. Microbial community stoichiometry can be attributed to the relative abundance of constituent populations which are hypothesized to have more constrained biomass stoichiometry. Plasticity in population stoichiometry is due to the heterogeneity of stoichiometry among its individual constituent members. Variance in cellular stoichiometry can be attributed to shifts in the relative abundance of macromolecule suites (for example, carbohydrates, nucleic acids, and proteins) each with a relatively constrained stoichiometry. The biomass stoichiometry at each level of organization is a composite (indicated by a blending of *colors*) of the level directly below.

composition, reflect an organism's physiology (for example, growth rate or accumulation of storage compounds), the in situ environmental conditions (for example, resource stoichiometry, temperature), evolutionary history (Baudouin-Cornu and others 2004) or some combination of these. Thus, understanding biomass stoichiometry can give additional information on an organism's life history and ecology. For example, positive relationships between rRNA content, biomass P:C and growth rate have been formalized as the growth rate hypothesis, GRH (Elser and others 2000) and have been shown to hold for a wide range of organisms (Elser and others 2003). Growth rate has been shown to be a major determinant of microbial biomass stoichiometry of single species (Makino and others 2003) and mixed communities (Makino and Cotner 2004) grown in culture. However, GRH relationships do not hold under non-P limitation (Makino and Cotner 2004) or pulsed resource supply (Binder and Liu 1998). A recent theoretical study demonstrated that the deviation from GRH relationships in bacteria can be explained by the optimization of the relative abundance of cellular machinery (for example, proteins, RNA) to maximize growth in environments with different resource stoichiometries (Franklin and others in press). The model was able to show mechanistically why GRH relationships would be expected under P limitation but not under N limitation. This last result illustrates the value of being able to deconstruct biomass stoichiometry into its constituent macromolecules to understand the underlying mechanisms behind such important ecological relationships as the coupling of resource availability and growth.

The GRH provides one example of a well-developed illustration of the link between constituent macromolecules, physiology, and biomass stoichiometry. Deconstructing whole organism stoichiometry into additional constituent macromolecules should allow for better understanding of the interaction between microbial physiology and biomass stoichiometry. For example, there exists a clear relationship between C storage as poly-3-hydroxybutyrate (PHB), potentially a significant component of total biomass C, and survivorship of soil bacteria under carbon limitation (Ratcliff and others 2008). The lipid content of aquatic bacteria has been shown to be taxon specific (Nichols and others 1993), closely related to the level of thermal adaptation or acclimation (Nichols and others 1997 and can be a significant pool of cellular P in marine bacteria (Van Mooy and others 2009). This has led to differences in resource availability affecting the composition of lipids in marine bacteria (Van Mooy and others 2009), which have been shown experimentally to affect the ability of bacteria to acclimate to temperature changes (Hall and others 2010a). Substitutions of elements under nutrient limitation have also been proposed to occur at the level of the proteome. Analysis of the sulfur (S) and C assimilatory pathways in a model bacterium and eukaryote revealed depletion of S and C in each pathway relative to the total proteome and to the enzymes involved in the other pathway (C or S assimilation, respectively) for both organisms (Baudouin-Cornu and others 2001). Furthermore, synthesis of extracellular enzymes, an essential phenotype of most soil microorganisms, requires significant amounts of C and N, influencing an organism's resource partitioning and ultimately its biomass stoichiometry.

Each of these examples illustrates how an organism's ecology and function are linked to the relative abundance of constituent macromolecules and their atomic composition that ultimately determines cellular biomass stoichiometry. Previously, evaluation of stoichiometric theory for microorganisms has been empirically elusive due to technological constraints. However, recent application of single-cell methods to microbial ecology allows empirical analysis of important stoichiometric parameters (described above) at a level of resolution previously unobtainable.

Empirical Analysis of Microbial Stoichiometry

To date, most of the studies on bacterial stoichiometry in the environment are observational (Tezuka 1990; Cleveland and Liptzin 2007; Hall and others 2009), focused at the community level and incorporate none of the molecular tools now available to microbial ecologists (Figure 1, above the dotted line). Experimental studies, although instructive (for example, Vrede and others 2002; Makino and others 2003; Makino and Cotner 2004), are limited to the small fraction of environmental organisms that can be cultured. Each of these limitations is primarily due to two key methodological constraints. First, it is difficult to separate microorganisms from the complex abiotic matrix they inhabit (for example, soils or sediments) or from other organisms (for example, algae, microzooplankton) to independently analyze their biomass composition. Second, traditional analytical methods require relatively large amounts of biomass to analyze the elemental and macromolecular composition of microbial biomass.

