CARBON, NITROGEN, AND PHOSPHORUS STOICHIOMETRY OF A MIXOTROPHIC PROTIST

by

NATALIE C. LUKOMSKI

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ABSTRACT

CARBON, NITROGEN, AND PHOSPHORUS

STOICHIOMETRY OF A MIXOTROPHIC

PROTIST

Natalie C. Lukomski, MS

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Supervising Professor: Thomas H. Chrzanowski

The microbial loop is one of two major pathways in aquatic systems in which nutrient and energy flow from dissolved nutrients to higher trophic levels. Protozoan-bacteria predator-prey relationships lie at the base of this food web. It is through this interaction that dissolved nutrients sequestered in the bacterial biomass become available to higher trophic levels.

Heterotrophic nanoflagellates are the main consumers of bacteria in aquatic systems (Porter et al.1985). Consequently, limitation of dissolved nutrients in the bacterial biomass could effect protozoan stoichiometry and the amounts of excreted waste products. These excreted waste products are a valuable resource to bacteria;

therefore protists play a critical role not only in the transfer of nutrients and energy to higher trophic level but also in nutrient resource regeneration. In this work, we explored how bacteria prey (*Pseudomonas fluorescens*) grown in four different nutrient conditions (Balanced, C-Limited, N-Limited and P-Limited media) effected growth dynamics, nutrient quotas (concentrations of particulate: carbon, nitrogen, and phosphorous) and consequently nutrient recycling when fed to a protozoan predator (*Ochromonas danica*).

Protozoan nutrient quotas are difficult to determine due to the presence of undigested bacteria in protozoan food vacuoles. Knowledge of the bacterial carbon, nitrogen and phosphorus quotas are required to distinguish undigested bacterial biomass from protozoan biomass. Filtration methods were used to separate the protozoan predator from their bacterial prey population to acquire more accurate quota measures. By doing so, four analytical methods were used to calculate the protozoan quotas. Each method revealed a similar tend in that *Ochromonas* contained the highest element quotas when feeding on P-Limited bacteria, and the lowest element quotas when feeding on C-Limited bacteria. Element ratios based on nutrient concentrations revealed that predator element ratios varied as a linear function of prey element ratios. Estimates of nutrient recycling were based on protozoan and bacterial nutrient quotas and dissolved nutrient measurements. Nitrogen and phosphorus recycling by the flagellate was greatest when feeding on C-Limited bacteria.

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INTRODUCTION

1.1 The Microbial Loop

1.1.1 Overview

Considerable effort was made in the mid-1970s to understand the trophodynamics of aquatic systems and much of this work focused upon food webs of marine waters. Less attention was given to freshwater systems since only minor differences in the trophodynamics were thought to exist between marine and freshwater systems (Hobbie 1988). Bacteria, protozoa and viruses were largely ignored in such studies since they were considered to be of low abundance and metabolically inactive most of the time (Steele 1974). Contrary to prevailing opinion, Pomeroy (1974) argued that a significant amount of carbon and energy must flow through microorganisms because of their higher metabolic rate per unit mass. He suggested that the classic linear food chain where phytoplankton are grazed upon by zooplankton, which are in turn eaten by fish, might be complemented by microbial based food chain consisting largely of bacteria grazed upon by protozoa. Azam et al. (1983) later formalized this concept in what has now become known as the "microbial loop" (Figure 1.1). In this construct, the movement of organic materials and energy originating in dissolved organic matter and subsequently sequestered into bacteria could be transferred to organisms in higher trophic levels. The inclusion of a microbial loop in the aquatic food web would also decrease the efficiency

of nutrient and energy transfer due to the increased number of trophic level transfers (Berninger et al. 1991). Estimates of the magnitude of specific interactions between each level and the resultant carbon and energy flow led to the conclusion that processes occurring within the microbial loop are responsible for major fluxes of organic matter; on average, half the phytoplankton primary production appears to be channeled through bacteria (Cole et al. 1988, White et al. 1991).

1.1.2. Major Players: Bacteria and Protozoa

The primary role of heterotrophic bacteria in marine food webs is the consumption of mineral nutrients (Goldman et al. 1985. Andersen et al. 1986). Bacteria are osmotrophs, obtaining nutrition by absorbing nutrients and organic matter from their surroundings. Bacteria are very efficient at nutrient uptake. Their membrane-bound permeases possess half-saturation constants in the nanomolar to millimolar range (Nissen et al. 1984). Two forms of organic compounds are present in aquatic systems: dissolved (DOM) and particulate (solid/semi-solid) (POM) organic matter. DOM remains in system surface waters longer while POM is usually lost by sinking and is more influential to dynamics in deep waters and the benthos. Both forms of organic compounds are utilized by bacteria and the nutrients produced by the breakdown or dissolution of some POM in the pelagic zones are unavailable to aquatic organisms other than bacteria.

The primary grazers of bacteria in aquatic systems appear to be heterotrophic nanoflagellates (HNF) (Porter et al.1985). In recent years, application of epifluorescent microscopy coupled to image analysis systems and improved culturing methods has led to more studies on a variety of protozoa, lending to a deeper understanding of the role of protozoa in ecosystem dynamics. Heterotrophic nanoflagellates are abundant and found

in marine, freshwater, coastal and lotic systems. They prey not only on bacteria, but also on phytoplankton and other small protists. Assimilation efficiencies of HNF have been estimated to approach 60% (Fenchel et al. 1982), thus, subsequent predation upon HNF by larger pelagic organisms links the nutrients and energy sequestered in bacteria to higher trophic levels (Azam et al 1983). Additionally, since phytoplankton and bacteria compete for dissolved mineral nutrients, HNF predation on bacteria has the potential to influence the outcome of algal-bacteria nutrient competition and alter overall trophic dynamics.

Predation upon flagellates by higher trophic levels clearly creates a feedback loop in which DOM and nutrients once contained within bacteria are now recycled back to the dissolved pool, replenishing and creating the "microbial loop" (Fenchel et al. 1982, Azam et al. 1983). Phytoplankton also replenishes the DOM pool by excretion of excess organic carbohydrates from photosynthetic mechanisms (Sterner et al. 1997). Thus, it appears that phytoplankton and bacteria seem to form a complex relationship: phytoplankton at times supplying bacteria with necessary organic material and at subsequent times competing with bacteria for essential mineral nutrients. Clearly the flow of carbon and mineral nutrients are tightly coupled and therefore quantification of nutrient flow between bacteria and flagellates seems essential for understanding the dynamics involved in the microbial loop.

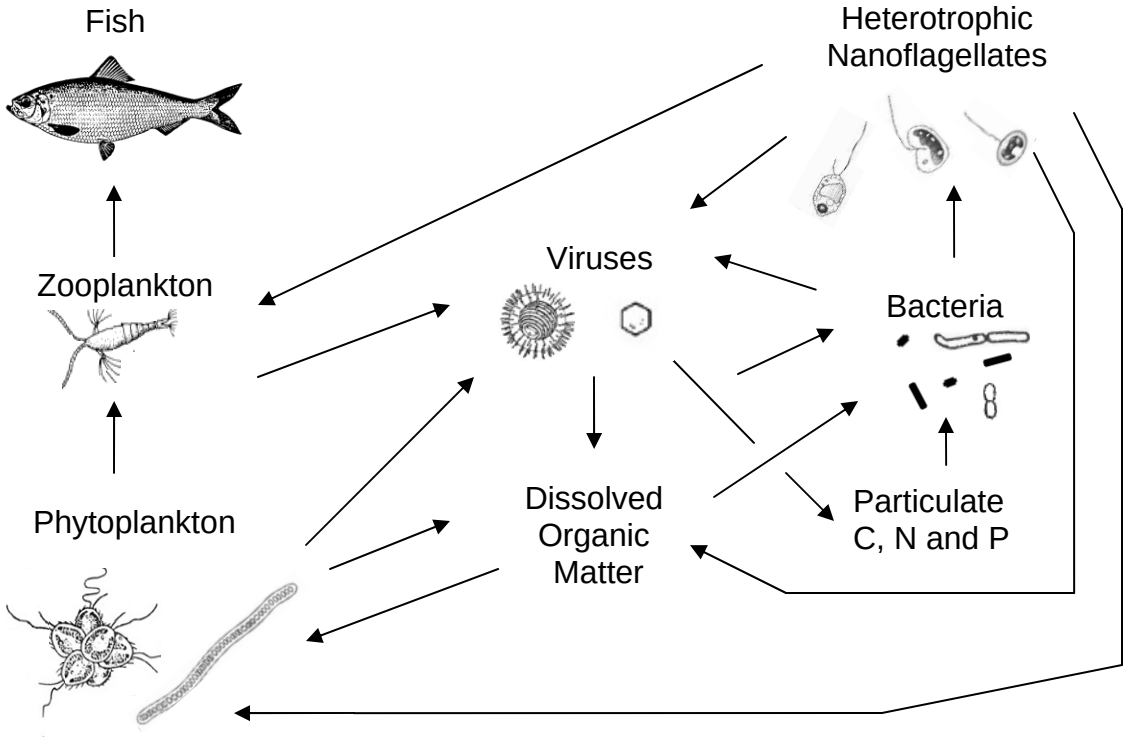


Figure 1.1 The Microbial Loop and its Relationship with the Classic Food Web.

1.1.3 Variable Structural Stoichiometry in Prey Organism

All organisms have requirements for nutrients, and depending upon the organism, the relative proportions of essential elements may vary as a function of growth rate, temperature, and nutrients in the environment (Elser et al. 1996). The chemical composition of organisms appears to be governed by the law of conservation of matter. Thus, the relative proportions (ratios) of key elements (Carbon:Nitrogen:Phosphorus, hereafter C:N:P) consumed from resources must appear in balance with the key elements in consumer biomass and wastes (Sterner and Elser 2002). This conservation of matter integrates with, and explains how the element content of an individual affects the flow of energy and nutrients through systems. The ratios of critical elements in cells are considered the organism's element stoichiometry, which is derived from its structural

stoichiometry (Klausmeier 2004). Structural molecules such as nucleic acids, proteins, lipids and carbohydrates dominate an organism's biomass and since each of these molecules has a distinct element composition, the proportion in which they appear will define an organism's structural stoichiometry. For example, structural components of cells, as some proteins, are poor in P but abundant in C (53%) and N (17%) (Sterner and Elser 2002) whereas relative to proteins, some nucleic acids are rich in P. RNA is structurally 9% P by weight and an important contributor of organismal P (Elser 1996). Thus, growing cells, containing a large amount of RNA relative to that of non-growing cells, would be expected to have lower C:P and N:P ratios (Chrzanowski et al 1996).

