ECOLOGICAL STOICHIOMETRY OF MARINE BACTERIA: RELATIONSHIP TO GROWTH RATE, PROTOZOAN PREDATION, AND ORGANIC MATTER DEGRADATION

By

DAVID F. GRUBER

A Dissertation submitted to the

Graduate School-New Brunswick

Rutgers, The State University of New Jersey

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

Graduate Program of Oceanography

written under the direction of

Professor Gary L. Taghon

and approved by

_Gary Taghon_____

_Sybil Seitzinger _____

_Kay Bidle_____

_Peter Morin_____

_David Caron_____

New Brunswick, New Jersey

October, 2007

ABSTRACT OF THE DISSERTATION

Ecological Stoichiometry of Marine Bacteria: Relationship to Growth Rate, Protozoan Predation and Organic Matter Degradation

By DAVID F. GRUBER

Dissertation Director

Professor Gary L. Taghon

The cycling of carbon between inorganic and organic compounds is an underlying process that drives all life forms. While the rates of production of organic matter have been extensively examined, the degradation rates and kinetics remain poorly understood. Microbial organisms, highly efficient recyclers, play a pivotal role in the degradation process in the ocean. This thesis explores the interactions among microbes (bacteria and protozoa) and how the competitive and predatory interactions affect the rate of organic matter degradation and regeneration. Emphasis is placed both on the structure and dynamics of the particulate and dissolved organic reservoir. Also, prey C:N:P stoichiometry is examined (both experimentally and in a model) to assess the role of elemental ratio relationships in population dynamics and organic matter cycling. It was found that under low growth rates, there is extensive variability of cell C:P and N:P, dependent on bacterial species, but at high growth rates, most species have similar C:P and N:P due to the necessity of P-rich ribosomes. Using clonal species of bacteria tagged

with red and green fluorescent proteins, this thesis provides evidence that protozoan predators may prefer slower growing bacterial cells (with higher C:P and N:P), possibly because their cellular stoichiometry closer resembles that of eukaryotic consumers and less energy would need to be expended on processing the excess nutrients.

Data from this thesis suggests that the ultimate bulk percentage of carbon remineralized or respired is primarily dependent on predator/prey interactions and trophic inefficiency, regardless of the limiting nutrient. It has long been debated how the addition of protisian predators stimulates the degradation of organic matter. Here, evidence is provided that the trophic inefficiency of converting bacteria cells to protist cells may account for much of this stimulation.

ACKNOWLEDGEMENTS

There are many people for whom I owe thanks for this thesis. Firstly, I would like to thank my advisor, Dr. Gary Taghon, for his unwavering guidance throughout my time at the Institute of Marine and Coastal Sciences.

Thank you also to my committee, Drs. Sybil Seitzinger, Kay Bidle, Peter Morin and Dave Caron, for overseeing my doctoral work and helping to ensure that I followed the correct line of questioning and took the necessary steps to attack the questions at hand.

I also thank Drs. Dan Tchernov and Tom Bibby for their friendship and intellectual encouragement throughout this thesis. Thanks to Vincent Pieribone for teaching me the ropes of molecular biology and for distracting me with fluorescent proteins --which inevitably became a pivotal tool for this thesis. Also, this research benefited from the astute comments of my officemate/colleague, Steve Tourto.

I would like to extend a very special thanks to Yana Zeltser. Without her encouuragement and assistance, this thesis may never have come to fruition.

Of course, I am grateful to my family, Marlaine, Edward and Lori, for their unconditional support.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS

CHAPTER 1

Introduction

Over the past few decades, a major focus of oceanography has been to quantify the amount of carbon being shuttled from inorganic to organic forms via photosynthesis. A small percentage of solar energy reaching marine primary producers (phytoplankton, cyanobacteria, macroalgae) is transformed by the photosynthetic apparatus into reduced organic matter. These photosynthetic organisms synthesize complex organic substances from simple inorganic molecules –providing the energetic foundation for most marine food webs. While a portion of the "producers" energy is expended on catabolic processes, a significant surplus of organic material is accumulated and available to heterotrophic "consumers." Net marine primary production, the amount available to heterotrophic consumers, is estimated at 45 Pg C/year –almost half the global primary production (2003), while accounting for less than 1% of Earth's photosynthetic biomass (Field et al. 1998).

Up until the mid-1970's, the fate of organic carbon was thought to be determined as it escalated through the tiers of the food chain (Steele 1974). Studies analyzing the sinks and sources of organic matter neglected to include bacteria, protozoa, and viruses, as they were considered to play a minor role. Now it is well established that microorganisms play a dominant role in organic matter cycling. This process, known as the microbial loop (Pomeroy 1974), refers to the bacterial recovery of dissolved organic matter (DOM) through uptake and metabolism (Azam et al. 1983), otherwise lost from the trophic system via excretion, exudation and diffusion (Jumars et al. 1989). In the ocean, the DOM pool is 10 times larger than the particulate organic matter (POM) pool

and 50 times larger than the pool sequestered in living biota. The rates and mechanisms at which this organic carbon is oxidized, transformed and returned to its inorganic form are poorly understood.

Dissolved organics originate from several sources –such as excretion from algae in the form of sugars, amino acids and glycolate. In addition, bacteria have enzymatic systems that induce rapid hydrolysis of particulate organic matter, significantly influencing the quantity and composition of the DOM pool (Azam and Worden 2004). Some labile organic molecules have extremely rapid turnover rates (glucose) of hourly to daily timescales, while others are recalcitrant (humic substances) and have turnover rates of thousands of years. The mechanism by which certain components of bacterial cells remain recalcitrant to microbial degradation remain unknown (Benner 1998). Yet, these refractory components accumulate in oceans, forming one of the largest pools (700 x 10^{15} g C) of organic carbon on earth (Hedges et al. 2000). It is not fully understood what role biology plays in the formation of refractory DOC and cycling of this large carbon reservoir. This thesis addresses the following three objectives:

Objective 1: *Determine how interactions among aerobic heterotrophic bacteria and protozoa influence carbon regeneration.*

It is established that the presence of protistan grazers increases the rate at which reduced organic carbon –derived from phytoplankton– is mineralized as CO_2, yet the roles of species diversity and habitat complexity are largely unexplored (Fenchel and Blackburn 1979). Heterotrophic bacteria (0.5 to 2.0 μm in diameter) perform three major

functions in the transformation of organic matter: they produce new bacterial biomass (bacterial secondary production), respire organic carbon to inorganic carbon (bacterial respiration) and produce dissolved organic compounds. Bacteria are mostly obligate osmotrophs that consume 10 to 50% of carbon fixed by photosynthesis via uptake of dissolved, low molecular weight compounds (less than 300 Da) (Bidle, personal communication).

Ducklow (1983) suggested that primarily ciliated protozoa (ubiquitous in the marine environment), within the microzooplankton (20-200 μm diameter) and nanozooplankton (2-20 μm diameter), control standing stocks and metabolic activity of bacteria – and form an important link between nanoplankton and larger consumers of the food web. The simple life history and short generation times of ciliates enable them to respond rapidly to changes in phytoplankton and bacterial populations, strongly influencing food web structure and function. By regulating bacterial activity, ciliates and other protists indirectly impact the dissolved and particulate carbon flux.

Early studies demonstrated that bactivorous protozoan grazers accelerate remobilization of phosphorus from organic matter and decomposition of detritus (Fenchel and Harrison 1976; Johannes 1964). If protozoa are excluded from the microbial communities under experimental conditions, bacterial density increases 2 to10 times, but the rate of mineralization decreases relative to systems where bacteria are grazed (Fenchel and Jorgensen 1977). Fenchel and Blackburn (1979) later summarized: “The reason for this stimulation of bacterial activity by grazing protozoa is not quite clear.” For many years, few studies addressed the impact of protozoa on the rate of organic matter mineralization. This thesis revisits this longstanding question and demonstrates that

under different nutrient conditions, protozoa stimulate organic matter degradation by varying mechanisms (including prosing a classic ecological concept as a mechanism for organic degradation stimulation, tropic inefficiency). Since Lindeman (1942) proposed the concept of trophic dynamics, ecologists have had great success quantifying individuals to ecosystems in terms of energy. Trophic dynamics is the use of energy to define the feeding relationships especially from one trophic level to another. *"*Lindeman's Efficiency of the System" states that 10% of the energy is passed to each escalating trophic level.

Objective 2: *Examine the relationship of elemental stoichoimetry of prey content to foraging and elemental cycling.*

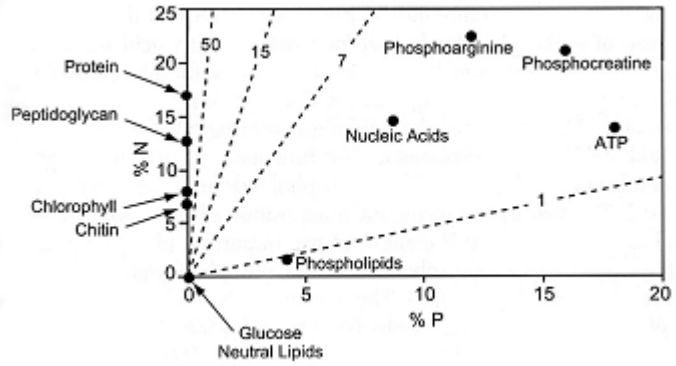
> "The decomposition of organic matter presumably is always liberating some of this phosphorus and nitrogen. Within limits, the more organic matter present the easier will be such regeneration. It is probable that benthic animals and anion exchange play a part in such processes." Hutchinson (1942)

Stoichiometry in Greek translates to "measuring elements." In biology it refers to the patterns of proportions of elements in reactants and products (Lotka 1956). This branch of chemistry deals with the application of the law of definite proportions and conservation of mass, first defined by Richter (1792). There is a major discontinuity between the abundance of molecules in non-living and living entities. For example, the stoichoimetry of total phytoplankton in the ocean generally conforms to the canonical redfied ratio of C:N:P at 106:16:1 (Redfield 1958), while bacteria fall in the average C:N:P range of 50:10:1 (Fagerbakke et al. 1996). Single species of both phytoplankton and bacteria can vary significantly.

Bacteria are obligate osmotrophs that require a variety of dissolved materials for the synthesis of all cellular components so that they may grow and divide, producing

progeny. There are several different classes of biological materials containing carbon, nitrogen and phosphorus that are not evenly distributed in their stoichoimetry. For instance, phosphorus plays a role in the biology of nucleic acids, but is virtually absent from proteins. Therefore, the abundance of various cellular components can have a significant effect on the total cell stoichiometry. A rapidly dividing cell may have increased RNA content and hence decreased C:P and N:P ratios (Fig 1).

Fig. 1.1: Percent N and P of important biomolecules (Sterner and Elser 2002)



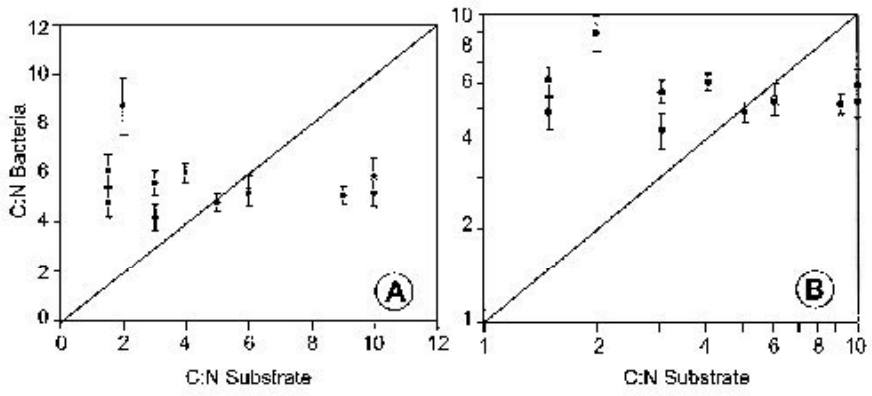
The "you are what you eat" hypothesis (Herbert 1961) states:

> "There are few characteristics of microorganisms which are so directly and so markedly affected by the environment as their chemical composition. So much is this the case that it is virtually meaningless to speak of chemical composition of a microorganism without at the same time specifying the environmental conditions that produce it."

While there is some validity in this hypothesis for eukaryotic plankton possessing food vacuoles (Klausmeier et al. 2004) (Rhee 1978), it is not steadfast for bacteria. Goldman et al (1987), for example, grew natural assemblages of marine bacteria on substances of differing C:N from 1.5 to 10 (Fig. 2). The bacteria biomass C:N remained constant and did not vary with medium. In this scenario, bacteria can be thought of as regulators and cells with food vacuoles as conformers. But, bacteria collected from

coastal waters and grown in the laboratory have been shown to have biomass C:P that vary from 8 to 500 (Tezuka 1990). This adheres the growth rate hypothesis (GRH), suggesting that growth and elemental stoichiometry of organisms are coupled through variation in nucleic acid composition, specifically variation in ribosomal RNA. While this flexible stoichoimetry does not rigidly apply to C:N (Goldman et al 1987). This thesis demonstrates that each bacterial strain regulates its elemental composition within a relatively narrow range of biomass C:N ratios and hence a shift bacterial populations may cause variations in bacterial stoichiometry. There is also the underlying possibility that studies reporting large variations are laden with experimental artifacts resulting from bacterial contamination or the difficulty in separating living versus non-living POM. Bacteria from the marine environments are reported to vary within a C:N range of 2.4 to 14.2 (Fagerbakke et al. 1996), but only a few bacterial isolates have been recorded.

Figure 1.2: C:N of substrate does not impact C:N of bacteria biomass of single species (Goldman et al 1987).



Bacterial grazing is a concept that has remained poorly defined, largely because the factors that determine prey selection are numerous, diverse, and difficult to directly

observe. Factors that influence grazing selection are both predator and prey specific. These include taxonomy, physiological condition, feeding capacity, nutrient content, motility, shape and size. Protists graze selectively on bacteria and may strongly influence the taxonomic composition, morphology, physiology and metabolic capacity of bacterial communities (Sherr and Sherr 2002). Grazing pressure produces morphological and compositional changes in bacterial communities by removing species and morphotypes that are less "grazing resistant" and by inducing morphological and physiological changes in pleomorphic species (Boenigk and Arndt 2002). Selective grazing may increase the abundance and physiological activity of certain bacterial species by removing competitors, releasing nutrients and selecting for strains capable of increased reproductive rates (Sherr et al. 1982; Sherr et al. 1987). Therefore, protistian bacterivory is hypothesized to be a major determinant of natural bacterial diversity. This thesis examines the role of stoichiometry in selective feeding by protists on bacteria. Evidence is provided that shows that protistian predators show a preference for slow growing bacteria. These slow growing bacteria have higher C:P and N:P ratios due to the fact that less of their cellular material is delegated to P-rich ribosomes. But, since prokaryotic cells, on average, have lower C:P and N:P that eukaryotic cells, it may be possible that eukaryotic predators are selecting prey due to cells that have more similar stoichiometry to their own, and hence, lowering the metabolic effort of processing the excess nitrogen or phosphorus.

Secondary Objective 3: *Determine how bacteria/protist interactions impact the formation, transformation, composition and utilization of the dissolved organic carbon pool.*

The assimilation of dissolved reduced organic carbon compounds is the most usual method for heterotrophic bacteria (except for a few predatory species) to obtain material for cell synthesis. Yet, not all dissolved organic matter is equal in lability. For example, freshly sampled water displays a relatively rapid rate of utilization of smaller molecules followed by a much lower rate of larger resistant compounds (Ogura 1975). Highly labile compounds such as amino acids, soluble sugars and fatty acids constitute only a few percent of the total pool of dissolved organics. The dominating fraction consists of high molecular weight components and colloidal matter (waxes, humic material and bound amino acids).

The role of bacteria on the rate and extent of DOM mineralization and their production of (semi-) refractory DOM is still relatively unknown. Some studies indicate that bacteria produce refractory DOM that is resistant to further utilization (Brophy and Carlson 1989; Heissenberger and Herndl 1994; Stoderegger and Herndl 1998; Taylor et al. 1985). Ogawa et al. (2001) showed that a natural inoculum of marine bacteria (and undoubtedly nanoflagellates and viruses) growing on labile compounds (glucose and glutamate) produce new DOM compounds that appear to be refractory for at least a year. It was unknown if a single strain of bacteria could produce similar refractory material. Bacterioplankton can also be a source of photoreactive CDOM that is refractory to a natural bacteria assemblage following photochemical alteration (2004). What kinds and how many different compounds make up the refractory DOM pool has yet to be discerned.

In aquatic ecosystems, bacteria are consumed by protozoa and other zooplankton, which in turn release DOM as colloidal matter (Koike et al. 1990; Tranvik 1994) and

macromolecular organic complexes (Nagata and Kirchman 1992b). A substantial portion (>50%) of primary and bacterial production can be consumed by a single class of protozoa, the Ciliata (Fenchel 1987). Therefore, ciliates can act as trophic links, nutrient regenerators and DOM producers (Strom et al. 1997) – roles often overlooked in traditional food webs. Little is known about the effects of additional trophic levels on the production and composition of refractory DOM. Nagata and Kirchman (1992a) suggested that the release of DOM by protozoa is potentially important in aquatic food webs and nutrient cycles. Kujawinski et al (2004) used electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT ICR MS) to identify 80 new DOM compounds produced when a protozoan grazed on bacteria.

In this thesis, the effects on DOM dynamics of a pure bacteria culture and when grazed on by a ciliate is examined. Electrospray ionization mass spectrometry (ESI-MS) was utilized for molecular level characterization of compounds produced during bacterial growth and grazing. In the past, ESI-MS has been used for identification and quantification of specific compounds (Hua et al. 1996; Loo and Ogorzalek Loo 1997; Poon 1997; Saito et al. 2004). Terrestrial and marine DOM pools, for example, have been characterized by ESI-MS (Kim et al. 2003; Koch et al. 2005; Kujawinski et al. 2002; Leenheer et al. 2001). ESI-MS has also been used to gain insights into changes in DOM due to protozoan grazing (Kujawinski et al. 2004), for characterization of DOM in rainwater (Seitzinger et al. 2003), and to discriminate between possible refractory and labile DOM compounds in freshwater samples (Seitzinger et al. 2005). It has been suggested that the spatio-temporal variation of DOM can significantly influence the global carbon cycle. The dynamics of DOM is largely controlled by the microbial

community (i.e. the microbial food web), whose biomass and specific metabolic activity are greater than larger organisms. This thesis provides evidence that bacteria, and not ciliates, may be the main shapers of the DOM continuim.

While parasitic and predatory bacteria exist in the ocean (Rendulic et al. 2004), their role in the carbon cycle is thought to be trivial and will, therefore, not be examined. Viruses were not examined in ocean ecosystems until 1989 yet are the most abundant biological entities in the sea (10^7 ml^{-1}). Bacteriophages induce bacterial mortality, creating a cyclic carbon cycle in which dissolved organic matter assimilated by bacteria is released via bacterial lysis and metabolized by other bacteria, enhancing upper-ocean respiration (Fuhrman and Schwalbach 2003). While the viral component likely plays a significant role in carbon remineralization, it will not be examined in this thesis.

REFRENCES

Azam, F., T. Fenchel, J. C. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. Marine Ecological Progress Series **10:** 257-263.

Azam, F., and A. Z. Worden. 2004. Microbes, molecules, and marine ecosystems. Science **303:** 1622-1624.

Benner, R. 1998. Cycling of dissolved organic matter in the ocean., p. 317-331. *In* D. H. a. L. Tranvik [ed.], Aquatic Humic Substances: Ecology and Biogeochemistry. Springer-Verlag.

Boenigk, J., and H. Arndt. 2002. Bacterivory by heterotrophic flagellates: community structure and feeding strategies. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **81:** 465-480.

Brophy, J. E., and D. J. Carlson. 1989. Production of biologically refractory dissolved organic carbon by natural seawater microbial populations. Deep-Sea Res. **36:** 497-507.

Ducklow, H. W. 1983. Production and fate of bacteria in the oceans. Bioscience **33:** 494-501.

Fagerbakke, K. M., M. Heldal, and S. Norland. 1996. Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. Aquatic Microbial Ecology **10:** 15-27.

Falkowski, P. G., E. A. Laws, R. T. Barber, and J. W. Murray. 2003. Phytoplankton and their role in Primary, New, and Export Production., p. 99-121. *In* M. Fasham [ed.], Ocean Biogeochemistry: The Role of the Ocean Carbon Cycle in Global Change. Global Change – The IGBP Series.

Fenchel, T. 1987. Ecology of Protozoa. Science Tech Publishers.

Fenchel, T., and T. H. Blackburn. 1979. Bacteria and Mineral Cycling. Academic Press.

Fenchel, T., and P. Harrison. 1976. The significance of bacterial grazing and mineral decomposition of particulate detritus, p. 285-299. *In* J. M. Anderson and A.

Macfadyen [eds.], The role of Terrestrial and Aquatic Organisms in Decomposition Processes. Blackwell Scientific Publications.

Fenchel, T., and B. B. Jorgensen. 1977. Detritus food chains of aquatic ecosystems: the role of bacteria. Advances in microbial ecology **1**.

Field, C. B., M. J. Behrenfeld, J. T. Randerson, and P. Falkowski. 1998. Primary production of the biosphere: Integrating terrestrial and oceanic components. Science **281:** 237-240.

Fuhrman, J. A., and M. Schwalbach. 2003. Viral Influence on Aquatic Bacterial Communities. Biol Bull **204:** 192-195.

Goldman, J. C., D. A. Caron, and M. A. Dennett. 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnol. Oceanogr. **32:** 1239-1252.

Hedges, J. I. and others 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Organic Geochemistry **31:** 945-958.

Heissenberger, A., and G. J. Herndl. 1994. Formation of high molecular weight material by free-living marine bacteria. Mar. Ecol. Prog. Ser. **111:** 129-135.

Hua, Y., W. Lu, M. S. Henry, R. H. Pierce, and R. B. Cole. 1996. On-line liquid chromatography-electrospray ionization mass spectrometry for determination of the brevetoxin profile in natural "red tide" algae blooms. J. Chromatogr. A **750:** 115-125.

Johannes, R. E. 1964. Influence of marine protozoa on nutrient regeneration. Univ.Georgia Marine Institute **85:** 434-442.

Jumars, P. A., D. L. Penry, J. A. Baross, M. J. Perry, and B. W. Frost. 1989. Closing the microbial loop: dissolved carbon pathway to heterotrophic bacteria from incomplete ingestion, digestion and sbsorption in animals. Deep-Sea Research **36:** 483-495.

Kim, S., A. J. Simpson, E. B. Kujawinski, M. A. Freitas, and P. G. Hatcher. 2003. High resolution electrospray ionization mass spectrometry and 2D solution NMR for

the analysis of DOM extracted by C-18 solid phase disk. Org. Geochem. **34:** 1325-1335.

Klausmeier, C. A., E. Litchman, T. Daufresne, and S. A. Levin. 2004. Optimal nitrogen-to-phosphorus stoichiometry of phytoplankton. Nature **429:** 171-174.

Koch, B. P., M. R. Witt, R. Engbrodt, T. Dittmar, and G. Kattner. 2005. Molecular formulae of marine and terrigenous dissolved organic matter detected by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Geochimica Et Cosmochimica Acta **69:** 3299-3308.

Koike, I., S. Hara, K. Terauchi, and K. Kogure. 1990. Role of sub-micrometre particles in the ocean. Nature **345:** 242-244.

Kramer, G. D., and G. J. Herndl. 2004. Photo- and bioreactivity of chromophoric dissolved organic matter produced by marine bacterioplankton. Aquat. Microb. Ecol. **36:** 239-246.

Kujawinski, E. B., R. Del Vecchio, N. V. Blough, G. C. Klein, and A. G. Marshall. 2004. Probing molecular-level transformations of dissolved organic matter: insights on photochemical degradation and protozoan modification of DOM from electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Mar. Chem. **92:** 23-37.

Kujawinski, E. B., M. A. Freitas, X. Zang, P. G. Hatcher, K. B. Green-Church, and R. B. Jones. 2002. The application of electrospray ionization mass spectrometry (ESI MS) to the structural characterization of natural organic matter. Org. Geochem. **33:** 171-180.

Leenheer, J. A., C. E. Rostad, P. M. Gates, E. T. Furlong, and I. Ferrer. 2001. Molecular resolution and fragmentation of fulvic acid by electrospray ionization/multistage tandem mass spectrometry. Anal. Chem. **73:** 1461-1471.

Lindeman, R. L. 1942. The Trophic-Dynamic Aspect of Ecology. Ecology **23:** 399-417.

Loo, J. A., and R. R. Ogorzalek Loo. 1997. Electrospray ionization mass spectrometry of peptides and proteins. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Lotka, A. J. 1956. Elements in Mathematical Biology. Dover Publications, Inc.

Nagata, T., and D. L. Kirchman. 1992a. Release of dissolved organic matter by heterotrophic protozoa: implications for microbial food webs. Arch. Hydrobiol. Beih. Ergebn. Limnol. **35:** 99-109.

---. 1992b. Release of macromolecular organic complexes by heterotrophic marine flagellates. Mar. Ecol. Prog. Ser. **83:** 233-240.

Ogawa, H., Y. Amagai, I. Koike, K. Kaiser, and R. Benner. 2001. Production of refractory dissolved organic matter by bacteria. Science **292:** 917-920.

Ogura, N. 1975. Further studies on decomposition of dissolved organic matter in coastal seawater. Marine Biology **31:** 101-111.

Pomeroy, L. R. 1974. The Ocean's Food Web, A Changing Paradigm. Bioscience **24:** 499-504.

Poon, G. K. 1997. Drug metabolism and pharmacokinetics, p. 499-525. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Redfield, A. C. 1958. The biological control of chemical factors in the environment. Am. Sci. **46:** 205-221.

Rendulic, S. and others 2004. A predator unmasked: Life cycle of Bdellovibrio bacteriovorus from a genomic perspective. Science **303:** 689-692.

Rhee, G.-Y. 1978. Effects of N:P atomic ratios and nitrate limitation on algal growth, cell composition and nitrate uptake. Limnol. Oceanogr. **23:** 10-25.

Richter, B. J. 1792. Ueber die neueren gegenstande in der chemie.

Saito, K., T. Toyo'oka, T. Fukushima, M. Kato, O. Shirota, and Y. Goda. 2004. Determination of psilocin in magic mushrooms and rat plasma by liquid chromatography with fluorimetry and electrospray ionization mass spectrometry. Anal. Chim. Acta **527:** 149-156.

Seitzinger, S. P. and others 2005. Molecular-level chemical characterization and bioavailability of dissolved organic matter in stream water using electrospray-ionization mass spectrometry. Limnol. Oceanogr. **50:** 1-12.

Seitzinger, S. P., R. M. Styles, R. Lauck, and M. A. Mazurek. 2003. Atmospheric pressure mass spectrometry: A new analytical chemical characterization method for dissolved organic matter in rainwater. Environ. Sci. Technol. **37:** 131-137.

Sherr, B. F., E. B. Sherr, and T. Berman. 1982. Decomposition of organic detritus: a selective role for microflagellate protozoa. Limnology and Oceanography **27:** 765-769.

Sherr, B. F., E. B. Sherr, and R. D. Fallon. 1987. Use of Monodispersed, Fluorescently Labeled Bacteria to Estimate In Situ Protozoan Bacterivory. Applied and Environmental Microbiology **53:** 958-965.

Sherr, E. B., and B. F. Sherr. 2002. Significance of predation by protists in aquatic microbial food webs. Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology **81:** 293-308.

Steele, J. H. 1974. The Structure of Marine Ecosystems. Harvard Univ. Press.

Stoderegger, K., and G. J. Herndl. 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol. Oceanogr. **43:** 877-884.

Strom, S. L., R. Benner, S. Ziegler, and M. J. Dagg. 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol. Oceanogr. **42:** 1364-1374.

Taylor, G. T., R. Iturriaga, and C. W. Sullivan. 1985. Interactions of bactivorous grazers and heterotrophic bacteria with dissolved organic matter. Mar. Ecol. Prog. Ser. **23:** 129-141.

Tezuka, Y. 1990. Bacterial regeneration of ammonium and phosphate as affeted by the carbon: nitrogen: phosphorus ratio of organic substrates. Microbial Ecology **19:** 227-238.

Tranvik, L. 1994. Colloidal and Dissolved Organic-Matter Excreted by a Mixotrophic Flagellate During Bacterivory and Autotrophy. Appl. Environ. Microbiol. **60:** 1884-1888.

CHAPTER 2

Bacterial Stoichiometry and the Growth Rate Hypothesis: Variability in C:N:P and Comparison to a Growth Rate Model

ABSTRACT

C:N:P stoichiometry of phytoplankton, zooplankton and larger consumers affects the structure and function of food webs, but the bacterial component, with its staggering functional diversity, is often neglected. We measured the carbon, nitrogen, and phosphorus content of pure cultures of four marine heterotrophic bacteria species during time-points along logarithmic growth and into the stationary phase under differing nutrient conditions. In all but one case, there were decreases in C:P, N:P and C:N ratios with increasing growth rate. These results demonstrate that some, but not all, strains of bacteria conform to the Growth Rate Hypothesis (GRH). It was also found that among these species, variation in growth rate corresponds with at least three fold variations in C:P and N:P. A model was developed based on the extensive literature on growth rates and chemical composition of bacteria. Overall, the stoichiometry of the bulk of growing cells reported in the literature and in this study correspond well to the model. While it has been hypothesized that the large variations in the C:P and N:P of bacteria reported in the literature is due to a breakdown of the GRH under P-replete conditions due to accumulation of inorganic polyphosphate, we found no evidence to support this claim. Variations in stoichiometry due to species and growth rate may have implications for community bacteria species composition via selective feeding.

INTRODUCTION

The elements carbon (C), nitrogen (N) and phosphorus (P) are present in all living matter, but their distribution is unevenly distributed throughout cellular components. Nucleic acids, for example, are rich in phosphorus while proteins are devoid of that element. Based on the direct relationship between phosphorus content and growth rate in animals, Elser et al. (1996) proposed the Growth Rate Hypothesis (GRH), which states that differences in N:P ratios of organisms are related to differences in their growth rate, due to differential allocations to P-rich ribosomal RNA necessary for protein (which are N-rich) synthesis (Elser et al. 1996). The GRH has received considerable attention because it predicts a tight coupling between ecosystem processes and evolutionary biology. Since the productivity of aquatic environments may be nitrogen limited, phosphorus limited, or both (Tyrrell 1999), the GRH provides a framework to study interactions among cell biology, nutrient cycling, and food webs (Sterner and Elser 2002).

Most tests of the GRH are based on experiments with eukaryotic, multicellular animals in which growth rate, P content, and N:P are measured (Elser et al. 2000; Main et al. 1997; Vrede et al. 1999). The applicability of the GRH to prokaryotes has been explicitly examined in only two studies of the relationship between growth rate and cell C:N:P stoichiometry, one with *Escherichia coli* (Makino et al. 2003) and one with a natural assemblage of lake bacteria (Makino and Cotner 2004). Although the results of both of these studies support the GRH, it is not known how generally applicable the GRH is to aquatic heterotrophic bacteria. The functional importance of bacteria in aquatic food webs and nutrient regeneration have been topics of intense study over the past few

decades (Azam et al. 1983; Pomeroy 1974). While only about 4500 prokaryotic species have been characterized, there may be millions (Torsvik et al. 2002) that represent a vast and relatively undefined diversity of organisms. Further information on both the species level and community level is needed.

Heterotrophic bacteria are obligate osmotrophs, requiring a suite of dissolved nutrients for growth. Bacteria have high contents of N-rich and P-rich macromolecules, in particular proteins and nucleic acids, respectively, and can rapidly adjust growth rates to respond to environmental conditions (Kirchman 2000). A priori, then, we might predict that heterotrophic bacteria should conform to the predictions of the GRH, and in particular expect that N:P and C:P ratios should be inversely related to growth rate. In this study we focused on how the elemental stoichiometry of four marine bacteria strains (from three different phylogenetic groups) varied with short-term (hours to days) fluctuations in growth rate. We also developed a model that includes the major components of the bacterial cell and used it to predict how growth rate-related shifts in the allocation of cell mass to different macromolecules influences cellular C:N:P stoichiometry. Experimental results were generally consistent with predictions from the GRH and the model. C:P and N:P ratios of bacteria decreased sharply, and non-linearly, with growth rate. The C:P ratio was more sensitive to variation in growth rate than the N:P ratio. The C:N ratio also decreased with growth rate, but only gradually.

MATERIALS AND METHODS

Elemental composition of bacteria during non-steady-state growth

We grew four strains of marine heterotrophic bacteria in batch cultures: BBFL7 (#AY028207), a Sphingobacteriales Flavobacteriales that was recently fully sequenced; TW7 (#AY028202) and BB2AT2 (#AY028206), both Gammaproteobacteria Alteromonadaceae; TW9 (#AY028204), a Gammaproteobacteria Marinomonas. These species were first isolated and characterized in Bidle and Azam (2001). Bacteria were grown on modified ZoBell liquid medium, consisting of aged 0.22-µm-filtered seawater with peptone (BioChemika #70178) and yeast extract (Sigma Y-4000). The C:N:P of the medium was 202:41:1 (molar basis). Each strain was grown separately under high and low nutrient concentration. The high nutrient medium contained 5 g peptone L^{-1} and 1 g yeast extract L^{-1} and the low nutrient medium contained 0.25 g peptone L^{-1} and 0.05 g yeast extract L^{-1}. Prior to autoclaving, media were filtered through Whatman GF/F filters to remove particulate matter.

Cultures were started by inoculating flasks with exponential-phase cells (centrifuged and resuspended in sterile seawater three times) at an initial concentration of 2000 cells mL^{-1}. Three replicate flasks were prepared for each combination of bacterial strain and nutrient concentration. Flasks were fit with sterile foam stoppers and placed on a rotary shaker at 100 rpm and incubated at 20°. Cultures were sampled repetitively over the next 2 to 100 hours, depending on growth rates of the different strains. Cells were collecting by filtering 3 to 20 mL of culture medium, depending on cell density, through precombusted (500°C for 4 h), 25-mm diameter Whatman GF/F filters. Each filter was rinsed with two, 2-ml volumes of Na_2SO_4 (0.17 mol L^{-1}) to remove residual media. Procedural filter blanks were prepared using sterile media to account for dissolved C, N, and P that remained adsorbed to the filters and these values were

subtracted from the bacterial samples. Filters were dried and analyzed for carbon and nitrogen (Verardo et al. 1990) and for phosphorus (Solórzano and Sharp 1980).