Therefore, using traditional methods it is necessary either to enrich organisms in culture or for environmental samples to analyze undifferentiated bulk communities. Fortunately, both of these problems can be largely avoided by employing a series of methods that allow for analysis of elemental and macromolecular composition at the level of a single-cell.

X-ray microanalysis (XRMA) allows for elemental analysis of single bacterial cells (Norland and others 1995). For XRMA, cells are first visually detected using a transmission electron microscope (TEM) then selected for analysis of elemental content. The quantity of virtually any element of interest (lower limit = atomic number ≥ 5 with heavier elements being easier to detect), can then be determined for the entire cell based on the X-ray emission spectra. Direct visualization allows for exclusion of abiotic particles and extra-cellular polymeric substances that might otherwise bias the elemental biomass signal. XRMA has been used to successfully evaluate the elemental composition of different bacterial isolates during different growth phases and under different nutrient limitations (Vrede and others 2002) and from both cultured and environmental samples (Fagerbakke and others 1996).

A second highly versatile approach to analyze elemental composition of microbial cells is secondary ion mass spectrometry (SIMS). Here a high-energy primary ion beam is used to expel secondary particles, neutral or charged, from the sample surface. Charged particles of one polarity are then collected by an electric field and focused, and the resulting secondary ion beam is analyzed by mass spectrometry. Recently, ion microscopy based on SIMS has found numerous applications in microbial ecology as it combines the quantification and visualization of elements and isotopes in microorganisms (Wagner 2009). NanoSIMS imaging devices offer elemental analysis at a spatial resolution between 50 and 150 nm and have been used to successfully identify and analyze individual cells from artificial and natural communities (Behrens and others 2008). In this manuscript we focus on the movement of C, N, and P between abiotic and microbial biomass pools. However, it is important to note that both XRMA and NanoSIMS have the potential to evaluate a suite of biogeochemically relevant elements (Wackett and others 2004) and thus can be used to evaluate the role of microbes in a wide range of biogeochemical cycles.

Complementary to elemental analysis of individual cells with XRMA and NanoSIMS is analysis of macromolecular composition using Raman microspectroscopy (MS). Although not new in medical microbiology (Maquelin and others 2002), Raman MS analysis has only recently been applied to environmental bacterial communities (Huang and others 2007a). In Raman MS incident monochromatic radiation (that is, laser light) interacts with molecules in the target cell. Based on the types of bonds present in certain macromolecules, pronounced peaks in the complex Raman MS spectra can be assigned to major compound classes such as proteins, carbohydrates, nucleic acids, and lipids (Schuster and others 2000). The intensity (peak height) of Raman MS bands is proportional to the concentration of the respective molecule. Therefore, because the identified macromolecule classes comprise the major pools of cellular C, N, and P (Elser and others 1996), shifts in macromolecular composition as determined by Raman MS correlate well with whole cell stoichiometry (Hall and others 2010b). Macromolecular signals as determined by Raman MS have been successfully applied to identify which resources plant associated bacteria utilized *in plantae* (Huang and others 2007b) as well as to differentiate the growth phase of the cells in heterogeneous populations (Hall and others 2010b).

It is important to note that these methods can produce abundant data of complex spectra that ecologists may be unaccustomed to. For example, a single Raman MS spectrum (that is, from a single-cell) may contain around 1200 data points, the peak height at each Raman MS band. There are examples of various computationally intensive approaches to deal with the data from the entire Raman spectrum (for example, see Wagner 2009 and references therein for examples). However, we found that extraction of about 15 identified peaks from complete spectra and analysis with traditional multivariate statistics (that is, principal components and canonical discriminant analyses) yielded powerful and ecologically relevant output (Hall and others 2010b). With respect to the ideas discussed in this manuscript, this subset of 15 peak heights accounted for a large proportion of variance in biomass N:C and P:C (up to 90 and 50%, respectively) suggesting that most proteins, nucleic acids, and carbohydrates were accounted for by this limited subset of spectral data. Taken together these approaches (XRMA, NanoSIMS, and Raman MS) to quantify cellular composition, both elemental and macromolecular, significantly increase the level of resolution of stoichiometric analyses in microbial ecology.