Ecological stoichiometry links structural stoichiometry to ecosystem process by balancing overall C:N:P flow through the interacting trophic levels (Sterner and Elser 2002). Therefore, an understanding of an organism's stoichiometry allows for predictions of ecological stoichiometry.

Predators of microbial cells face an interesting dilemma: single-cell prey-items represent discrete food packages of some specific proportion of essential elements (C:N:P) that may not match their own nutritional needs, and this may have consequences that cascade through the trophic structure. For instance, HNF will incorporate nutrients at a rate needed for maintenance, growth and reproduction. Assimilated nutrients not needed for growth will be excreted back into the dissolved pool. Nutrients excreted by HNF are again available for bacterial uptake and the ratio of elements excreted by the HNF may affect the element composition of bacteria. Nutrient limitation creates a threshold on the amount of molecules produced within a cell. Structural stoichiometry constrained by nutrient limitation effects bacterial stoichiometry. In turn, protozoa

feeding on nutrient limited prey populations are consequently affected, assimilating nutrients (protozoan stoichiometry) and recycling products in excess. These recycled products may promote greater nutrient limitation of bacteria, in turn "looping back" to affect the rate of recycled nutrients. For that reason, prey chemical composition undoubtedly affects consumer-driven nutrient recycling thru imbalanced growth between a predator's resource supply and the predator's demand thus, effecting food-web growth dynamics and nutrient regeneration.

1.1.4 Food Selection by Protozoa

Heterotrophic nanoflagellates, the major consumers of bacteria in aquatic systems (Porter et al.1985) serve as a driving force for bacterial assemblage diversity. HNF have a variety of life strategies: free swimming, temporarily attached, and attached. Free swimming protozoa cannot feed on all sizes of bacteria with equal efficiency and appear to preferentially consume bacteria (food selection) with cell lengths ranging from 1.1-1.3 μm (Chrzanowski and Šimek 1990). Consequently, the effect of protozoan grazing upon bacteria having a specific cell size has known to shift the bacterial assemblage toward the larger and/or smaller bacteria (Hahn and Hölfe 1999). However, abundant taxonomic groups of bacteria belonging to the preferred size range are preferentially ingested, allowing less dominant taxomonic groups outside this range to flourish affecting bacterial diversity (Fenchel 1986, Jezbera et al. 2005).

In response to grazing pressure, bacteria may alter phenotypic characteristics to avoid predation. To escape protozoan grazing pressure, bacteria have been shown to form filaments (Hahn et al. 1999, Šimek et al. 1997, Corno and Jürgens 2006), synthesize high-speed motility apparatus (Matz et al. 2002), and revert to cell miniaturization (Hahn

et al. 2000, Posh et al. 1999). Filamentous bacteria exceed the preferential prey-size range of protists and are grazed at lower rates due to contact or ingestion failure (Wu et al. 2004). High swimming speeds allow bacteria to avoid or escape predation (Matz and Jürgens 2003). Posch et al. (1999) described a chemostat study in which a morphological shift to smaller bacteria cell size (0.3-0.9 μm) reduced protozoan ingestion rate. It has been suggested that these reported morphological shifts may be a response to allelochemicals released by flagellates (Kuhlmann and Heckman 1985, Kusch and Kuhlmann 1994, Pernthaler 2005). Clearly, flagellate predation can directly impact the structural, phenotypic, and taxonomic composition of bacterial communities.

Food selection by protozoa is complex and mechanisms of selection (active or passive) are the subject of considerable debate (Jürgens and DeMott 1995, Boenigk el al. 2001 and 2002). Clearly, food selection and the attendant C, N, and P, will impact protozoa growth, reproduction, and the nature of elements excreted. Investigations into protozoan element composition are needed to quantify nutrient and energy flow between prey and consumer, explain the shifts in protozoan food selection, and to deduce how prey structural stoichiometry may affect that of the protozoa.

There have been few studies addressing the element stoichiometry of protozoans grazing on prey of different food quality (bacteria or phytoplankton of varying C:N:P ratios) (Nakano 1994, Goldman et al 1987). There have been no studies addressing the potential synergistic effects of predator recycling on prey growth while also accounting for the presence of undigested prey within the predator food vacuole.

In this work, a model microbial predator-prey system was used to assess the growth, stoichiometry, and potential nutrient regeneration of a common protozoan

predator. The element stoichiometry of the protozoan *Ochromonas danica* was assessed when feeding upon bacteria of varying food quality. Bacteria have a short half-life within the flagellate food vacuole (~75 minutes, Shannon et al. 2007), nevertheless, during steady state growth, the presence of undigested bacteria in food vacuoles at the time of sampling could, theoretically, alter estimates of the protozoan C:N:P ratio. The experimental design employed here attempts to account for the presence of undigested bacteria within predator food vacuoles and independently assess the element stoichiometry of the protozoan.

Protozoa may not strictly regulate their element composition. Thus there is the potential for differential regeneration of nutrients when feeding upon prey of varying element composition. In any system, recycled nutrients may release the prey from nutrient limitation and dynamically bring about a shift in prey element composition. Such shifts may cascade upward and compound interpretation of protozoan feeding behavior.

CHAPTER 2

MATERIALS AND METHODS

2.1 Overview

The overall goal of this research was to determine if the element composition of protozoa is variable. Model organisms were chosen for study: a cosmopolitan protozoan, *Ochromonas danica*; and a common bacterium known to support the growth of *O. danica*, *Pseudomonas fluorescen*s.

Below, I describe the approach to create culture conditions that would subsequently yield four populations of *P. fluorescens* each having a characteristic element composition. I characterize the growth of *P. fluorescens* on these media and describe their element composition. In turn, I use the different populations of *P. fluorescens* to grow *O. danica* and characterize the resulting element composition of the protozoan.

2.1.1 Organisms and Maintenance

The predator in this system is *Ochromonas danica*. Species of the chrysophyte genus *Ochromonas* are well known unicellular protozoa found in both freshwater and marine systems. *Ochromonas* is characterized by a single chloroplast and two anterior flagella of unequal lengths, with mastigonemes present on the longer. *Ochromonas danica* lacks siliceous scales and varies in shape from pyriform (pear-shaped) to spherical (Pringsheim 1952). Generally, *Ochromonas danica's* nutritional mode is mixotrophic;

capable of autotrophic growth through photosynthesis, osmotrophic growth with uptake of DOM in the dark, or by phagotrophic growth thru the ingestion of food particles. However, our lab stain was an obligate heterotroph.

Ochromonas danica is a broadly distributed voracious bacterivore using interception feeding. In this process, food items are transported toward the cell by a current created by the beating of the large flagellum. The small flagellum assists by directing the prey toward the ventral furrow, a sensitive area of the cell where phagocytosis occurs. Once prey makes contact, the large flagellum stops beating and a phagocytic vesicle forms around the bacterial cells.

Pseudomonas fluorescens is a common Gram-negative bacterium found in soils, associated with plant surfaces (roots and leaves), and in aquatic systems. This organism secretes a fluorescent pigment, fluorescein, as a by-product of metabolism. It is capable of thriving in diverse environments due to the versatile metabolism of different carbon sources and minute mineral salt requirements. *P. fluorescens* is a motile rod-shaped bacterium having multiple flagella.

Pseudomonas fluorescens (ATCC 17386) stock cultures were maintained at 28°C on Standard Mineral Base (SMB) (White and Hegeman 1998) with glucose added to 60 mM. The strain of *Ochromonas danica* (UTEX 1298) used in this study was maintained axenically at 22°C on *Ochromonas* Medium (Star 1978) and was not capable of autotrophic growth.

In the sections that follow I describe the methods used to establish: (1) that *P. fluorescens* could be grown in media that would result in cells of varying element content (section 2.2); and (2) that *Ochromonas* could grow using these bacteria as prey (section

2.3). I then describe the methods used to conduct grazing studies from which I determined the element composition of *Ochromonas* when preying on bacteria of differing element content (section 2.4)

2.2 Preparation of *Pseudomonas fluorescens* of Varying Element Composition

2.2.1. Growth of Pseudomonas fluorescens and Determining Element Composition in Media of Varying Element Composition

Bacteria were grown in triplicate batch cultures at 28°C (Precision Model 50 water bath, 65 opm) where the element composition of SMB was adjusted to yield four nutrient conditions; Balanced, C-Limited, N-Limited, and P-Limited (Table 2.1).

Table 2.1 Element composition of media

Medium	Composition Glucose (g L^{-1}) / mM C	NH_4Cl (g L^{-1}) / mM N	KH_2PO_4 (g L^{-1}) / mM P	Element ratio (atomic C:N:P)
Balanced	1.8 / 60	20 / 6.0	6.8 / 0.5	120:12:1
C-Limited	1.8 / 60	66.6 / 20	27.2 / 2.0	30:10:1
N-Limited	3.6 / 100	8.4 / 2.5	6.8 / 0.5	200:5:1
P-Limited	3.6 / 100	16.9 / 5.0	1.36 / 0.1	1000:50:1

Approximately half of a 75 mL culture of *Pseudomonas fluorescens* grown to stationary phase in Balanced medium was concentrated by centrifugation (Eppendorff 5804R, 5000 rpm, 5 min, 4ºC), washed 2X with SMB-buffer (SMB less components containing C, N, or P) and resuspended in 15 mL of SMB-buffer. Batch cultures containing 330 mL of each medium were inoculated with washed cells (1 mL). Growth in all cultures was monitored by changes in optical density (540 nm). At stationary phase,

samples were removed and preserved (5 mL sample in 1 mL of 25% glutaraldehyde) for determination of cell abundance and cell size (as volume). An additional set of samples was collected for determination of element content (see below).

Bacteria were collected on a 25-mm 0.22 μm-black polycarbonate filters (Osmonics Inc.), stained with diamidinophenylindole (DAPI) (Porter and Feig 1980), and enumerated using epifluorescence microscopy (Olympus BH-2, 1250X). Bacterial cell volumes (V_{bact}) were determined from length and width of at least 200 cells and were assumed to be cylinders with two half-spheres at each end. Volumes were calculated following the formula:

$$V_{bact} = [\pi (0.5W^2) (L - W)] + [(4/3) \pi (0.5W^3)]$$

where W is the maximum cell width and L is the maximum cell length in μm. Length and width of individual cells were determined from digital images (Olympus DP70 camera) and Simple PCI imaging software (Compix, Inc., Brandywine, PA, USA).