Average specific growth rate between sampling times, $\bar{\mu}$, was calculated as $\bar{\mu} = \frac{\ln C_2 - \ln C_1}{t_2 - t_1}$ where C_2 is the carbon biomass at time t_2, and C_1 is the carbon biomass at time t_1. Average particulate carbon, nitrogen, and phosphorus concentrations (molar basis) were computed for each corresponding time interval.

Model of growth rate and C:N:P stoichiometry

We based our model on the extensive literature on growth rates and chemical composition of bacteria (Bremer and Dennis 1996; Cox 2004; Herbert 1961; Neidhardt et al. 1990). Because protein (*Pr*) comprises the majority of bacterial cell biomass, the specific growth rate (μ) was set to the rate of protein synthesis,

$$\mu = \frac{1}{Pr}\frac{d\,Pr}{dt} \tag{1}$$

The rate of protein synthesis depends on the number of ribosomes (N_r), the fraction of ribosomes that are active (β_r), and the peptide chain elongation rate (c_p) (Bremer and Dennis 1996):

$$\mu = \frac{1}{Pr} N_r \beta_r c_p \tag{2}$$

In the microbial physiology literature, c_p typically has units, amino acid residues ribosome^{-1} s^{-1}. Because we wanted to model how the mass fractions of different cell components varied with bacterial specific growth rate (units h^{-1}), we converted the units of c_p to mass protein (mass RNA)$^{-1}$ h^{-1} by assuming an average molecular weight of 108 for amino acids, 4566 RNA nucleotide residues per ribosome, and an average molecular

weight of 324 for nucleotides (Bremer and Dennis 1996). N_r was set to the mass fraction of rRNA per cell, calculated as mass fraction of total RNA × 0.98 (representing the fraction of total RNA that is stable, i.e., rRNA and tRNA) × 0.86 (representing the fraction of stable RNA that is rRNA), and β_r was set to 0.8 (Bremer and Dennis 1996). Substituting these values into Eq. 1 gives

$$\mu = \frac{0.177R \times c_p}{Pr} \tag{3}$$

where R is the mass fraction of total RNA.

Using Eq. 2 to calculate growth rate is problematic because c_p is not a constant but is itself a function of growth rate (Bremer and Dennis 1996; Cox 2004). To avoid terms involving the specific growth rate on both sides of Eq. 2, we used data on the chemical composition and protein synthesis rate of *Mycobacterium bovis* (Cox 2004) and *Escherichia coli* (Bremer and Dennis 1996) to derive an empirical relationship between c_p and R. We used a Q_{10} of 2 to adjust the literature c_p values to 20ºC. As the (dry) mass fraction of RNA increases from 0.04 to 0.23, c_p increases nonlinearly from 0.6 to 6.5 aa residues ribosome^{-1} s^{-1} as $c_p = 3.6 \ln(R) + 12$ ($r^2 = 0.99$). Bremer and Dennis (1996) indicate that the maximum value for c_p (6.5) occurs when the ribosome is saturated with substrates. Thus,

$$\mu = \frac{0.177R \times \left(3.6\ln(R) + 12\right)}{Pr} \text{ for } 0.04 < R \leq 0.23 \tag{4a}$$

$$\mu = \frac{1.2R}{Pr} \text{ for } R > 0.23 \tag{4b}$$

Most existing models relate bacterial growth rates to absolute amounts of various macromolecules per cell (Cox 2004; Marr 1991). Because we wanted to see how growth

rate and bulk C:N:P stoichiometry were related, we used the approach of Vrede et al. (2004), which relates the allocation of C, N, and P to macromolecular pools and how the relative sizes of these pools change with growth rate. This approach was developed for metazoans, in particular crustacean zooplankton, and thus contains some macromolecular pools not present in bacteria (e.g., chitin) while omitting others (e.g., cell wall components). The values for some parameters are applicable to eukaryotes, not prokaryotes. We modified their model to make it applicable to bacteria (Table 1). Values for the distribution of cell dry mass among the major macromolecular pools were taken primarily from the extensive literature on *E. coli* (Bremer and Dennis 1996; Neidhardt et al. 1990). The fractional mass allocations to lipids (as phospholipids), DNA, glycogen, peptidoglycan, and lipopolysaccharide were kept constant (Table 1) and together accounted for 0.206 of total cell mass. R, the fractional allocation of cell mass to RNA, varies from as low as 0.015 in cells in stationary phase to 0.51 in cells in log phase (Herbert 1961). In our model, we varied R from 0.04 to 0.5. Protein made up the remainder of the cell mass and therefore varied inversely with RNA as

$$Pr = 0.794 - R \tag{5}$$

An inverse relationship between RNA and protein content of bacteria is well known (Herbert 1961; Marr 1991; Neidhardt et al. 1990).

The C, N, and P contents were calculated as

$$\%C = \left(Pr \times C_{Pr}\right) + \left(D \times C_N\right) + \left(R \times C_N\right) + \left(Pl \times C_{Pl}\right) + \left(LPS \times C_{LPS}\right) + \left(Pg \times C_{Pg}\right) + \left(Gl \times C_{Gl}\right) \tag{6}$$

$$\%N = \left(Pr \times N_{Pr}\right) + \left(D \times N_N\right) + \left(R \times N_N\right) + \left(Pl \times N_{Pl}\right) + \left(LPS \times N_{LPS}\right) + \left(Pg \times N_{Pg}\right) \tag{7}$$

$$\%P = \left(D \times P_N\right) + \left(R \times P_N\right) + \left(Pl \times P_{Pl}\right) + \left(LPS \times P_{LPS}\right) \tag{8}$$

Values for C, N and P as percent of dry mass were converted to moles of each element to calculate predicted C:P, N:P, and C:N ratios.

We performed a sensitivity analysis of the model by varying the mass fractions of phospholipid, DNA, glycogen, peptidoglycan, and lipopolysaccharide from 0 to 0.25. The RNA mass fraction was kept constant at 0.05 (results of the sensitivity analysis were qualitatively similar across the entire range of RNA mass fraction). The mass fraction of protein was set to [1 – Σ(mass fraction of all other macromolecules)]. The model was re-run and new values of C:P, N:P, and C:N were calculated as a function of the mass fractions of each macromolecule.

Reconstruction of Phylogeny

We compared the relatedness of the four strains of bacteria by reconstructing the 16S rDNA gene phylogeny using the Maximum Likelihood (ML) method (Felsenstein 1981), PhyML (software package that estimates maximum likelihood phylogenies from DNA and protein sequences) (Guindon et al. 2005), and the Bayesian approach. All 17 species (Fig.. 6) were retrieved from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) using NCBI's Basic Local Alignment Search Tool (BLAST) to select bacterial species related to strains used in this study. The analysis was performed to reconfirm the results of Bidle and Azam (2001) using different bacterial strains for comparison. In addition, an archaeon (*Phrococcus abyssi* ST549) was selected as an outgroup. For ML and PHYML analysis, the MODELTEST program (Posada and Crandall 1998) was used to compare the likelihood scores while applying different nucleotide substitution models. The Bayesian approach was implemented in MrBayes ver. 3.1.2. (Ronquist and Huelsenbeck 2003). To assess robustness of our

estimates, we used two substitution models: HKY + Γ_5 (Hasegawa et al. 1985; Yang 1994) and GTR + Γ_5 (Tavare 1986). Each Markov chain Monte Carlo (MCMC) sampler was run for five million steps; autocorrelation was decreased by sampling every 1,000 other steps; mixing was improved by using tampering with 3 heated chains and two independent chains were run in parallel. Four independent samplers were run to check convergence under each model of evolution and split frequencies were < .015. The first one million steps were discarded as burn-in.

RESULTS

Elemental composition of bacteria during non-steady-state growth

Cultures generally showed the growth curves expected under non-steady-state conditions, with clear exponential and stationary phases (Fig. 1-4). All strains reached a higher biomass, typically by a factor of 10 or more, when grown on the high nutrient medium.

The C:P ratios and N:P ratios of cells increased with the age of the culture in most cases (Fig. 1-4). In strain BB2AT2 (Fig. 2) grown on high nutrients, C:P was low (~20-30) during exponential growth, then increased linearly to a value of 67 after 46 h as cells entered stationary phase. Under low nutrient conditions, strain BB2AT2 again had a constant C:P of about 30 during exponential growth, but after increasing to a high value of 64 during early stationary phase the C:P then decreased gradually to a value of 44 after 46 h. Strain BBFL7 (Fig. 1) showed a qualitatively similar response under high nutrient conditions, but the C:P ratios were greater. In fact, C:P ratios of BBFL7 were the highest of all strains tested, peaking at 91. During exponential growth the C:P ratio fluctuated

around 50-60, then increased during stationary phase to 83-91. An opposite pattern occurred during growth on the low nutrient medium (Fig. 1), as C:P ratios were 76-90 during exponential and early stationary phases and then decreased to a minimum of 62 at 48 h. Strain TW7 (Fig. 3) grown on high nutrients showed a substantial increase in biomass, from 7.8×10^{-4} to 4.4×10^{-2} mmol C mL^{-1}, and a steady but slight increase in C:P ratio, from 27 to 40. A less dramatic growth response but more dramatic C:P response occurred when TW7 grew on low nutrients (Fig. 3). During the brief exponential phase and into early stationary phase, during which biomass only increased from 4.9×10^{-4} to 1.2×10^{-3} mmol C mL^{-1}, the C:P increased from 23 to 52. Strain TW9 (Fig. 4) grown on high nutrients showed little change in C:P ratio, from 21 at 5 h to 28 after 30 h. On the other hand, when grown on low nutrients the C:P ratio of TW9 increased from 34 during exponential growth to 64 in late stationary phase.

In all strains, patterns in N:P ratios during batch growth were essentially identical to those for C:P (Fig. 1-4), with the only difference that N:P ratios were numerically lower than C:P ratios.

C:N ratios were less variable than C:P or N:P, but there were discernable patterns over time (Fig. 1-4). In strain BB2AT2 on high nutrients C:N decreased from an initial value of 4.2 to 3.6 during exponential growth, then gradually increased to 4.2 during stationary phase (Fig.2). The pattern was similar for this strain grown on low nutrients. Strain BBFL7 (Fig.1) also showed a decrease in C:N during exponential growth followed by an increase in stationary phase, reaching final values around 5 after 100 h. C:N ratios for strain TW7 (Fig. 3) increased from about 3.7 during early growth to 4.2-4.4 after 70 h. In contrast, there were no obvious trends in C:N ratio over time for strain TW9 (Fig.

4). C:N was around 3.9 for TW9 under high nutrient conditions and 4.2 when grown on low nutrients.

When data for all strains were combined, there were significant negative correlations between the three ratios and growth rate (Fig. 5). Spearman rank correlation coefficients were –0.385 between μ and C:P ($p = 0.0095$), –0.378 between μ and N:P ($p = 0.011$) and –0.333 between μ and C:N ($p = 0.026$). There was more scatter in all three ratios at lower growth rates than at higher growth rates.

Model of growth rate and C:N:P stoichiometry

When the model was run with the standard values for mass fractions of phospholipid, DNA, glycogen, peptidoglycan, and lipopolysaccharide (Table 1), as the fraction of RNA increased from 0.04 to 0.5 the fraction of protein decreased from 0.75 to 0.29 and specific growth rate increased from 0.004 to 2 h^{-1}. Correspondingly, the C:P ratio decreased from 122 to 21.5, N:P decreased from 29 to 5.7, and C:N decreased from 4.2 to 3.8. The model values for C:P and N:P ratios fit the actual values for strain BBFL7 reasonably well, but were consistently greater than the values for the other strains (Fig. 5A and 5B). In contrast, model values for C:N ratio were consistently less than actual values for strain BBFL7 but close to the values for the other three strains (Fig. 5C).

The model output for C:N:P stoichiometry was more sensitive to variations in the mass fractions of some macromolecules away from their baseline values than others. The mass fractions of DNA, phospholipid, and lipopolysaccharide had the strongest impact on C:P ratio (Fig. 6A). The C:P decreased as the fraction of these macromolecules increased because they contain appreciable amounts of P (Table 1). Increased amounts of glycogen and peptidoglycan only slightly decreased the C:P ratio. Like protein, neither of these

macromolecules contains P; the slight decrease in C:P was due to the lower C content of glycogen and peptidoglycan, relative to protein (Table 1).

Similar patterns occurred in the model output for N:P ratio (Fig. 6B). Even though DNA and especially phospholipids and lipopolysaccharides have lower concentrations of N than protein (Table 1), increased amounts of DNA, phospholipids and lipopolysaccharides at the expense of protein led to sharp declines in N:P because protein does not contain P. Peptidoglycan content had little effect on N:P, because it has an N content similar to protein. Glycogen content had a moderate negative effect on N:P because it has no N or P, therefore increasing the amount of glycogen decreased the total N content of the cell.

Patterns in the C:N ratio were different than C:P and N:P (Fig. 6C). C:N increased as lipopolysaccharides and phospholipid increased because both these macromolecules have C content similar to protein, but much lower N content (Table 1). C:N also increased as glycogen increased because even though glycogen has a lower C content than protein, it has no N. In contrast, C:N decreased as DNA and peptidoglycan increased. Both of these macromolecules have N content similar to protein, but lower C content.

DISCUSSION

The Growth Rate Hypothesis has been the focus of studies mainly involving metazoans (Elser et al. 2000; Main et al. 1997) and has shown promise in connecting cellular biochemical allocation, animal growth, and C:N:P stoichiometry within a broad evolutionary context. Since body C:N:P stoichiometry greatly impacts processes such as

nutrient regeneration and food web dynamics, the GRH provides the potential to connect the cellular biology of growth to nutrient cycling. Yet, few studies have investigated the applicability of the GRH to bacteria. In the marine environment, heterotrophic bacteria take up 10-50% of the estimated 45 Pg C/year (Falkowski et al. 2003) of primary production and convert it to biomass. Additionally, as bacteria outnumber all other organisms and are the dominant organisms capable of taking up and regenerating dissolved N and P, they are key players in community trophic dynamics and play a major role in nutrient cycling. Therefore, testing the applicability of the GRH in the prokaryotic domain provides greater context and potential applicability to the theory.

In this study we found that variations in C:N:P stoichiometry of bacteria during growth in batch cultures generally conform to predictions of the Growth Rate Hypothesis. At high specific growth rates, the bacteria species that we tested and others reported in the literature have low C:P and N:P ratios. This dynamic is indicative of high phosphorus content of rapidly growing cells due to increased proportions of rRNA. Yet, this was not always the case for all bacteria species under all conditions. The GRH was not supported by the data from BBFL7 grown under the lower nutrient conditions. The C:P ratios were 76-90 during exponential and early stationary phases and then decreased to a minimum of 62 after reaching the stationary growth phase, directly opposite to what the GRH predicts. BBFL7 was the most distantly related strains we examined (Fig. 6) and as a member of the Cytophaga group has also been reported to degrade recalcitrant organic matter (Haukka et al. 2005). Scanning electron microscope images of BBFL7 revealed a dark, electron-dense material that took up a significant portion of the cell (Fig. 12), but it is not known if this morphological feature played a role in this anomaly without further

examining and analyzing specific macromolecular pools during growth. While TW7 and BB2AT2 are both members of Alteromonadaceae and showed similar patters in growth and responses to C:P and N:P, there is not yet enough data to conclude that phylogeny plays a major role in bacteria response to stoichiometry. This highlights the need to better characterize the macromolecular pools of a diverse range of bacteria under varying growth conditions. Currently, several thousand bacteria species have been described (Hawksworth and Colwell 1992), but it has been estimated that the number of bacterial species is in the millions (Colwell 1997). Since BBFL7 did not conform to the GRH predictions under lower nutrient conditions, it highlights the importance of examining a wide diversity of bacteria to understand how the genetic and physiological variability impacts bulk bacterial cellular stoichiometry.

Makino et al. (2003) tested the GRH using *E. coli* grown in continuous culture and found that C:P ratios (Fig. 8) and N:P ratios (Fig. 9) decrease as growth rate increases. We found similar trends in the marine strains we studied, although their C:P and N:P values were lower than those of *E. coli*. C:N ratios (Fig. 10) do not vary as much as C:P and N:P because protein (accounting for about 80% of cellular N) as a fraction of cell mass changes little with environmental conditions. While changes in nucleic acid content are likely to be small relative to protein, the GRH does predict a slight decrease of C:N with growth rate. In all four species we examined, we observed a decrease in the C:N ratio with increasing specific growth rate (Fig 4c).

Makino and Cotner (2004) also explicitly tested the GRH using a natural assemblage of freshwater bacteria grown in continuous culture. Again, C:P and N:P ratios of this bacterial community decreased with growth rates under most culture

conditions (Figs. 8 and 9) and were greater than our marine strains. C:N ratios of these freshwater bacteria were slightly higher than our marine strains and decreased slightly with growth rate, as predicted by the GRH (Fig. 10). Chrzanowski and Kyle (1996) found that *Pseudomonas fluorescens* in continuous culture at very low growth rates has high C:P (Fig. 8), N:P (Fig. 9), and C:N (Fig. 10) ratios that decrease slightly as growth rate increases over a narrow range 0.03-0.09 h^{-1}. Vadstein and Olsen (1989) report that a natural assemblage of freshwater bacteria, in continuous culture at very low growth rates, also had C:P and N:P ratios that decreased as growth rate increased from 0.014 to 0.068 h^{-1} (Figs. 9 and 9), but in this case the C:N ratio was constant at 6.9 (Fig. 10). Goldman and Dennett (2000) report that C:N ratios of natural assemblages of marine bacteria in continuous culture decreased slightly, but significantly, with growth rate when cultures were carbon limited and decreased more strongly when cultures were nitrogen limited (Fig. 10).

Overall, the stoichiometry of the bulk of growing cells reported in the literature and in this study correspond surprisingly well to the model. We found no evidence to support the idea that the coupling of stoichiometry to growth rate is only applicable under P limited conditions (Makino and Cotner 2004) and calculate that these cultures may not have been P limited (Fig. 11). It has been hypothesized that accumulation of inorganic polyphosphate by bacteria growing under P-replete (Elser et al. 2003; Makino and Cotner 2004; Vrede et al. 2002) causes the large variations seen in the C:P and N:P of bacteria. For instance, bacteria C:P ratio has been reported to vary 58-fold, from as low as 8 (Bratbak 1985) to as high as 464 (Tezuka 1990). One difficulty with this explanation is that polyphosphate content is usually low in rapidly growing cells and begins to

accumulate when growth rate slows under conditions of nutrient imbalance (Harold 1966). If polyphosphate storage represents a large pool of cellular P, then C:P and N:P should be directly related to growth rate, rather than inversely related as seen in most studies (Figs. 8 and 9). There is the underlying possibility that studies reporting large variations are laden with experimental artifacts resulting from the difficulty in separating living versus non-living particulate organic matter in samples from natural environments. It should also be stressed that although the model (Fig. 8) can be run with all macromolecules other than RNA and protein at 25% to account for studies that report bacteria C:P up to the 220, this may not reflect reality and may be due to contamination or errors in analysis.

The canonical Redfield ratios (Redfield 1958) for phytoplankton have been ubiquitously cited as C:N:P of 106:16:1 over the past half century. Fagerbakke et al (1996) introduced a "typical" bacteria molar C:N:P ratio of 50:10:1. In our model a C:P above 50 corresponds to bacteria that have specific growth rates less than 0.2 h^{-1}. As with the Redfield ratio for plankton, these numbers refer to the bulk average bacteria at low growth rates. As it is reported that the majority of bacteria in the ocean are inactive, a typical bacteria molar C:N:P ratio of 50:10:1 may correspond to bacteria that are senescent or growing slowly, rather than actively growing cells. This typical ratio also does not account for variability of C:N:P among bacterial species.

This study showed that bacteria stoichiometry can vary at least three-fold among species, depending on growth rate (Fig. 5). This supports the hypothesis that each bacterial strain regulates its elemental composition within a relatively narrow range of biomass C:P and N:P ratios and hence a shift in bacterial populations could cause

variations in bacterial stoichiometry (Makino et al. 2003). Therefore, depending on nutrient conditions, certain bacteria species may be growing faster than others and, hence, have lower C:P and N:P ratios. More than most organisms, bacteria grow at vastly different rates, depending on their nutrition (Neidhardt et al. 1990). Variations in stoichiometry due to species-specific differences in physiology and growth rate may influence their "food value" to consumers and therefore could have implications for predicting bacterial species composition via selective feeding.

Table 2.1. Symbols and parameter values used in the growth rate-stoichiometry model.

Symbol	Parameter (units)	Value
μ	Specific growth rate (h^{-1})	Output from model
β_r	Fraction of ribosomes that are active	0.8[¶]
Pl	Fractional allocation of cell dry mass to phospholipid	0.091[#]
D	Fractional allocation of cell dry mass to DNA	0.031[#]
Gl	Fractional allocation of cell dry mass to glycogen and other storage polysaccharides	0.025[#]
Pg	Fractional allocation of cell dry mass to peptidoglycan	0.025[#]
LPS	Fractional allocation of cell dry mass to lipopolysaccharide	0.034[#]
R	Fractional allocation of cell dry mass to RNA	Variable, 0.05-0.3[#]
Pr	Fractional allocation of cell dry mass to protein	By difference
C_{Pl}	C content of phospholipid (%)	65[§]
C_N	C content of nucleic acids (%)	32.7[§]
C_{Gl}	C content of glycogen (%)	37[§]
C_{LPS}	C content of lipopolysaccharide (%)	58[¥]
C_{Pg}	C content of peptidoglycan (%)	32.3[§]
C_{Pr}	C content of protein (%)	53[§]
N_{Pl}	N content of phospholipid (%)	1.6[§]
N_N	N content of nucleic acids (%)	15.4[§]
N_{LPS}	N content of lipopolysaccharide (%)	1.2[¥]
N_{Pg}	N content of peptidoglycan (%)	12.7[§]
N_{Pr}	N content of protein (%)	17[§]
P_{Pl}	P content of phospholipid (%)	4.2[§]
P_N	P content of nucleic acids (%)	8.7[§]
P_{LPS}	P content of lipopolysaccharide (%)	2.8[¥]

[¶] (Bremer and Dennis 1996)
[#] (Neidhardt et al. 1990)
[§] (Sterner and Elser 2002)
[¥] (Hayter et al. 1987)

Figure 2.1. BBFL7 particulate organic carbon biomass, C:P molar ratio, N:P molar ratio, C:N molar ratio and percent substrate in cell biomass over time in bacterial cultures grown with high and low initial nutrient concentrations. Note logarithmic scale for POC and breaks in abscissae.

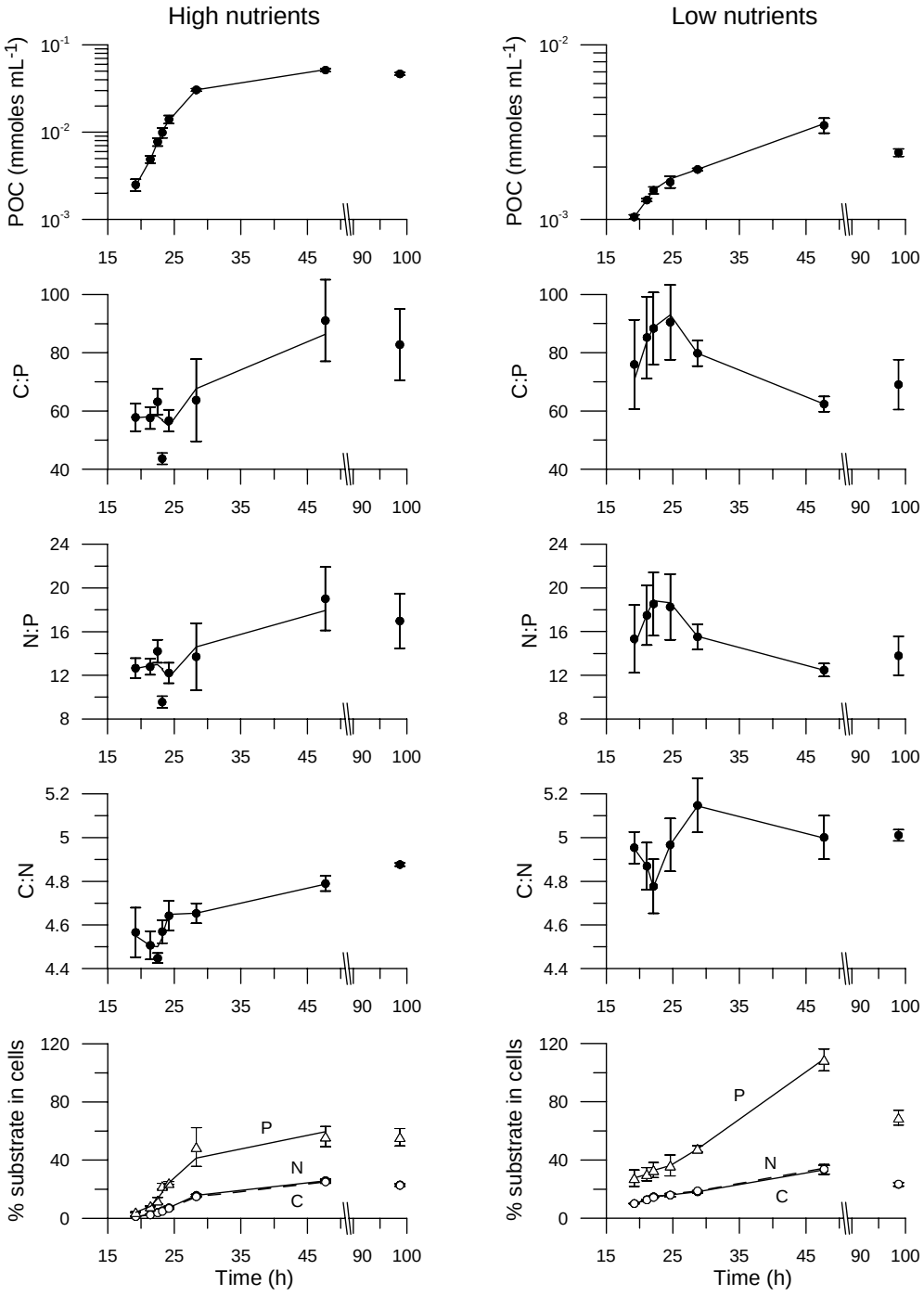


Figure 2.2 BB2AT2 particulate organic carbon biomass, C:P molar ratio, N:P molar ratio, C:N molar ratio and percent substrate in cell biomass over time in bacterial cultures grown with high and low initial nutrient concentrations. Note logarithmic scale for POC and breaks in abscissae.

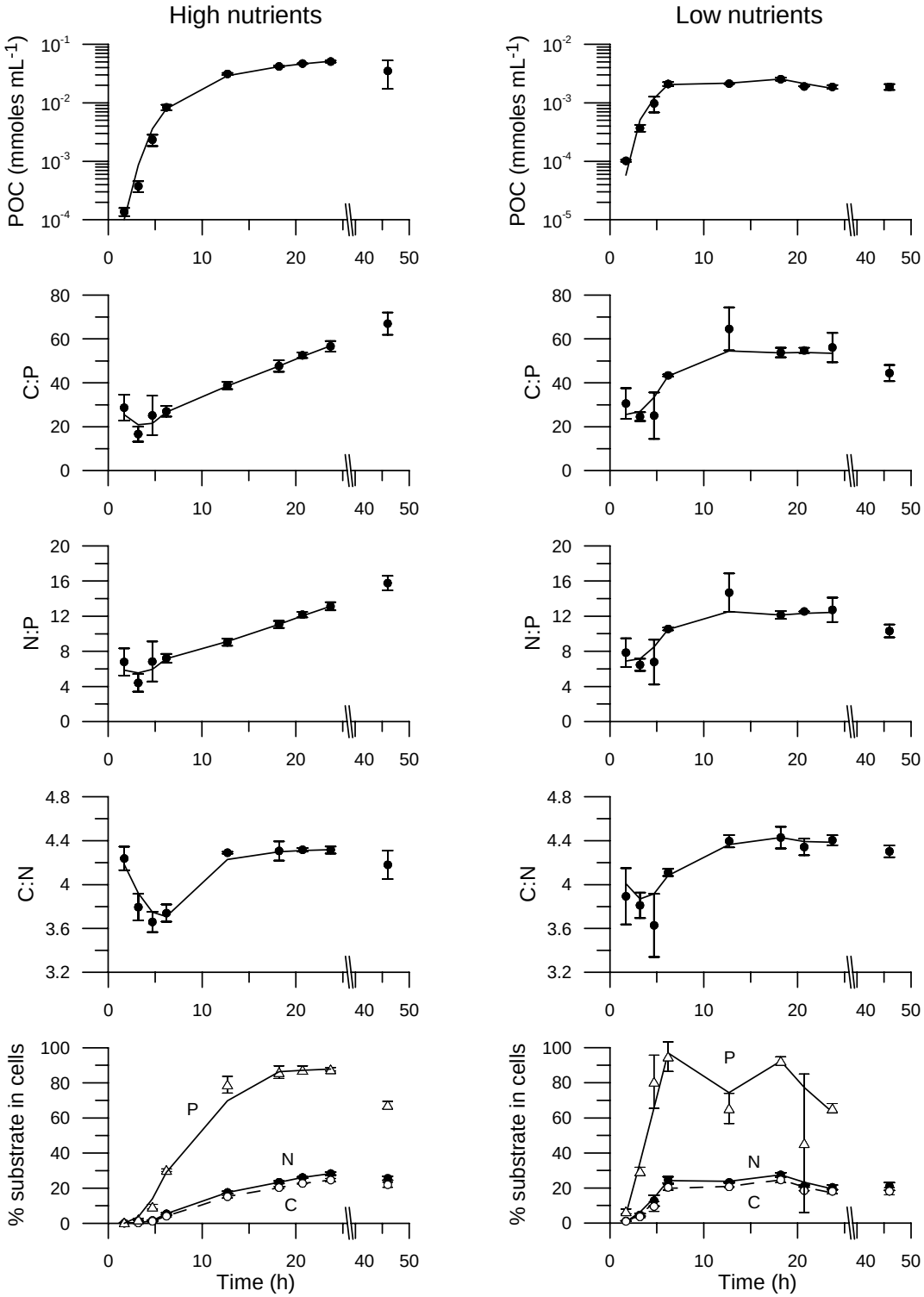


Figure 2.3. TW7 particulate organic carbon biomass, C:P molar ratio, N:P molar ratio, C:N molar ratio and percent substrate in cell biomass over time in bacterial cultures grown with high and low initial nutrient concentrations.

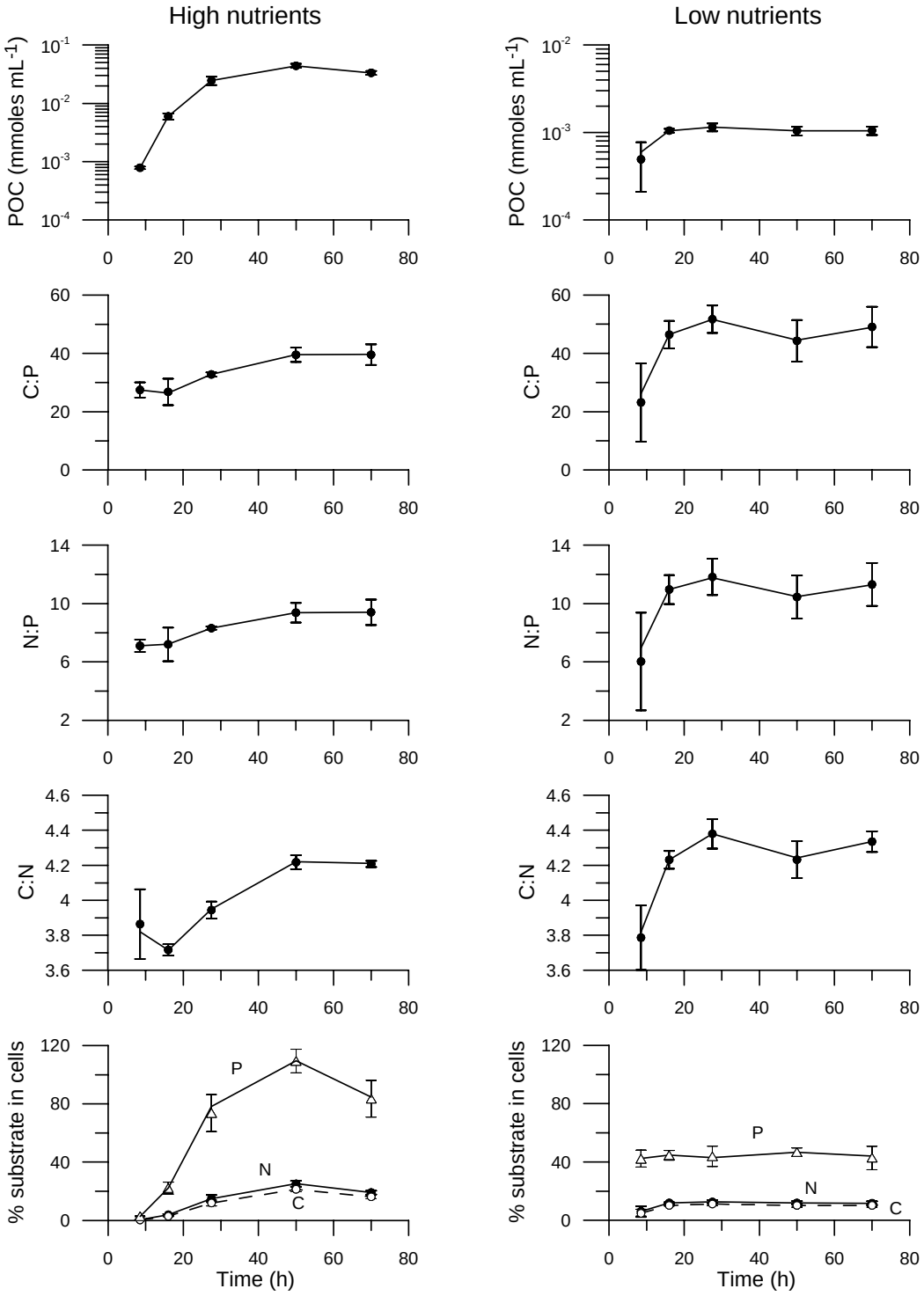


Figure 2.4. TW9 particulate organic carbon biomass, C:P molar ratio, N:P molar ratio, C:N molar ratio and percent substrate in cell biomass over time in bacterial cultures grown with high and low initial nutrient concentrations.