Although the elemental analysis of microbial biomass relative to the stoichiometry of the

resource pool may indicate the direction of nutrient cycling (for example, whether N is likely to be retained in microbial biomass or recycled to the environment), it does not address the rate at which the biogeochemical process of interest occurs. This can be partially addressed by applying stable isotope studies in concert with NanoSIMS or Raman MS analysis. In addition to the ability to analyze biomass composition, both NanoSIMS and Raman provide the opportunity to track the incorporation of stable isotopes (for example, from labeled substrates) to regions of microbial cells (NanoSIMS) or to specific macromolecules (Raman MS). For example, using NanoSIMS the incorporation of stable isotope labeled substrates into single cells can be visualized and quantified at the sub-cellular level (Behrens and others 2008). Such analyses with NanoSIMS can be conducted at a sensitivity approximately 1000× higher than that achieved by microautoradiography for detecting labeled carbon (Lechene and others 2006). Similarly, Raman MS can identify the incorporation of stable isotope labeled compounds into macromolecules by observing the shifts in the Raman spectra of defined peaks (Huang and others 2007a). Thus, this approach links elemental analysis with NanoSIMS and macromolecular analysis with Raman MS to the rate of use of specific substrates in situ. Furthermore, the tracking of labeled substrates combined with the methods described below allows for identification of the active members of the community. This helps to reduce the complexity of the microbial community by focusing the analyses on the members of the community that are most likely to be influencing biogeochemical processes.

In addition to these analytical capabilities, each of the aforementioned single-cell methods can be combined with in situ hybridization (for example, fluorescent in situ hybridization, FISH) thus linking analysis of biomass composition to specific phylogenetic units within a microbial community. FISH with rRNA-targeted probes can be applied at multiple phylogenetic resolutions (Amann and others 1995) and can target functional genes (Pernthaler and Amann 2004; Wagner and others 1998). FISH also has a long list of successful syntheses with other analytical methods that increase the breadth of its applicability (Wagner and others 2003). For example, FISH has been successfully coupled with Raman MS (Huang and others 2007a). More recently, rRNA-targeted in situ hybridization was combined with NanoSIMS by labeling phylogenetic groups with halogenated rRNA probes (SIMSISH; Li and others 2008). Another approach uses rRNA probes linked to halogen-containing fluorochromes, effectively combining signal amplification with labeling for both fluorescence microscopy and NanoSIMS, EL-FISH (Behrens and others 2008). This allows application of in situ hybridization techniques to cells with limited ribosome count, traditionally a drawback to FISH analyses, by amplifying the signal (in this case a halogen label). Because XRMA can detect a wide range of elements, and sensitivity increases with molecular weight, EL-FISH and SIMSISH with the heavier halogens (for example, Br or I) should be applicable to XRMA analysis, although this has yet to be tested. Using these methods, the ability to scale phylogenetic information from single species to the level of genera, phyla and whole domains can be coupled to the evaluation of elemental and macromolecular composition, and activity.

The combination of these empirical techniques allows for empirical validation of the conceptual framework outlined in this manuscript, as well as the testing of many of the extant stoichiometric mechanisms developed for larger organisms but previously empirically elusive for microorganisms. Furthermore, the application of stable isotope labeling with Raman-FISH, SIMSISH, and EL-FISH provides a bridge between community structure, microbial function, and elemental and macromolecular composition in the context of ecological stoichiometry.

Challenges to Single-Cell Stoichiometric Analyses

Although single-cell analyses have several advantages, one key disadvantage is that natural microbial communities are composed of too many cells to evaluate each one independently. Therefore, it is not possible (or necessarily desirable) to independently analyze the elemental and macromolecular composition of all cells in a natural microbial assemblage. Rather, the approach outlined here is best suited for hypotheses focused on specific biogeochemical pathways and/or specific phylogenetic groups within the microbial community. For example, an a priori survey of the microbial community, such as a deep sequencing of the rRNA amplicon from community DNA, or transcriptomic and/or proteomic analysis, are ways to identify groups that are most likely to be the dominant members of the community and therefore responsible for key biogeochemical processes. After community analysis, microbial groups of interest can be targeted with designated probes and their role (for example, whether they are acting as a sink or

source of mineral nutrient) in nutrient cycling more clearly defined following the conceptual framework outlined here. In addition, a pre-sorting of 'populations' may aid in covering the heterogeneity present in any given microbial community. For example, it is possible to subject a mixed community to cell sorting by cell size or fluorescence signal after incubation with a FISH probe using flow cytometry before subsequent stoichiometric analyses with the methods discussed here. It is also possible to use an activity stain, such as cyanoditolyl tetrazolium chloride (CTC), to aid in focusing these analyses on the active portion of the community.