Cells (4 mL) were collected on 25-mm precombusted (475°C for 2 hrs) glass-fiber filters (Whatman GF/F) and dried (66ºC, 48 hrs). Carbon and N content of cells were determined using a CNH analyzer (Perkin-Elmer series 2200 CHN Analyzer). Particulate phosphate (P) was determined from persulfate digestions (2 mL) followed by soluble reactive phosphate (SRP) analysis (Strickland and Parsons 1972). All samples were assayed in triplicate. Element ratios are reported as mole:mole.

2.3 *Ochromonas danica* Growing on *Pseudomonas fluorescens*

Ochromonas danica was preconditioned to graze on *P. fluorescens* of a given element composition (Table 2.1). *Ochromonas danica* growing axenically on *Ochromonas* Medium (1 mL) was concentrated by centrifugation (VWR Galaxy 16DH

centrifuge, 10,000 rpm, 1 min), washed 2X in SMB-buffer, and resuspended (1 mL) in SMB-buffer. Washed cells (250 μL) were inoculated into a stationary phase culture of *P. fluorescens* (200 mL) growing in one of the four media described above (Table 2.1).

Samples of the cultures were removed daily, preserved (5 mL in 1 mL of 25% glutaraldehyde) and stored refrigerated for later determination of abundance by direct count (Olympus BH-2, 500X *O. danica*, 1250X *P. fluorescens*). Protozoa were collected on 25-mm 1.0-μm black polycarbonate-filters (Osmonics Inc.) and stained with primulin (Caron 1983). Bacterial cells were enumerated as above. The abundance of both *Ochromonas* and *Pseudomonas* was monitored for 100 hrs.

2.4 Experiment Design of Grazing Experiments

Below I describe how each population of *P. fluorescens* was used to grow *O. danica* and to characterize the resulting element composition of the protozoan. The overriding difficulty faced in such experimental designs lies with quantifying the element content of both the predator and prey when they are difficult to physically separate. Here, we employ a series of cultures and make several assumptions to simplify the task.

2.4.1. Experimental Design Overview

Each experiment consisted of nine cultures: three containing bacteria only (BO) and six containing bacteria and flagellates (BF and BFF). All nine cultures were inoculated with *P. fluorescens* which was allowed to grow until stationary phase. *Ochromonas danica* was inoculated into six of the cultures and allowed to reach exponential growth phase. The element content of *P. fluorescens* alone was determined from BO cultures. Element content of the *P. fluorescens* and *O. danica* in combination was determined from three of the BF cultures. There is the possibility that *O. danica*

grazing on *P. fluorescens* would release nutrients that would in turn foster re-growth of *P. fluorescens*. To examine this possibility, *P. fluorescens* and *O. danica* in the remaining three cultures (BFF) were separated by filtration and the element content of each determined by mass balance and the dissolved nutrients contain in BFF filtrates were compared to dissolved nutrients remaining in BO filtrates.

2.4.2. Growth of O. danica Grazing on P. fluorescens of Varying Element Composition

Nine cultures (200 mL) of *P. fluorescens* were prepared for each of the media describe in Table 2.1 using a 1:6.6 dilution of stock medium (30 mL of medium with 170 mL of deionized water), inoculated as above and grown to stationary phase (Precision Model 50 water bath, 65 opm) at 28°C. Preconditioned *Ochromonas* (250 µL) was inoculated into six of the cultures (BF and BFF cultures). Cell abundances and volumes for both *O. danica* and *P. fluorescens* were determined as above. Element composition of cells and the medium were determined for *P. fluorescens* in stationary phase (BO cultures) and for *P. fluorescens and O. danica* in stationary phase (BF and BFF cultures). Samples of BFF cultures were filtered through a 47-mm 3.0µm pore-size polycarbonate filter (Osmonics Inc.) to remove the larger flagellates from the bacteria (Emerson vacuum pump, 2.5 Hg). The filtrate containing bacteria and small flagellates was collected in acid-washed bottles (Nalgene).

2.4.3. Determination of O. danica Cell Size

Flagellate cells were stained and enumerated as above. Flagellate cell volume (V_{flag}) was determined from length and width of at least 50 cells (Olympus BH-2, 500X) and were assumed to be spindle-shaped using the formula (Eccleston-Parry and Leadbeater):

$$V_{flag} = \pi \frac{\left(W^2 * L\right)}{6}$$

where W is the maximum cell width and L is the maximum cell length in μm. Length and width of individual cells were determined from digital images (Olympus DP70 camera) and Simple PCI imaging software (Compix, Inc., Brandywine, PA, USA)

2.4.4. Determination of O. danica element composition

Carbon, N, and P of *P. fluorescens* and *O. danica* growing in combination in BF cultures were determined as described above for *P. fluorescens* growing alone and reported as femtomoles (fmoles) per volume, unless specified. Element ratios are reported as mole:mole.

2.4.5. Dissolved Chemical Analyses

Dissolved nutrients in each culture were determined at end of each experiment. Approximately 150 mL of culture was filtered through a 47-mm precombusted glass-fiber filter (Whatman GF/F), the filtrate collected and stored frozen (-4°C) in acid-washed bottles (Quorpak) until analysis. Ammonium (NH_4^+), total dissolved phosphorus (TDP), and SRP was determined according to the methods of Strickland and Parsons (1972). Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were analyzed using a Shimadzu TOC-V_{CSH} Analyzer. Dissolved organic nitrogen (DON) was calculated as the difference between TDN and NH_4^+. Total organic phosphate (TOP) was calculated as the difference between TDP and SRP.

All media contained MOPS buffer, as indicated in the recipe by White and Hegeman (1998). Carbon and nitrogen present from MOPS was accounted for with dissolved nutrients.

Regeneration or released nutrients by the flagellate was originally hypothesized to be the difference between dissolved nutrients in BFF cultures and BO cultures. However, due to incomplete separation of the flagellate from the bacteria by filtration in BFF cultures as described above, released nutrients could not be specified to a specific organism and was estimated using the formula:

$$N_R = \overline{(\overline{X}_{BF} + \overline{X}_{BFF})} - \overline{X}_{BO}$$

Mean dissolved nutrients in BFF cultures ($\overline{X}_{BFF}$) were similar to that of BF cultures ($\overline{X}_{BF}$) values and used estimate the nutrients released (N_R) back to the dissolved pool by either bacteria or flagellates.

2.5 Calculating Element Composition for *Ochromonas*

2.5.1. Determining Protozoan Element Composition

The experimental design allowed us to determine the element composition of *O. danica* using four approaches. We can determine the element content of *O. danica* based by normalizing the quota of each element in both predator and prey to cell abundance. *Ochromonas* is very plastic and easily deforms and passes through filters that should, in theory, retain the cells. Consequently, it was impossible to completely separate the predator from its prey. Since predator populations are difficult to separate from prey populations, we can expand this analysis to account for the efficiency at which the populations were separated (cross-contamination). Both *P. fluorescens* and *O. danica* varied in size when growing in the various media, consequently we can determine the element content of both organisms by normalizing cell quota of each element to cell volume and repeating the analyses described above. Low pressure filtration used to separate the flagellate fraction from the bacterial fraction and preliminary experiments

demonstrated that optimal separation efficiency was obtained with a 3.0-µm pore size filter.

2.5.1.1. Protozoan Element Composition Based on Quota Normalized to Cell Abundance

The concentration of element x in *P. fluorescens* alone (BO cultures) may be determined directly:

Equation 1: $$Tx_{BO} = N_{bactBO} * Qx_{bactBO}$$

where N_{bactBO} is the number of bacteria (cells mL^{-1}), Qx_{bactBO} is the bacterial quota for element x (fmole $cell^{-1}$) and Tx_{BO} is the total particulate amount of element x (fmole mL^{-1}) within cells. Rearranging Equation 1 and solving for the bacterial cell quota, Qx_{bactBO} yields:

Equation 1a: $$Qx_{bactBO} = \frac{Tx_{BO}}{N_{bactBO}}$$

The concentration of element x in *P. fluorescens* and *O. danica* growing together (Tx_{BF}) in BF cultures may be determined from:

Equation 2: $$Tx_{BF} = \left[N_{bactBF} * Qx_{bactBF}\right] + \left[N_{flagBF} * Qx_{flagBF}\right].$$

Here N_{bactBF} is the number of bacteria (cells mL^{-1}), Qx_{bactBF} is the bacterial quota of element x (fmole $cell^{-1}$), and N_{flagBF} is the number of protozoa (cells mL^{-1}) found in the BF cultures. Substituting Qx_{bactBO} from Equation 1a into Equation 2 and solving for Qx_{flagBF} yields:

Equation 2a: $$Qx_{flagBF} = \frac{\left[N_{bactBO} * Tx_{BF}\right] - \left[N_{bactBF} * Tx_{BO}\right]}{\left[N_{bactBO} * N_{flagBF}\right]}$$

This approach assumes there is no change in the bacterial quota when growing alone or in the presence of the flagellate predator.

2.5.1.2. Protozoan Element Composition Based on Quota Normalized to Cell Abundance and Accounting for Separation Efficiency

An expanded approach to determine the concentration of element x in *P. fluorescens* and *O. danica* growing together in BFF cultures (Tx_{BFF}) considers the efficiency of flagellate separation:

Equation 3: $$Tx_{BFF} = \left[E_{bact} * N_{bactBFF} * Qx_{bactBFF}\right] + \left[E_{flag} * N_{flagBFF} * Qx_{flagBFF}\right].$$

Here, E_{bact} represents the proportion of bacteria passing through a 3.0 µm pore-size polycarbonate filter, $N_{bactBFF}$ is the total number of bacteria (cells mL^{-1}), and $Qx_{bactBFF}$ is the bacterial quota (fmole $cell^{-1}$) in the BFF cultures. E_{flag} is the proportion of protozoa that pass through a 3.0 µm pore-size filter, $N_{flagBFF}$ is the total number of flagellates (cells mL^{-1}), and $Qx_{flagBFF}$ is the flagellate quota (fmole $cell^{-1}$) in BFF cultures. By repeating the same procedure as above and solving for the flagellate quota yields:

Equation 3a: $$Qx_{flagBFF} = \frac{\left[N_{bactBO} * Tx_{BFF}\right] - \left[E_{bact} * N_{bactBFF} * Tx_{BO}\right]}{\left[E_{flag} * N_{bactBO} * N_{flagBF}\right]}$$

2.5.1.3. Protozoan Element Composition Based on Quota Normalized to Cell Volume

An alternative way to report the flagellate quota of element x in BF cultures is to normalize to cell volume (fmole μm^{-3}). Thus:

Equation 4: $$Qx_{flagBF} = \frac{\left[N_{bactBO} * Tx_{BF} * V_{bactBO}\right] - \left[N_{bactBF} * Tx_{BO} * V_{bactBF}\right]}{\left[N_{bactBO} * N_{flagBF} * V_{bactBO} * V_{flagBF}\right]}.$$

Here V_{bactBO} is the average volume of bacteria in the BO cultures (μm^3), V_{bactBF} is the average volume of bacteria in BF cultures (μm^3), while V_{flagBF} is the average volume of flagellates in the BF cultures (μm^3).