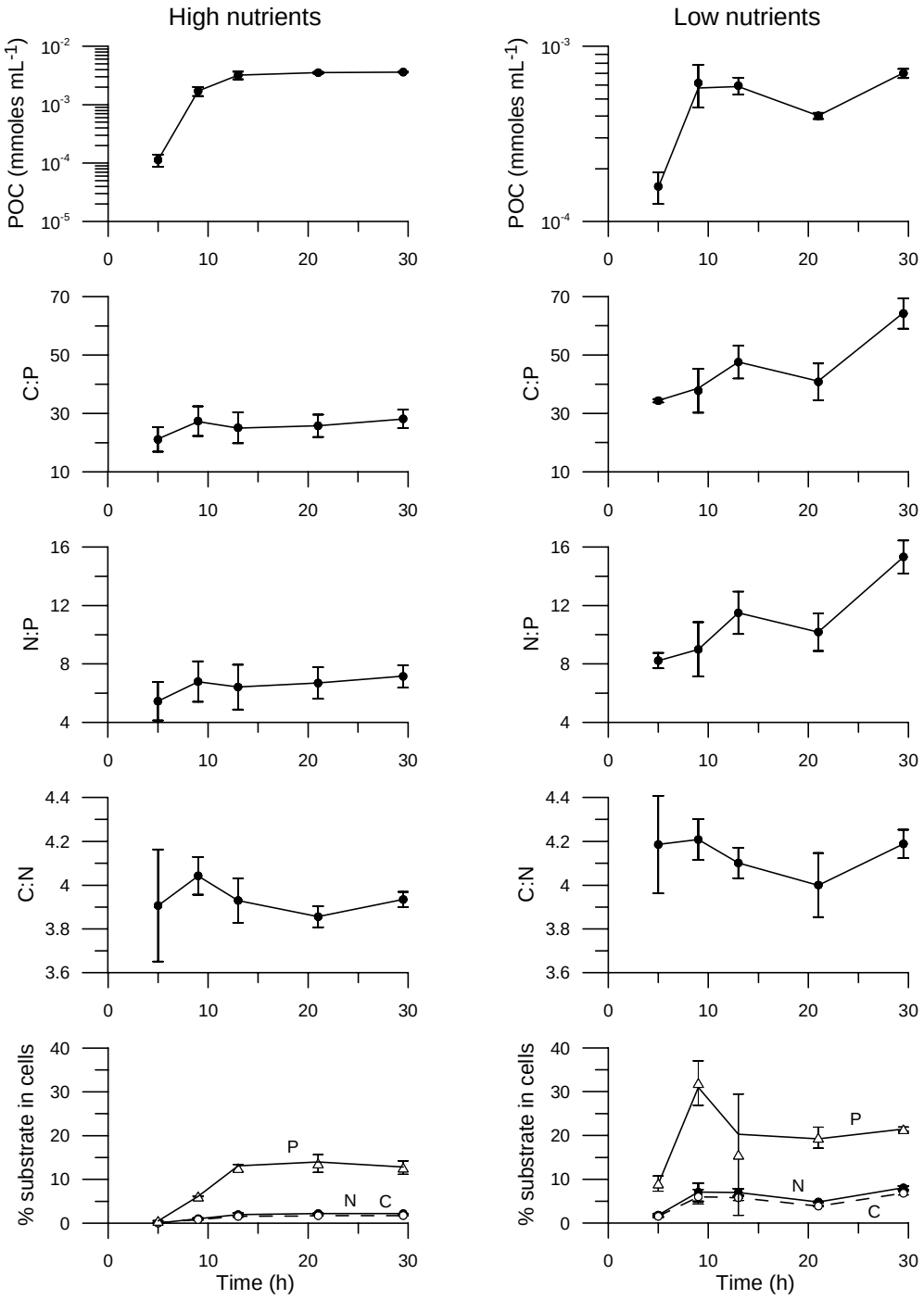


Figure 2.5. Scatterplots of C:P, N:P, and C:N ratios and specific growth rates of all strains. Solid symbols are for bacteria grown on high nutrient media and open symbols are for bacteria grown on low nutrient media. ■ = BB2AT2, ★ = BBFL7, ▲ = TW7, ● = TW9.

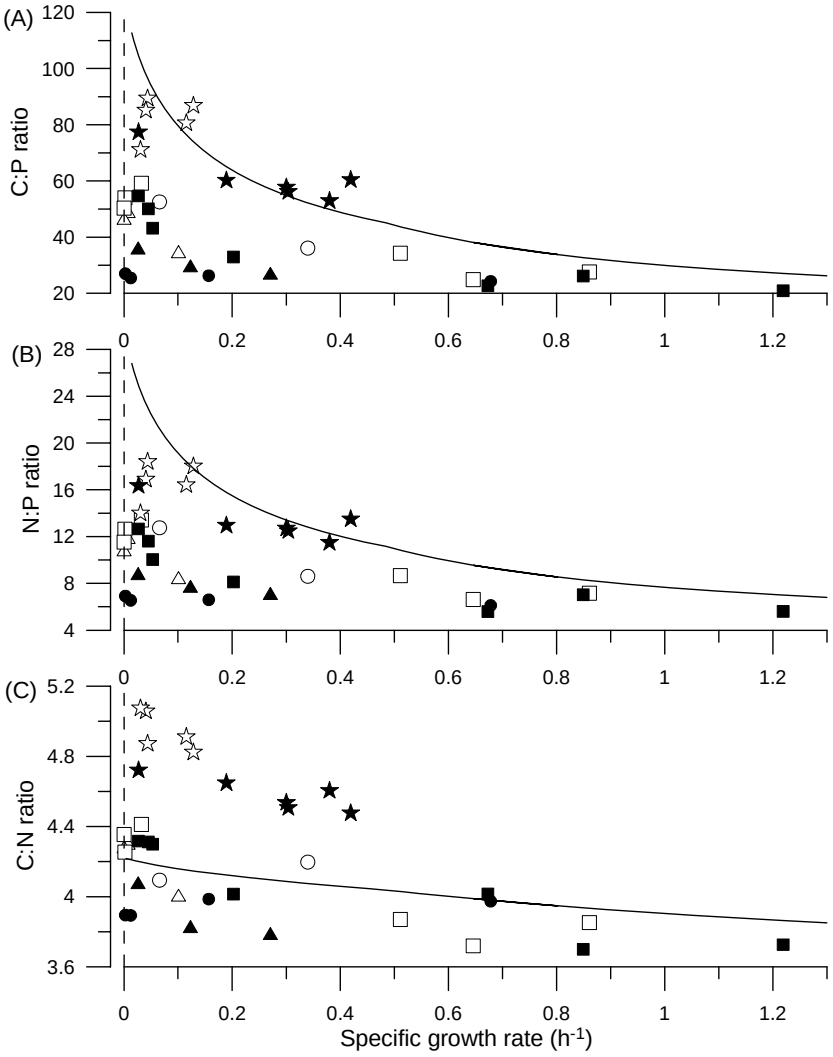


Figure 2.6. Model results of varying the mass fraction of different macromolecules on cell stoichiometry. The solid part of the line for each macromolecule represents the range of 25% to 300% of its baseline value in Table 1.

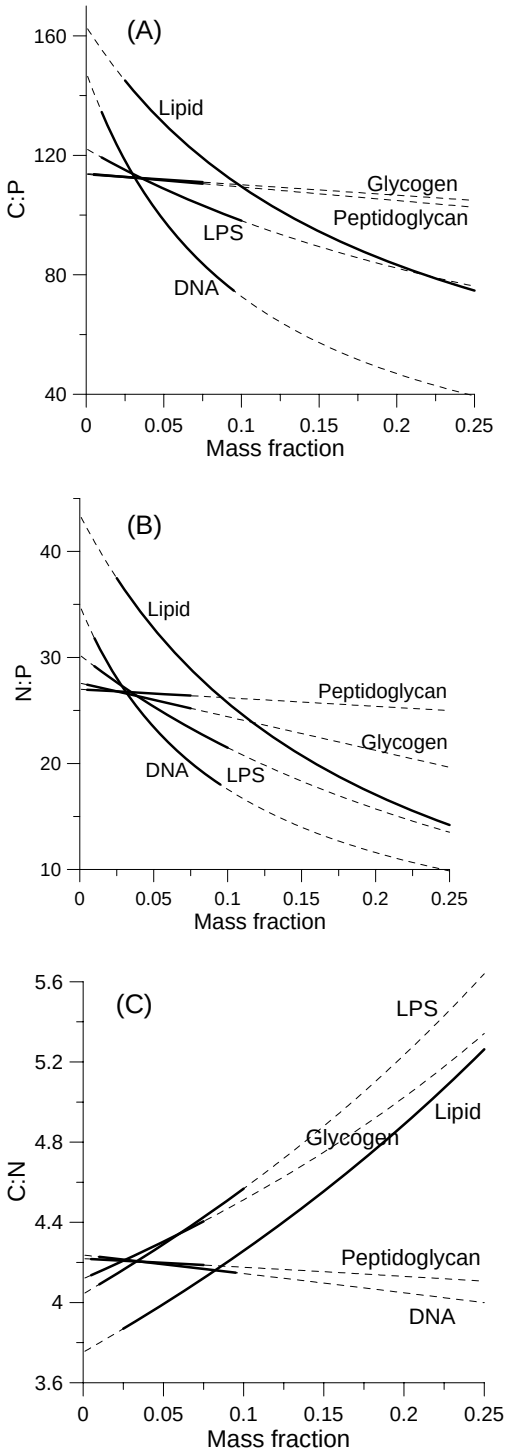


Figure 2.7. Phylogenetic tree reconstructed using Bayesian analyses (consensus tree based on 50% majority rule). Posterior probabilities were indicated above relevant nodes. An archaeon (*Pyrococcus abyssi* ST549) was used as an outgroup.

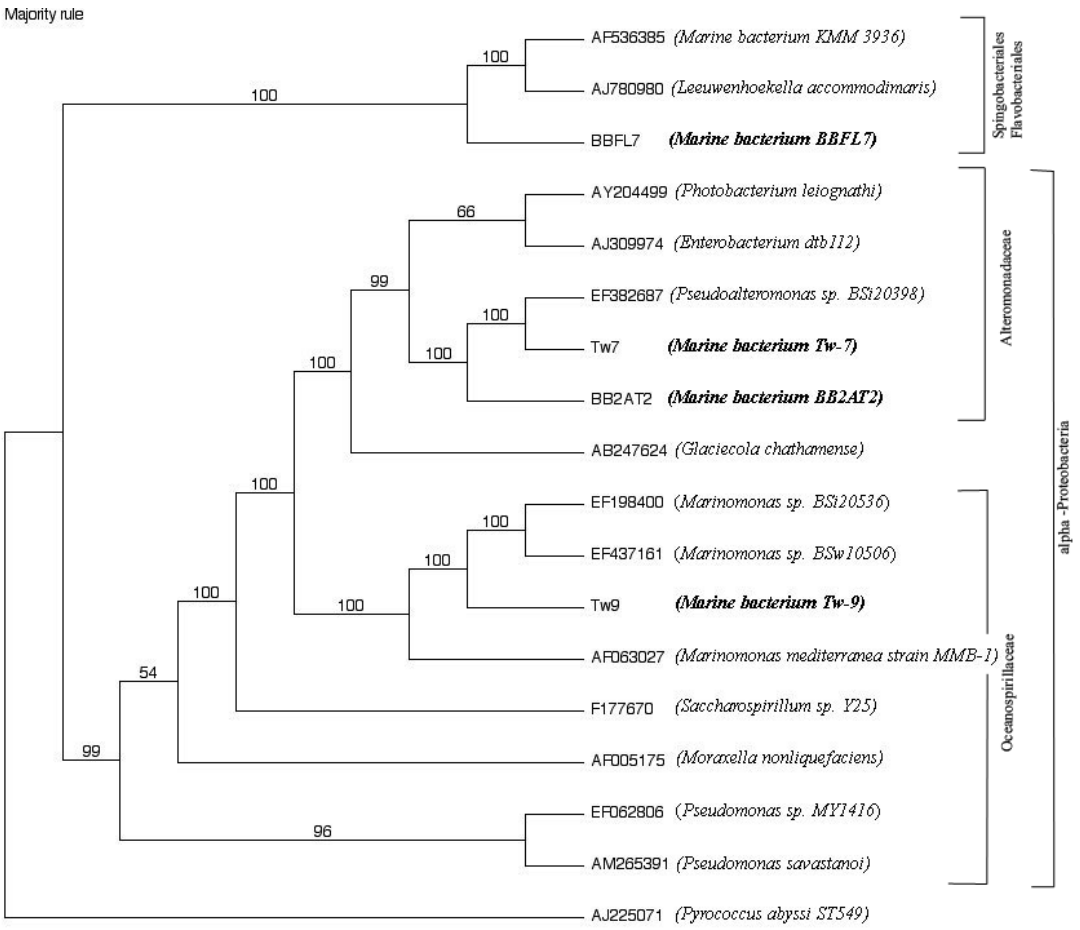


Figure 2.8. Data and model predictions of C:P ratio in bacteria at different growth rates. 'Baseline' curve is model output with all macromolecules other than RNA and protein at initial values given in Table 1, '0.25×' curve is model output with all macromolecules other than RNA and protein at 25% of their initial values, '2×' curve is model output with all macromolecules other than RNA and protein at 200% of their initial values, and '3×' curve is model output with all macromolecules other than RNA and protein at 300% of their initial values.

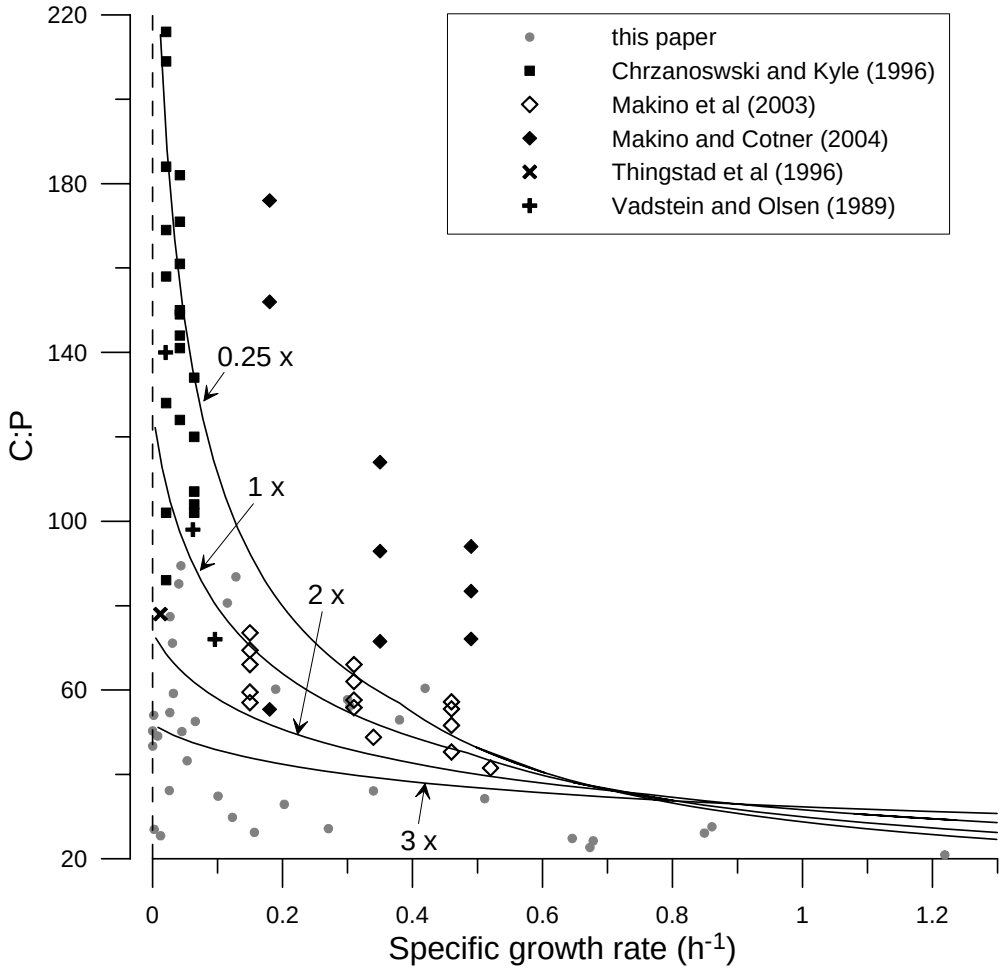


Figure 2.9. Data and model predictions of N:P ratio in bacteria at different growth rates. 'Baseline' curve is model output with all macromolecules other than RNA and protein at initial values given in Table 1, '0.25×' curve is model output with all macromolecules other than RNA and protein at 25% of their initial values, '2×' curve is model output with all macromolecules other than RNA and protein at 200% of their initial values, and '3×' curve is model output with all macromolecules other than RNA and protein at 300% of their initial values.

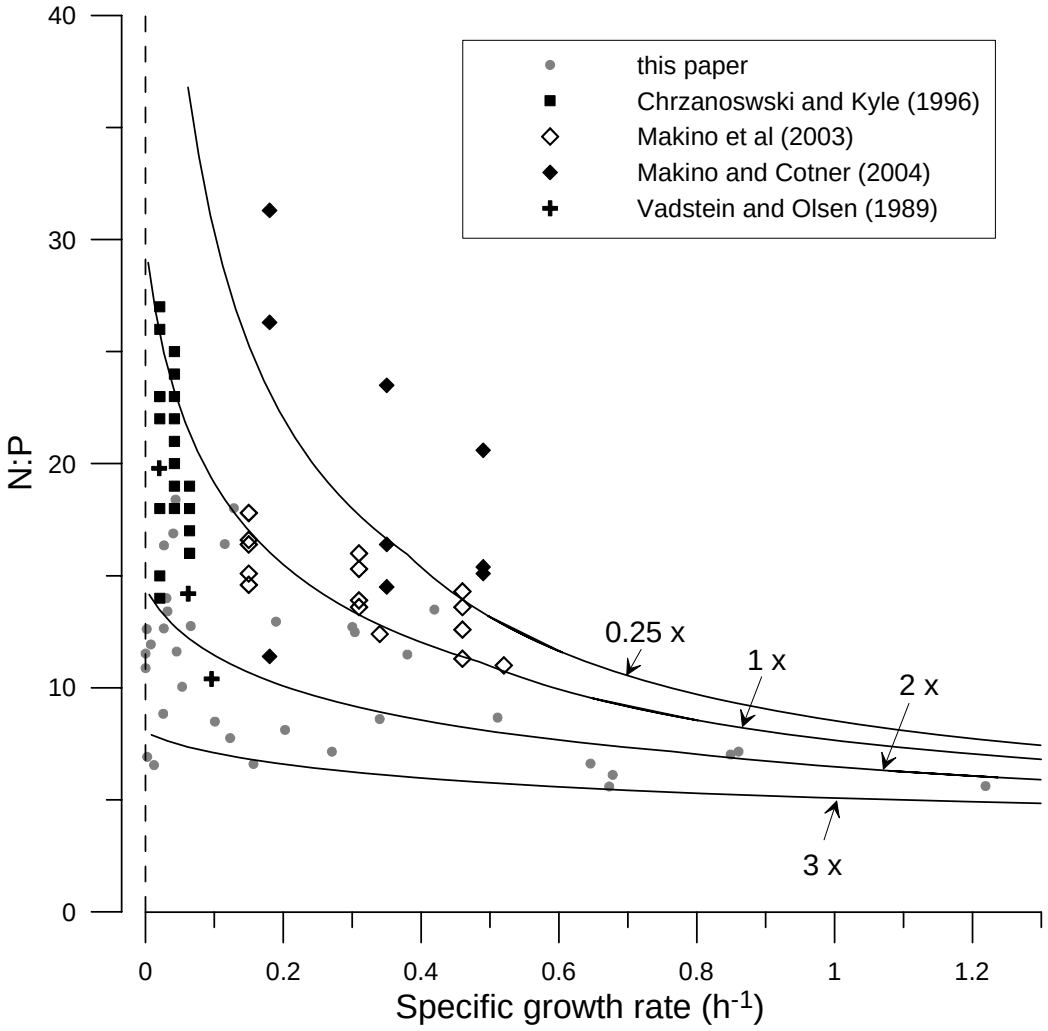


Figure 2.10. Data and model predictions of C:N ratio in bacteria at different growth rates. 'Baseline' curve is model output with all macromolecules other than RNA and protein at initial values given in Table 1, '0.25×' curve is model output with all macromolecules other than RNA and protein at 25% of their initial values, '2×' curve is model output with all macromolecules other than RNA and protein at 200% of their initial values, and '3×' curve is model output with all macromolecules other than RNA and protein at 300% of their initial values.

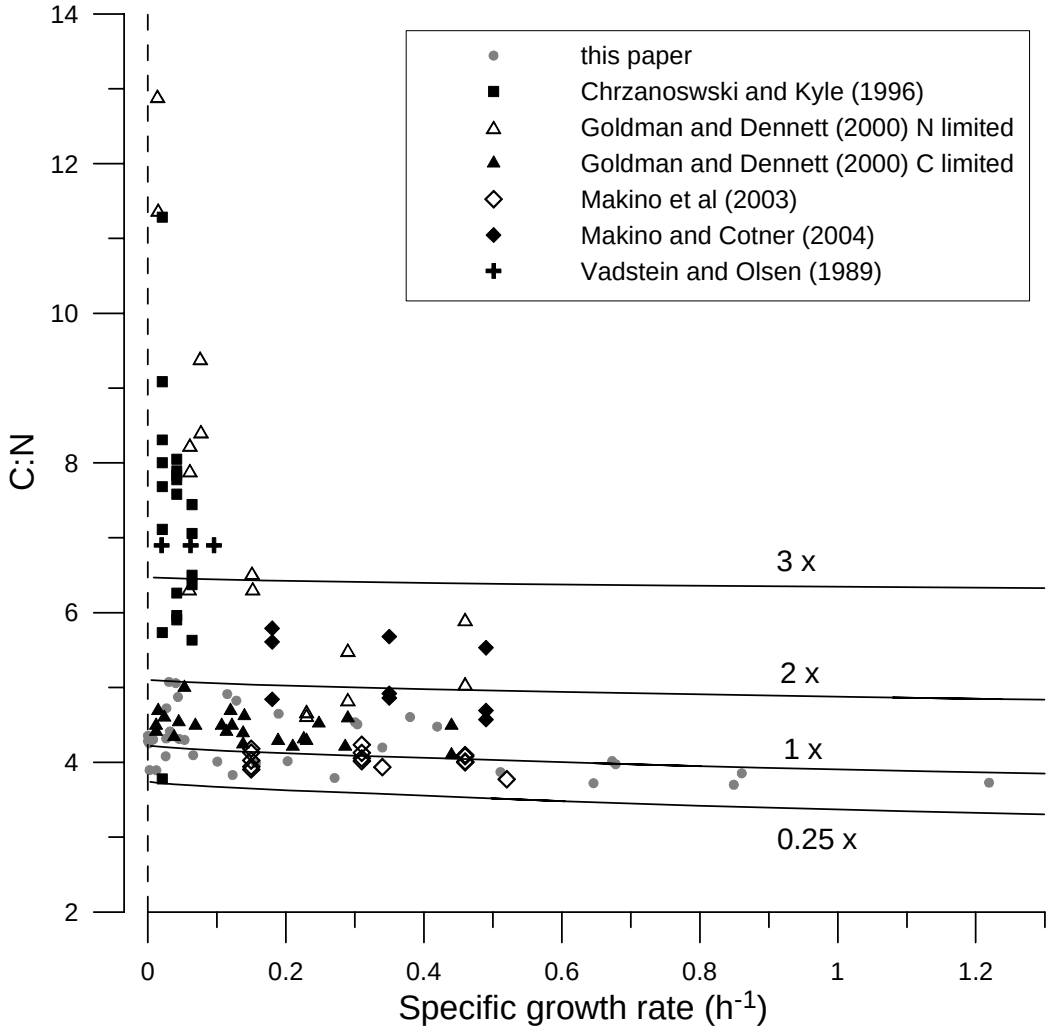


Figure 2.11. The calculated percentage of phosphorus remaining in culture experiments. The size of the circle refers to the percentage P remaining. Open circles are data from this experiment; light shaded circles (Makino et al. 2004) and solid circles (Makino and Cotner 2004).

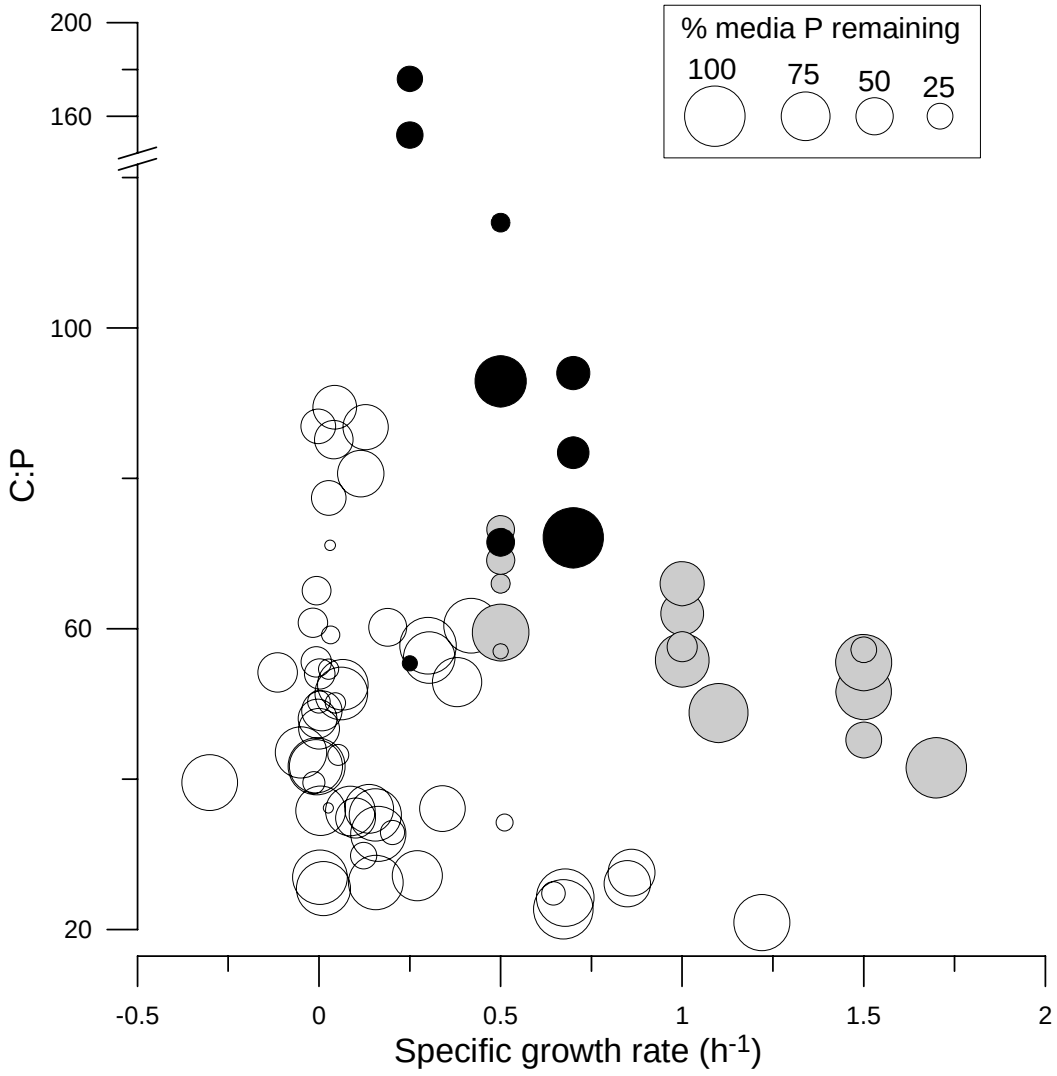
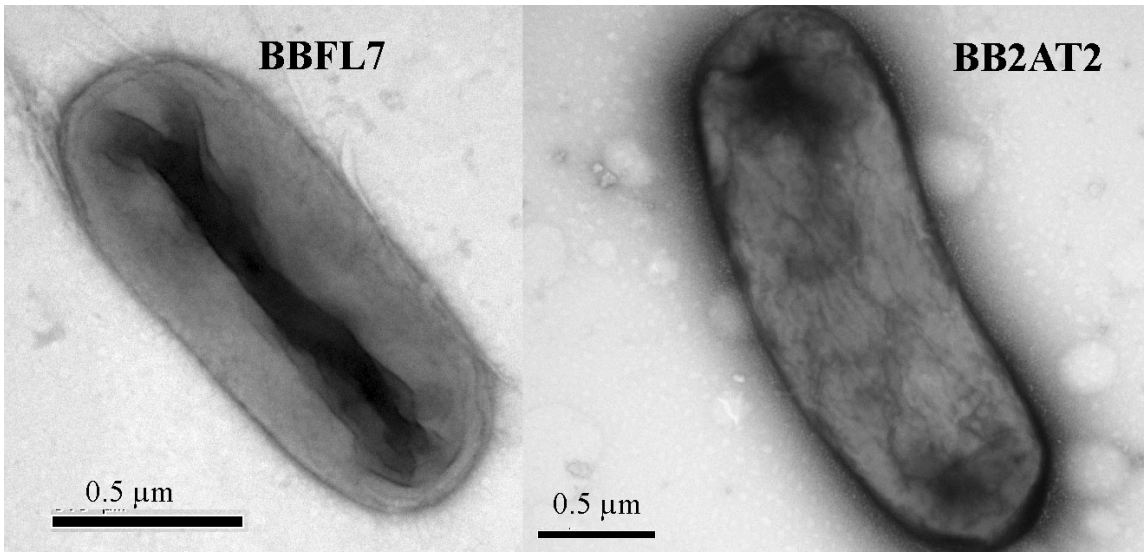


Figure 2.12. Scanning Electron Microscope photograph of BBFL7 and BB2AT2.



ACKNOWLEDGEMENTS

This work was supported by Biocomplexity grant OCE0120453 from the National Science Foundation.

We thank Kay Bidle for providing the bacteria strains used in this study. Hui Liu assisted in the creation of the phylogenic tree. Gary Taghon created the model and ran sensitivity analysis.

REFERENCES

Azam, F., T. Fenchel, J. C. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. Marine Ecological Progress Series **10:** 257-263.

Bidle, K. D., and F. Azam. 2001. Bacterial control of silicon regeneration from diatom detritus: Significance of bacterial ectohydrolases and species identity. Limnology and Oceanography **46:** 1606-1623.

Bratbak, G. 1985. Bacterial Biovolume and Biomass Estimations. Appl. Environ. Microbiol. **49:** 1488–1493.

Bremer, H., and P. P. Dennis. 1996. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1553-1569. *In* F. C. Neidhardt et al. [eds.], Escherichia coli and Salmonella: Cellular and Molecular Biology. ASM Press.

Chrzanowski, T. H., and M. Kyle. 1996. Ratios of carbon, nitrogen and phosphorus in Pseudomonas fluorescens as a model for bacterial element ratios and nutrient regeneration. Aquat Microb Ecol **10:** 115-122.

Colwell, R. R. 1997. Microbial biodiversity and biotechnology. *In* M. L. Reaka-Kudla, D. E. Wilson and E. O. Wilson [eds.], Biodiversity II: Understanding and protecting our biological resources. Joseph Henry Press.

Cox, R. A. 2004. Quantitative relationships for specific growth rates and macromolecular compositions of Mycobacterium tuberculosis, Streptomyces coelicolor A3(2) and Escherichia coli B/r: an integrative theoretical approach. Microbiol-Sgm **150:** 1413-1426.

Elser, J. J. and others 2003. Growth rate-stoichiometry couplings in diverse biota. Ecol Lett **6:** 936-943.

Elser, J. J., D. R. Dobberfuhl, N. A. Mackay, and J. H. Schampel. 1996. Organism size, life history, and N:P stoichiometry. Bioscience **46:** 674-684.

Elser, J. J., W. J. O'brien, D. R. Dobberfuhl, and T. E. Dowling. 2000. The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. J Evolution Biol **13:** 845-853.

Fagerbakke, K. M., M. Heldal, and S. Norland. 1996. Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. Aquat Microb Ecol **10:** 15-27.

Falkowski, P. G., E. A. Laws, R. T. Barber, and J. W. Murray. 2003. Phytoplankton and their role in Primary, New, and Export Production., p. 99-121. *In* M. Fasham [ed.], Ocean Biogeochemistry: The Role of the Ocean Carbon Cycle in Global Change. Global Change – The IGBP Series.

Felsenstein, J. 1981. Evolutionary trees from gene-frequencies and quantitative characters finding maximum likeihood estimates. Evolution **35:** 1229-1242.

Goldman, J. C., and M. R. Dennett. 2000. Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation. Limnology and Oceanography **45:** 789-800.

Guindon, S., F. Lethiec, P. Duroux, and O. Gascuel. 2005. PHYML Online--a web server for fast maximum likelihood-based phylogenetic inference. Nucl. Acids Res. **33:** W557-559.

Harold, F. M. 1966. Inorganic polyphosphates in biology: structure, metabolism, and function. Bacteriological Reviews **30:** 772-794.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution **22:** 160–174.

Haukka, K., E. Heikkinen, T. Kairesalo, H. Karjalainen, and K. Sivonen. 2005. Effect of humic material on the bacterioplankton community composition in boreal lakes and mesocosms. Environmental Microbiology **7:** 620-630.

Hawksworth, D. L., and R. R. Colwell. 1992. Biodiversity amongst microorganisms and its relevance. Biodiv. Conserv. **1:** 221-345.

Hayter, J. B., M. Rivera, and E. J. Mcgroarty. 1987. Neutron scattering analysis of bacterial lipopolysaccharide phase structure. Changes at high pH. Journal of Biological Chemistry **262:** 5100-5105.

Herbert, D. 1961. The chemical composition of micro-organisms as a function of their environment, p. 391-416. *In* G. G. Meynell and H. Goodet [eds.], Symposium of the Society for General Microbiology. Cambridge University Press.

Kirchman, D. L. 2000. Uptake and Regeneration of Inorganic Nutrients by Marine Heterotrophic Bacteria, p. 261-288. *In* D. L. Kirchman [ed.], Microbial Ecology of the Oceans. Wiley-Liss.

Main, T. M., D. R. Dobberfuhl, and J. J. Elser. 1997. N : P stoichiometry and ontogeny of crustacean zooplankton: A test of the growth rate hypothesis. Limnol. Oceanogr. **42:** 1474-1478.

Makino, W., and J. B. Cotner. 2004. Elemental stoichiometry of a heterotrophic bacterial community in a freshwater lake: implications for growth- and resource-dependent variations. Aquat Microb Ecol **34:** 33-41.

Makino, W., J. B. Cotner, R. W. Sterner, and J. J. Elser. 2003. Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C : N : P stoichiometry. Funct Ecol **17:** 121-130.

Marr, A. G. 1991. Growth rate of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **55:** 316-333.

Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the bacterial cell. A molecular approach. Sinauer Associates.