A separate disadvantage to focusing only on elemental ratios is the loss of information on chemical diversity, especially organic carbon compounds, present in the environment. This is especially relevant when dealing with terrigenous organic matter where the presence of complex structural compounds and secondary metabolites are much more prevalent than in organic matter produced autochthonously in aquatic ecosystems. For example, although glucose and lignin have similar elemental composition, they also have vastly different biological lability owing to the differences in their molecular structure. This deficiency can be partly addressed using Raman MS, which gives more detailed information on the molecular composition of organisms than elemental analysis alone. For example, PHB has a well-defined peak in the Raman spectra (De Gelder and others 2008) and is a common carbohydrate reserve found in mycorrhizal symbionts (Ratcliff and others 2008). In this example, Raman MS gives information not only on the contribution of the constituent macromolecule to total biomass C but also specific information on the nature of the chemical compound that drives that signal. Although Raman does not provide a comprehensive approach to describing the chemical diversity of microbial cells or their environment, it does provide more information than elemental analysis alone.

Overview and Future Directions

The coupling of ecological theory with culture-free microbiological methods is an essential step in understanding how microorganisms affect ecosystem processes. Observational studies linking microbial community composition and ecosystem processes result in only spatially and temporally limited understanding of environmental processes and their controlling factors. Therefore, it is important to organize the abundance of information on environmental microorganisms now available into clear mechanistic frameworks that are directly related to ecosystem function. The presence of environmental controls on microbial stoichiometry (for example, temperature, Hall and others 2009), and stoichiometric thresholds that govern microbial nutrient recycling (for example, Taylor and Townsend 2010; Elser and others 1995) suggests that understanding the drivers of microbial biomass stoichiometry is one way to increase the ability to predict whether microbes act as a sink or a source of limiting nutrients in a wide variety of ecosystems. We conclude with two specific examples of how the concepts and methods discussed in this manuscript could be applied to elucidate the influence of microorganisms on ecosystem processes.

Although the genetic diversity of natural microbial communities is exceptionally high recent research points to ecological sorting of microbial taxa at relatively coarse levels of phylogenetic resolution (Phillippot and others 2010). This includes differences in the relative abundance of broad phylogenetic groups among habitats and across environmental gradients. Recently, the relationship between biomass stoichiometry and carbon use efficiency of bacteria and fungi was shown to correspond to the distribution of bacterial:fungal ratios across a terrestrial resource gradient (Keiblinger and others 2010). By coupling NanoSIMS or XRMA with in situ hybridization similar analyses can now be extended to virtually any phylogenetic group of interest. For example, the relative abundance of the Gammaproteobacteria and the Firmicutes has been shown to vary between cropland, hayed pasture, grazed pasture, and forest soil (Phillippot and others 2010). By coupling XRMA or NanoSIMS with rRNA probes (in situ hybridization) it is now possible to evaluate whether differences in the relative abundance of these groups is linked to differences in their biomass stoichiometry and if their resource demands correlate with their resource environment. Further analysis using Raman MS and in situ hybridization could determine the driver of these hypothesized differences in biomass stoichiometry between groups. These approaches may explain differences in the relative abundance of various microbial groups in time and space. More importantly, comparison of the biomass stoichiometry of each group with the resource stoichiometry of each environment would allow insight into how different microbial groups are likely affecting nutrient recycling in a given environment.

Similarly, surveys of aquatic bacterial community composition indicate that the Betaproteobacteria compose a significant proportion of freshwater bacterial communities but are virtually absent from marine habitats (Zwart and others 2002). A recent analysis of bacterial stoichiometry from a wide range of aquatic ecosystems concluded that lake bacteria were depleted in N and P relative to their marine counterparts (Cotner and others 2010). These two studies taken together suggest that the Betaproteobacteria may have a generally low nutrient content compared to other bacterial classes. This idea could be tested by targeting the Betaproteobacteria with in situ hybridization and simultaneously analyzing biomass composition (XRMA) and macromolecular composition (Raman MS) relative to other members of the bacterioplankton community. This same approach could also be extended to evaluate the seasonal cycles of microbial community structure that have been noted in a wide range of aquatic habitats (Crump and Hobbie 2005; Shade and others 2007; Hullar and others 2006; Fuhrman and others 2006). Changes in the abundance of specific microbial groups between seasons may alter community stoichiometry due to differences in their group-specific biomass stoichiometry. If so this would suggest a seasonally variable role for the microbial community in nutrient recycling. Taken together these methods and concepts provide yet another set of important tools to unravel the complex interaction between microorganisms and their environment.

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