2.5.1.4. Protozoan Element Composition Based on Quota Normalized to Cell Volume and Accounting for Separation Efficiency

Equation 5 modifies Equation 4 by accounting for the efficiency at which O. danica was separated from *P. fluorescens* using a 3.0µm pore-size filter: This was accomplished by accounting for the and flagellate

$$\text{Equation 5:} \qquad Qx_{flag_{BFF}} = \frac{\left[N_{bact_{BO}} * Tx_{BFF} * V_{bact_{BO}}\right] - \left[E_{bact} * N_{bact_{BFF}} * Tx_{BO} * V_{bact_{BFF}}\right]}{\left[E_{flag} * N_{bact_{BO}} * N_{flag_{BF}} * V_{bact_{BO}} * V_{flag_{BFF}}\right]}$$

Where (Tx_{BFF}) is the total particulate element content of *P. fluorescens* and *O. danica* cells when growing together; E_{bact} is the efficiency at which the bacteria were separated from the protozoan; and E_{flag} is the efficiency at which the protozoan was separated from the bacteria. Although, these equations are used to decipher the element composition of a flagellate in the presence of a bacterial population, their weakness stems from the assumption that the bacterial element composition is invariant even with the possibility of protozoan nutrient recycling.

Data were analaysed using one-way ANOVA or Factorial ANOVA. Means were analysed using Tukey's post hoc test. Cell quota and volume measurements were log transformed prior to analysis to reduce heteroscedasticity and skewedness. All analyses were conducted using Statistica 7.0; graphs were created using Sigma Plot 9.0.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Determination of *Ochromonas* Exponential Growth Phase

3.1.1. Characteristics of Pseudomonas fluorescens Growth in Various Media

Pseudomonas fluorescens grew equally well in each of the four media. Growth rates and generation times were approximately 0.4 hr^{-1} and 1.6 h (Figure 3.1 and Table 3.1). Media had no affect on bacterial growth rate (ANOVA: $F_{3,8} = 1.11$; $p = 0.4$). At the onset of stationary phase, greater cell abundance was found in P- and N-Limited media and likely resulted from the larger glucose concentration in these media (Table 2.1).

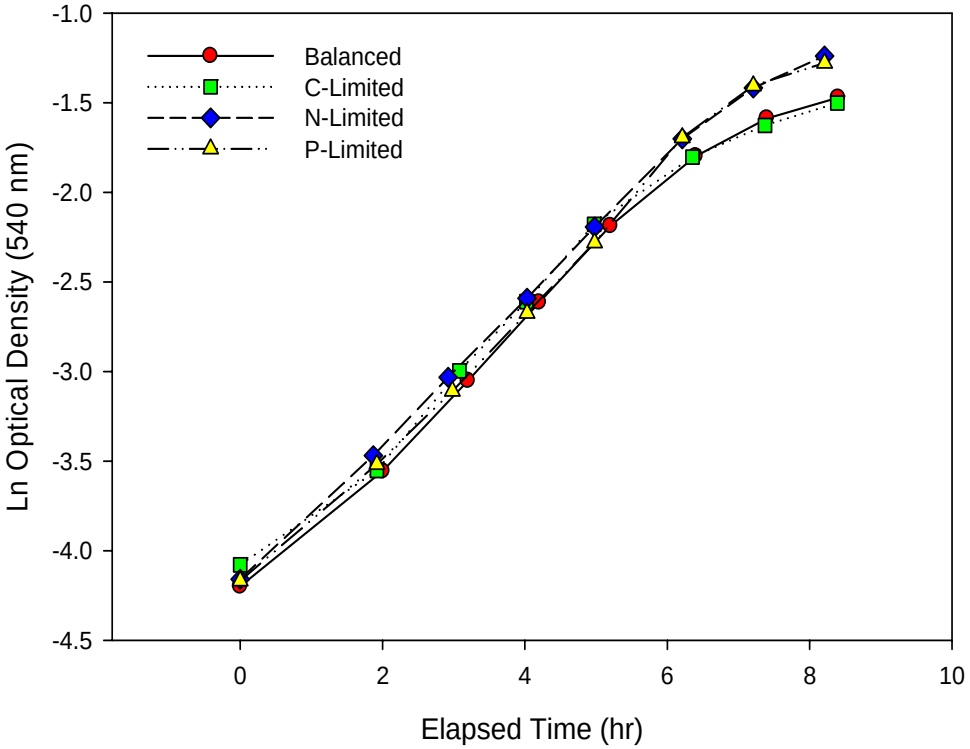


Figure 3.1 *Pseudomonas fluorescens* growth in each of the four media.

Table 3.1 Growth of *P. fluorescens* in varying media type.

Medium	Growth Rate (hr^{-1})	Generation Time (hr)
Balanced	0.43	1.61
C-Limited	0.44	1.58
N-Limited	0.42	1.65
P-Limited	0.41	1.69

Cell size did not vary regardless of the medium in which the cells were grown (Table 3.2; ANOVA: $F_{3,8} = 1.73$; $p < 0.0005$).

Table 3.2 Comparison of *P. fluorescens* mean cell sizes grown in different media. S.E. = standard error and N = number of cells measured.

Medium	Mean Cell Size (μm^3) ± S.E. (N)
Balanced	0.217 ± 0.0610 (257)
C-Limited	0.137 ± 0.0168 (268)
N-Limited	0.204 ± 0.0091 (287)
P-Limited	0.152 ± 0.0225 (259)

The element content of cells (Quota, $Q_{element}$) grown in each of the four types of media were normalized to volume and is presented in Figure 3.2 and Table 3.3. Media type had a significant effect on QC and QN (ANOVA: QC: $F_{3,8} = 4.09$, QN: $F_{3,8} = 4.29$; $p < 0.05$). There were statistically significant differences between individual element concentrations when cells were grown in C-Limited and N-Limited media (Tukey's post hoc: QC: $p = 0.037$; QN: $p = 0.031$; and QP: $p = 0.042$). Cells grown in C-Limited medium contained the highest concentrations of each element (QC, QN and QP) while cells grown in N-Limited medium contained the lowest concentrations of each element (Table 3.3).

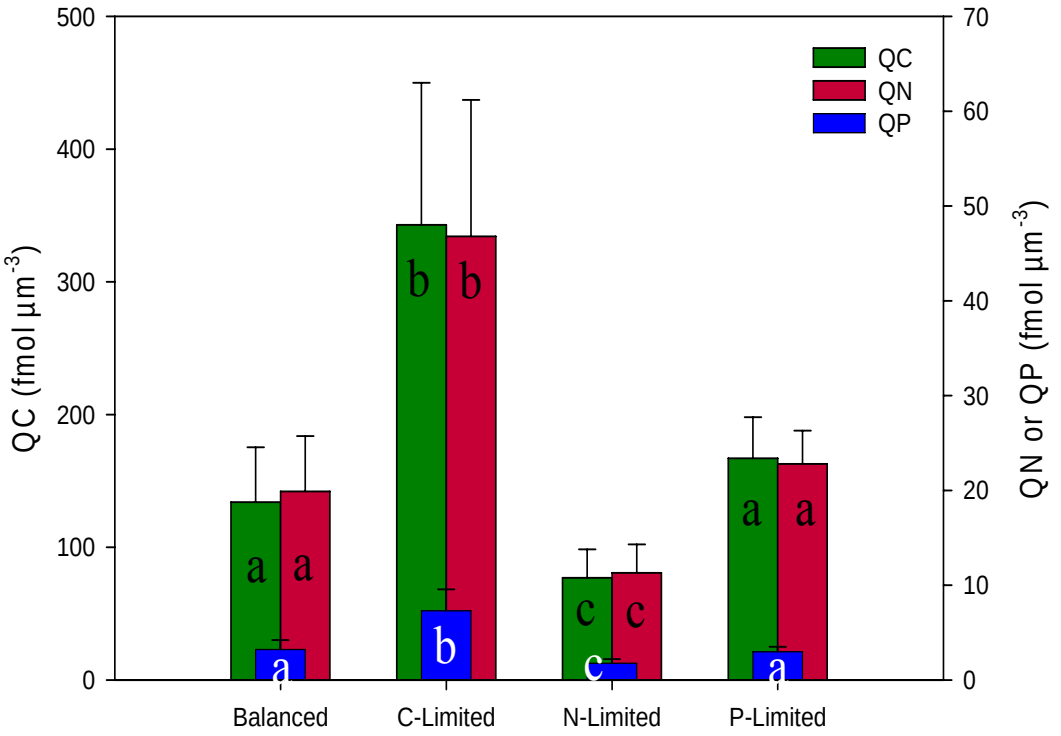


Figure 3.2 Cell quota of carbon (QC), nitrogen (QN), and phosphorus (QP) for *P. fluorescens* grown in different media types. Mean values and standard errors reported in Table 3.3. Error bars represent the SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Table 3.3 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Pseudomonas fluorescens* grown in different media types. (Mean ± SE, n = 3 triplicate batch cultures)

Medium	Element (fmol µm^{-3})		
	C	N	P
Balanced	134 ± 41.4	19.9 ± 5.84	3.22 ± 1.00
C-Limited	343 ± 107	46.8 ± 14.4	7.30 ± 2.25
N-Limited	76.9 ± 21.4	11.3 ± 2.99	1.75 ± 0.431
P-Limited	167 ± 31.0	22.8 ± 3.49	2.98 ± 0.505

Element ratios (C:N, C:P, and N:P) for cells grown in each media type are represented in Figure 3.3 and Table 3.4. Media type had a significant effect on C:P and N:P ratios (Factorial ANOVA main effect of media: C:P: $F_{3,8} = 10.63$, N:P: $F_{3,8} = 4.86$; $p < 0.05$). Cells grown in C-Limited medium had higher C:P and N:P ratios (Table 3.4)