Pomeroy, L. R. 1974. The Ocean's Food Web, A Changing Paradigm. Bioscience **24:** 499-504.

Posada, D., and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:** 817-818.

Redfield, A. C. 1958. The biological control of chemical factors in the environment. Am. Sci. **46:** 205-221.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572-1574.

Solórzano, L., and J. H. Sharp. 1980. Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol. Oceanogr. **25:** 754-758.

Sterner, R. W., and J. J. Elser. 2002. Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere.

Tavare, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the life Sciences **17:** 57-86.

Tezuka, Y. 1990. Bacterial regeneration of ammonium and phosphate as affected by the carbon: nitrogen: phosphorus ratio of organic substrates. Microbial Ecol **19:** 227-238.

Torsvik, V., L. Ovreas, and T. F. Thingstad. 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science **296:** 1064-1066.

Tyrrell, T. 1999. The relative influences of nitrogen and phosphorus on oceanic primary production. Nature **400:** 525-531.

Vadstein, O., and Y. Olsen. 1989. Chemical compositon and phosphate uptake kinetics of limnetic bacterial communities cultured in chemostats under phosphorus limitation. Limnol. Oceanogr. **34:** 939-946.

Verardo, D. J., P. N. Froelich, and A. Mcintyre. 1990. Determination of organic carbon and nitrogen in marine sediments using the Carlo Erba NA-1500 Analyzer. Deep-Sea Research **37:** 157-165.

Vrede, K., M. Heldal, S. Norland, and G. Bratbak. 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. Appl. Environ. Microbiol. **68:** 2965-2971.

Vrede, T., T. Andersen, and D. O. Hessen. 1999. Phosphorus distribution in three crustacean zooplankton species. Limnology and Oceanography **44:** 225-229.

Vrede, T., D. R. Dobberfuhl, S. A. L. M. Kooijman, and J. J. Elser. 2004. Fundamental connections among organism C : N : P stoichiometry, macromolecular composition, and growth. Ecology **85:** 1217-1229.

Yang, Z. 1994. Estimating the pattern of nucleotide substitution. Journal of Molecular Evolution **39:** 105-111.

CHAPTER 3

Selective Grazing by Ciliates Based on the Elemental Stoichiometry of Bacteria Labeled with Fluorescent Proteins

ABSTRACT

Marine protists are known to selectively graze live, versus dead, bacteria and can differentiate prey based on size, but there is less known about how chemical cues or cellular composition of prey affect selective feeding by protozoa. In this study we found that two species of protozoa (*Euplotes vannus* and *Cyclidium* sp.) selectively consumed *E. coli* which had different elemental stoichiometry and growth stage. Bacterial prey cells were tagged, in vivo, with Green Fluorescent Protein (GFP) and Red Fluorescent Protein (RFP) and harvested at differing points in their growth in batch cultures. In cells from exponential growth and after two days at stationary growth, C:P ratio increased from 39 to 106 and N:P increased from 10 to 26, respectively, while C:N was invariant at 4.0. When offered a mixture of equal concentrations of both types of bacteria for 135 minutes, *Cyclidium* grazed the high C:P/high N:P cells down to 22% of their initial abundance, while *Euplotes* reduced these cells to 33% of their initial abundance. Neither ciliate decreased the abundance of the low C:P/low N:P cells, relative to control treatments without predators present. These results are surprising, since predator-prey dynamics among eukaryotic organisms generally show the opposite trend, with predators choosing prey with lower C:P or C:N ratios. But, protozoa have higher N:P and C:P ratios than their prokaryotic prey and by selectively consuming bacteria more similar to their own cellular stoichiometry, protozoa may possibly spend less energy metabolizing, converting, and excreting excess nutrients by ingesting lower N:P and C:P bacterial cells. This finding may help to better understand microbial community dynamics and nutrient regeneration/cycling in aquatic and marine environments.

INTRODUCTION

The ecological role of heterotrophic protozoa has attracted increased attention in recent decades due to their effectiveness in consuming bacteria in aquatic environments (Azam et al. 1983; Fenchel 1980a; Porter et al. 1985; Sherr et al. 1987; Sherr et al. 1989). Predation by bactivorous protists is now identified as the primary source of bacterial mortality (Cole 1999; Pace 1986). The most numerous and important predators are protozoa, especially flagellates and ciliates (Fenchel 1987). In the ocean, bacterial abundance is remarkably constant at around 10^6 ml^{-1} (Hobbie et al. 1977) and accounts for most oceanic biomass and metabolism (Azam 1998). This bacterial constancy is attributed to a threshold concentration, below which protozoa cannot obtain sufficient energy (Fenchel 1984). Besides controlling bacterial abundance (Berninger et al. 1991), protozoan grazing also influences phenotypic (Hahn et al. 1999; Matz et al. 2002a) and species composition (Simek et al. 1997) of bacterial communities.

Protozoan grazing is most often quantified by measuring changes in bacteria abundance over time (Enzinger and Cooper 1976; Mccambridge and Mcmeekin 1979; Mccambridge and Mcmeekin 1980) or by measuring the grazing rates on bacterial analogs, mainly fluorescent microspheres or fluorescently labeled bacteria (FLB) (Borsheim 1984; Mcmanus and Fuhrman 1988; Sherr et al. 1987). These bacteria analogs have limitations. FLBs are often non-viable bacteria that have been chemically modified by the staining process in ways that have unknown effects on their detection by protozoa and their palatability as prey. Microspheres are usually composed of inorganic silica beads with no food value to consumers. Predators can discriminate between motile versus non-motile (Gonzalez et al. 1993) and live versus dead prey (Landry 1991).

Growth rates for both ciliates (Taylor and Berger 1976) and flagellates (Sherr et al. 1983) vary depending on the species of bacteria grazed. Different bacterial species are grazed at varying efficiencies (Mitchell et al. 1988). Cell surface hydrophobicity can also influence prey selection by protists (Monger et al. 1999).

The physical and chemical properties of prey and their role in selective predation have been extensively studied in phytoplankton and metazoan zooplankton (Demott 1995) but less so in bacteria and protozoa (Matz et al. 2002b). Protozoan selective feeding appears to be an important mechanism determining community structure of planktonic food webs and influencing rates of organic matter remineralization and nutrient cycling. The purpose of this study was to examine if protozoa selectively graze a clonal species of bacterium based on cell C:N:P stoichiometry. Both red and green fluorescent proteins (RFP and GFP) were cloned from coral animals and inserted into the plasmid DNA of a single strain of bacteria. By growing the bacteria separately, harvesting them at different phases of growth (which results in differing cellular C:P and N:P) and then feeding these labeled bacteria to protistian predators, it was possible to test if there was preferential feeding based on bacterial growth stage and cellular stoichiometry.

MATERIALS AND METHODS

Bacteria containing plasmid with RFP and GFP

A single *Escherichia coli* strain was recombinantly modified using the vector pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA) to contain a gene for red fluorescent protein (RFP). The RFP, dsRED, was originally found in the corallimorph *Discosoma*

sp. (Matz et al. 1999). The RFP coding sequence from pDsRed (Clontech, Palo Alto, Calif.) was excised by PvuII/StuI digestion (positions 55 to 1041), and the resulting 986-bp fragment was inserted into vector pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA). The same *E. coli* strain was transfected using the same vector, but containing a gene for green fluorescent protein (GFP). The GFP (Gp2) was obtained from the coral, *Montastraea cavernosa,* as described in (Kao et al. 2007). To clone GFP from *M. cavernosa*, PCR was used to amplify the fluorescent protein gene. A conserved 5' primer was designed corresponding to a region of the mRNA that included the start codon (CTTACTTACGTCTACCATCATGAGTGTG), and a 3' primer that included the stop codon (TTGGCTTTTCGTTAAGCCTTTACTTGGCC). This amplification was then cloned into the vector pCR4Blunt-TOPO.

Grazing Experiments

Cultures of *E. coli* expressing RFP and *E. coli* expressing GFP were grown in 250 ml flasks containing 50 ml Luria-Bertani media prefiltered filtered through Whatman GF/F filters to remove particulate matter. After autoclaving, the antibiotic kanamycin (final concentration 10 μg/ml) was sterile filtered into the media. All cultures were grown at 37°C (the optimal folding temperature for dsRED) on a rotary shaker at 50 rpm. *E. coli*-RFP was harvested after 66 hours (OD_{600nm} of 2.14) and *E. coli*-GFP after 8 hours (OD of 1.11). Cultures were filtered through a 5μm mesh to remove large particulates and diluted using sterile-filtered seawater at (salinity 22 PPT) to a concentration of 10^7 cells/ml according to direct cell counts. Equal volumes of *E. coli*-RFP and *E. coli*-GFP preparations were combined to make a mixed prey suspension.

Two species of ciliates were used as predators. *Euplotes vannus* is a large hypotrich ciliate (82 µm long, 47 µm wide, 26 µm high) and was obtained from the Culture Collection of Algae and Protozoa (Dunstaffnage Marine Laboratory, UK). *Cyclidium* sp. is a small scuticociliate (26 µm long, 10 µm wide, 10 µm high) and was isolated from a New Jersey salt marsh. Both ciliates were maintained on natural populations of bacteria in a soil extract media at salinity of 22. Prior to an experiment, ciliates were harvested by passing cultures through plastic screens (105 µm mesh for *Euplotes* and 21 µm for *Cyclidium*) to remove larger particulates, then subsequently gravity drained twice through polycarbonate filters (10 µm pore size for *Euplotes*, 5 µm for *Cyclidium*) to remove smaller particles and extant bacteria. The abundance of ciliates was determined from direct counts in a Sedgewick Rafter counting cell and adjusted to 2,400 ml^{-1} for *Euplotes* and 3,000 ml^{-1} for *Cyclidium*. Ciliates then sat for 3-4 hours to clear food vacuoles.

Separate grazing experiments with each predator were conducted in 50 ml-capacity flasks (not shaken, kept at 27ºC) by combining 8 ml of a ciliate preparation and 2 ml the mixed bacterial prey preparation. This resulted in a final total concentration of 2E+6 bacteria/ml (half *E. coli*-RFP and half *E. coli*-GFP). A control treatment without protozoa was included to account for non-predatory changes in bacterial abundance. Each treatment was replicated ten times. Each flask was sampled at time 0 and 135 minutes and immediately fixed to 1% glutaraldyhyde (final concentration).

Bacteria were enumerated by sampling1-ml from each flask at each time point and fixing to 1% glutaraldyhyde (final concentration). Samples were appropriately diluted and filtered onto a 0.22µm, black Millipore membrane filter. Cell concentration

was determined from averaging counts from 10 grids per sample with epifluorescence microscopy using an Olympus BH-2 microscope (100W mercury lamp, BP490 excitation filter/17AFC + 170515 barrier filter).

Elemental Composition of Bacteria

Eight separate flasks, four containing *E. coli*-RFP only and four containing *E. coli*-GFP only were also established and sampled at the beginning and end of the experiment for particulate organic carbon (POC), particulate nitrogen (PN) and particulate phosphorus (PP). Samples were filtered through precombusted (500ºC for 5 h) 25-mm-diameter Whatman GF/F filters. The filters were dried and analyzed for C and N using a Carlo Erba Instruments NA 1500 series 2 elemental analyzer (Mortlock and Froelich 1989). Separate samples were filtered and analyzed for particulate phosphorus (Solórzano and Sharp 1980). Results for POC, PN, and PP were corrected based on filters processed from sterile controls.

Statistical Analyses

Statistical analyses were performed using Statistix v7 (Analytical Software, Tallahassee FL).

RESULTS

The average abundances of *E. coli*-GFP and *E. coli*-RFP at the start of the experiment varied from 1.08E+6 to 1.28E+6 cells/ml in all treatments (Table 1). There was no significant difference in abundance of either strain in any treatment ($p > 0.05$ by one-way analysis of variance and comparison of means by Bonferroni, Scheffe, and Tukey HSD methods). After 135 minutes, the abundance of both strains in the control

treatment had declined slightly but was still at 98% of the initial abundance for *E. coli*-GFP and 99% of the initial abundance for *E. coli*-RFP.

Neither ciliate species significantly changed the abundance of *E. coli*-GFP relative to the change in the control (Table 2). In contrast, both species significantly reduced the abundance of *E. coli*-RFP (Fig. 1). The population of *E. coli*-RFP was reduced to 22% of its initial size by *Cyclidium* and to 33% by *Euplotes*.

The C:N ratio of both *E. coli*-GFP and *E. coli*-RFP remained fairly constant at 4 (Table 3). The *E. coli*-GFP cells, taken at late exponential growth and transferred to sterile, aged seawater with no added nutrients, started with a C:P of 39 and rose to 50 by the end of 135 minutes. The N:P of *E. coli*-GFP cells also increased from 10 to 15. The *E. coli*-RFP harvested after remaining at stationary growth for two days had already adjusted to low growth conditions and thus showed less of a change in elemental stoichiometry. The C:P began at 106 and declined to 86 and the N:P began at 26 and ended at 22. Two-sample t tests on the C, N, and P contents of both strains revealed no significant differences for any element between samples at the beginning of the experiment and after 135 minutes.

DISCUSSION

It is well documented that bactivorous protozoa selectively graze based on cell size of bacteria (Gonzalez et al. 1990; Wilks and Sleigh 1998). While size-based selectivity has received the most attention, recent studies illustrate that planktonic ciliates and flagellates can use other criteria to discriminate among prey, such as prey density

(Fenchel 1980a), prey mobility (Gonzalez et al. 1993), cell surface hydrophobicity (Monger et al. 1999), and C:N ratio (John and Davidson 2001; Tso and Taghon 1999).

Generally, larger bacterial cells are more rapidly grazed by ciliates (Epstein and Shiaris 1992; Gonzalez et al. 1990; Simek and Chrzanowski 1992). While ciliates have a maximum, minimum, and optimal prey-size range depending solely on their anatomical feeding structure (Fenchel 1980b; Verni and Gualtieri 1997), higher contact probability for larger particles has been assumed to be the primary reason for this selectivity (Fenchel 1987) with morphological limitations of feeding also recognized as an important determinant (Boenigk and Arndt 2000). Since the larger-sized bacteria are under greater grazing pressure and the larger cells in the assemblage are mainly cells which are actively growing and dividing (Krambeck and Krambeck 1984), researchers have indirectly assumed that protozoa are selectively grazing on the faster growing cells (Gonzalez et al. 1990; Krambeck 1988; Sherr et al. 1992) and are cropping the production rather than the standing stock of suspended bacteria. For example, Krambeck (1988) states "grazing obviously imposes an upper limit to increases in cell size and thereby specific growth rate and keeps specific bacterial properties constant from a certain productivity level on." Sherr et al. (1992) also theorized that "selective grazing of dividing cells may be an important factor in maintaining taxonomic and metabolic diversity within bacterioplankton assemblages."

The data from this laboratory study strongly suggest the opposite trend, that protozoa selectively graze the older standing stock, rather than the faster-growing cells. In the past, it has been difficult to differentiate and determine the growth state of the bacteria that the protozoa were grazing upon. Grazing rates are mainly obtained by

measuring changes in bacteria populations, and it was not possible to determine which of these cells were actively growing and which were not. Also, while protozoa tend to mechanically select for larger sized cells, it was not possible to determine the species composition or growth status of the different-sized cells. By using a single clonal species of bacterium, we were able to rule out any species-specific grazing patterns. Also, by utilizing newly available recombinant DNA markers and labeling the faster growing cells with GFP and slower growing cells with RFP, we were able to assess the impact of grazing on different bacterial growth phases. This approach also reduced the risk of altering bacterial cell surface properties, which may occur when external staining techniques are used to make cells fluorescent (Epstein and Rossel 1995).

We had originally also hypothesized that the protozoa would graze faster on growing cells more rapidly since such bacteria have lower C:P and N:P ratios (Gruber and Taghon, in prep) and may be more "nutritional" to the protozoan predators. Optimal foraging theory predicts a generally positive selection for food of a higher nutritional value (Krivan 1996; Macarthur and Pianka 1966). But both *Euplotes* and *Cyclidium* showed strong preferential removal of the cells that had been in stationary growth and had higher C:P and N:P ratios than the cells in late exponential growth.

Another study that examined stoichiometry and prey suitability was conducted using the predatory microflagellate, *Paraphysomonas vestita*, feeding on two phytoplankton species of similar cell size with C:N ranging from 4.8 to 14 (John and Davidson 2001). The flagellate fed at a greater rate on phytoplankton with lower C:N (N-replete). Similar results were reported by Jones and Flynn (2005), who show that prey with lower C:N support higher copepod production, but also that mixed prey diets

are necessary for copepods as there are other growth limiting factors (possibly differences in essential fatty acid or amino acid composition) when fed only one species of diatom. In our study, the C:N was relatively invariant at 4 in both the late exponential and stationary growth phase cells, while there was much more variation in C:P and N:P.

In most previous studies examining selective feeding based on elemental stoichiometry of prey and prederator in eukaryotes. It is well established that prokaryotes have lower C:N, N:P, and C:P ratios than eukaryotes (Fagerbakke et al. 1996; Sterner and Elser 2002). Therefore, it would be plausible for protozoa to select prey that more closely match their own cellular stoichiometry, such as the *E. coli*-RFP cells in our experiment. By doing so, protozoa would have lower metabolic costs of digesting, processing, and excreting 'extra' nitrogen and phosphorus in their prey. Johansen (1976) reported that the specific ammonia excretion of tintinnids, a class of ciliates, is one to two orders of magnitude greater than that of macrozooplankton. It may be that ciliates' role in regenerating nutrients is based on the growth stage of the bacteria they are gazing. There are different hypothesis concerning the importance of protozoans in planktonic systems. Some believe that small size and high specific metabolism of protozoa make them primary players in the remineralization (conversion from organic to inorganic form) of nutrients (Harrison 1980) and an important link between bacterial production and higher trophic levels (Porter et al. 1979). Another theory is that protozoa are more efficient than higher trophic levels in converting food into biomass, therefore making their role in nutrient regeneration less than suggested (Fenchel 1977). This has given rise to the arguments that protozoa constitute an important trophic "link" for the transfer of nutrients from bacteria to higher organisms, or they act as a "sink," or source of

remineralized nutrients. It may be that due to certain nutrient limitations, the elemental stoichiometry of bacteria will increase and hence the protists will selectively choose these higher C:P and N:P cell and thus regenerate less nutrients. Therefore, the bacteria elemental stoichiometry may determine if protozoa constitute a "link" or a "sink."

While our experiments were not designed to identify possible chemical cues that might play a role in selective feeding by the ciliates, it is likely that chemoreception plays a role. Evidence of considerable flexibility and complexity in chemoperceptive feeding (Verity 1991) suggests that we have only glimpsed the more detailed features of feeding behavior in aquatic protozoans. Recently, for example, a 40-kDa glycoprotein was isolated from the heliozoon, *Actinophrys sol*, which acts as an adhesive substance to immobilize and ingest prey flagellates (Sakaguchi et al. 2001). When this glycoprotein was coated to agarose beads, the beads adhered to flagellates and induced phagocytosis after *Actinophrys* ingested the coated beads. An interesting follow-up study would be to subject the argose-coated bead to prey of varying elemental stoichiometry to determine if this specific glycoprotein differs in its affinity. It is likely that the mechanisms of selecting prey based on cellular physiology or elemental stoichiometry are more complex, however, and identifying specific proteins involved are only a first, albeit important, step.

Our results also indicate the potential consequences of encountering patches of different prey types at varying growth states and different nutritional status – and the potential importance of selective feeding to ensure if protozoa are to maximize their growth. The effect of nutritional status and stoichiometric composition of the prey are substantial, not only for the initial step of prey selection, but also for growth efficiency.

ACKNOWLEDGEMENTS

This work was supported by Biocomplexity grant OCE0120453 from the National Science Foundation.

We thank Hung-Teh Kao, Yutao Fu and Daniel Distel for providing DNA of fluorescent proteins. Steven Tuorto assisted with experimental design and bacteria counts. We also thank Anna Mei for assistance with protozoa counts.

Table 3.1. Abundance of bacteria (cells ml^{-1}) at the beginning and end of the grazing experiments and in the control treatment. Data are averages (SD; n).

Treatment	*E. coli*-GFP	
	t = 0 min	t = 135 min
Control	1.26E+6 (0.209E+6; 9)	1.24E+6 (0.156E+6; 10)
Cyclidium sp.	1.19E+6 (0.163E+6; 10)	1.16E+6 (0.346E+6; 10)
E. vannus	1.28E+6 (0.137E+6; 10)	1.26E+6 (0.116E+6; 9)
	E. coli-RFP	
	t = 0 min	t = 135 min
Control	1.16E+6 (0.214E+6; 9)	1.15E+6 (0.332E+6; 10)
Cyclidium sp.	1.08E+6 (0.173E+6; 10)	0.237E+6 (0.088E+6; 10)
E. vannus	1.09E+6 (0.121E+6; 10)	0.361E+6 (0.123E+6; 9)

Table 3.2. Changes in average abundance of bacteria (cells ml^{-1}, SD in parentheses), during the grazing experiments, and results of 2-sample t-tests testing the null hypotheses that changes in abundance were the same in the control and when each predator was present versus the one-sided alternative hypotheses that the changes were greater when predators were present. For *E. coli*-RFP the sample variances were not equal and the t-statistics and degrees of freedom were calculated assuming unequal sample variances.

Treatment	Difference in *E. coli*-GFP abundance	Difference in *E. coli*-RFP abundance
Control	-13,900 (281,000)	-12,100 (386,000)
Cyclidium sp.	-24,700 (355,000)	-844,000 (198,000)
	H_0: $dif_{control} = dif_{Cyclidium}$	H_0: $dif_{control} = dif_{Cyclidium}$
	$t_{17} = 0.07$, $p = 0.47$	$t_{11.7} = 5.81$, $p < 0.0001$
E. vannus	-13,000 (158,000)	-733,000 (118,000)
	H_0: $dif_{control} = dif_{Euplotes}$	H_0: $dif_{control} = dif_{Euplotes}$
	$t_{16} = -0.01$, $p = 0.50$	$t_{9.5} = 5.36$, $p = 0.0002$

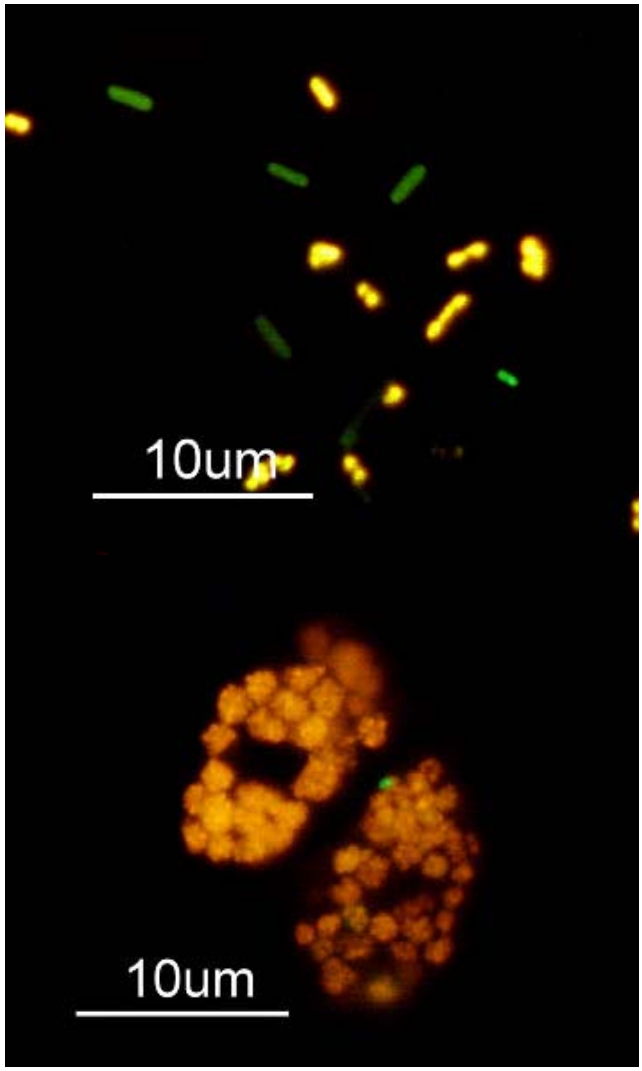
Table 3.3. Elemental stoichiometry of *E. coli*-GFP and *E. coli*-RFP at the start and the end of the experiment. Data are averages (SD).

	Time (min)	C:N	C:P	N:P
E. coli-GFP	0	4.08 (0.06)	39.1 (8.59)	9.60 (2.19)
	135	4.04 (0.09)	49.9 (17.7)	15.4 (4.58)
E. coli-RFP	0	4.02 (0.01)	106 (14.9)	26.4 (3.68)
	135	3.99 (0.03)	86.2 (5.55)	21.6 (1.29)

Figure 3.1. *E. coli*-RFP and *E.coli*-GFP at time 0 min (A) and inside food vacuole of *Cyclidium* sp. after 135 min (B).

A

B



REFRENCES

Amon, R. M. W., and R. Benner. 1996. Bacterial utilization of different size classes of dissolved organic matter. Limnol. Oceanogr. **41:** 41-51.

Azam, F. 1998. Microbial Control of Oceanic Carbon Flux: The Plot Thickens. Science **280:** 694-696

Azam, F., T. Fenchel, J. C. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. Marine Ecological Progress Series **10:** 257-263.

Azam, F., and R. E. Hodson. 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. **22:** 492-501.

Azam, F., D. C. Smith, G. F. Steward, and A. Hagstrom. 1993. Bacteria-organic matter coupling and its significance for oceanic carbon cycling. Microbial Ecol **28:** 167-179.

Baines, S. B., and M. L. Pace. 1991. The production of dissolved organic matter by phytoplankton and its importance to bacteria: Patterns across marine and freshwater systems. Limnol. Oceanogr. **36:** 1078-1090.

Barsdate, R. J., R. T. Prentki, and T. Fenchel. 1974. Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers. Oikos **25:** 239-251.

Benner, R., and M. Strom. 1993. A critical evaluation of the analytical blank associated with DOC measurements by high-temperature catalytic oxidation. Mar. Chem. **41:** 153-160.

Berninger, U.-G., B. F. Finlay, and P. Kuuppo-Leinikki. 1991. Protozoan control of bacterial abundances in freshwater. Limnol. Oceanogr. **36:** 139-147.

Bidle, K. D., and F. Azam. 2001. Bacterial control of silicon regeneration from diatom detritus: Significance of bacterial ectohydrolases and species identity. Limnology and Oceanography **46:** 1606-1623.

Boenigk, J., and H. Arndt. 2000. Particle handling during interception feeding by four species of heterotrophic nanoflagellates. J Eukaryot Microbiol **47:** 350-358.

Borsheim, K. Y. 1984. Clearance rates of bacteria-sized particles by freshwater ciliates, measured with monodisperse fluorescent latex beads. Oecologia **63:** 286-288.

Bratbak, G. 1985. Bacterial Biovolume and Biomass Estimations. Appl. Environ. Microbiol. **49:** 1488–1493.

Bremer, H., and P. P. Dennis. 1996. Modulation of chemical composition and other parameters of the cell by growth rate, p. 1553-1569. *In* F. C. Neidhardt et al. [eds.], Escherichia coli and Salmonella: Cellular and Molecular Biology. ASM Press.

Brophy, J. E., and D. J. Carlson. 1989. Production of biologically refractory dissolved organic carbon by natural seawater microbial populations. Deep-Sea Res. **36:** 497-507.

Caron, D. A. 1991. Evolving Role of Protozoa in Aquatic Nutrient Cycles, p. 387-414. *In* P. C. Reid, C. M. Turley and P. H. Burkill [eds.], Protozoa and Their Role in Marine Processes. Ecological Sciences. Springer-Verlag.

Caron, D. A., E. L. Lim, G. Miceli, J. B. Waterbury, and F. W. Valois. 1991. Grazing and utilization of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory cultures and a coastal plankton community. Mar. Ecol. Prog. Ser. **76:** 205-217.

Chrzanowski, T. H., and M. Kyle. 1996. Ratios of carbon, nitrogen and phosphorus in Pseudomonas fluorescens as a model for bacterial element ratios and nutrient regeneration. Aquat Microb Ecol **10:** 115-122.

Cole, J. J. 1999. Aquatic microbiology for ecosystem scientists: New and recycled paradigms in ecological microbiology. Ecosystems **2:** 215-225.

Cole, J. J., S. Findlay, and M. L. Pace. 1988. Bacterial production in fresh and saltwater ecosystems: A cross-system overview. Mar. Ecol. Prog. Ser. **43:** 1-10.

Colwell, R. R. 1997. Microbial biodiversity and biotechnology. *In* M. L. Reaka-Kudla, D. E. Wilson and E. O. Wilson [eds.], Biodiversity II: Understanding and protecting our biological resources. Joseph Henry Press.

Cox, R. A. 2004. Quantitative relationships for specific growth rates and macromolecular compositions of Mycobacterium tuberculosis, Streptomyces coelicolor A3(2) and Escherichia coli B/r: an integrative theoretical approach. Microbiol-Sgm **150:** 1413-1426.

Del Giorgio, P. A., and J. J. Cole. 1998a. Bacterial growth efficiency in natural aquatic systems. Annu. Rev. Ecol. Syst. **29:** 503-541.

---. 1998b. Bacterial growth efficiency in natural aquatic systems. Annual Review of Ecology and Systematics **29:** 503-541.

Demott, W. R. 1995. Food selection by calanoid copepods in response to between-lake variation in food abundance. Freshwat. Biol. **33:** 171-180.

Ducklow, H. W. 1983. Production and fate of bacteria in the oceans. Bioscience **33:** 494-501.

Elser, J. J. and others 2003. Growth rate-stoichiometry couplings in diverse biota. Ecol Lett **6:** 936-943.

Elser, J. J., D. R. Dobberfuhl, N. A. Mackay, and J. H. Schampel. 1996. Organism size, life history, and N:P stoichiometry. Bioscience **46:** 674-684.

Elser, J. J., W. J. O'brien, D. R. Dobberfuhl, and T. E. Dowling. 2000. The evolution of ecosystem processes: growth rate and elemental stoichiometry of a key herbivore in temperate and arctic habitats. J Evolution Biol **13:** 845-853.

Enzinger, R. M., and R. C. Cooper. 1976. Role of bacteria and protozoa in the removal of *Escherichia coli* from estuarine waters. Appl. Environ. Microbiol. **31:** 758-763.

Epstein, S. S., and J. Rossel. 1995. Methodology of in situ grazing experiments: evaluation of a new viral dye for preparation of fluorescently labeled bacteria. Mar. Ecol. Prog. Ser. **128:** 143-150.

Epstein, S. S., and M. P. Shiaris. 1992. Size-selective grazing of coastal bacterioplankton by natural assembledges of pigmented flagellates, colorless flagellates, and ciliates. Microb. Ecol. **23:** 211-225.

Fagerbakke, K. M., M. Heldal, and S. Norland. 1996. Content of carbon, nitrogen, oxygen, sulfur and phosphorus in native aquatic and cultured bacteria. Aquat Microb Ecol **10:** 15-27.

Falkowski, P. G., E. A. Laws, R. T. Barber, and J. W. Murray. 2003. Phytoplankton and their role in Primary, New, and Export Production., p. 99-121. *In* M. Fasham [ed.], Ocean Biogeochemistry: The Role of the Ocean Carbon Cycle in Global Change. Global Change – The IGBP Series.

Felsenstein, J. 1981. Evolutionary trees from gene-frequencies and quantitative characters finding maximum likeihood estimates. Evolution **35:** 1229-1242.

Fenchel, T. 1977. The significance of bacterivorous protozoa in the microbial community of detrital particles, p. 529-544. *In* J. Cairns [ed.], Aquatic Microbial Communities. Garland Publishing, Inc.

---. 1980a. Suspension feeding in ciliated protozoa: functional response and particle size selection. Microb. Ecol. **6:** 1-11.

---. 1980b. Suspension feeding in ciliated protozoa: structure and function of feeding organelles. Archiv für Protistenkunde **123:** 239-260.

---. 1984. Suspended marine bacteria as a food source, p. 301-305. *In* M. J. R. Fasham [ed.], Flows of energy and materials in marine ecosystems. Plenum.

---. 1987. Ecology of Protozoa. Science Tech Publishers.

Fenchel, T., and P. Harrison. 1976. The significance of bacterial grazing and mineral decomposition of particulate detritus, p. 285-299. *In* J. M. Anderson and A. Macfadyen [eds.], The role of Terrestrial and Aquatic Organisms in Decomposition Processes. Blackwell Scientific Publications.

Goldman, J. C. 1984. Conceptual role for microaggregates in pelagic waters. Bull. Mar. Sci. **35:** 462-476.

Goldman, J. C., D. A. Caron, and M. A. Dennett. 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnol. Oceanogr. **32:** 1239-1252.

Goldman, J. C., and M. R. Dennett. 2000. Growth of marine bacteria in batch and continuous culture under carbon and nitrogen limitation. Limnology and Oceanography **45:** 789-800.

Gonzalez, J. M., E. B. Sherr, and B. F. Sherr. 1990. Size-selective grazing on bacteria by natural assemblages of estuarine flagellates and cilliates. Appl. Environ. Microbiol. **56:** 583-589.

---. 1993. Differential feeding by marine flagellates on growing versus starving, and on motile versus nonmotile, bacterial prey. Mar. Ecol. Prog. Ser. **102:** 257-267.

Gruber, D. F., J. P. Simjouw, S. P. Seitzinger, and G. L. Taghon. 2006. Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. Appl Environ Microb **72:** 4184-4191.

Guindon, S., F. Lethiec, P. Duroux, and O. Gascuel. 2005. PHYML Online--a web server for fast maximum likelihood-based phylogenetic inference. Nucl. Acids Res. **33:** W557-559.

Hadas, O., N. Malinsky-Rushansky, R. Pinkas, and T. E. Cappenberg. 1998. Grazing on autotrophic and heterotrophic picoplankton by ciliates isolated from Lake Kinneret, Israel. Journal of Plankton Research **20:** 1435-1448.

Hahn, M. W., E. R. B. Moore, and M. G. Hofle. 1999. Bacterial filament formation, a defense mechanism against flagellate grazing, is growth rate controlled in bacteria of different phyla. Appl Environ Microb **65:** 25-35.

Harold, F. M. 1966. Inorganic polyphosphates in biology: structure, metabolism, and function. Bacteriological Reviews **30:** 772-794.

Harrison, W. G. 1980. Nutrient regeneration and primary production in the sea, p. 433-460. *In* P. G. Falkowski [ed.], Primary Productivity in the Sea. Brookhaven Symposia in Biology. Plenum Press.

Hasegawa, M., H. Kishino, and T. Yano. 1985. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. Journal of Molecular Evolution **22:** 160–174.

Haukka, K., E. Heikkinen, T. Kairesalo, H. Karjalainen, and K. Sivonen. 2005. Effect of humic material on the bacterioplankton community composition in boreal lakes and mesocosms. Environmental Microbiology **7:** 620-630.

Hawksworth, D. L., and R. R. Colwell. 1992. Biodiversity amongst microorganisms and its relevance. Biodiv. Conserv. **1:** 221-345.

Hayter, J. B., M. Rivera, and E. J. Mcgroarty. 1987. Neutron scattering analysis of bacterial lipopolysaccharide phase structure. Changes at high pH. Journal of Biological Chemistry **262:** 5100-5105.

Hedges, J. I. and others 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Org. Geochem. **31:** 945-958.

Heissenberger, A., and G. J. Herndl. 1994. Formation of high molecular weight material by free-living marine bacteria. Mar. Ecol. Prog. Ser. **111:** 129-135.

Herbert, D. 1961. The chemical composition of micro-organisms as a function of their environment, p. 391-416. *In* G. G. Meynell and H. Goodet [eds.], Symposium of the Society for General Microbiology. Cambridge University Press.

Hobbie, J. E., R. J. Daley, and S. Jasper. 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:** 1225-1228.

Hua, Y., W. Lu, M. S. Henry, R. H. Pierce, and R. B. Cole. 1996. On-line liquid chromatography-electrospray ionization mass spectrometry for determination of the brevetoxin profile in natural "red tide" algae blooms. J. Chromatogr. A **750:** 115-125.

Johannes, R. E. 1964. Influence of marine protozoa on nutrient regeneration. Univ.Georgia Marine Institute **85:** 434-442.

Johansen, P. L. 1976. A study of tintinnids and other protozoa in eastern Canadian waters with special references to tintinnid feeding, excretion and reproduction rates. PhD. Dalhousie Univ.

John, E. H., and K. Davidson. 2001. Prey selectivity and the influence of prey carbon : nitrogen ratio on microflagellate grazing. Journal of Experimental Marine Biology and Ecology **260:** 93-111.

Jones, R. H., and K. J. Flynn. 2005. Nutritional status and diet composition affect the value of diatoms as copepod prey. Science **307:** 1457-1459.

Jumars, P. A., D. L. Penry, J. A. Baross, M. J. Perry, and B. W. Frost. 1989. Closing the microbial loop: dissolved carbon pathway to heterotrophic bacteria from incomplete ingestion, digestion and sbsorption in animals. Deep-Sea Res. **36:** 483-495.

Kao, H.-T., S. Sturgis, R. Desalle, D. F. Gruber, and V. A. Pieribone. 2007. Dynamic Regulation of Fluorescent Proteins from a Single Species of Coral. Marine Biotechnology **In press**.

Kaunzinger, C. M. K., and P. J. Morin. 1998. Productivity controls food-chain properties in microbial communities. Nature **395:** 495-497.

Kim, S., A. J. Simpson, E. B. Kujawinski, M. A. Freitas, and P. G. Hatcher. 2003. High resolution electrospray ionization mass spectrometry and 2D solution NMR for the analysis of DOM extracted by C-18 solid phase disk. Org. Geochem. **34:** 1325-1335.

Kirchman, D. L. 2000. Uptake and Regeneration of Inorganic Nutrients by Marine Heterotrophic Bacteria, p. 261-288. *In* D. L. Kirchman [ed.], Microbial Ecology of the Oceans. Wiley-Liss.

Koch, B. P., M. R. Witt, R. Engbrodt, T. Dittmar, and G. Kattner. 2005. Molecular formulae of marine and terrigenous dissolved organic matter detected by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Geochimica Et Cosmochimica Acta **69:** 3299-3308.

Koike, I., S. Hara, K. Terauchi, and K. Kogure. 1990. Role of sub-micrometre particles in the ocean. Nature **345:** 242-244.

Krambeck, C. 1988. Control of bacterioplankton structures by grazing and nutrient supply during the decline of an algal bloom. Verh. Int. Verein. Limnol. **23:** 496-502.

Krambeck, C., and H.-J. Krambeck. 1984. Morphometric analysis of cell-cycle responses in bacterioplankton. Arch. Hydrobiol. Beih. Ergebn. Limnol. **19:** 111-118.

Kramer, G. D., and G. J. Herndl. 2004. Photo- and bioreactivity of chromophoric dissolved organic matter produced by marine bacterioplankton. Aquat. Microb. Ecol. **36:** 239-246.

Krivan, V. 1996. Optimal foraging and predator-prey dynamics. Theoretical Population Biology **49:** 265-290.

Kujawinski, E. B., R. Del Vecchio, N. V. Blough, G. C. Klein, and A. G. Marshall. 2004. Probing molecular-level transformations of dissolved organic matter: insights on photochemical degradation and protozoan modification of DOM from electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Mar. Chem. **92:** 23-37.

Kujawinski, E. B., M. A. Freitas, X. Zang, P. G. Hatcher, K. B. Green-Church, and R. B. Jones. 2002. The application of electrospray ionization mass spectrometry (ESI MS) to the structural characterization of natural organic matter. Org. Geochem. **33:** 171-180.

Landry, M. R. 1991. Discrimination between living and heat-killed prey by a marine zooflagellate, Paraphysomonas vestita (Stokes). J. Expt. Mar. Biol. and Ecol. **146:** 139-151.

Leenheer, J. A., C. E. Rostad, P. M. Gates, E. T. Furlong, and I. Ferrer. 2001. Molecular resolution and fragmentation of fulvic acid by electrospray ionization/multistage tandem mass spectrometry. Anal. Chem. **73:** 1461-1471.

Loo, J. A., and R. R. Ogorzalek Loo. 1997. Electrospray ionization mass spectrometry of peptides and proteins. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Macarthur, R. H., and E. R. Pianka. 1966. On Optimal Use of a Patchy Environment. The American Naturalist **100:** 603-609.

Main, T. M., D. R. Dobberfuhl, and J. J. Elser. 1997. N : P stoichiometry and ontogeny of crustacean zooplankton: A test of the growth rate hypothesis. Limnol. Oceanogr. **42:** 1474-1478.

Makino, W., and J. B. Cotner. 2004. Elemental stoichiometry of a heterotrophic bacterial community in a freshwater lake: implications for growth- and resource-dependent variations. Aquat Microb Ecol **34:** 33-41.

Makino, W., J. B. Cotner, R. W. Sterner, and J. J. Elser. 2003. Are bacteria more like plants or animals? Growth rate and resource dependence of bacterial C : N : P stoichiometry. Funct Ecol **17:** 121-130.

Marr, A. G. 1991. Growth rate of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **55:** 316-333.

Matz, C., J. Boenigk, H. Arndt, and K. Jurgens. 2002a. Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate Spumella sp. Aquat Microb Ecol **27:** 137-148.

Matz, C., P. Deines, and K. Jurgens. 2002b. Phenotypic variation in Pseudomonas sp CM10 determines microcolony formation and survival under protozoan grazing. Fems Microbiology Ecology **39:** 57-65.

Matz, C., and K. Jurgens. 2005. High motility reduces grazing mortality of planktonic bacteria. Appl Environ Microb **71:** 921-929.

Matz, M. V. and others 1999. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology **10:** 969-973.

Mccambridge, J., and T. A. Mcmeekin. 1979. Relative effects of bacterial and protozoan predators on survival of Escherichia coli in estuarine waters. Water Res. **13**.

---. 1980. Relative effects of the bacterial and protozoan predators on survival of Escherichia coli in estuarine water samples. Appl. Environ. Microbiol. **40**.

Mcmanus, G. B., and J. A. Fuhrman. 1988. Clearance of bacteria-sized particles by natural populations of nanoplankton in the Chesapeake Bay outflow plume. Mar. Ecol. Prog. Ser. **42:** 199-206.

Mitchell, G. C., J. H. Baker, and H. A. Sleigh. 1988. Feeding of a freshwater flagellate Bodo saltans, on diverse bacteria. J. Protozool. **35:** 219-222.

Monger, B. C., M. R. Landry, and S. L. Brown. 1999. Feeding selection of heterotrophic marine nanoflagellates based on the surface hydrophobicity of their picoplankton prey. Limnology and Oceanography **44:** 1917-1927.

Mortlock, R. A., and P. N. Froelich. 1989. A simple method for the rapid determinatoin of biogenic opal in pelagic marine sediments. Deep-Sea Res. **36:** 1415-1426.

Nagata, T., and D. L. Kirchman. 1992a. Release of dissolved organic matter by heterotrophic protozoa: implications for microbial food webs. Arch. Hydrobiol. Beih. Ergebn. Limnol. **35:** 99-109.

---. 1992b. Release of macromolecular organic complexes by heterotrophic marine flagellates. Mar. Ecol. Prog. Ser. **83:** 233-240.

Neidhardt, F. C., J. L. Ingraham, and M. Schaechter. 1990. Physiology of the bacterial cell. A molecular approach. Sinauer Associates.

Odum, E. P. 1971a. Fundamentals of ecology, 3rd ed. ed. W. B. Saunders.

---. 1971b. Fundamentals of Ecology, 3 ed. W. B. Saunders.

Ogawa, H., Y. Amagai, I. Koike, K. Kaiser, and R. Benner. 2001. Production of refractory dissolved organic matter by bacteria. Science **292:** 917-920.

Olesen, M., and C. Lundsgaard. 1995. Seasonal Sedimentation of Autochthonous Material from the Euphotic Zone of a Coastal System. Estuar Coast Shelf S **41:** 475-490.

Pace, M. L. 1986. An empirical analysis of zooplankton community size-structure across lake trophic gradients. Limnol. Oceanogr. **31:** 45-55.

Payne, W. J. 1970. Energy yields and growth of heterotrophs. Annu Rev Microbiol. **24:** 17-52.

Pomeroy, L. R. 1974. The Ocean's Food Web, A Changing Paradigm. Bioscience **24:** 499-504.

Poon, G. K. 1997. Drug metabolism and pharmacokinetics, p. 499-525. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Porter, K. G., M. L. Pace, and J. F. Battey. 1979. Ciliate protozoans as links in freshwater planktonic food chains. Nature **277:** 563-565.

Porter, K. G., E. B. Sherr, B. F. Sherr, M. Pace, and R. W. Sanders. 1985. Protozoa in planktonic food webs. J. Protozool. **32:** 409-415.

Posada, D., and K. A. Crandall. 1998. MODELTEST: testing the model of DNA substitution. Bioinformatics **14:** 817-818.

Redfield, A. C. 1958. The biological control of chemical factors in the environment. Am. Sci. **46:** 205-221.

Ronquist, F., and J. P. Huelsenbeck. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. Bioinformatics **19:** 1572-1574.

Saito, K., T. Toyo'oka, T. Fukushima, M. Kato, O. Shirota, and Y. Goda. 2004. Determination of psilocin in magic mushrooms and rat plasma by liquid chromatography with fluorimetry and electrospray ionization mass spectrometry. Anal. Chim. Acta **527:** 149-156.

Sakaguchi, M., H. Murakami, and T. Suzaki. 2001. Involvement of a 40-kDa glycoprotein in food recognition, prey capture, and induction of phagocytosis in the protozoon Actinophrys sol. Protist **152:** 33-41.

Schmidt, J. L., J. W. Deming, P. A. Jumars, and R. G. Keil. 1998. Constancy of bacterial abundance in surficial marine sediments. Limnology and Oceanography **43:** 976-982.

Seitzinger, S. P. and others 2005. Molecular-level chemical characterization and bioavailability of dissolved organic matter in stream water using electrospray-ionization mass spectrometry. Limnol. Oceanogr. **50:** 1-12.

Seitzinger, S. P., and R. W. Sanders. 1997. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication. Mar. Ecol. Prog. Ser. **159:** 1-12.

Seitzinger, S. P., R. M. Styles, R. Lauck, and M. A. Mazurek. 2003. Atmospheric pressure mass spectrometry: A new analytical chemical characterization method for dissolved organic matter in rainwater. Environ. Sci. Technol. **37:** 131-137.

Sharp, J. H., R. Benner, L. Bennett, C. A. Carlson, R. Dow, and S. E. Fitzwater. 1993. Re-evaluation of high temperature combustion and chemical oxidation measurements of dissolved organic carbon in seawater. Limnol. Oceanogr. **38:** 1774-1782.

Sherr, B. F., E. B. Sherr, and T. Berman. 1983. Grazing, growth, and ammonium excretion rates of a heterotrophic microflagellate fed with four species of bacteria. Appl. Environ. Microbiol. **45:** 1196-1201.

Sherr, B. F., E. B. Sherr, and R. D. Fallon. 1987. Use of Monodispersed, Fluorescently Labeled Bacteria to Estimate In Situ Protozoan Bacterivory. Appl Environ Microb **53:** 958-965.

Sherr, B. F., E. B. Sherr, and J. Mcdaniel. 1992. Effect of protistan grazing on the frequency of dividing cells in bacterioplankton assemblages. Appl. Environ. Microbiol. **58:** 2381-2385.

Sherr, B. F., E. B. Sherr, and C. Pedros-Alio. 1989. Simultaneous measurement of bacterioplankton production and protozoan bactivory in estuarine water. Mar. Ecol. Prog. Ser. **54:** 209-219.

Simek, K., and T. H. Chrzanowski. 1992. Direct and indirect evidence of size-selective grazing on pelagic bacteria by freshwater nanoflagellates. Appl. Environ. Microbiol. **58:** 3715-3720.

Simek, K. and others 1997. Morphological and compositional shifts in an experimental bacterial community influenced by protists with contrasting feeding modes. Applied and Environmental Microbiology **63:** 587-595.

Solórzano, L., and J. H. Sharp. 1980. Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol. Oceanogr. **25:** 754-758.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:** 159-271.

Steele, J. H. 1974. The Structure of Marine Ecosystems. Harvard Univ. Press.

Stenson, A. C., W. M. Landing, A. G. Marshall, and W. T. Cooper. 2002. Ionization and fragmentation of humic substances in electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry. Anal. Chem. **74:** 4397-4409.

Stenson, A. C., A. G. Marshall, and W. T. Cooper. 2003. Exact masses and chemical formulas of individual Suwannee River fulvic acids from ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectra. Anal. Chem. **75:** 1275-1284.

Sterner, R. W., and J. J. Elser. 2002. Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere.

Stoderegger, K., and G. J. Herndl. 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol. Oceanogr. **43:** 877-884.

Strom, S. L., R. Benner, S. Ziegler, and M. J. Dagg. 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol. Oceanogr. **42:** 1364-1374.

Tavare, S. 1986. Some probabilistic and statistical problems in the analysis of DNA sequences. Lectures on Mathematics in the life Sciences **17:** 57-86.

Taylor, G. T., R. Iturriaga, and C. W. Sullivan. 1985. Interactions of bactivorous grazers and heterotrophic bacteria with dissolved organic matter. Mar. Ecol. Prog. Ser. **23:** 129-141.

Taylor, W. D., and J. Berger. 1976. Growth responses of cohabitating ciliate protozoa to various prey bacteria. Can. J. Zool. **54:** 1111-1114.

Tezuka, Y. 1990. Bacterial regeneration of ammonium and phosphate as affeted by the carbon: nitrogen: phosphorus ratio of organic substrates. Microbial Ecol **19:** 227-238.

Torsvik, V., L. Ovreas, and T. F. Thingstad. 2002. Prokaryotic diversity - Magnitude, dynamics, and controlling factors. Science **296:** 1064-1066.

Tranvik, L. 1994. Colloidal and Dissolved Organic-Matter Excreted by a Mixotrophic Flagellate During Bacterivory and Autotrophy. Appl. Environ. Microbiol. **60:** 1884-1888.

Tso, S. F., and G. L. Taghon. 1999. Factors affecting predation by Cyclidium sp. and Euplotes sp. on PAH-degrading and nondegrading bacteria. Microbial Ecol **37:** 3-12.

Tyrrell, T. 1999. The relative influences of nitrogen and phosphorus on oceanic primary production. Nature **400:** 525-531.

Vadstein, O., and Y. Olsen. 1989. Chemical compositon and phosphate uptake kinetics of limnetic bacterial communities cultured in chemostats under phosphorus limitation Limnol. Oceanogr. **34:** 939-946.

Verardo, D. J., P. N. Froelich, and A. Mcintyre. 1990. Determination of organic carbon and nitrogen in marine sediments using the Carlo Erba NA-1500 Analyzer. Deep-Sea Research **37:** 157-165.

Verity, P. G. 1991. Feeding in Planktonic Protozoans - Evidence for Nonrandom Acquisition of Prey. Journal of Protozoology **38:** 69-76.

Verni, F., and P. Gualtieri. 1997. Feeding behaviour in ciliated protists. Micron **28:** 487-504.

Vrede, K., M. Heldal, S. Norland, and G. Bratbak. 2002. Elemental composition (C, N, P) and cell volume of exponentially growing and nutrient-limited bacterioplankton. Appl. Environ. Microbiol. **68:** 2965-2971.

Vrede, T., T. Andersen, and D. O. Hessen. 1999. Phosphorus distribution in three crustacean zooplankton species. Limnology and Oceanography **44:** 225-229.

Vrede, T., D. R. Dobberfuhl, S. A. L. M. Kooijman, and J. J. Elser. 2004. Fundamental connections among organism C : N : P stoichiometry, macromolecular composition, and growth. Ecology **85:** 1217-1229.

Whitman, W. B., D. C. Coleman, and W. J. Wiebe. 1998. Prokaryotes: The unseen majority. P Natl Acad Sci USA **95:** 6578-6583.

Wilks, S. A., and M. A. Sleigh. 1998. Grazing rates in Euplotes mutabilis: Relationship between particle size and concentration. Microbial Ecol **36:** 165-174.

Yang, Z. 1994. Estimating the pattern of nucleotide substitution. Journal of Molecular Evolution **39:** 105-111.

Zubkov, M. V., and M. A. Sleigh. 1995. Ingestion and assimilation by marine protists fed on bacteria labeled with radioactive thymidine and leucine estimated without separating predator and prey. Microb. Ecol. **30:** 157-170.

CHAPTER 4

Nutrient Dynamics and Organic Carbon Remineralization Kinetics in Cultures of Marine Bacteria and Protozoa under Carbon and Nitrogen Limited Conditions

ABSTRACT

In laboratory batch culture experiments using one species of heterotrophic marine bacterium (*Vibrio* strain-NAP) and one protozoan ciliate grazer species (*Cyclidium sp*.), we examined the rates and kinetics in which a simple carbon source, glucose, was remineralized to CO_2 in systems constrained by either carbon or nitrogen concentrations. Under both nitrogen and carbon limitation, similar percentages of carbon were remineralized following approx. a two-week time span (32% and 38% in bacteria-only cultures and ~71% and 73% in bacteria + ciliates, under nitrogen and carbon limitation, respectively), yet the dynamics and nutrient partitioning among the particulate and dissolved pools varied greatly depending on the nutrient that was limited. In the carbon limited trials, the increased tropic level (i.e. introduction of protozoa) resulted in more than a three-fold decrease in the particulate organic carbon and particulate nitrogen pool, with the particulate C:N remaining relatively constant, averaging 4.1. Alternatively, under nitrogen limitation, the presence of protozoan grazers nearly doubled the particulate organic carbon pool and elevated the particulate C:N ratio to a maximum value of 13.6. This increase in C:N in the nitrogen-limited, bacteria + ciliates trial (averaged 10.6 compared to 9.1 with bacteria only), is possibly because *Cyclidium*, like all protozoa, have higher C:N ratios than bacteria. An interesting finding in the nitrogen-limiting experiment was the production of what appeared to be relatively constant concentration of semi-refractory dissolved organic nitrogen (~100 μM) that persisted throughout both bacteria and bacteria + ciliate experiments. This study shows that the rate at which the percentage of dissolved carbon is remineralized is dampened in a nitrogen-limited system versus a carbon-limited system. Yet this data suggests that the ultimate

bulk percentage of carbon remineralized is primarily dependent on predator/prey interactions and trophic inefficiency, regardless of the limiting nutrient.

INTRODUCTION

The estimated 1.2×10^{29} bacterial cells (Whitman et al. 1998) found in the global marine environment undoubtedly play a significant role in biogeochemical nutrient cycling. But, over the past few decades, the research impetus has focused primarily on the production of organic matter, and to a much lesser extent on the reverse transformation back into inorganic matter. Bacteria are one of the most efficient converters of dissolved nutrients into particulate biomass (Azam et al. 1993) and the vast majority of bacterial species are obligate osmotrophs that consume dissolved low molecular weight compounds (less than 300 Da). In all ecosystems, bacteria outnumber all other organisms and play a prominent role in shuttling organic matter up the food chain. Within the marine environment, bacteria are responsible for taking up 10-50% of the estimated 45 Pg C/year (Falkowski et al. 2003) produced via primary production, which is released into the dissolved pool by excretion, exudation and diffusion (Jumars et al. 1989).

The fate of bacterial cells prominently dictates rates of nutrient cycling. Predation is the primary mechanism responsible for bacteria mortality in aquatic ecosystems (Cole 1999) and recently the viral component has also been given increased attention. It has been suggested by Ducklow (1983) that primarily ciliated protozoa, that are grouped with the microzooplankton (20-200 μm diameter) and nanozooplankton (2-20 μm diameter), control standing stocks and metabolic activity of bacteria and form an important link

between nanoplankton and larger consumer of the food web. Their simple life history and short generation times enables protozoa to respond rapidly to changes in bacterial populations. Studies have shown that more than half of primary and bacterial production can be consumed by marine ciliates (Fenchel 1987). In this capacity, ciliates function as trophic links, nutrient regenerators, and also transform prey material into DOM (Strom et al. 1997), processes previously overlooked in traditional food webs (Steele 1974).

While it is known that protozoan grazing regulates bacterial activity and mobilizes nutrients (Goldman 1984), the mechanism by which grazers expedite the remineralization of organic matter is unknown. Early studies by Johannes (1964) demonstrated that bactivorous protozoan grazers accelerate remineralization of phosphorus and decomposition of detritus. Johannes hypothesized that protozoan grazing maintains bacteria in a state of "physiological youth" and prevents them from reaching self-limiting numbers. Barsdate et al. (1974), using aquatic microcosms, also showed that phosphorus cycled much more rapidly in systems with bacteria and protozoan grazers than with bacteria alone and theorized grazers may qualitatively change the bacterial population by selecting for rapidly growing bacteria or by "stirring" and releasing growth stimulating substances such as ammonia. Fenchel and Harrison (1976) also performed a batch culture experiment using ^{14}C labeled barley hay in seawater and showed that after 37 days of incubation, bacteria alone decomposed ~20% of the barley hay and bacteria mixed with protozoa decomposed 80%. They raised the possibility that the increased rate of decomposition may be due to "some growth promoting substance" excreted by the protozoa other than orthophosphate. Fenchel (1977) also hypothesized that protozoa create "microturbulence" zones which expose fresh organic matter surfaces for bacteria.

Matz and Jurgens (2005) recently reported that protozoan predators increased bacteria motility, and hence it is also possible that added respiratory motility costs of evading predators could result in increased carbon mineralization.

The purpose of this study was to analyze the effects of grazing on mechanisms of particulate organic carbon (POC), particulate nitrogen (PN), dissolved organic carbon (DOC), total dissolved nitrogen (TDN), dissolved inorganic nitrogen (DIN) and CO_2 production/respiration during a stepwise increase in trophic level, under both carbon and nitrogen limitation. To investigate the effects of bacterial-protist interaction on the remineralization of organic carbon (conversion to CO_2), we established batch culture systems where the bacterium, *Vibrio* strain-NAP, was grown on a simple source of dissolved carbon (glucose) with excess phosphorus, but limited by either labile nitrogen or carbon. We used concentrations of carbon much higher than found in the water column to create a significant particulate pool (operationally defined as material captured on a GF/F filter, pore size ~0.7 μm) with bacterial abundance comparable to the estimated 10^9/ml in estuarine sediment porewater (Schmidt et al. 1998). Replicate microcosms were established with the same nutrient concentrations and bacterial inoculum, but a small (~25 μm) marine scuticociliate, *Cyclidium sp*., was introduced once the bacteria established stationary growth phase.

MATERIALS AND METHODS

Experimental setup

Production and dynamics of DOM and POM were examined using glucose and ammonia as the sole utilizable carbon and nitrogen sources with initial concentrations of

18 mM C, 2.66 mM N (carbon-limited experiment) and 32 mM C, 640 μM N (nitrogen-limited experiment). There were three treatments in each experiment: sterile control, bacteria only and bacteria plus ciliate. Experiments took place in 125-ml flasks (baked at 500C for 5 h before use) containing 25 ml sterile, low carbon and nitrogen 62.8 μM-C and 10.5 μM-N from Nitrilotriacetic acid and 0.8 μM C and 0.2 μM N from Na_2EDTA) modified mineral basal solution (MBS). (Table 1)

All flasks (except the sterile controls) were initially inoculated with the bacterium *Vibrio* sp.-NAP. Initial samples were taken directly after inoculation (day 0). Half the remaining flasks were inoculated with the bactiverous protozoan, *Cyclidium sp.* just after day 1 time-point was taken (~24 hours after inoculation with bacteria), when the bacteria had achieved, or were approaching, stationary growth. Flasks were capped with aluminum foil to maintain sterility, but to permit air exchange. All cultures were incubated in the dark on a shaker table at 50 rpm and 20°C. Triplicate sacrificial flasks were used at each time point to prevent contamination from repeated sampling of the same flasks.

Bacterial strain.

We used a single marine heterotrophic bacterial strain, *Vibrio sp*.-NAP, a gamma proteobacterium first isolated by Tso and Taghon (1999). The 16s rRNA gene of this strain was sequenced before performing this study to confirm bacterium identity. Flasks were inoculated with 10 μl of triple-washed cells harvested from cultures at the end of the log growth phase. At each time point, abundance was estimated by measuring the optical density (1-cm path length) at 550 nm of a 1-ml sample.

Grazer.

We used a bactivorous scuticociliate, *Cyclidium sp.,* isolated from Piles Creek (40˚36.53N, 74˚13.60W) within the New York/New Jersey Harbor. *Cyclidium* is a dominant member of protozoan communities in pelagic and benthic habitats and was also reported a dominant grazer by Tso and Taghon (1999). The ciliate is about 25 µm long in its longest dimension, 10 µm wide, and 10 µm high. Prior to the experiments, ciliates were maintained in culture using protozoan pellets (Carolina Biological Supply) as a nutrient source. Before being introduced to *Vibrio sp.*- NAP cultures, ciliates were filtered through a polyester mesh (41-µm openings) to remove detritus. Ciliates in the <41 µm fraction were triple rinsed through with low-carbon MBS and then concentrated onto a 5-µm Nuclepore filter and re-suspended to a final density of 40,000 cells/ml. Flasks were inoculated with 25 µl of concentrated ciliate suspension (resulting in media concentration of ~40 cells/ml). At each time point, abundance was determined by direct counts of a 1-ml sample using a Sedgewich Rafter counting cell.

Bulk chemical analysis.

At each time point, samples were taken from each flask for particulate organic carbon (POC), particulate nitrogen (PN), dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and dissolved inorganic nitrogen (DIN).

For the POC and PN analysis, varying volumes of sample (depending on cell density) were filtered through precombusted (500C for 5 h) 25-mm-diameter Whatman GF/F filters. Filters were dried and analyzed using a Carlo Urba Instruments NA 1500

series 2 elemental analyzer (Mortlock and Froelich 1989). Results were corrected based on filters processed from sterile controls. The filtrate containing the dissolved fraction was transferred to acid-washed capped polypropylene vials and stored frozen (-20C) in the dark, for less than 1-month, until needed for all other analysis.

DOC and TDN were measured using a Shimadzu 5000 high temperature combustion analyzer for DOC (Benner and Strom 1993; Sharp et al. 1993) with an inline NOx detector (Antek model 7000 total N analyzer) for TDN analysis (Seitzinger and Sanders 1997). DIN was measured using a Lachet, Inc., autoanalyzer (ammonium; QuikChem 31-107-06-1-A; nitrate plus nitrite, QuickChem 31-107-04-1-A). DON was determined as the difference between TDN and DIN.

RESULTS

Bulk DOC, POC, and population dynamics

(i) Bacteria-only treatments.

Carbon-limited experiment: Glucose was rapidly utilized during the exponential growth phase of the bacteria as indicated by changes in optical density (Fig. 1a) and DOC concentration (Fig. 1b). The initial day 1 decrease in DOC concentration from 18 mM to 3.46 mM was accompanied by a 10 mM rise on POC as the bacteria achieved stationary growth, corresponding to a 56% bacteria growth efficiency. The efficiency at which bacteria convert DOC into bacterial biomass, BGE, can be expressed as: BGE = (ΔPOC/ΔDOC) × 100%. The DOC concentration decreased further to 2.1 mM by day 2 and then steadily declined to 1.5 mM after two weeks (1.46 mM higher than the culture medium background of 67 μM). POC remained constant at ~10 mM from day 1 to day

14 (Fig. 1b). The C:N ratio of the particulate matter remained relatively constant throughout the entire two weeks, between 4.0 and 4.2 (Fig. 1d).

The total dissolved nitrogen concentration remained high throughout the experiment (Fig. 1c). From day 0 to day 1, TDN decreased from 2.66 mM to its lowest concentration of 0.85 mM (0.57 mM of this being ammonium). Throughout the following 13 days of the experiment, both TDN and ammonium displayed a gradual increase to a final concentration of 1.18 mM and 1.13 mM respectively. Nitrate plus nitrate concentrations were 1-2 μM throughout the experiment. PN rose to 2.39 mM at day 1 and remained almost constant through day 14.

Nitrogen-limited experiment:

Glucose was utilized much less rapidly and the exponential growth phase of the bacteria extended for 3 days (Fig. 3a). The initial day 1 decrease in DOC concentration from 36 mM to 33.4 mM was accompanied by a 1.7 mM rise on POC, corresponding to a 67% bacteria growth efficiency. By day 3, the DOC concentration had decreased to 25.6 mM, with a POC of 4.1 mM. Over the following 13 days, DOC steadily decreased to 20 mM and POC rose slightly to 4.46 mM. During the exponential growth phase, the C:N ratio rose from 4 to 6.5 from day 1 to day 2 and to 8.7 upon reaching stationary growth at day 3. Over the following 13 days, C:N gradually rose to a value of 10.1 (Fig. 3d).

The total dissolved nitrogen dropped from 0.64 mM to 0.14 mM (0.068 mM of which was ammonium) at day 1. By day 2, TDN had declined further to 0.086 mM (the lowest recorded concentration) and the ammonium concentration decreased to ~0.005 mM (the concentration it remained for the following two weeks). TDN fluctuated between 0.09 and 0.14 mM. Nitrate plus nitrate concentrations were extremely low, 1-2

μM, throughout the experiment. PN rose to a high of 0.49 mM on day 2 and slowly decreased to 0.44 mM at day 16.

(ii) Bacteria plus bactivorous protozoan treatments.

Carbon-limited experiment:

Following the introduction of *Cyclidium* at day 1, the optical density of the cultures continued to rise from 0.33 at day 1 to 0.44 on day 2, but then sharply decreased to 0.08 on day 4 (Fig. 2a). *Cyclidium* abundance increased from 20 to 24,600 cells/ml during that same period. From day 4 onwards, the optical density of the remaining cultures was only slightly above the sterile control values. Ciliate abundance varied cyclically from day 4 through day 14, with highs of 31,000 cells/ml to lows of 18,000 cells/ml. Ciliates at these cell densities have negligible impact on optical density and points of maximal *Cyclidium* population did not result in pulses of POC (Fig. 2b).

Throughout the experiment, there was no significant production of DOC due to the grazing activity of *Cyclidium*. After *Cyclidium* was added, DOC decreased from 3.8 mM to 2.1 mM between day 2 and 3 and then slowly declined to 1.64 mM at the conclusion of the experiment at day 16, corresponding to ~9-10% of the initial DOC value and averaged 0.12 mM higher than the bacteria-only experiment during the same time period.

POC concentration steadily decreased following *Cyclidium* addition, from 10.6 mM at the end of the exponential growth phase of *Vibrio sp*.-NAP, to values of 3.64 to 5.38 mM during the interval of greatest *Cyclidium* abundance (days 4 to 9), to a final value of 2.93 mM on day 14 (Fig. 2b).

As with the bacteria-only trial, the C:N ratio of the particulate matter also remained relatively constant throughout the entire two weeks, between 4.0 and 4.2 (Fig. 3d).

The dissolved nitrogen concentration remained high throughout the experiment (Fig. 2c). The ammonium concentration reached its lowest level of 0.52 mM on day 2 and than more than tripled to 1.74 mM on day 9. After the addition of the ciliate, the ammonium concentration averaged 0.47 mM higher than the bacteria only experiment. PN rose to 2.53 mM at day 1 and steadily decreased after the addition of *Cyclidium* to 0.73 mM on day 14.

Nitrogen-limited experiment:

Following the addition of *Cyclidium* on day 1, the bacteria were still in the early portion of their exponential growth phase and had optical density of 0.23. Yet, even as *Cyclidium* reached its highest abundance of 143,600/ml at day 5 (almost 5 times higher than the highest value of the carbon-limited experiment), the optical density continued to rise to 0.39 (Fig. 4a). Even at day 16, with 99,900 ciliates/ml, the optical density was 0.17, indicating that the ciliates were unable to effectively clear the bacteria, as they had in the carbon-limited experiment. The ciliate population also did not display the oscillating population dynamics displayed in the carbon-limited experiment and often observed in closed-system trophic dynamics experiments (Kaunzinger and Morin 1998). Instead, ciliate population peaked at day 5 and then stabilized at ~100,000 cells/ml at day 8, and remained at that abundance through the conclusion of the experiment at day 16. A visual inspection of the cells under a dissecting microscope showed the cells to be relatively inactive, compared to their usual “swarming” behavior.

Throughout the experiment, there was no witnessed production of DOC due to the grazing activity of *Cyclidium*. Alternatively, the presence of *Cyclidium* resulted in 2 fold decrease in DOC concentration compared to the bacteria-only experiment (Fig. 4b). Between day 2 and day 3, for example, as the bacteria were still approaching stationary growth in the bacteria-only trial, the DOC decreased 2.75 mM in the bacteria-only trial and 8.16 mM in the bacteria + ciliate trial, yet the POC rose less dramatically, 0.90 mM and 1.21 mM, respectively. From day 3 to day 16, DOC continued to decline an additional 11.42 mM to a final concentration of 6.46 mM (more then three times this amount remained in the bacteria-only experiment).