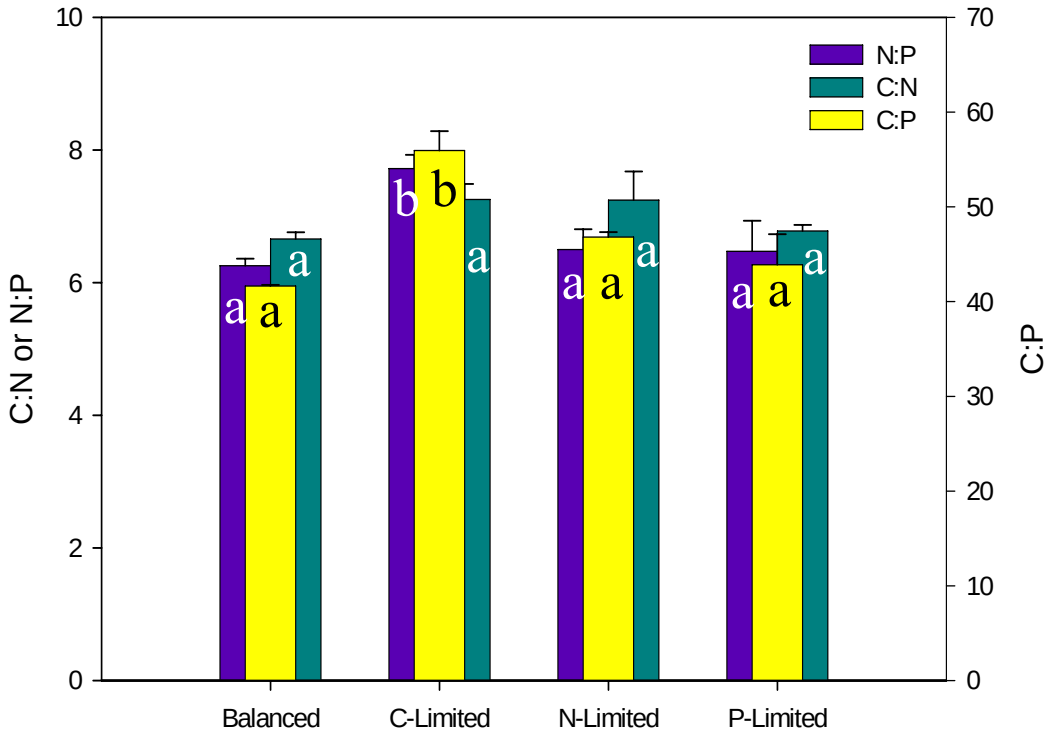


Figure 3.3 Element ratios of *Pseudomonas fluorescens* grown in different media types at full strength. Mean values and standard errors are reported in Table 3.4. Error bars represent the SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

whereas C:P and N:P ratios of cells grown in all other media types were statistically similar. Variations among C:N ratios for *Pseudomonas* populations were not statistically different, regardless of nutrient conditions.

Table 3.4 Element ratios in *Pseudomonas fluorescens* grown in different media types. (Mean ± SE, n = 3 triplicate batch cultures)

Medium	Ratio (mole:mole)		
	C:N	C:P	N:P
Balanced	6.66 ± 0.100	41.63 ± 0.119	6.25 ± 0.107
C-Limited	7.25 ± 0.234	55.95 ± 2.04	7.72 ± 0.209
N-Limited	7.24 ± 0.431	46.80 ± 0.522	6.50 ± 0.307
P-Limited	6.78 ± 0.091	43.89 ± 3.23	6.47 ± 0.462

3.1.2 Feasibility Study: Ochromonas Growth Kinetics

Ochromonas was preconditioned to feed on a given prey type prior to its use in experiments to confirm prey would support growth and permit *Ochromonas* to obtain a stable stoichiometry. Growth rates and generation times of *Ochromonas* varied greatly (Figure 3.4 and Table 3.5) despite feeding upon prey of similar element ratios. Growth was worst when *Ochromonas* was feeding upon bacteria produced in Balanced or P-Limited media and intermediate when feeding upon bacteria produced in C-Limited medium. Growth was best when *Ochromonas* was feeding on cells produced in N-Limited medium ($\mu = 0.22$ hr^{-1} and $g = 3.2$ hr) resulting in a significantly higher growth rate than was found for Balanced (Tukey's post hoc: $p = 0.004$), C-Limited (Tukey's post hoc: $p = 0.011$) or P-Limited media (Tukey's post hoc: $p = 0.004$). *Ochromonas* feeding upon N-Limited cells grew 3X faster when feeding on bacteria produced in Balanced or P-Limited media (Figure 3.4), even though bacteria grown in N-Limited medium contained the lowest concentration of elements compared with other bacterial prey grown in other media types.

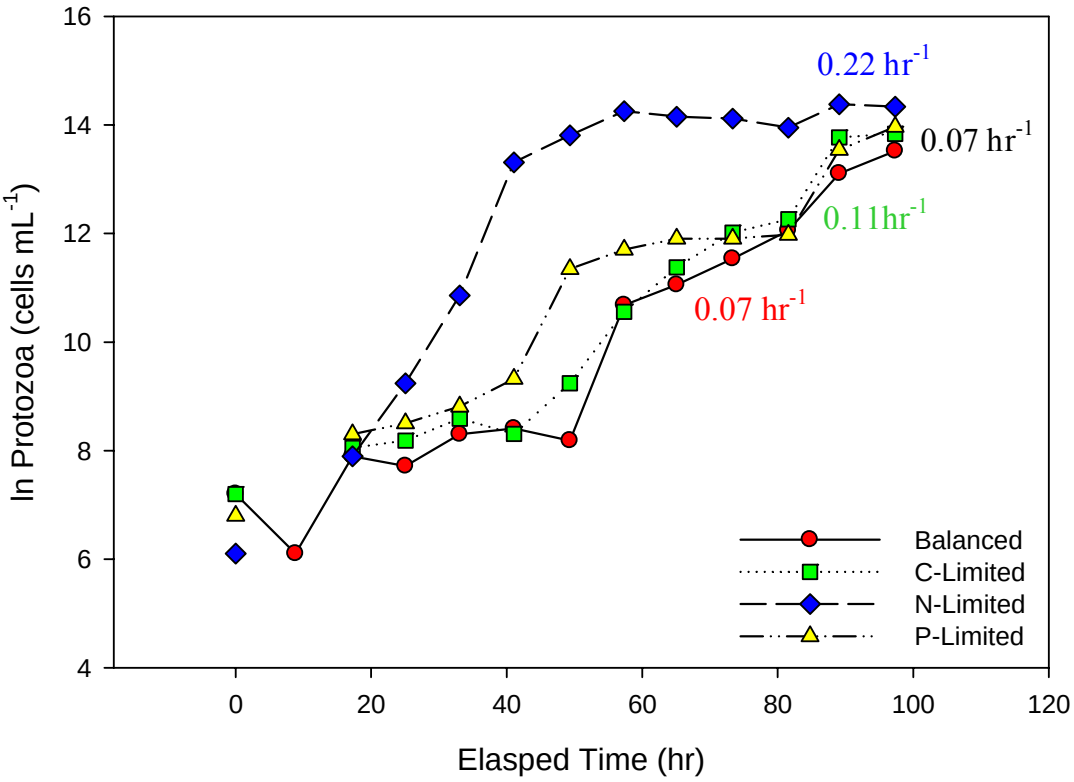


Figure 3.4 *O. danica* growth curve feeding on *P. fluorescens* produced in different media types.

Table 3.5 Growth of *O. danica* grazing on *P. fluorescens* in varying media type.

Medium	Growth Rate (μ) (hr^{-1})	Generation Time (g) (hr)
Balanced	0.07	9.9
C-Limited	0.11	6.3
N-Limited	0.22	3.2
P-Limited	0.07	9.9

It is conceivable that the differences among *Ochromonas* growth rates resulted from an inadequate supply of bacteria. However, at the beginning of each experiment, the abundance of bacteria exceeded $4x10^8$ cells mL^{-1} (Figure 3.5). Bacterial abundance

remained at this level for the entire time *Ochromonas* was in exponential growth and only declined in all cultures after 49 hours, suggesting there was ample prey in all treatments.

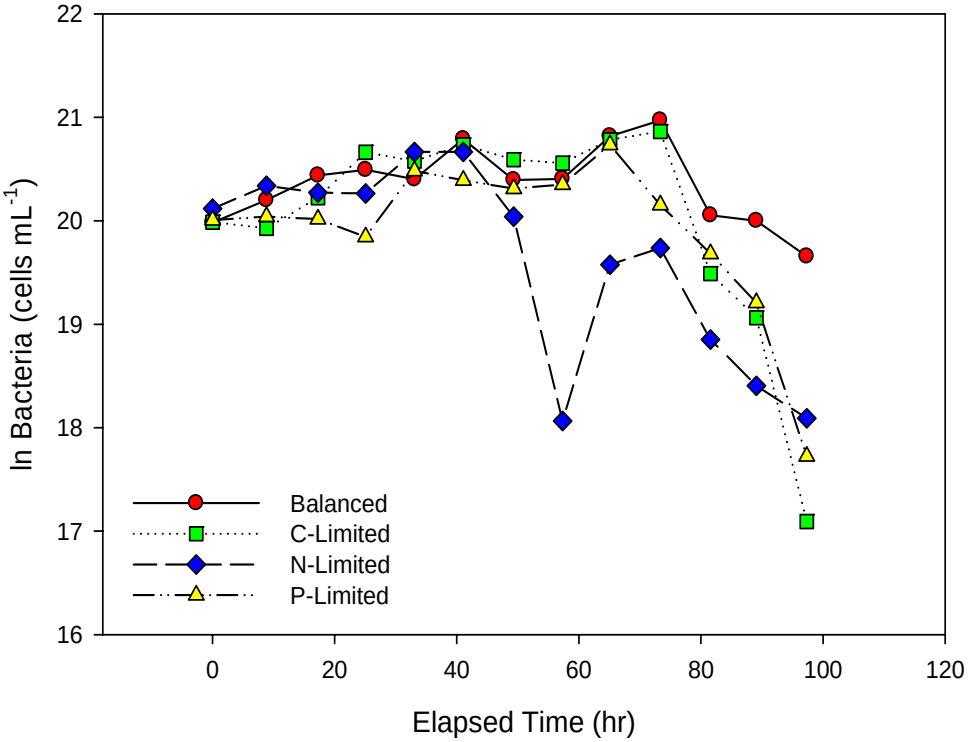


Figure 3.5 Abundance of bacteria after the introduction of *Ochromonas* in different media types.