POC concentration steadily increased following *Cyclidium* addition to 6 mM at day 5, when the ciliate population reached its highest abundance (143,600/ml). The POC then hovered around this concentration for the following week, before dropping from 5.46 to 4.05 mM between day 12 and 16, yet the ciliate population remained nearly the same from day 8 to 16.

The C:N ratio essentially followed the same dynamics as the bacteria-only trial through day 3, rising to 9.3. C:N continued to rise to 13.6 at day 5, however, then decreased to the bacteria-only value of 9.7 at day 8 and remained constant at ~10 for the remainder of the experiment (Fig. 4d).

The dissolved nitrogen concentration was immediately drawn down to 0.069 mM (0.005 mM of which was ammonia) at day 1 (Fig. 4c). When the *Cyclidium* were in exponential growth at day 3, there was a pulse of dissolved nitrogen, 0.17 mM (0.010 mm of which was ammonia). For the rest of the experiment TDN, fluctuated between 0.068 and 0.15 mM, with ammonia averaging ~0.006 mM. PN rose to 0.49 mM,

decreased slightly while the *Cyclidium* were in logarithmic growth (day 1-5) with an average of 0.44 mM and then pulsed from day 6 to 12 with an average of 0.53 mM, before declining to 0.42 mM at the conclusion of the experiment on day 16.

DISCUSSION

The conversion of DOM to bacterial biomass has been studied in many natural aquatic and laboratory systems, and has resulted in numerous published values of yield or growth efficiency (Del Giorgio and Cole 1998a; Payne 1970). The extent to which limitation of either labile carbon or nitrogen impacts growth efficiency and rate of remineralization is less studied. In this study, we found that the percentage of organic carbon remineralized was nearly the same after two weeks under either carbon or nitrogen limitation. The addition of a bactivorous protozoan increased the extent of organic carbon remineralization from 38% to 73% in carbon-limited and 32% to 71% in nitrogen-limited systems.

Changes in bulk organic carbon and nitrogen pools

The results of our carbon-limited bacteria-only experiment are generally similar to those of Ogawa (2001) and Gruber et al (2006), studies that focused on the production of dissolved organic matter and used glucose as the sole labile DOC source. Ogawa (2001) used a natural, marine bacterial assemblage as innoculum and Gruber et al. (2006) used a single bacterial strain, *Pseudomonas chlororaphis*. After two days in Ogawa's experiment, bacteria converted 7% of the initial glucose carbon to POC, 15% to other forms of DOC, and the remaining 78% was respired, for a bacterial growth efficiency of

8%. After two days in (Gruber et al. 2006), *P. chlororaphis* converted 31% of glucose carbon to POC, 5% to other forms of DOC, and the remaining 64% was presumably lost as CO_2, for a growth efficiency of 33%. After two days in the present carbon-limited experiment, *Vibrio sp.* –NAP converted 51% glucose carbon to POC, 11% into other forms of DOC, and the remaining 38% was presumably lost as CO_2 (Fig. 5), for a bacterial growth efficiency of 57% – a value typical of cultured bacteria. It should be noted that roughly 10% of the initial DOC remains throughout the carbon-limited experiments. It was determined in a similarly designed experiment (Gruber et al. 2006), that all the glucose DOC was rapidly utilized and this remaining 10% was refractory to the single strain of bacteria. Therefore, while there are still significant concentrations of DOC, it is not bioavailable to the bacteria, and hence growth is limited by labile carbon. It was also determined by electrospray ionization mass spectrometry (ESI-MS) that the small background of carbon in EDTA and nitriltriacetic acid is not bioavailable to *Vibrio sp.* NAP (Georgina Spyres, personal communication). A similar pattern emerged in the N-limited experiment. While the NH_4 concentrations declined to nearly undetectable concentrations, DON was produced from the initial NH_4 and remained at background concentrations of ~100 uM, but this was shown to not be available to *Vibrio sp*.-NAP. Therefore, both carbon and nitrogen limitation are defined in this study as limitation of labile carbon and nitrogen.

DOC continued to decrease in Ogawa's et al.'s (2001) experiment and after 365 days represented 5% of the initial glucose carbon. In Gruber et al. (2006), DOC decreased only slightly over days 2-36, reaching a final level of 3% of the initial glucose carbon. In the present carbon-limited bacteria-only experiment, DOC decreased slightly

over days 2-14 reaching a final level of 8% of the initial glucose carbon. It has been shown that bacteria growing on labile carbon, such as glucose, will convert 10% or less (Ogawa et al. 2001) into refractory compounds that are not easily accessible to bacteria – and this percentage of refractory dissolved organic carbon produced is almost the same in cultures that have protozoan grazers (Gruber et al. 2006). ESI-MS data showed that these compounds are produced mainly by bacteria, and not ciliates – although ciliates greatly impact the rate at which the carbon is remineralized.

Initially, our carbon-limited culture medium had a glucose C:NH_4-N ratio of 5.9 and a glucose C:PO_4 ratio of 2.6. The substrate C:N ratio was about the same and the substrate C:P ratio was much lower than typical values of ~5 and 50, respectively, for bacterial biomass (Sterner and Elser 2002). Dissolved ammonia concentrations were always in excess, and we also observed ammonium regeneration of 0.56 mM in the bacteria-only and 1.22 mM in the bacteria + ciliate trial between day 2 and day 14. We did not measure P concentration, but given the high initial P concentration and low C:P of the culture medium, we concluded that P also was not limiting. In contrast, the initial substrate glucose C:NH_4-N ratio in the nitrogen-limited experiment was 42 and the glucose C:PO_4 ratio was 5.1, making nitrogen the limiting nutrient. This was confirmed as the added NH_4 rapidly decreased to near undetectable levels after day 1 and no ammonia regeneration occurred throughout the experiment.

After two days in our nitrogen-limited experiment *Vibrio sp.* –NAP converted 10% glucose carbon to POC and 12% was presumably lost as CO_2 (Fig. 6). This growth efficiency of 42% is lower than the 57% calculated in the carbon-limited experiment. As

bacterial growth efficiency is inversely related to substrate C:N (Goldman et al. 1987), these BGE fall within the range expected.

Introduction of a small population of a bactiverous protozoan (*Cyclidium* sp.) after the bacteria had reached stationary growth in the carbon-limited experiment changed the distribution of carbon among the particulate, dissolved, and gaseous pools. The *Cyclidium* population size rapidly increased (Fig. 2a). While we did not measure the grazing rate directly, the size of the transients in DOC of 105 μM between day 6 and 7 and 44 μM between day 8 and 9 was consistent with published data on grazing rates. Assuming a bacterial carbon content of 5.8×10^{-9} μmol C/cell (70 fg C/cell) (Caron et al. 1991) and assuming between 20 and 88% of bacterial carbon content is released by ciliate grazing (Taylor et al. 1985), then the population of 21,000 ciliates/ml would have to graze at a rate of 41 to 173 bacteria/ciliate/h to account for the 105 μM increase in DOC. This range of grazing rates is within values reported in other studies (Hadas et al. 1998; Zubkov and Sleigh 1995).

The main effect of grazing on the bulk carbon pools in the carbon-limited experiment was to increase the amount of glucose–C converted to CO_2, at the expense of POC. The POC concentration steadily declined from day 2 to day 14 at an average of 0.59 mM C/day (Fig. 2b). DOC concentration also declined over this time interval, although at a much slower rate (average of 0.040 mM C/day). We assume the differences between the amounts of carbon originally added as glucose and subsequently measured as DOC plus POC represent carbon respired as CO_2 and lost from the system. The mass balance for nitrogen (added NH_4-N = particulate nitrogen + dissolved nitrogen), an element that should not have been lost from the system, showed that almost all nitrogen

was accounted for in the carbon-limited experiment and ~87% was accounted for in the nitrogen-limited experiment.

The most likely explanation for the increase in carbon conversion to CO_2 when the ciliate was present is the inefficiency of energy and carbon transfer between successive trophic levels. Typically, 10-20% of food energy is transferred between successive trophic levels (Odum 1971a). On day 14 in the carbon-limited experiment, after the population of *Cyclidium* had been at 18,700 to 31,300 ml^{-1} for 11 days (Fig. 2), the percentage of glucose-C present as POC was 30% of the bacteria-only value. On day 16 in the nitrogen-limited experiment, after the population of *Cyclidium* had been at 93,600 to 143,600 mil^{-1} for 13 days, the percentage of glucose-C present as POC was 32% of the bacteria-only value. These slightly higher percentages than those predicted by Odum (1971a) also agrees with Fenchel's hypothesis (1977) that protozoa are more efficient than organisms at higher trophic levels in converting food into biomass (therefore making their role in nutrient regeneration less than others suggested). As unicellular organisms, protozoa do not have to maintain energetically expensive organ systems. As further evidence of stimulation of carbon remineralization due to trophic inefficiency, we note that the primary divergences in bacteria-only and bacteria + protozoa respiration rates occurs while the ciliates are in their primary growth phase (days 2-5, Fig. 2a). At this point, the metabolic cost of converting bacterial cells to ciliate cells is responsible for the majority of the divergence in remineralization percentages.

The presence of *Cyclidium* did not change the C:N ratio of the particulate matter in the carbon-limited experiment; the ratio was almost invariant (between 4 and 4.2) with and without the protozoa present, indicating that nitrogen was not limiting. But

Cyclidium did have a marked impact on the C:N ratios in the N-limited experiment. On day 5, when *Cyclidium* peaked at its highest population density, 143,600/ml, the C:N also peaked at 13.4. Protozoa have higher C:N ratios than their bacterial prey (Caron 1991) and it is possible that the *Cyclidium* dominated the particulate pool, and hence spiked the C:N. It has also been shown (Gruber et al. 2006) that grazers increase the C:N of the particulate pool by releasing excess nitrogen from the bacteria into the dissolved phase. This type of dynamic between C and N has also been observed in sinking particulate matter (Olesen and Lundsgaard 1995) that ages and increases in C:N as it sinks and NH_4 is released. But, as NH_4 levels were extremely low in our nitrogen-limited experiment, this was not the case. Conversely, the PN increased over time in the nitrogen-limited experiment.

It is somewhat surprising that the *Cyclidium* population in the nitrogen-limited experiment was almost five times the size of that of the carbon-limiting experiment, especially as the bacteria-only POC in the nitrogen-limited experiment was less than half (41%) the carbon-limited POC. We offer two possible explanations for this large protist population. Bacteria grown on nitrogen-limited media are forced to maintain their C:N at higher values than those grown under carbon-limitation (Fig. 1c versus 3c). These higher C:N bacteria (~10 versus 4) may be more "palatable" for eukaryotic protozoan, such as *Cyclidium*, because their higher C:N may more closely resembles the C:N of *Cyclidium*. It is well known that bacteria have much lower C:N than organisms higher up the food chain (Sterner and Elser 2002). By consuming bacterial prey closer to their own C:N stoichiometry, *Cyclidium* may expend less energy metabolizing, converting, and excreting the excess nitrogen. A second possibility is that there may have been a higher

turnover of bacteria in the nitrogen-limited experiment, as initial DOC was doubled (to ensure nitrogen limitation). Due to the nitrogen limitation, grazing may have provided a slow, but constant, source of dissolved organic nitrogen that allowed bacteria to steadily maintain their abundance at high numbers. This would prevent the "boom and bust" dynamics seen when ciliates are exposed to a single pulse of bacteria and carbon and may also explain the lack of "swarming" behavior exhibited by ciliates in the nitrogen-limited experiment. This may also help at least partly to explain the nearly double POC concentrations seen in the bacteria + ciliate nitrogen limited experiment, as opposed to bacteria-only.

There are different hypothesis concerning the importance of protozoa in planktonic systems. Some hold that the small size and high specific metabolism of protozoans make them primary players in the remineralization (conversion from organic to inorganic form) of nutrients (Harrison 1980) and forge an important link between bacterial production and higher trophic levels (Porter et al. 1979). Another idea is that protozoa are more efficient than higher trophic levels in converting food into biomass, therefore making their role in nutrient regeneration less than might otherwise be expected (Fenchel 1977). These alternative views have given rise to arguments that protozoa constitute an important trophic "link" for the transfer of nutrients from bacteria to higher organisms, or they act as a "sink," or source of remineralized nutrients. This study shows that protozoa can take on different functions (both "link" and "sink") depending on the composition of nutrients available to the specific system. It may also provide some insight into the longstanding question of how protozoa cause the stimulation of remineralization of organic matter.

Table 4.1: Composition of the modified MBS media used in this study.

Chemical	Media conc. (M)
NaCl	3.08 E-01
$MgCl_2$	2.99 E-04
Na_2CO_3	4.00 E-06
$NaHCO_3$	4.60 E-05
$C_6H_9NO_6$ (nitrilotriacetic acid)	1.04 E-05
KOH	2.60 E-05
$MgSO_4$	2.41 E-05
$CaCl_2$	4.59 E-04
$(NH_4)_6Mo_7O_2$	1.50 E-09
$FeSO_4$	7.12 E-08
$C_{10}H_{14}N_2O_8Na_2$ (EDTA)	7.66 E-08
$ZnSO_4$	3.83 E-07
$FeSO_4$	1.80 E-07
$MnSO_4$	9.11 E-08
$CuSO_4$	1.60 E-08
$Co(NO_3)$	8.59 E-09
$Na_2B_4O_7$	4.72 E-09
$(NH_4)_2SO_4$	1.51 E-03 (C-limit) and 3.78 E-04 (N-limit)
K_2HPO_4	4.02 E-03
KH_2PO_4	2.20 E-03

Figure 4.1. *Vibrio sp*-NAP-only carbon-limitation experiment. (A) Averages ± SD of optical density over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) Concentration of nitrogen in dissolved and particulate phases. (D) C:N ratio of particulate matter.

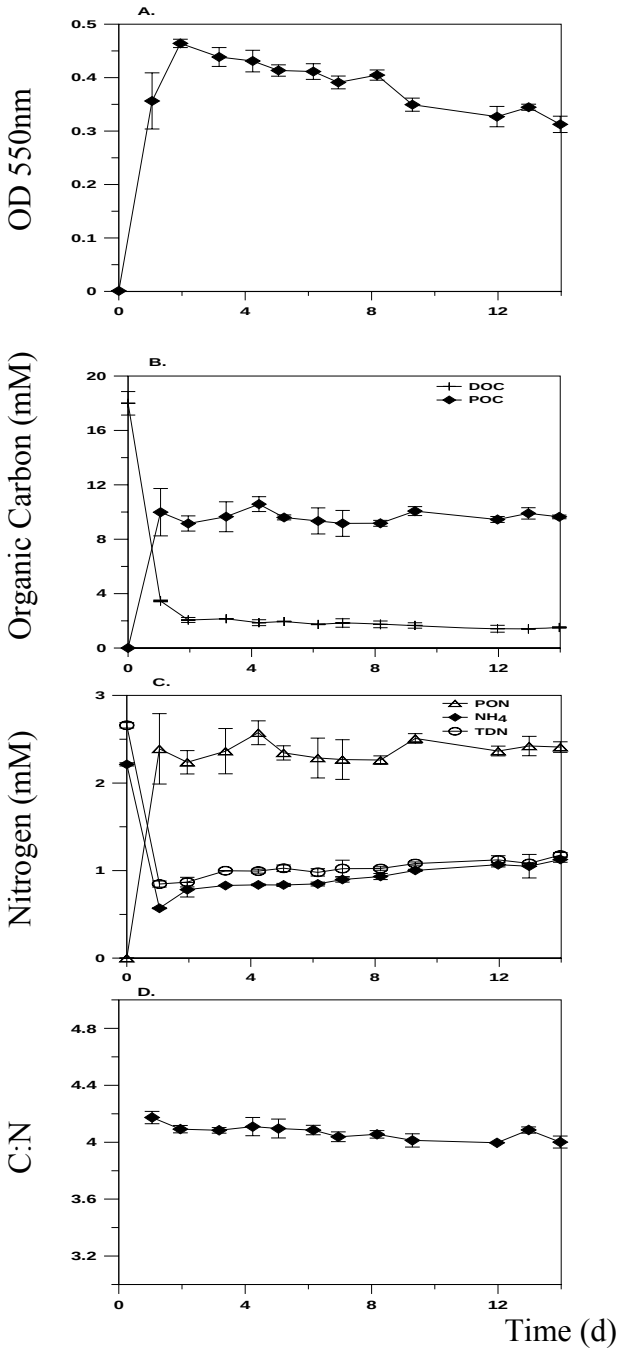


Figure 4.2. *Vibrio sp*-NAP plus *Cyclidium sp.* carbon-limitation experiment. (A) Averages ± SD of optical density and ciliate abundance over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) Concentration of nitrogen in dissolved and particulate phases. (D) C:N ratio of particulate matter.

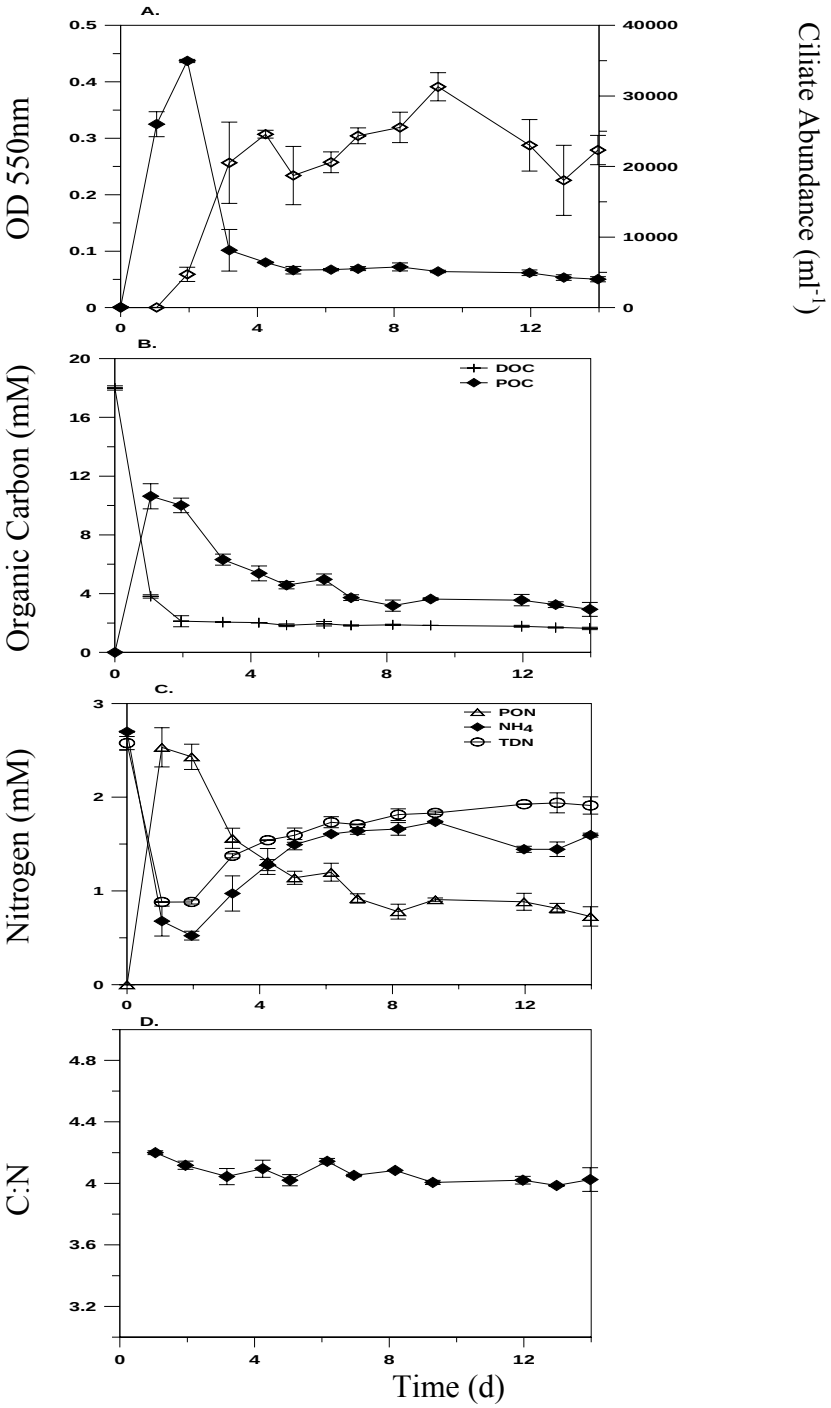


Figure 4.3. *Vibrio sp*-NAP-only nitrogen-limitation experiment. (A) Averages ± SD of optical density over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) Concentration of nitrogen in dissolved and particulate phases. (D) C:N ratio of particulate matter.

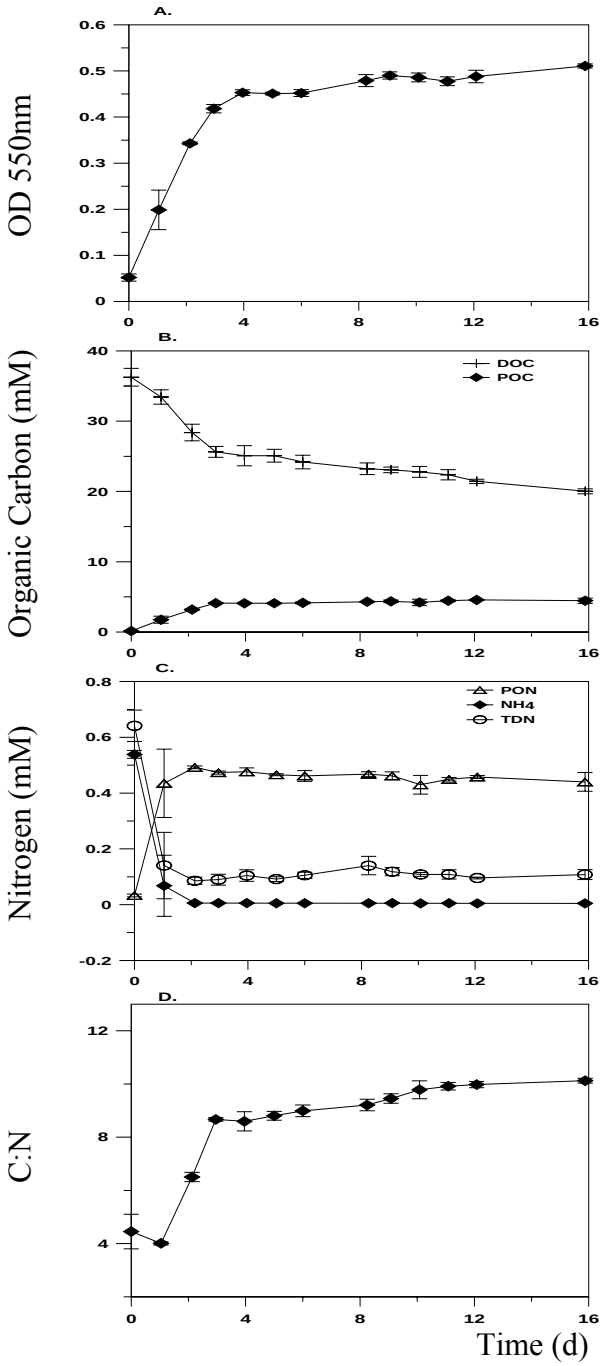


Figure 4.4. *Vibrio sp*-NAP plus *Cyclidium sp.* nitrogen-limitation experiment. (A) Averages ± SD of optical density and ciliate abundance over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) Concentration of nitrogen in dissolved and particulate phases. (D) C:N ratio of particulate matter.

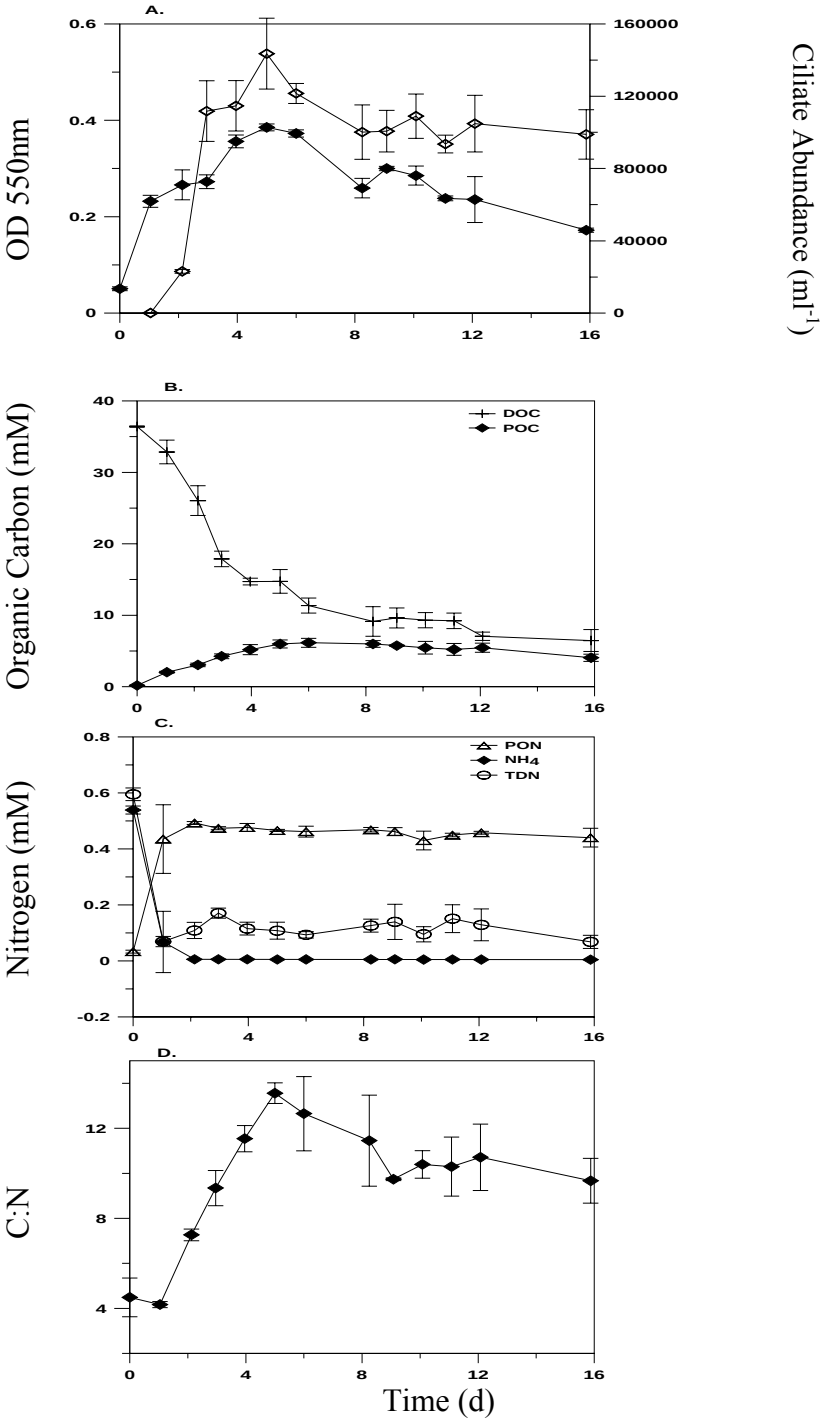


Figure 4.5. Carbon budgets for the *Vibrio sp*-NAP and *Vibrio sp*-NAP plus *Cyclidium* carbon-limited treatments, expressed as percentage of carbon originally added as glucose.

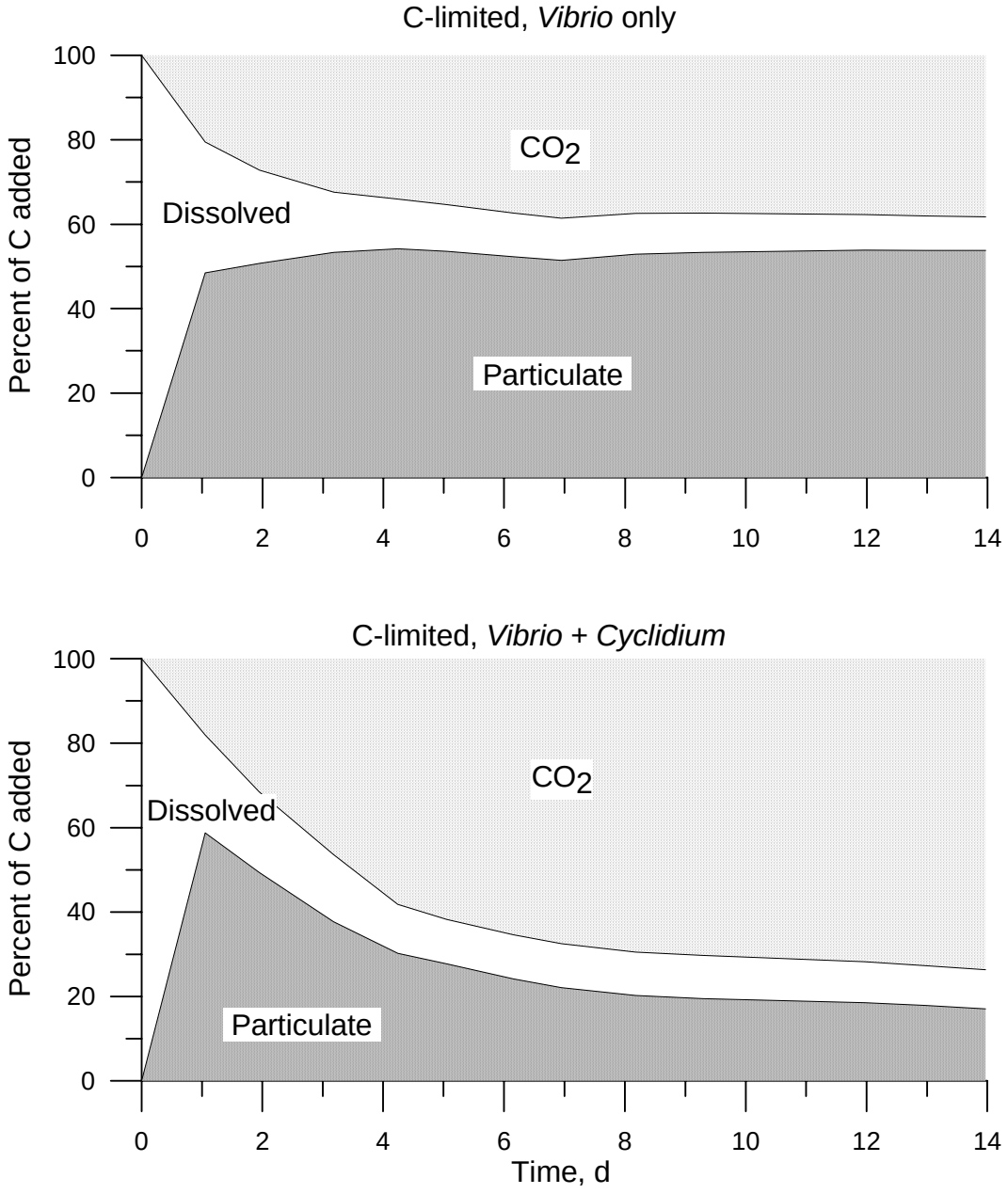
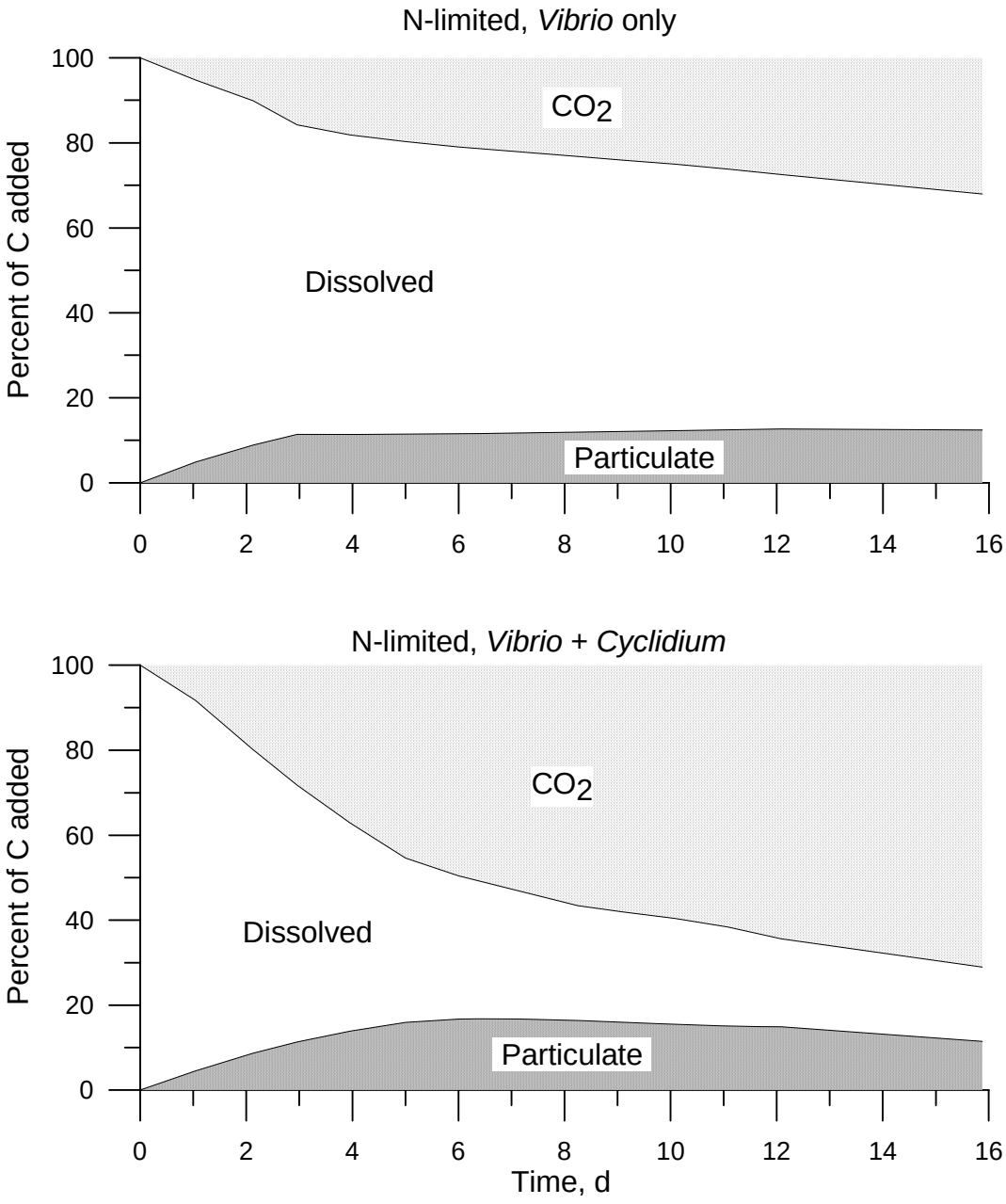


Figure 4.6. Carbon budgets for the *Vibrio sp*-NAP and *Vibrio sp*-NAP plus *Cyclidium* nitrogen-limited treatments, expressed as percentage of carbon originally added as glucose.