3.1.3 Conclusions from Feasibility Study

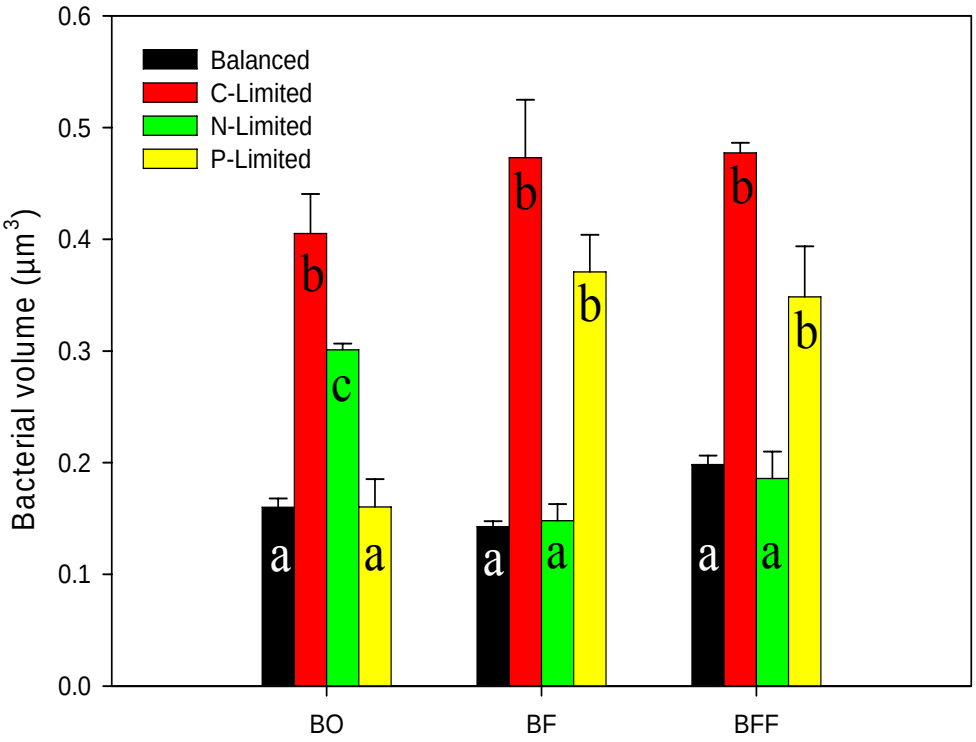
Results confirmed that by altering the element content of media supplied to bacteria, the resulting bacterial cells differed in element content even though the elemental ratios did not vary greatly. Each prey type successfully supported growth of *Ochromonas* over 100 hrs. Evaluating differences in *Ochromonas* growth when feeding on different prey types, suggests a stoichiometric component could account for protozoan growth. At the end of the feasibility experiment all cultures were still very dense with bacteria and protozoan cell growth due to the concentration of the media, consequently it was necessary to dilute the media. A 1:6.6 dilution was found to limit bacterial and protozoan abundance and therefore would be used in all future experiments.

3.2 Determining the Element Content of *Ochromonas danica*

3.2.1 Characteristics of the Prey: Pseudomonas fluorescens

Pseudomonas fluorescens was grown in each media type as in the feasibility studies, with the exception that all media were diluted 1:6.6. The characteristics of *Pseudomonas fluorescens* were slightly different in the diluted media than in full strength media. Bacteria varied in size depending on nutrient condition (Factorial ANOVA: $F_{3,24} = 27.66$, $p < 0.05$; Figure 3.6 and Table 3.6) and the interaction of nutrient condition and culture type also had a significant effect on bacterial volume (Factorial ANOVA: $F_{6,24} = 6.15$, $p < 0.05$). The smallest cells in BO cultures were associated with Balanced and P-Limited media (0.160 ± 0.043 μm^3) and were not significantly different (Tukey's post hoc: $p = 1.00$; Figure 3.6A). Bacterial cells grown in P-Limited medium resulted in intermediate cell size. The largest cells in BO culture types were grown in C-Limited medium (Figure 3.6A and Table 3.6) and were significantly larger than cells growing in all other media (Tukey's post hoc: Balanced: $p < 0.005$; N-Limited: $p < 0.005$; P-Limited: $p = 0.007$). The smallest cells in BF and BFF cultures were associated with Balanced and N-Limited media (Figure 3.6A and Table 3.6) and were not significantly different (Tukey's post hoc: BF: $p = 0.98$, BFF: $p = 1.00$; Figure 3.6A). In BF and BFF culture types, the largest cells were associated with C-Limited and P-Limited media which were also not significantly different from each other (Tukey's post hoc: BF: $p = 0.63$, BFF: $p = 0.45$; Figure 3.6A).

A.



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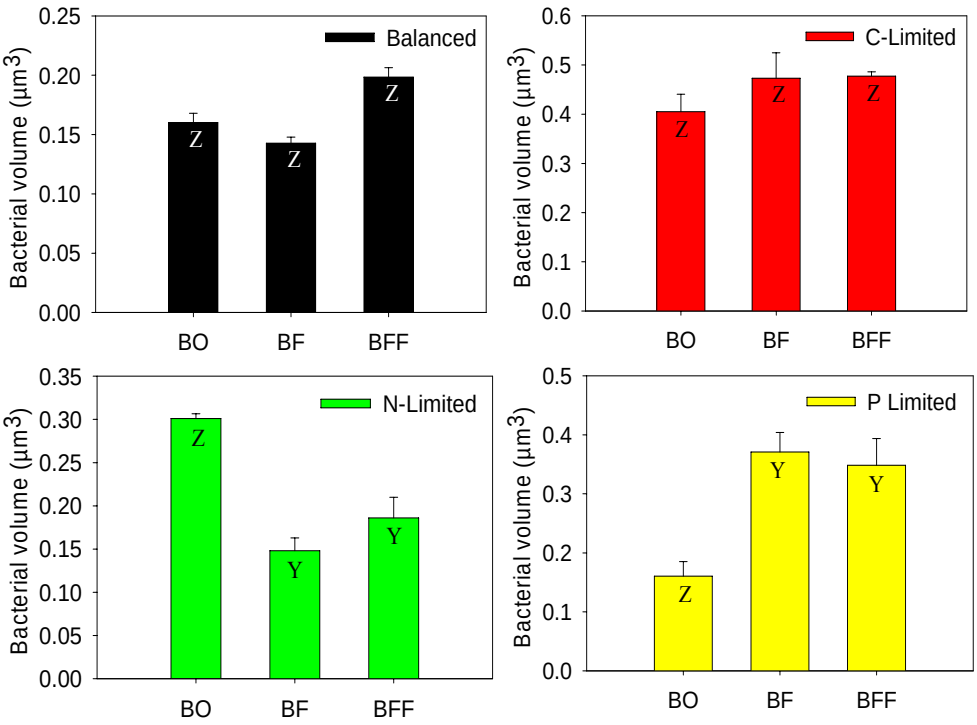

Figure 3.6 Volume of *Pseudomonas fluorescens* grown in each media and culture type. Mean values and standard errors are reported in Table 3.6. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

Upon introduction of flagellate, there was a significant shift in bacterial mean cell size. This shift is best seen in P-Limited and N-Limited media by comparing the differences between mean cell volume in BO cultures with BF and BFF cultures (Figure 3.6B). In P-Limited medium, when comparing BO to BF or BFF cultures, mean bacterial cell size doubled in the presence of the flagellate (Tukey's post hoc: $p < 0.005$). When comparing BO cultures with BF or BFF cultures, mean cell size decreased by half in N-Limited medium (Tukey's post hoc: $p < 0.05$). Cell size distribution graphs were constructed for further analysis using the entire bacterial population volume measurements in BO and BF cultures types (Figure 3.7). A statistically significant shift in bacterial cell size distribution between culture types of N-Limited medium (χ^2: $p < 0.05$) appeared to result in a reduction in the number of larger cells in the distribution and increased the number of smaller cells, thus decreasing the average bacterial volume. The reverse appeared to occur between the culture types of P-Limited medium (χ^2: $p < 0.05$), where the portion of smaller size bacterial cells had been reduced from the population and increased in the number of larger cell size in the bacterial distribution, thus increasing the average bacterial volume.

Table 3.6 Comparison of *P. fluorescens* mean cell sizes grown in different media and culture types. S.E. = standard error and N = number of cells measured.

Medium	Mean Cell Size (μm^3) ± S.E. (N)		
	BO culture	BF culture	BFF culture
Balanced	0.160 ± 0.043 (234)	0.143 ± 0.009 (202)	0.198 ± 0.019 (235)
C-Limited	0.405 ± 0.035 (235)	0.473 ± 0.052 (74)	0.477 ± 0.009 (31)
N-Limited	0.301 ± 0.006 (368)	0.148 ± 0.015 (204)	0.186 ± 0.024 (223)
P-Limited	0.160 ± 0.025 (446)	0.371 ± 0.033 (81)	0.348 ± 0.045 (99)