ACKNOWLEDGEMENTS

This work was supported by Biocomplexity grant OCE0120453 from the National Science Foundation.

We thank Charlotte Fuller and Ron Lauck for their assistance with nutrient analysis and Anna Mei for assistance counting protozoa and filtering samples.

REFERENCES

AZAM, F., D. C. SMITH, G. F. STEWARD, and A. HAGSTROM. 1993. Bacteria-organic matter coupling and its significance for oceanic carbon cycling. Microbial Ecology **28:** 167-179.

BARSDATE, R. J., R. T. PRENTKI, and T. FENCHEL. 1974. Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers. Oikos **25:** 239-251.

BENNER, R., and M. STROM. 1993. A critical evaluation of the analytical blank associated with DOC measurements by high-temperature catalytic oxidation. Mar. Chem. **41:** 153-160.

CARON, D. A. 1991. Evolving Role of Protozoa in Aquatic Nutrient Cycles, p. 387-414. *In* P. C. Reid, C. M. Turley and P. H. Burkill [eds.], Protozoa and Their Role in Marine Processes. Ecological Sciences. Springer-Verlag.

CARON, D. A., E. L. LIM, G. MICELI, J. B. WATERBURY, and F. W. VALOIS. 1991. Grazing and utilization of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory cultures and a coastal plankton community. Mar. Ecol. Prog. Ser. **76:** 205-217.

COLE, J. J. 1999. Aquatic microbiology for ecosystem scientists: New and recycled paradigms in ecological microbiology. Ecosystems **2:** 215-225.

DEL GIORGIO, P. A., and J. J. COLE. 1998. Bacterial growth efficiency in natural aquatic systems. Annu. Rev. Ecol. Syst. **29:** 503-541.

DUCKLOW, H. W. 1983. Production and fate of bacteria in the oceans. Bioscience **33:** 494-501.

FALKOWSKI, P. G., E. A. LAWS, R. T. BARBER, and J. W. MURRAY. 2003. Phytoplankton and their role in Primary, New, and Export Production., p. 99-121. *In* M. Fasham [ed.], Ocean Biogeochemistry: The Role of the Ocean Carbon Cycle in Global Change. Global Change – The IGBP Series.

FENCHEL, T. 1977. The significance of bacterivorous protozoa in the microbial community of detrital particles, p. 529-544. *In* J. Cairns [ed.], Aquatic Microbial Communities. Garland Publishing, Inc.

---. 1987. Ecology of Protozoa. Science Tech Publishers.

FENCHEL, T., and P. HARRISON. 1976. The significance of bacterial grazing and mineral decomposition of particulate detritus, p. 285-299. *In* J. M. Anderson and A. Macfadyen [eds.], The role of Terrestrial and Aquatic Organisms in Decomposition Processes. Blackwell Scientific Publications.

GOLDMAN, J. C. 1984. Conceptual role for microaggregates in pelagic waters. Bull. Mar. Sci. **35:** 462-476.

GOLDMAN, J. C., D. A. CARON, and M. A. DENNETT. 1987. Regulation of gross growth efficiency and ammonium regeneration in bacteria by substrate C:N ratio. Limnol. Oceanogr. **32:** 1239-1252.

GRUBER, D. F., J. P. SIMJOUW, S. P. SEITZINGER, and G. L. TAGHON. 2006. Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. Applied and Environmental Microbiology **72:** 4184-4191.

HADAS, O., N. MALINSKY-RUSHANSKY, R. PINKAS, and T. E. CAPPENBERG. 1998. Grazing on autotrophic and heterotrophic picoplankton by ciliates isolated from Lake Kinneret, Israel. Journal of Plankton Research **20:** 1435-1448.

HARRISON, W. G. 1980. Nutrient regeneration and primary production in the sea, p. 433-460. *In* P. G. Falkowski [ed.], Primary Productivity in the Sea. Brookhaven Symposia in Biology. Plenum Press.

JOHANNES, R. E. 1964. Influence of marine protozoa on nutrient regeneration. Univ.Georgia Marine Institute **85:** 434-442.

JUMARS, P. A., D. L. PENRY, J. A. BAROSS, M. J. PERRY, and B. W. FROST. 1989. Closing the microbial loop: dissolved carbon pathway to heterotrophic bacteria from incomplete ingestion, digestion and sbsorption in animals. Deep-Sea Res. **36:** 483-495.

KAUNZINGER, C. M. K., and P. J. MORIN. 1998. Productivity controls food-chain properties in microbial communities. Nature **395:** 495-497.

MATZ, C., and K. JURGENS. 2005. High motility reduces grazing mortality of planktonic bacteria. Applied and Environmental Microbiology **71:** 921-929.

MORTLOCK, R. A., and P. N. FROELICH. 1989. A simple method for the rapid determinatoin of biogenic opal in pelagic marine sediments. Deep-Sea Res. **36:** 1415-1426.

ODUM, E. P. 1971. Fundamentals of ecology, 3rd ed. ed. W. B. Saunders.

OGAWA, H., Y. AMAGAI, I. KOIKE, K. KAISER, and R. BENNER. 2001. Production of refractory dissolved organic matter by bacteria. Science **292:** 917-920.

OLESEN, M., and C. LUNDSGAARD. 1995. Seasonal Sedimentation of Autochthonous Material from the Euphotic Zone of a Coastal System. Estuarine Coastal and Shelf Science **41:** 475-490.

PAYNE, W. J. 1970. Energy yields and growth of heterotrophs. Annu Rev Microbiol. **24:** 17-52.

PORTER, K. G., M. L. PACE, and J. F. BATTEY. 1979. Ciliate protozoans as links in freshwater planktonic food chains. Nature **277:** 563-565.

SCHMIDT, J. L., J. W. DEMING, P. A. JUMARS, and R. G. KEIL. 1998. Constancy of bacterial abundance in surficial marine sediments. Limnology and Oceanography **43:** 976-982.

SEITZINGER, S. P., and R. W. SANDERS. 1997. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication. Mar. Ecol. Prog. Ser. **159:** 1-12.

SHARP, J. H., R. BENNER, L. BENNETT, C. A. CARLSON, R. DOW, and S. E. FITZWATER. 1993. Re-evaluation of high temperature combustion and chemical oxidation measurements of dissolved organic carbon in seawater. Limnol. Oceanogr. **38:** 1774-1782.

STEELE, J. H. 1974. The Structure of Marine Ecosystems. Harvard Univ. Press.

STERNER, R. W., and J. J. ELSER. 2002. Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere.

STROM, S. L., R. BENNER, S. ZIEGLER, and M. J. DAGG. 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol. Oceanogr. **42:** 1364-1374.

TAYLOR, G. T., R. ITURRIAGA, and C. W. SULLIVAN. 1985. Interactions of bactivorous grazers and heterotrophic bacteria with dissolved organic matter. Mar. Ecol. Prog. Ser. **23:** 129-141.

TSO, S. F., and G. L. TAGHON. 1999. Factors affecting predation by Cyclidium sp. and Euplotes sp. on PAH-degrading and nondegrading bacteria. Microbial Ecology **37:** 3-12.

WHITMAN, W. B., D. C. COLEMAN, and W. J. WIEBE. 1998. Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences of the United States of America **95:** 6578-6583.

ZUBKOV, M. V., and M. A. SLEIGH. 1995. Ingestion and assimilation by marine protists fed on bacteria labeled with radioactive thymidine and leucine estimated without separating predator and prey. Microb. Ecol. **30:** 157-170.

CHAPTER 5

Dynamics and Characterization of Refractory Dissolved Organic Matter Produced by a Pure Bacterial Culture in an Experimental Predator-Prey System

(The majority of this chapter is published in Gruber et al. (2006))

ABSTRACT

We studied the effects of a bacterium (*Pseudomonas chlororaphis*) and a bactivorous protozoan (*Uronema* sp.) on transformations of labile dissolved organic carbon (DOC). In 36-day time series experiments, bacteria were grown on glucose both with and without protozoa. We measured bulk organic carbon pools and used electrospray ionization mass spectrometry (ESI-MS) to characterize dissolved organic matter on a molecular level. Bacteria rapidly utilized glucose, depleting it to non-detectable levels and producing new DOC compounds of higher molecular weight within two days. Some of these new compounds, representing 3-5% of the initial glucose-C, were refractory and persisted for over a month. Other new compounds were produced and subsequently used by bacteria during the lag and exponential growth phases, pointing to a dynamic cycling of organic compounds. Grazers caused a temporary spike in the DOC concentration, consisting of labile compounds subsequently utilized by the bacteria. Grazing did not increase the complexity of the DOC pool already established by the bacteria but did continually decrease the particulate organic carbon pool and expedited the conversion of glucose-C to CO_2. After 36 days, 29% of initial glucose-C remained in pure bacteria cultures, while only 6% remained in cultures where a grazer was present. In this study the bacteria were the primary shapers of the complex DOC continuum, suggesting higher trophic levels possibly have less of an impact on the qualitative composition of DOC than previously assumed.

INTRODUCTION

Dissolved organic matter (DOM) comprises the largest, yet least characterized, reservoir of reduced organic carbon in aquatic systems, estimated at 700 x 10^{15} g C (Hedges et al. 2000). DOM is important in the carbon and nitrogen cycles, the scavenging and solubilization of trace contaminants, and biogeochemical cycles of other elements (Baines and Pace 1991; Jumars et al. 1989). Heterotrophic bacteria process and reprocess some of this DOM (Azam and Hodson 1977), channeling about one-half of oceanic primary production through the microbial loop (Cole et al. 1988).

The role of bacteria on the rate and extent of DOM mineralization and their production of (semi-) refractory DOM have received less attention. Some studies indicate that bacteria produce refractory DOM that is resistant to further utilization (Brophy and Carlson 1989; Heissenberger and Herndl 1994; Stoderegger and Herndl 1998; Taylor et al. 1985). Ogawa et al. showed that a natural inoculum of marine bacteria (and undoubtedly nanoflagellates and viruses) growing on labile compounds (glucose and glutamate) produce new DOM compounds that appear to be refractory for at least a year. It was not known if a single strain of bacteria could produce similar refractory material. Bacterioplankton can also be a source of photoreactive CDOM that is refractory to a natural bacteria assemblage following photochemical alteration (2004). What kinds and how many different compounds make up the refractory DOM pool are largely unknown.

In aquatic ecosystems, bacteria are consumed by protozoa and other zooplankton, which in turn release DOM as colloidal matter (Koike et al. 1990; Tranvik 1994) and macromolecular organic complexes (Nagata and Kirchman 1992b). A substantial portion (>50%) of primary and bacterial production can be consumed by a single class of

protozoa, the Ciliata (Fenchel 1987). Therefore, ciliates can act as trophic links, nutrient regenerators and DOM producers (Strom et al. 1997) – roles often overlooked in traditional food webs. Little is known about the effects of additional trophic levels on the production and composition of refractory DOM. Nagata and Kirchman (1992a) suggested that the release of DOM by protozoa is potentially important in aquatic food webs and nutrient cycles. Kujawinski et al (2004) used electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry (ESI FT ICR MS) to identify 80 new DOM compounds produced when a protozoan grazed on bacteria.

Here, we analyzed the effects on DOM dynamics of a pure bacteria culture alone and when grazed on by a ciliate. We used conventional, bulk analysis to examine how trophic structure influenced DOC transformations. We used electrospray ionization mass spectrometry (ESI-MS) for molecular level characterization of compounds produced during bacterial growth and grazing. ESI-MS has been used for identification and quantification of specific compounds (Hua et al. 1996; Loo and Ogorzalek Loo 1997; Poon 1997; Saito et al. 2004). Terrestrial and marine DOM pools have been characterized by ESI-MS (Kim et al. 2003; Koch et al. 2005; Kujawinski et al. 2002; Leenheer et al. 2001). ESI-MS has also been used to gain insights into changes in DOM due to protozoan grazing (Kujawinski et al. 2004), for characterization of DOM in rainwater (Seitzinger et al. 2003), and to discriminate between possible refractory and labile DOM compounds in freshwater samples (Seitzinger et al. 2005).

MATERIALS AND METHODS

Experimental Setup

Production and dynamics of DOM were examined using glucose as the sole utilizable carbon source (10 mM-C initial concentration). There were three treatments: sterile control, bacteria only, and bacteria plus ciliate. Experiments took place in 125-ml flasks (baked at 500°C for 4 h before use) containing 25 ml sterile, low carbon (62.8 µM-C from nitrilotriacetic acid, 0.8 µM-C from Na_2EDTA) modified Mineral Basal Solution (MBS). In the modified MBS ammonia and phosphate concentrations were five times lower, with carbonate buffer concentration 50 times lower and metal concentrations a hundred times lower.

All flasks (except the sterile controls) were initially inoculated with the bacterium, *Pseudomonas chlororaphis*. Initial samples were taken directly after inoculation (day 0). Half of the remaining flasks were inoculated with the bactivorous protozoan *Uronema* sp. after 3 days, when the bacteria had reached stationary growth. Flasks were capped with aluminum foil to maintain sterility but permit air exchange. All cultures were incubated in the dark on a shaker table at 50 rpm and 20°C. Samples were taken at day 0, 1, 2, 3, 5, 6, 8, 9, 10, 13, 14, 17, 20, and 36. Triplicate sacrificial flasks were used for each time point to prevent contamination from repeated sampling of the same flasks.

Bacterial Strain

We used a single freshwater heterotrophic bacterial strain, *Pseudomonas chlororaphis* (ATCC#17418), a gamma proteobacterium (Stanier et al. 1966). Flasks were inoculated with 10µl of triple washed cells harvested from cultures at the end of log growth phase. At each time point, abundance was estimated by measuring the optical density (1 cm pathlength) at 550 nm of a 1 ml sample.

Grazer

We used a freshwater ciliate, *Uronema* sp. (Scuticociliatida), as the bactivorous protozoan grazer. This ciliate is about 20µm in longest dimension. Prior to the experiment, ciliates were maintained in culture using protozoan pellets (Carolina Biological Supply) as a nutrient source. Before being introduced into *P. chlororaphis* cultures, ciliates were filtered through a polyester mesh (41 µm openings) to remove detritus. Ciliates in the <41 µm fraction were triple rinsed with low carbon MBS, then concentrated onto a 5 µm Nuclepore filter and resuspended at a final density of 1,600 cells/ml. Flasks were inoculated with 25 µl of concentrated ciliate suspension (~40 cells). At each time point, abundance was determined by direct counts of a 1 ml sample using a Sedgewick Rafter counting cell.

Bulk chemical analysis

At each time point, samples were taken from each flask for particulate organic carbon (POC), particulate nitrogen (PN), DOC, total dissolved nitrogen (TDN), and dissolved inorganic nitrogen (DIN).

For the POC and PN analysis, 23 ml of sample was filtered through a precombusted (500°C for 4 h) 25 mm diameter Whatman GF/F filter. The filter was dried and analyzed using a Carlo Erba Instruments NA 1500 Series 2 elemental analyzer (Mortlock and Froelich 1989). Results for POC and PN were corrected based on filters processed from the sterile controls. The filtrate containing the dissolved fraction was transferred to acid-washed capped polypropylene vials and stored frozen (-20°C) in the dark until needed for all other analyses.

DOC and TDN were measured using a Shimadzu 5000 high temperature combustion analyzer for DOC (Benner and Strom 1993; Sharp et al. 1993) with an inline NOx detector (from Antek Model 7000 Total N Analyzer) for TDN analysis (Seitzinger and Sanders 1997). DIN was measured using a Lachat, Inc. auto analyzer (ammonium, QuikChem 31-107-06-1-A; nitrate plus nitrite, QuikChem 31-107-04-1-A). DON was determined as the difference between TDN and DIN.

Compound-level analysis

All analyses were performed using an Agilent 1100 liquid chromatography mass spectrometer equipped with an ESI source and a quadrupole mass selective detector, ESI-MS (Seitzinger et al. 2005; Seitzinger et al. 2003). An autosampler introduced 20 µl of individual samples into the LC mobile phase that went directly to the ESI source (no LC column was used). The mobile phase was 50:50 (v/v) methanol: 0.05% formic acid in DI water (pH 3.5) with a flow rate of 0.220 ml min^{-1}. Samples were analyzed in both the positive and negative ionization mode of the ESI source. The flow of the drying gas (N_2 at 350°C) in the source was 10 ml/min (24 psig). The ions were introduced in the mass spectrometer through a capillary (3000 V); the fragmentor voltage was 40V. The quadrupole temperature was 99°C and the quadrupole voltage was kept at the default voltage of 0V for both ionization modes. The scanned mass range was 50 to 1000 m/z.

For each DOM sample, six replicate injections were made to facilitate a robust interpretation of the data. The raw mass spectra were recorded on Agilent software (Chemstation version A.7.01) and then imported into ACCESS (Microsoft) for instrument blank correction and statistical analysis as described in Seitzinger et al. (Seitzinger et al. 2005; Seitzinger et al. 2003) and then into EXCEL (Microsoft) for data

interpretation. To determine the background spectra due to the media and bacteria inoculation, samples collected immediately after bacteria were added to the media (day 0 in the experiments) were analyzed in positive and negative ionization ESI modes. These background spectra were used to correct the subsequent time series ESI spectra. Glucose ions were identified in the background spectra (day 0), based on glucose standards in a media matrix, to track the glucose utilization in subsequent uncorrected time series ESI spectra.

ESI is a soft ionization technique and fragmentation of the molecular ions does not occur under standard instrument conditions (Loo and Ogorzalek Loo 1997). Studies using ESI coupled with high resolution MS (Fourier transformed ion cyclotron resonance MS) on terrestrial DOM and humic and fulvic acids also concluded that fragmentation was not a factor in the analysis (Kujawinski et al. 2002; Stenson et al. 2003). Previous analysis of DOM from aquatic systems have shown that DOM compounds are mostly detected as singly charged ions (Kujawinski et al. 2002; Stenson et al. 2002) with humic and fulvic material and with C_{18} extracted marine DOM (Koch et al. 2005). In addition, organic compound standards were detected in a singly charged state (our unpublished data). Therefore, in the current study we assume that the detected mass to charge ratios (m/z) can be considered as a good proxy for the molecular weight of the compounds.

Interpretation of ESI-MS data requires caution regarding the limitations as indicated above, as well as the potential detection of several different compounds within the one unit mass resolution of the ESI-MS used in this study. Different compounds have different ion efficiencies resulting in different ion abundances in the ESI-MS spectra unrelated to their individual concentration in solution. Ion abundance cannot be used as a

direct measure of concentration. Also, some DOM compounds in a sample may not be effectively ionized by the ESI technique, due to the polarity of the compound and the sample matrix, and would therefore not appear in the mass spectrum (Kujawinski et al. 2002).

RESULTS

Bulk DOC, POC and population dynamics

Bacteria-only experiment

Glucose was rapidly utilized during the exponential growth phase of the bacteria as indicated by changes in optical density and DOC concentration (Fig. 1). The initial decrease in DOC concentration from 10 mM to around 440 μM coincided with the approximate 10-fold increase in optical density by day 2. The DOC concentration decreased slightly after the initial steep DOC decline but stayed about 250 μM higher than the media background (67 μM) for the remainder of the experiment.

The experiment results were divided into 3 stages based on the bacterial abundance and the DOC trends: the lag phase where the bacteria abundance was low and the DOC was high (day 1, Stage I); the exponential growth phase where the bacteria abundance increased to a maximum and a steep decrease in DOC was observed (day 2, Stage II); and the stationary growth phase where a slight decrease in both bacteria abundance and DOC concentration occurred (day 3-36, Stage III). If first order kinetics is assumed for the utilization of the DOC, the decay constant during day 1 (Stage I) was 0.028 per day before increasing to 3.1 ± 0.1 per day in Stage II. In Stage III the decay constant decreased to 0.013 ± 0.002 per day, a 250 fold reduction.

The POC concentration rapidly increased to 3.1 mM by day 2, then remained constant during the rest of the experiment (Fig. 1). The C:N ratio of the particulate matter was quite uniform throughout the experiment (data not shown), with an overall mean value of 4.5 (S.D. = 0.9).

Dissolved nitrogen concentration remained high throughout the experiment (data not shown). Ammonium concentration on day 1 was 3150 μM, which compared well with the expected media concentration of 3000 μM. Ammonium decreased to about 2800 μM on day 2 and remained there for the remainder of the experiment. Dissolved N was present essentially exclusively as ammonium; DON was below detection and nitrite plus nitrate were about 1 μM.

Bacteria plus bactivorous protozoan experiment

Following the introduction of *Uronema* just after day 3 samples were taken, the optical density of the cultures decreased from 0.2 on day 5 to 0.006 on day 8. *Uronema* abundance increased from 6 to 5080 per ml in the same time period (Fig. 2). From day 8 onwards, the optical density of the remaining cultures was only slightly above the sterile control values. Ciliate abundance varied cyclically from day 8 through day 20, from highs of 9000-10600 per ml to lows of 5600-6000 per ml (Fig. 2). Ciliates at these cell densities contribute negligibly to optical density (Gruber, Tuorto and Taghon, unpublished data). Ciliate abundance declined sharply to 28 per ml between day 20 and day 36.

DOM was released during the increase in ciliate and decrease in bacteria abundance between day 5 and day 8 (Fig. 2). The maximum DOC increase was between day 5 and day 6 from 430 to 690 μM. During this period *Uronema* increased from 8 to

500 per ml, while OD decreased from 0.2 to 0.07. After this transient, DOC concentration decreased to an average value of 315 μM for the remainder of the experiment, a level slightly lower, by 53 μM, than the DOC concentration for the bacteria-only experiment during the same time period.

POC concentration steadily decreased following *Uronema* addition, from a value of 3100 μM at the end of the exponential growth phase of *P. chlororaphis*, to values of 620-1000 μM during the interval of greatest *Uronema* abundance (days 8-20), to a final value of 240 μM on day 36 (Fig. 2). Once *Uronema* was established in the cultures, the C:N ratio of the particulate matter gradually increased from its pre-*Uronema* value of 4.5 to 6.2 on day 36 (data not shown).

Dissolved nitrogen concentration again remained high throughout the experiment (data not shown). Ammonium concentration reached its lowest level of 2200 μM on day 5, then gradually increased to 2800 μM by day 20. DON was below detection and nitrate plus nitrate concentration was less than 1 μM.

Electrospray Ionization Mass Spectrometry Characterization

Characterization of DOM produced by Bacteria

The loss of glucose ions to an undetectable level in both positive and negative ESI spectra after day 2 also indicates that glucose was rapidly utilized during the exponential growth phase of the bacteria.

The ESI spectra changed from day 0 to day 1 and again from day 1 to day 2; it then remained relatively constant. New m/z values were detected at day 1 in both positive (118 masses) and negative (100 masses) ionization mode that were not there on day 0.

Most of these new detected masses (80 in positive mode, 81 in negative mode) did not occur beyond day 1. The remaining new m/z values identified at day 1 were present throughout the experiment. On day 2 additional new masses (45 in positive mode, 27 in negative) were detected, and all of these remained present throughout the experiment (Fig. 3).

After identifying the new m/z values in the ESI spectra from the different growth stages, the molecular weight distribution of these masses was examined. The m/z values were grouped into four categories (50-250, 251-500, 501-750, and 751-1000 m/z) to simplify the interpretation (Fig. 4). In positive ionization mode, 84% of the masses detected on day 1 only had an m/z lower than 500 (Fig. 4). This distribution is comparable to masses detected on day 1 that remained present through day 36 (Fig. 4). In contrast with day 1, only 38% of the new masses detected on day 2 had m/z less than 500 (Fig. 4).

There was a shift towards higher m/z values for compounds detected in negative ionization mode compared to positive mode (Fig. 4). In negative ionization mode, about 50% of the m/z values in the day 1 only and day 1 through day 36 distribution fall in the lower than 500 m/z categories (Fig. 4). This change is mostly due to the increase in the number of masses in the 751-1000 m/z category. For the day 2 through day 36 distribution, 85% of the masses are greater than 500 m/z, with the majority greater than 750 m/z (Fig. 4).

Characterization of DOM following bactivorous protozoan addition

Additional new compounds were detected three days after *Uronema* addition that were not present in the bacteria-only treatment (Fig. 5). No new m/z values due to

Uronema inoculation were detected in either ionization mode on day 5, 48 h after inoculation. For subsequent samples most new masses were identified in the positive ionization mode. At day 6, 30 new masses were detected all with a mass <400 m/z. In the day 8 sample, 15 new compounds with masses <400 m/z were identified. For day 9 when the transient DOC pulse decreased to the same DOC concentration as for the bacteria-only treatment, only one new compound was detected. Of the new m/z identified in the positive ionization mode on day 6, day 8, and day 9, ten occurred on day 6 and day 8 and one compound remained in all three samples (Fig. 5). Thus a total of 35 new compounds were detected in the grazer treatment in addition to the 132 compounds already present in the bacteria only spectra, an increase of ~27%.

In the negative mode, only three new compounds were detected on day 6, and two of those had m/z >400. Two new compounds were identified on day 8. In the subsequent sample after the DOC increase due to the ciliate addition (day 9), no new masses were detected. A total of four new compounds were identified in day 6 and day 8 samples of which one remained present both days (Fig. 5).

DISCUSSION

The conversion of DOM to bacterial biomass has been studied in many natural aquatic and laboratory systems, and has resulted in many published values of yield or growth efficiency (Del Giorgio and Cole 1998b; Payne 1970). The rate and extent of the transformation of labile DOM to refractory compounds is less studied, but is at the heart of the issue of carbon storage in biologically unavailable pools. In this study we found that a pure culture of bacteria rapidly produced a complex pool of DOM from a simple

labile compound. Many of the new DOM compounds (49-68%) were themselves used within one day, while the remainder formed a small refractory DOM pool (3% of the initial carbon). The addition of a bactivorous protozoan increased the extent of organic carbon remineralization from 71% to 94%, but had no effect on the complexity of the DOM pool.

Changes in bulk organic carbon and nitrogen pools

The results of our bacteria-only experiment are generally similar to those of Ogawa et al. (Ogawa et al. 2001), although there were substantial differences in experimental conditions. For example, they used glucose as a labile DOM source, but at much lower initial concentration than we did (208 μM-C vs. 10000 μM-C). They also used a natural, marine bacterial assemblage as inoculum while we used an isolated, freshwater strain. In both experiments, glucose was undetectable within two days. After two days in their experiment bacteria converted 7% of the initial glucose carbon to POC, 15% to other forms of DOC, and the remaining 78% was respired, for a growth efficiency (production/assimilation) of 8%. After two days in our experiment *P. chlororaphis* converted 31% of glucose carbon to POC, 5% to other forms of DOC, and the remaining 64% was presumably lost as CO_2 (Fig. 6). This is a growth efficiency of 33%, a value typical for cultured bacteria. DOC continued to decrease in Ogawa et al.'s experiment (Ogawa et al. 2001), and after 365 days represented 5% of the initial glucose carbon. They concluded this DOC was refractory to further bacterial transformation. In our experiment, DOC decreased only slightly over days 2-36, reaching a final level of 3% of the initial glucose carbon.

Introduction of a small population of the bactivorous protozoan *(Uronema sp.)* after the bacteria had reached stationary growth changed the distribution of carbon among the particulate, dissolved, and gaseous pools. The *Uronema* population rapidly increased (Fig. 2A). Although we did not directly measure grazing rate, the size of the transient increase in DOC between days 5 and 6 (Fig. 2B) is consistent with published data on ciliate grazing rates. Assuming a bacterial carbon content of 5.8×10^{-9} μmole C/cell [70 fg C/cell,(Caron et al. 1991)] and assuming between 20-88% of bacterial carbon is released by ciliate grazing (Taylor et al. 1985), then the population of 500 ciliates/ml would have had to graze at a rate of 4300-18000 bacteria/ciliate/h to account for the 260 μM increase in DOC (also assuming no bacterial uptake of the released DOC). While high, this estimated range of grazing rates is within values reported in other studies (Zubkov and Sleigh 1995).

The main effect of grazing on the bulk carbon pools was to increase the amount of glucose-C converted to CO_2 (Fig. 6). POC concentration steadily declined from day 5 to day 20 at an average of 130 μM C/d (Fig. 2B). DOC concentration also declined over this time interval, although at a slower rate (average of 11 μM C/d). We assume that any difference between the amounts of carbon originally added as glucose and measured as DOC or POC represents carbon respired as CO_2 and lost from the system. If there were significant amounts of carbon in another pool that we did not measure, such as growth of bacteria on flask surfaces, then this assumption would be incorrect. The mass balance for nitrogen, an element that should not have been lost from the system, was excellent. We accounted for 94% of the added NH_4-N as particulate or dissolved nitrogen throughout

the incubation period. Thus, we conclude that wall growth was not significant and that calculating CO2 production by difference is justified.

The most likely explanation for the increase in carbon conversion to CO_2 is the well-known inefficiency of energy and carbon transfer between trophic levels. Typically, 10-20% of food energy is transferred between successive trophic levels (Odum 1971b). On day 20, after the population of *Uronema* had been at 5600 to 10600 per ml for 13 days (Fig. 2A), the percentage of glucose-C present as POC was only 22% of the bacteria-only value. By the end of the experiment there was 2600 μM POC in the bacteria-only flasks and only 9% of that, 240 μM POC, in the bacteria plus bactivorous protozoan flasks. Thus, our results are quite consistent with 10-20% trophic transfer efficiency.

A puzzling result is the continued, albeit slight, decline in DOC concentration in the grazer treatment. After the transient pulse had subsided by day 9, DOC concentration continued to decline from 340 μM to 260 μM by day 20 (Fig. 2B), while DOC was constant at 370 μM in the bacteria-only treatment (Fig. 1B). If the residual DOC present after *P. chlororaphis* had depleted all the glucose were refractory, then we would expect similar residual levels of DOC in both treatments. One possibility is uptake of DOC by *Uronema*. Protozoa can actively take up low molecular weight compounds across the cell membrane, and larger dissolved compounds by pinocytosis (Fenchel 1987). While such DOC uptake may be insufficient to meet the cell's metabolic needs, it represents a sink for DOC. The increase in DOC to a level comparable to the bacteria-only treatment by day 36 (Fig. 2B), after the *Uronema* population had declined precipitously (Fig. 2A), is consistent with this explanation.

The presence of *Uronema* led to an increase in the C:N of the particulate matter (Fig. 2C). The C:N ratio of the bacteria-only treatment was invariant at 4.5, indicating that N was not limiting (Fig. 1C). Protozoa have higher C:N than their bacterial prey (Caron 1991), so grazing can result in a release of the excess nitrogen from the particulate to the dissolved phase. The average NH_4 concentration in the grazer treatment was 2650 μM, compared to 2250 μM in the bacteria-only treatment, consistent with this explanation.

Formation of DOM compounds during bacterial growth

P. chlororaphis changed the DOC pool from a single labile compound (glucose) to a more complex pool. We detected ~200 labile and refractory compounds (masses) in this study. Detection of masses in the day 1 sample (118 in positive and 100 in negative ESI mode) that were not present on day 0 indicates production or release of compounds associated with the bacterial utilization of glucose. Of these new masses, a majority no longer appear later in the experiment (Fig. 4), which could indicate complete remineralization of these labile compounds, incorporation into biomass by *P. chlororaphis* during the exponential growth phase, or release as new compounds on day 2. The utilization of newly released compounds suggests a dynamic cycling of organic compounds by *P. chlororaphis* during the lag and exponential growth phases. Increasing the sampling frequency during these phases could give additional insight into production and consumption of DOM compounds by *P. chlororaphis*.

A number of new masses were identified in the day 1 and day 2 samples that remained present for the remainder of the experiment (Fig. 4). These new masses appeared to be compounds that were refractory to utilization by *P. chlororaphis* for the

duration of the experiment. These refractory compounds were likely responsible for the relatively constant DOC concentration from day 2 through day 36. A larger percentage of masses detected in negative ESI mode exhibited higher molecular weights (m/z values) compared to positive mode (Fig. 4). This indicates that *P. chlororaphis* produced more, high molecular weight compounds with acidic functional groups (e.g. carboxyl and amino groups) than compounds with basic functional groups (e.g. hydroxyl and amine groups) during day 1 and day 2.

Earlier studies indicated that bacteria can transform labile compounds to compounds that are resistant to further mineralization. This transformation resulted in an increase of the average molecular weight of the initial substrate DOC pool (Brophy and Carlson 1989; Heissenberger and Herndl 1994). Molecular weight was determined in these studies by size exclusion/gel filtration, which can only be used to infer a molecular weight range based on elution time. In our study we were able to observe a shift to higher molecular weight with a mass unit resolution within the 50-1000 m/z bin (the LMW-DOM pool). It is possible that the labile compounds released on day 1 were taken up, transformed into new compounds, and released on day 2 as refractory compounds. This scenario is supported by the shift towards higher molecular weight for masses detected in day 1 and day 2 samples (Fig. 4). However, the mechanisms responsible for this "transformation" are still poorly understood. Analysis of the elemental composition of the formed compounds using high-resolution mass spectrometry might help to elucidate differences in elemental composition as well as number of functional groups (Kim et al. 2003; Kujawinski et al. 2004; Stenson et al. 2003). Kujawinski et al. (Kujawinski et al. 2004) found in their study of biological DOM that the most common mass difference

could be explained by the addition of multiples of $–CH_2CH_2O$ although the underlying chemistry was not clear.

Impact of bactivorous protozoan grazers on DOM

The addition of a grazer increased the complexity of the DOM pool as established by *P. chlororaphis*, but only temporarily. The DOC and ESI results from the *Uronema* experiment (Figs. 2 and 5) point to an initial release of DOM compounds that were utilized within three days. The compounds that were released due to grazing were mostly low molecular weight compounds (<400 m/z). These compounds appeared, like the compounds detected on day 1 only in the *P. chlororaphis* experiment, to be utilized by *P. chlororaphis*. Thus, the addition of *Uronema* did not change the composition or the molecular weight distribution of the DOM pool over a long time period. The utilization of these compounds could be due to a low availability of more labile organic compounds at this time point in the experiment. Taylor et al. (1985) also suggested that protozoan grazing enhanced DOC release and that the DOC released was composed of primarily low molecular weight compounds that were re-used relatively quickly.

Most previous studies investigating DOM produced by bacteria looked at shifts in ranges of molecular weight or overall changes in some functional groups (e.g. amino acids). With ESI-MS we could see production and subsequent utilization of compounds in time series experiments. The next step is to focus in on the elemental composition and structure of these compounds and find clues why these compounds are labile or refractory. The similar molecular weight distribution of labile and refractory compounds within the <1000 m/z bin identified in this study suggests that molecular weight is not the determining factor. The size-reactivity continuum model (Amon and Benner 1996)

suggests an overall low bioavailability (reactivity) of low molecular weight compounds. The degradation of reactive high molecular weight DOM (>1000 MW) by bacteria leads to the formation of more refractory LMW-DOM (<1000 MW) in this model. Implied in this approach is that, overall, refractory LMW-DOM is older than reactive HMW-DOM. In our study we found that within the LMW DOM pool bacteria released DOM with decreased reactivity (starting from labile glucose) even though this DOM was recently produced. The results from this study are still consistent with the size reactivity model (1) because the refractory compounds are still part of the LMW-pool, but with the addition that the refractory compounds can be formed within the LMW-pool and are not necessarily all breakdown products from HMW compounds. With tools like ESI-MS, the overall dynamics of individual compounds within the LMW-DOM pool can be investigated.

An important conclusion of this study is that the addition of a higher tropic level (i.e. grazer) to a single bacteria system has a substantial effect on the rate and extent at which carbon remineralization occurs, but it has a minimal impact on the quantity and quality of DOC. It has been hypothesized (Nagata and Kirchman 1992a) that ciliate grazers may also shape DOC as they release compounds that are unable to be re-used by bacteria. Our experiment suggests that bacteria could be the primary shapers of DOC and play the prominent role in converting labile organic carbon to refractory. Therefore, while the bacteria-ciliate interaction is crucial to determine the fate of particulate organic carbon, it possibly has a smaller influence on dissolved organic carbon in aquatic systems.

ACKNOWLEDGEMENTS

This work was supported by Biocomplexity grant OCE0120453 from the National Science Foundation.

D.F. Gruber and J.-P. Simjouw contributed equally to this work. Experimental design, sampling and particulate nutrient analysis was done by D. Gruber. R. Lauck and D. Gruber analyzed dissolved nutrients. Compound-level analysis was done by J-P Simjouw and S. Seitzinger. Manuscript was written by D. Gruber, J-P Simjouw, S. Seitzinger and G. Taghon.

We would like to thank Dr. Peter Morin for kindly providing the *Uronema* sp. used in this study and Dr. Dave Caron for additional ciliate cultures and advice on isolation and culturing methods. Dr. Georgina Spyres participated in preliminary experiments. Dr. Jim Ammerman and Dr. Kay Bidle provided useful dialogue on bacteria physiology.

Figure 5.1. Bacteria-only experiment. (A) Averages ± SD of optical density over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) C:N ratio of particulate matter. Note axis break between 20 and 35 days, and logarithmic scale for carbon concentration.

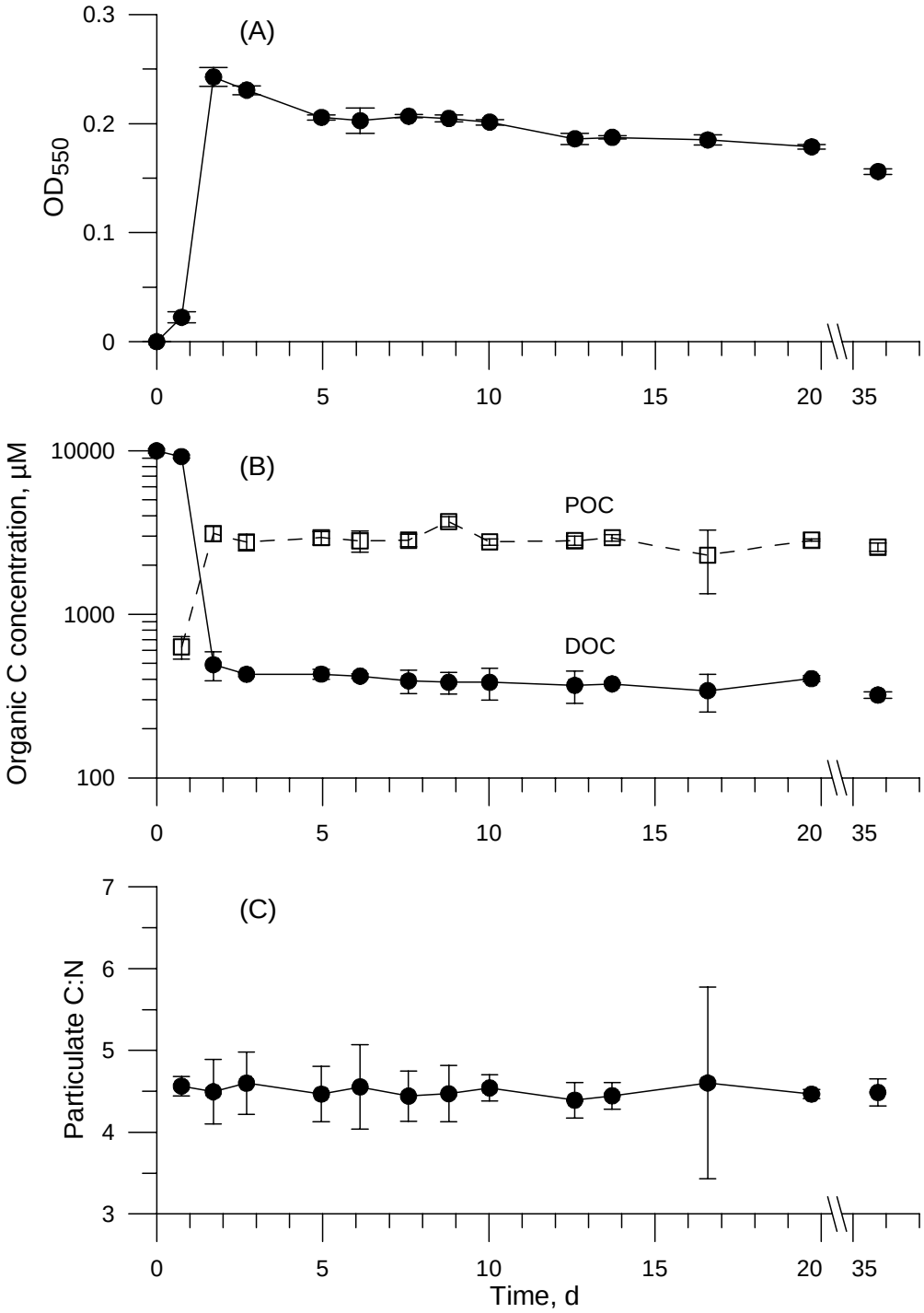


Figure 5.2. Bacteria plus bactivorous protozoan experiment. (A) Averages ± SD of optical density and ciliate abundance over time. (B) Concentration of organic carbon in dissolved and particulate phases. (C) C:N ratio of particulate matter. Note axis break between 20 and 35 days, and logarithmic scales for ciliate abundance and carbon concentration.

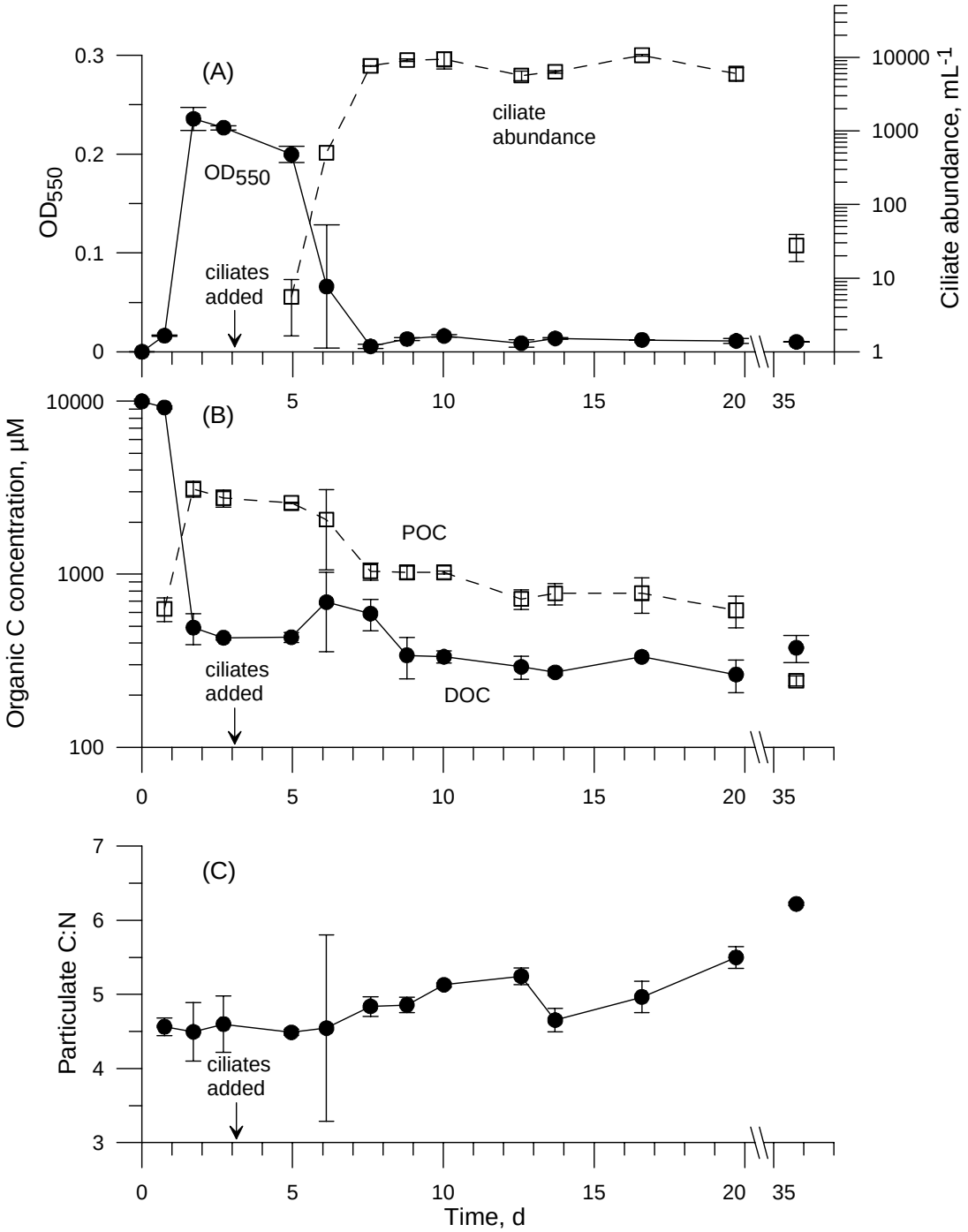


Figure 5.3. Examples of new m/z values and ion abundance identified in positive mode on day 1 (solid line) and on day 2 (dotted line) that remained present for the duration of the experiment. Note: Ion abundance is not a direct measure of concentration because ion abundance varies per compound in the ESI-MS technique.

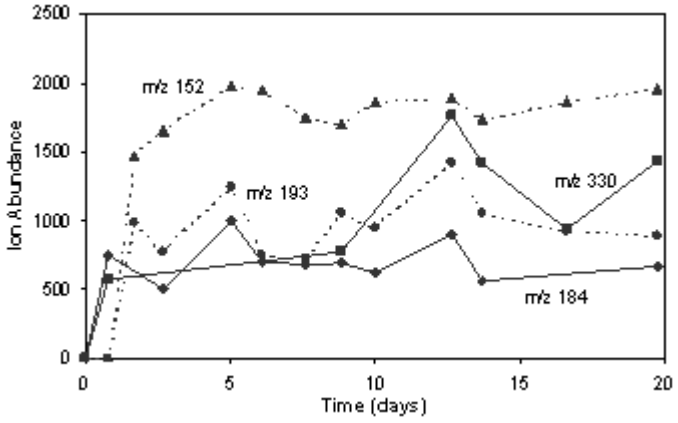


Figure 5.4. Molecular weight distribution for the bacteria-only experiment of newly detected masses at day 1 only, masses first detected on day 1 and present for the remainder of the experiment, and masses first detected on day 2 and present for the remainder of the experiment. (The newly detected masses were present in background corrected spectra; the mass distribution is therefore not influenced by glucose ions or any other ions present in the initial media)

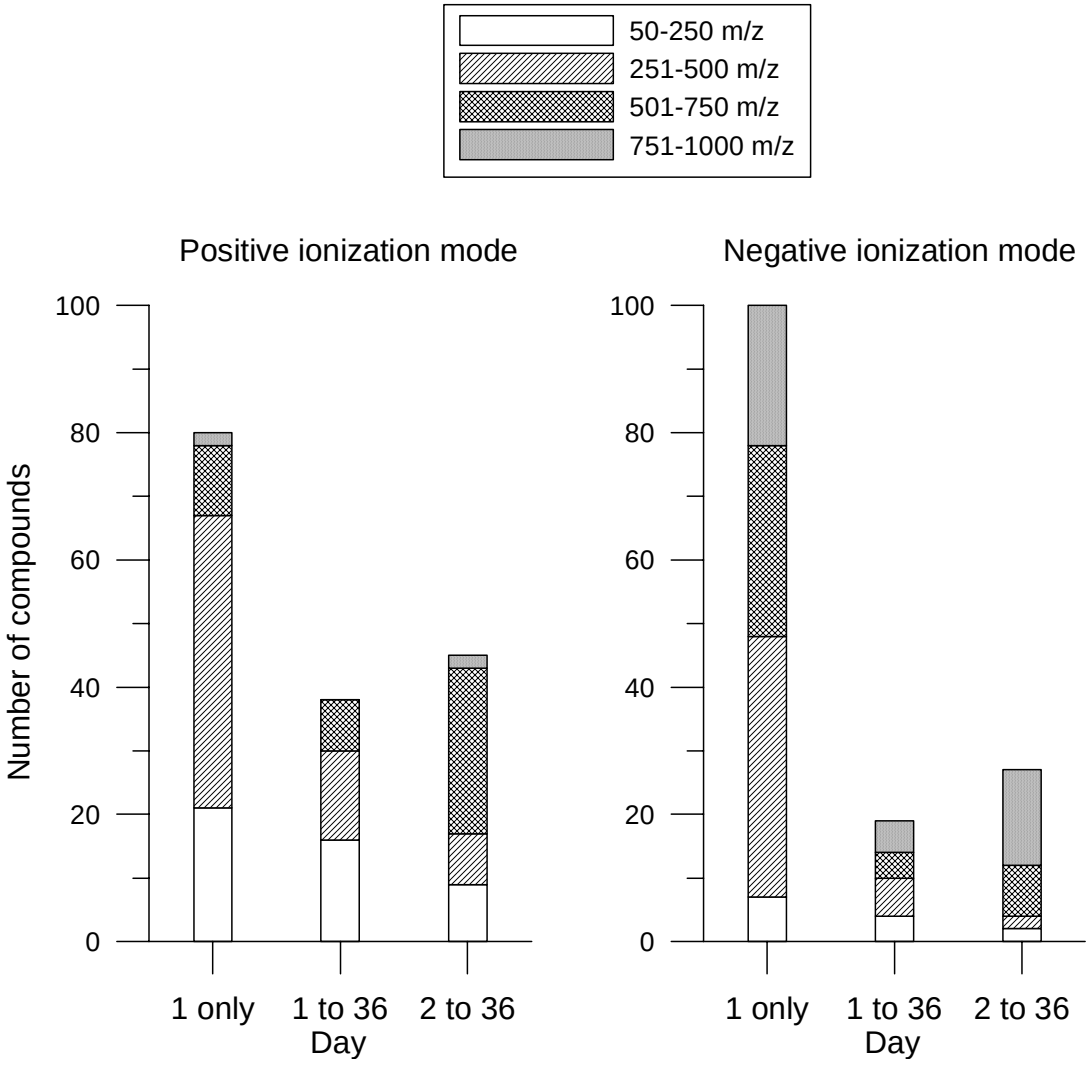


Figure 5.5. New masses detected in time point samples with positive and negative mode ESI-MS. This figure is a composite of results from the bacteria-only and bacteria plus bactivorous protozoan experiments. For comparison the dissolved organic carbon concentration of the bacteria plus bactivorous protozoan time points are included.

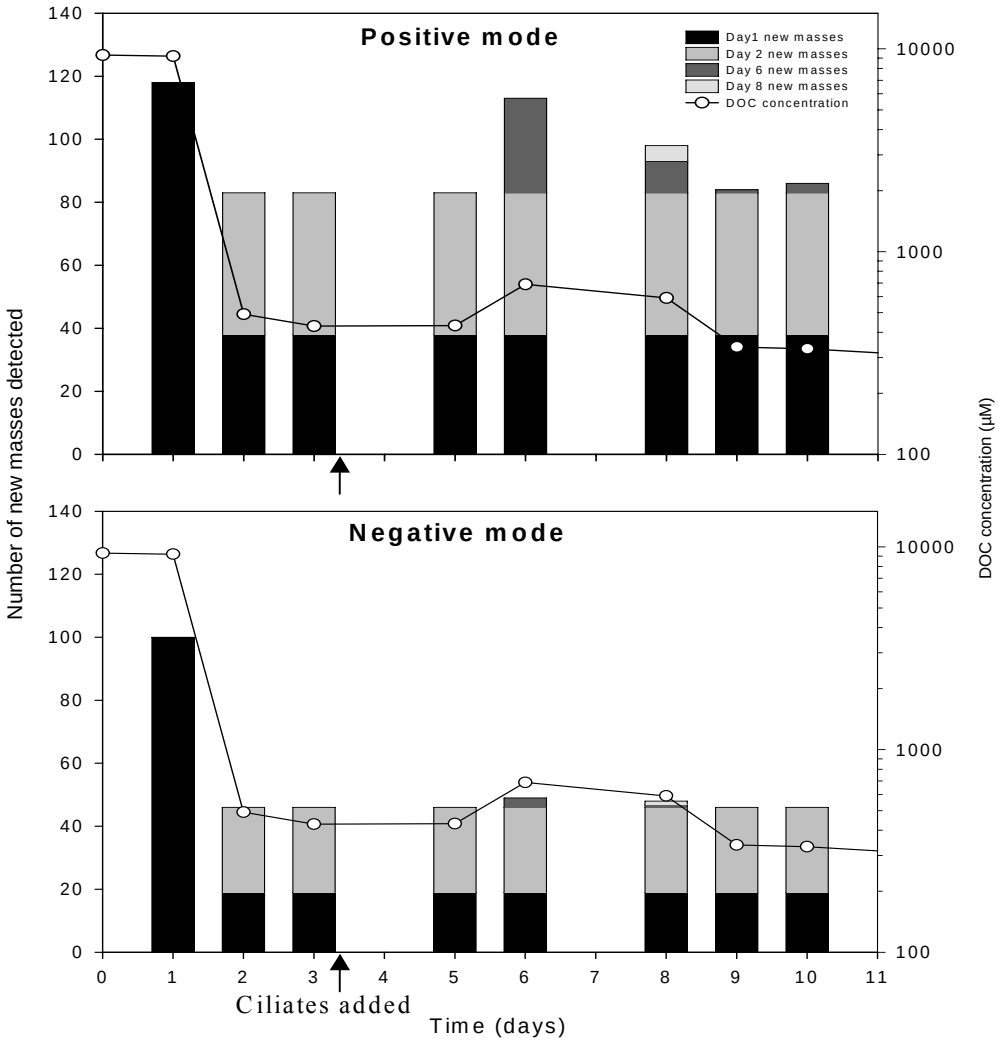
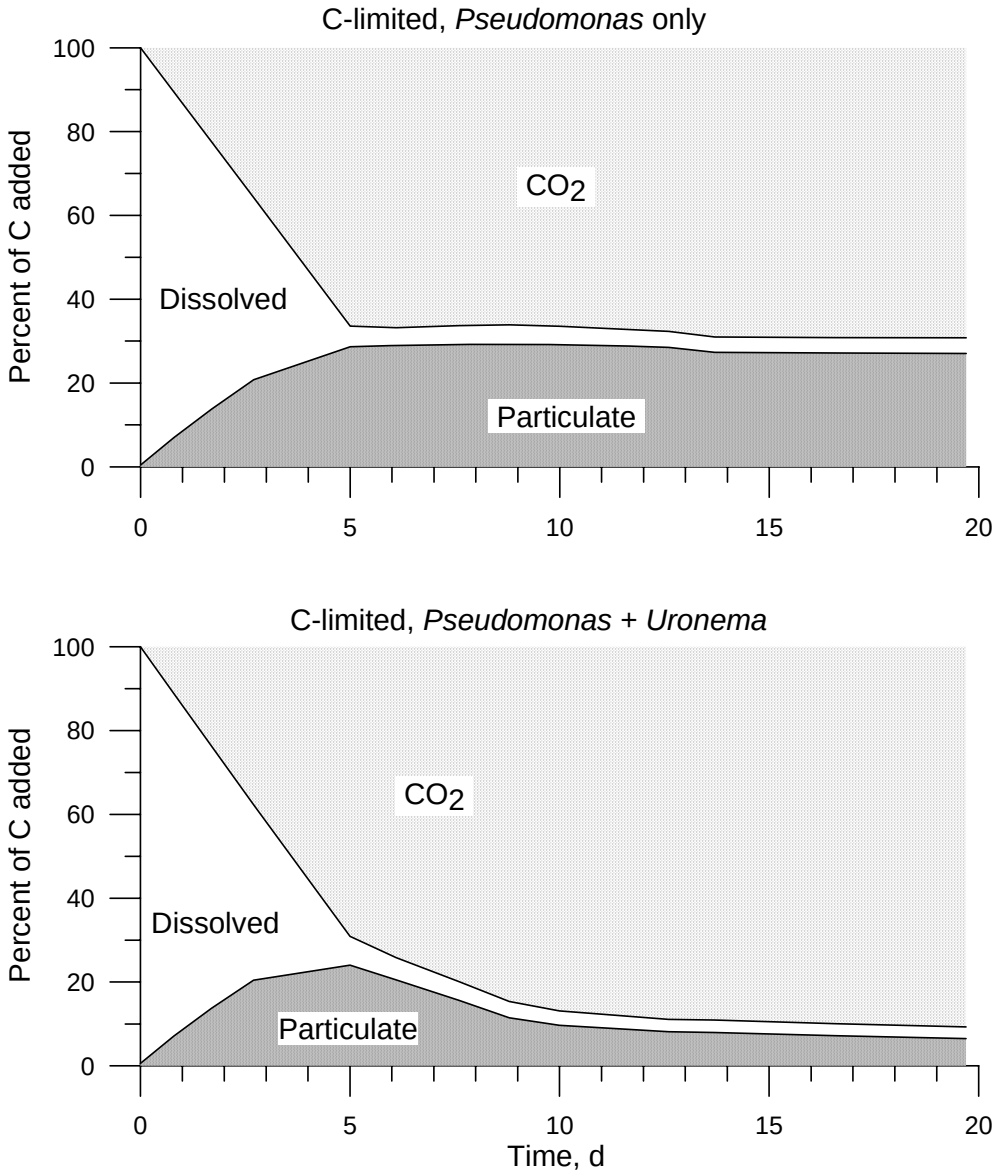


Figure 5.6. Carbon budgets for the *P.chlororaphis* (bacteria-only) and *P.chlororaphis* + *Uronema* (bacteria + bactivorous protozoan) treatments, expressed as percentage of carbon originally added as glucose. Note axis break between 20 and 35 days. Dashed lines connect values measured on days 20 and 36.



REFERENCES

Amon, R. M. W., and R. Benner. 1996. Bacterial utilization of different size classes of dissolved organic matter. Limnol. Oceanogr. **41:** 41-51.

Azam, F., and R. E. Hodson. 1977. Size distribution and activity of marine microheterotrophs. Limnol. Oceanogr. **22:** 492-501.

Baines, S. B., and M. L. Pace. 1991. The production of dissolved organic matter by phytoplankton and its importance to bacteria: Patterns across marine and freshwater systems. Limnol. Oceanogr. **36:** 1078-1090.

Benner, R., and M. Strom. 1993. A critical evaluation of the analytical blank associated with DOC measurements by high-temperature catalytic oxidation. Mar. Chem. **41:** 153-160.

Brophy, J. E., and D. J. Carlson. 1989. Production of biologically refractory dissolved organic carbon by natural seawater microbial populations. Deep-Sea Res. **36:** 497-507.

Caron, D. A. 1991. Evolving Role of Protozoa in Aquatic Nutrient Cycles, p. 387-414. *In* P. C. Reid, C. M. Turley and P. H. Burkill [eds.], Protozoa and Their Role in Marine Processes. Ecological Sciences. Springer-Verlag.

Caron, D. A., E. L. Lim, G. Miceli, J. B. Waterbury, and F. W. Valois. 1991. Grazing and utilization of chroococcoid cyanobacteria and heterotrophic bacteria by protozoa in laboratory cultures and a coastal plankton community. Mar. Ecol. Prog. Ser. **76:** 205-217.

Cole, J. J., S. Findlay, and M. L. Pace. 1988. Bacterial production in fresh and saltwater ecosystems: A cross-system overview. Mar. Ecol. Prog. Ser. **43:** 1-10.

Del Giorgio, P. A., and J. J. Cole. 1998. Bacterial growth efficiency in natural aquatic systems. Annual Review of Ecology and Systematics **29:** 503-541.

Fenchel, T. 1987. Ecology of Protozoa. Science Tech Publishers.

Gruber, D. F., J. P. Simjouw, S. P. Seitzinger, and G. L. Taghon. 2006. Dynamics and characterization of refractory dissolved organic matter produced by a pure bacterial culture in an experimental predator-prey system. Appl Environ Microb **72:** 4184-4191.

Hedges, J. I. and others 2000. The molecularly-uncharacterized component of nonliving organic matter in natural environments. Org. Geochem. **31:** 945-958.

Heissenberger, A., and G. J. Herndl. 1994. Formation of high molecular weight material by free-living marine bacteria. Mar. Ecol. Prog. Ser. **111:** 129-135.

Hua, Y., W. Lu, M. S. Henry, R. H. Pierce, and R. B. Cole. 1996. On-line liquid chromatography-electrospray ionization mass spectrometry for determination of the brevetoxin profile in natural "red tide" algae blooms. J. Chromatogr. A **750:** 115-125.

Jumars, P. A., D. L. Penry, J. A. Baross, M. J. Perry, and B. W. Frost. 1989. Closing the microbial loop: dissolved carbon pathway to heterotrophic bacteria from incomplete ingestion, digestion and sbsorption in animals. Deep-Sea Res. **36:** 483-495.

Kim, S., A. J. Simpson, E. B. Kujawinski, M. A. Freitas, and P. G. Hatcher. 2003. High resolution electrospray ionization mass spectrometry and 2D solution NMR for the analysis of DOM extracted by C-18 solid phase disk. Org. Geochem. **34:** 1325-1335.

Koch, B. P., M. R. Witt, R. Engbrodt, T. Dittmar, and G. Kattner. 2005. Molecular formulae of marine and terrigenous dissolved organic matter detected by electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Geochimica Et Cosmochimica Acta **69:** 3299-3308.

Koike, I., S. Hara, K. Terauchi, and K. Kogure. 1990. Role of sub-micrometre particles in the ocean. Nature **345:** 242-244.

Kramer, G. D., and G. J. Herndl. 2004. Photo- and bioreactivity of chromophoric dissolved organic matter produced by marine bacterioplankton. Aquat. Microb. Ecol. **36:** 239-246.

Kujawinski, E. B., R. Del Vecchio, N. V. Blough, G. C. Klein, and A. G. Marshall. 2004. Probing molecular-level transformations of dissolved organic matter: insights on

photochemical degradation and protozoan modification of DOM from electrospray ionization Fourier transform ion cyclotron resonance mass spectrometry. Mar. Chem. **92:** 23-37.

Kujawinski, E. B., M. A. Freitas, X. Zang, P. G. Hatcher, K. B. Green-Church, and R. B. Jones. 2002. The application of electrospray ionization mass spectrometry (ESI MS) to the structural characterization of natural organic matter. Org. Geochem. **33:** 171-180.

Leenheer, J. A., C. E. Rostad, P. M. Gates, E. T. Furlong, and I. Ferrer. 2001. Molecular resolution and fragmentation of fulvic acid by electrospray ionization/multistage tandem mass spectrometry. Anal. Chem. **73:** 1461-1471.

Loo, J. A., and R. R. Ogorzalek Loo. 1997. Electrospray ionization mass spectrometry of peptides and proteins. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Mortlock, R. A., and P. N. Froelich. 1989. A simple method for the rapid determinatoin of biogenic opal in pelagic marine sediments. Deep-Sea Res. **36:** 1415-1426.

Nagata, T., and D. L. Kirchman. 1992a. Release of dissolved organic matter by heterotrophic protozoa: implications for microbial food webs. Arch. Hydrobiol. Beih. Ergebn. Limnol. **35:** 99-109.

---. 1992b. Release of macromolecular organic complexes by heterotrophic marine flagellates. Mar. Ecol. Prog. Ser. **83:** 233-240.

Odum, E. P. 1971. Fundamentals of Ecology, 3 ed. W. B. Saunders.

Ogawa, H., Y. Amagai, I. Koike, K. Kaiser, and R. Benner. 2001. Production of refractory dissolved organic matter by bacteria. Science **292:** 917-920.

Payne, W. J. 1970. Energy yields and growth of heterotrophs. Annu Rev Microbiol. **24:** 17-52.

Poon, G. K. 1997. Drug metabolism and pharmacokinetics, p. 499-525. *In* R. B. Cole [ed.], Electrospray ionization mass spectrometry, fundamentals, instrumentation, and application. John Wiley & Sons, Inc.

Saito, K., T. Toyo'oka, T. Fukushima, M. Kato, O. Shirota, and Y. Goda. 2004. Determination of psilocin in magic mushrooms and rat plasma by liquid chromatography with fluorimetry and electrospray ionization mass spectrometry. Anal. Chim. Acta **527:** 149-156.

Seitzinger, S. P. and others 2005. Molecular-level chemical characterization and bioavailability of dissolved organic matter in stream water using electrospray-ionization mass spectrometry. Limnol. Oceanogr. **50:** 1-12.

Seitzinger, S. P., and R. W. Sanders. 1997. Contribution of dissolved organic nitrogen from rivers to estuarine eutrophication. Mar. Ecol. Prog. Ser. **159:** 1-12.

Seitzinger, S. P., R. M. Styles, R. Lauck, and M. A. Mazurek. 2003. Atmospheric pressure mass spectrometry: A new analytical chemical characterization method for dissolved organic matter in rainwater. Environ. Sci. Technol. **37:** 131-137.

Sharp, J. H., R. Benner, L. Bennett, C. A. Carlson, R. Dow, and S. E. Fitzwater. 1993. Re-evaluation of high temperature combustion and chemical oxidation measurements of dissolved organic carbon in seawater. Limnol. Oceanogr. **38:** 1774-1782.

Stanier, R. Y., N. J. Palleroni, and M. Doudoroff. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43:** 159-271.

Stenson, A. C., W. M. Landing, A. G. Marshall, and W. T. Cooper. 2002. Ionization and fragmentation of humic substances in electrospray ionization Fourier transform-ion cyclotron resonance mass spectrometry. Anal. Chem. **74:** 4397-4409.

Stenson, A. C., A. G. Marshall, and W. T. Cooper. 2003. Exact masses and chemical formulas of individual Suwannee River fulvic acids from ultrahigh resolution electrospray ionization Fourier transform ion cyclotron resonance mass spectra. Anal. Chem. **75:** 1275-1284.

Stoderegger, K., and G. J. Herndl. 1998. Production and release of bacterial capsular material and its subsequent utilization by marine bacterioplankton. Limnol. Oceanogr. **43:** 877-884.

Strom, S. L., R. Benner, S. Ziegler, and M. J. Dagg. 1997. Planktonic grazers are a potentially important source of marine dissolved organic carbon. Limnol. Oceanogr. **42:** 1364-1374.

Taylor, G. T., R. Iturriaga, and C. W. Sullivan. 1985. Interactions of bactivorous grazers and heterotrophic bacteria with dissolved organic matter. Mar. Ecol. Prog. Ser. **23:** 129-141.

Tranvik, L. 1994. Colloidal and Dissolved Organic-Matter Excreted by a Mixotrophic Flagellate During Bacterivory and Autotrophy. Appl. Environ. Microbiol. **60:** 1884-1888.

Zubkov, M. V., and M. A. Sleigh. 1995. Ingestion and assimilation by marine protists fed on bacteria labeled with radioactive thymidine and leucine estimated without separating predator and prey. Microb. Ecol. **30:** 157-170.

Curriculum Vita

David F. Gruber

Ph. D.	2007	Rutgers, The State University of New Jersey, New Brunswick, NJ Institute of Marine and Coastal Sciences Biological Oceanography
M.S.	2001	Columbia University, New York, NY Graduate School of Journalism
M.E.M.	1998	Duke University, Durham, NC Coastal Environmental Management
B.S.	1995	University of Rhode Island, Kingston, RI Environmental Management and Oceanography, *with distinction*

PUBLICATIONS:

Gruber, D.F, Simjouw, J-P. Seitzinger, S.P. and G. L. Taghon. 2006. Dynamics and Characterization of Refractory Dissolved Organic Matter Produced by a Pure Bacterial Culture in an Experimental Predator-Prey System. Applied and Environmental Microbiology 72(6): 4184-4191.

Pieribone, V., and D. F. Gruber. 2006. Aglow in the Dark: The Revolutionary Science of Biofluorescence. Harvard University Press, Cambridge, MA. 288 pp.

Nasar, S. and Gruber, D. 2006. Manifold Destiny. *The New Yorker*, Aug. 28 issue, p.44-57. [Also featured in the Ecco/Harper Perennial anthology, *The Best American Science Writing* 2007.]

Kao, H-T, S. Sturgis, R. DeSalle, J. Tsai, D. Davis, D. F. Gruber, and V. A. Pieribone. Dynamic Regulation of Fluorescent Proteins from a Single Species of Coral. In press, Marine Biotechnology.

Gruber, D.F., C.J. Madden and W.M. Kemp. 2001. Seagrass ecosystem responses to variable hydrologic and geographic conditions: A comparative simulation analysis of Thalassia testudinum in Florida Bay and the Caloosahatchee Estuary. Proceedings of the 1999 Florida Bay and Adjacent Marine Systems Science Conference.

Murray, L., D.F. Gruber and W.M. Kemp. 1999. Comparative studies of seagrass and epiphyte communities in Florida Bay and two other south Florida estuaries in relation to freshwater inputs. Proceedings of the 1999 Florida Bay and Adjacent Marine Systems Science Conference.

Gruber, D. F. 1998. Uncertainty and Climate Change: Implications of the thermohaline circulation on general circulation models. Master's Thesis. Duke University, Durham, NC. 98 pp.