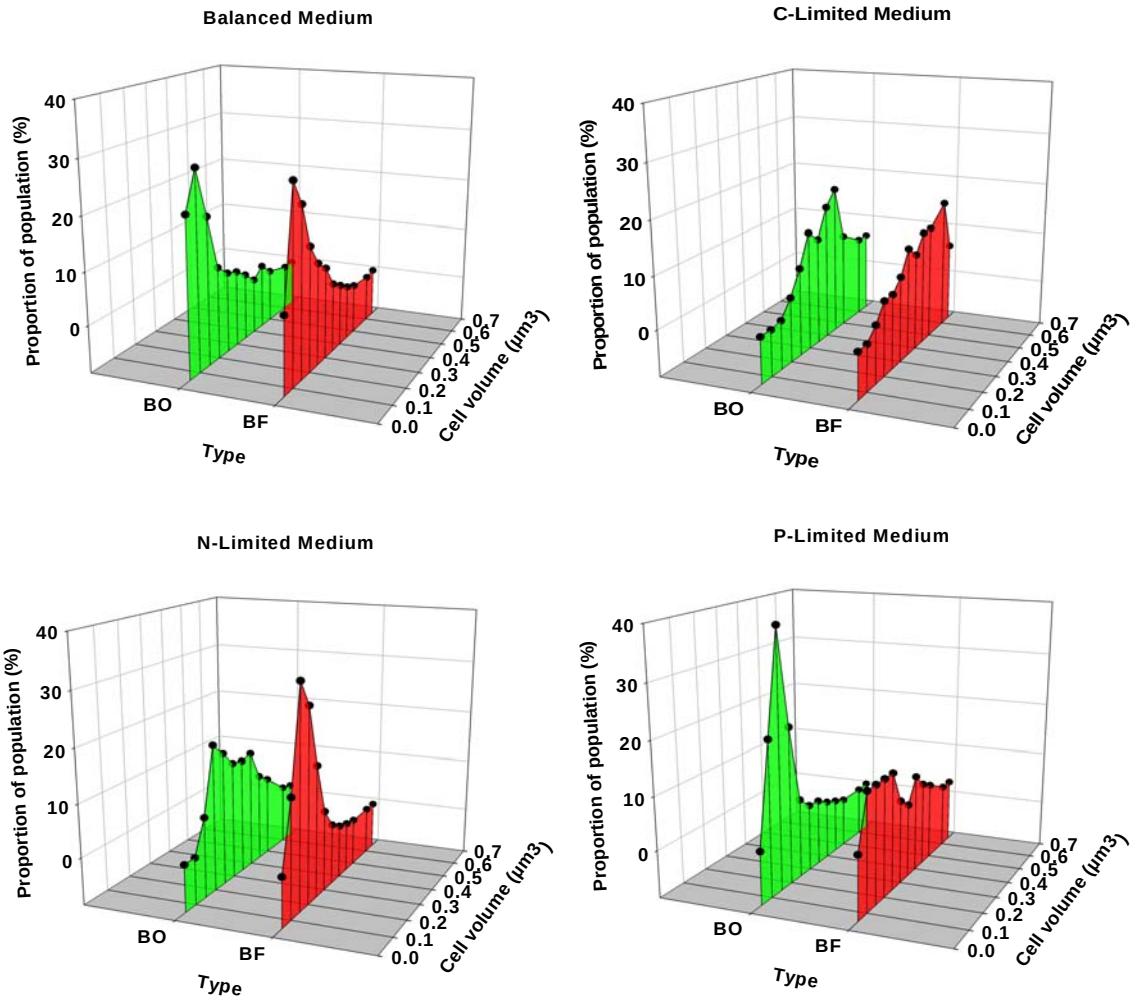

Figure 3.7 Comparison of bacterial volume (μm^3) distribution curves among media and culture types.

One possible explanation for the shift in the distribution towards a smaller bacterial cell size in N- and P-Limited media is the potential error for measuring the ejected remnants of partial digested bacteria. Staining methods used here are unable to differentiate between partial digested bacteria that have been excreted and bacterial cells of small size. Another possible explanation could be due to bacterial change in element composition. Since protozoa could theoretically amplify nutrient limitation in bacteria by excretion of non-limiting nutrients, this stress could alter bacteria stoichiometry. An

explanation for the shift towards a larger cell size distribution is the potential for bacterial re-growth on recycled nutrients. Bacteria cells capable of storing recycled nutrients could result in larger cells. Protozoan size selective grazing could possibly explain the shift in either direction.

Element concentrations (QC, QN and QP) of a bacteria varied depending on media type (ANOVA: QC: $F_{3,8} = 7.59$, $p < 0.05$; QN: $F_{3,8} = 6.25$, $p < 0.05$; QP: $F_{3,8} = 15.27$, $p < 0.005$). Despite their small size, cells growing in Balanced medium contained the absolute highest concentrations of each element (Figure 3.8 and Table 3.7). However due to high variability of quotas of cells grown in Balanced medium, bacterial carbon concentration was equivalent in Balanced to those measured in N-Limited and P-Limited media (Tukey's post hoc: N-Limited: $p = 0.65$; P-Limited: $p = 0.07$); bacterial QP measurements were equivalent in Balanced to that measured in N-Limited medium (Tukey's post hoc: N-Limited: $p = 0.87$). Lower element quotas were found in bacteria grown in C-Limited and P-Limited media compared to quotas obtained for cells grown in Balanced or N-Limited media and were not statistically different (Tukey's post hoc: QC: $p = 0.49$; QN: $p = 0.94$; QP: $p = 0.65$).

Overall, bacteria grown in C- and P-Limited media had lower N and P contents than bacteria grown in N-Limited medium. These results are similar to those found by Vrede (2002) for bacterial isolates grown in batch culture. Bacteria grown in Balanced medium had the highest nutrient levels yet were the same size as bacteria grown in P-Limited medium, which contained low nutrient levels. These results suggest that there may be a differential cost to a predator when selecting prey items: *Ochromonas* could

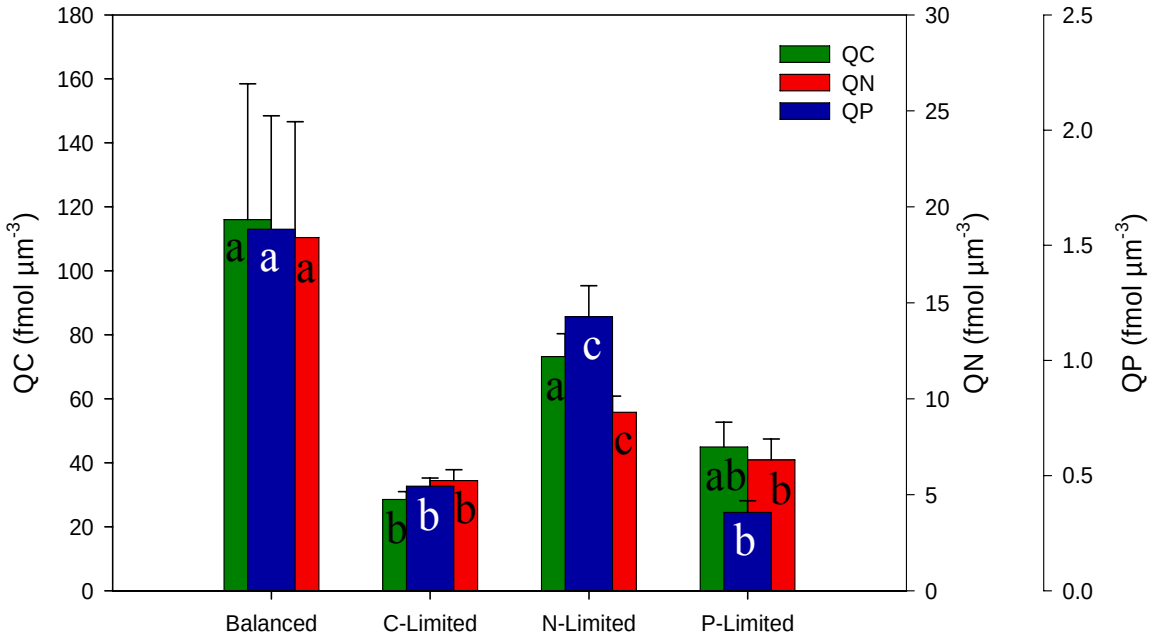

Figure 3.8 Cell quota of carbon (QC), nitrogen (QN), and phosphorus (QP) for *P. fluorescens* grown in different media types. Mean values and standard errors reported in Table 3.3. Error bars represent the SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Table 3.7 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Pseudomonas fluorescens* grown in different media types at 1:6.6 dilution. (Mean ± SE, n = 3 triplicate batch cultures)

Medium	Element (fmol µm^{-3})		
	C	N	P
Balanced	116 ± 42.5	18.4 ± 6.03	1.57 ± 0.492
C-Limited	28.5 ± 2.49	5.74 ± 0.568	0.454 ± 0.035
N-Limited	73.2 ± 7.17	9.30 ± 0.832	1.19 ± 0.134
P-Limited	44.9 ± 7.77	6.81± 1.10	0.340 ± 0.0502

receive 3x more C, N, P if it preys on a "well-nourished" prey item compared to what it would obtain if it fed upon a "poorly-nourished" prey item. Similarly, C-Limited bacteria cells had the largest cell volume but contained the lowest nutrient levels making them poor food quality to predators. *Ochromonas* would have to consume more C-Limited bacteria to receive the same amount of nutrients than if it grazed on an

alternative food source. N-Limited bacteria were intermediate in size and had intermediate concentrations of each element. Therefore, the available nutrient supply to prey could have consequential effects on predator growth and nutrient regeneration.

Alternatively food quality may be considered in terms of nutrient element ratios (C:N, C:P, and N:P), which also varied depending on media type (ANOVA: C:N: $F_{3,8}$ = 35.60, p <0.0005; C:P: $F_{3,8}$ = 144.25, p <0.00001; N:P: $F_{3,8}$ = 267.30, p <0.00001). Mean element ratios for *Pseudomonas* grown in each media type are shown in Figure 3.9 and Table 3.8. The C:N ratio varied from 4.97 to 7.87, with the lowest ratio found in cells growing in C-Limited medium and the highest ratio found in cells growing in N-Limited medium. C:N ratios of cells grown in each medium were significantly different from each other (Tukey's post hoc: p <0.05) with the exception of those obtained for cells grown in Balanced and P-Limited media (Tukey's post hoc: p = 0.55). C:P values varied two-fold with the highest ratio associated with bacteria grown in P-Limited medium (Tukey's post hoc: Balanced: p <0.0005; C-Limited: p <0.0005; N-Limited: p <0.0005). Bacteria grown in N-Limited and C-Limited media had the lowest C:P ratios, but these ratios were not significantly different from the C:P ratio of cells grown in Balanced medium (Tukey's post hoc: p >0.1). N:P values also varied approximately two-fold. The N:P ratio of P-Limited bacteria was 19.9 ± 0.318 fmol μm^{-3} while the N:P ratio associated with cells grown in Balanced medium was 11.67 ± 0.217 fmol μm^{-3}. N:P ratios of cells grown in Balanced and C-Limited media were similar (Tukey's post hoc: p = 0.2), however the N:P ratio of cells from these media were significantly greater than that of cells grown in N-Limited medium (Tukey's post hoc: p <0.005) and significantly less than that of cells grown in P-Limited medium (Tukey's post hoc: p <0.003).

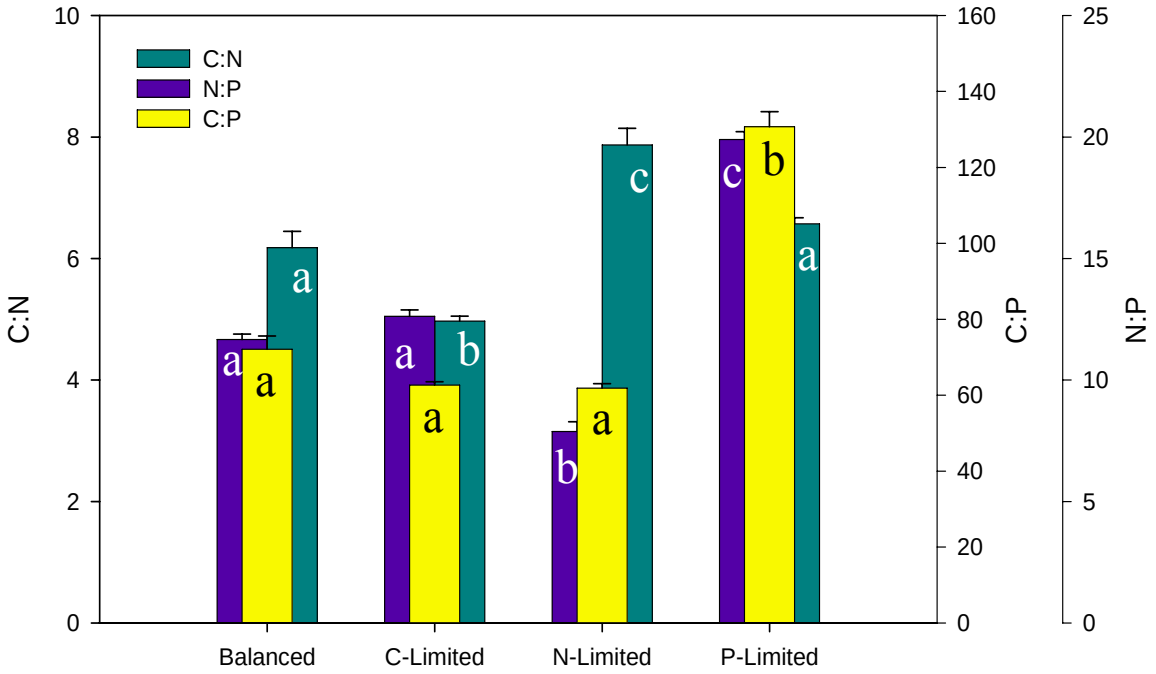

Figure 3.9 Element ratios of *Pseudomonas fluorescens* grown in different media types. Mean values and standard errors are reported in Table 3.4. Error bars represent the SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Table 3.8 Element ratios in *Pseudomonas fluorescens* grown in different media types. (Mean ± SE, n = 3 triplicate batch cultures)

Medium	Ratio (mole:mole)		
	C:N	C:P	N:P
Balanced	6.18 ± 0.266	72.07 ± 3.54	11.67 ± 0.217
C-Limited	4.97 ± 0.079	62.64 ± 0.883	12.62 ± 0.261
N-Limited	7.87 ± 0.272	61.85 ± 1.16	7.88 ± 0.402
P-Limited	6.57 ± 0.100	130.74 ± 3.93	19.9 ± 0.318

Comparison of Figures 3.7 and 3.8 reveals that bacteria grown in Balanced medium not only contain absolutely more C, N and P than bacteria grown in other media, but they also contained more P relative to the concentrations of other elements. In terms of food quality, protozoa with high demands for P might expect to grow well on N-Limited bacteria. Similarly, P-Limited bacteria might be good food quality for predators with

high carbon demands and meager phosphorus demands. Clearly, each medium allowed bacteria to obtain a different element stoichiometry.

3.2.2 Characteristics of the Predator: Ochromonas danica

The growth rates of *Ochromonas* varied depending upon the type of bacteria on which it was feeding (ANOVA: $F_{3,8} = 332.10$, $p < 0.00001$; Figure 3.10 and Table 3.9) and were reflected by the loss of bacteria in each of the cultures (Figure 3.11). The fastest growth rate, 0.069 hr^{-1}, was obtained when *Ochromonas danica* was growing on bacteria harvested from P-Limited medium. Growth was slowest, 0.016 hr^{-1}, when feeding on bacteria produced in Balanced medium. All growth rates were significantly different from each other (Tukey's post hoc: $p < 0.0005$).

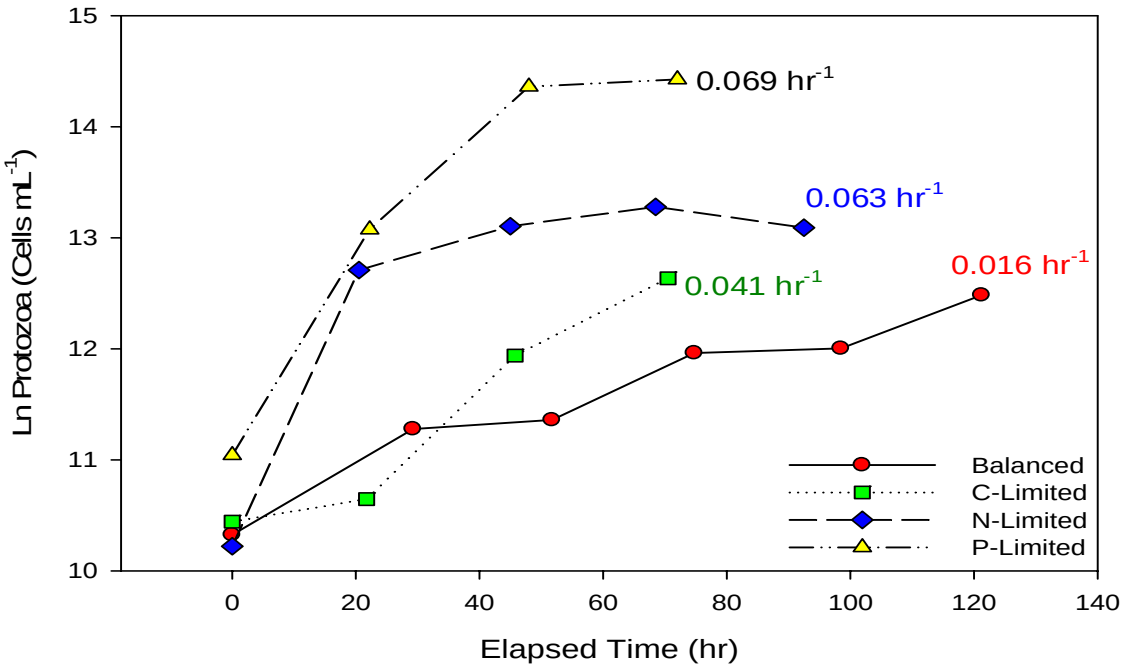

Figure 3.10 *O. danica* growth curve feeding on *P. fluorescens* produced in different media types at 1:6.6 dilution.

Table 3.9 Growth of *O. danica* grazing on *P. fluorescens* in varying media type.

Medium	Growth Rate (hr^{-1})	Generation Time (hr)
Balanced	0.016	42.39
C-Limited	0.041	17.02
N-Limited	0.063	11.09
P-Limited	0.069	10.01

Exponential growth of *Ochromonas* growing on bacteria in Balanced medium was calculated over a time interval of 121.5 hrs, 70.5 hrs in C-Limited medium, 45 hrs in N-Limited medium, and 48 hrs in P-Limited medium. Depletion of bacteria during these growth studies is represented in Figure 3.11.

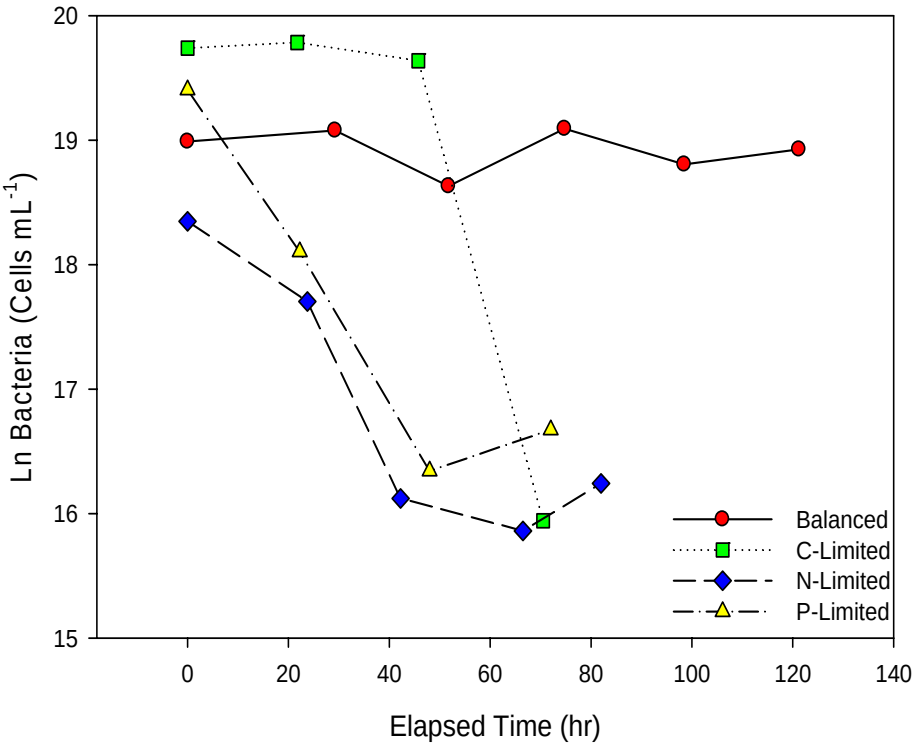

Figure 3.11 Abundance of bacteria after the introduction of *Ochromonas* in different media types.

Ochromonas cell size appeared to vary as a function of prey nutritional status (Factorial ANOVA: $F_{3,15} = 13.07$, $p < 0.0005$) and culture type (Factorial ANOVA: $F_{1,15} = 151.96$, $p < 0.00001$) (Figure 3.12). *Ochromonas* was large (Figure 3.12A and Table 3.10) when feeding upon P-Limited bacteria but these cells were not significantly larger than those grown on Balanced or N-Limited bacteria (Tukey's post hoc: Balanced: $p = 0.998$; N-Limited: $p = 1.00$). On the other hand, *Ochromonas* were considerably smaller when feeding upon C-Limited bacteria than when growing on P-Limited bacteria (Tukey's post hoc $p = 0.014$). C-Limited bacteria had the largest cell volume (Table 3.6) but the lowest element quotas (Table 3.7) and the lowest element ratios suggesting that C-Limited bacteria contained large amounts of water within their cells. Thus, *Ochromonas* grazing upon C-Limited bacteria resulted in the protozoa having small cell size. *Ochromonas* had the largest cell volume and grew best when grazing on P-Limited bacteria. P-Limited bacteria were small, did not contain large concentrations of each element and had the largest ratios of C:P and N:P. These data suggest that P-Limited bacteria contained less water per unit volume and provided a small packet of relatively high quality food for *Ochromonas*.

Filtration of BFF cultures, resulted in a significant shift in flagellate mean cell size. This shift is seen in all media by comparing the differences between mean cell volume in BF cultures with BFF cultures (Figure 3.12B and Table 3.10). In all media, when comparing BF to BFF cultures, mean flagellate cell size decreased by half (Tukey's post hoc: Balanced: $p < 0.005$; C-Limited: $p < 0.005$; N-Limited: $p < 0.007$; P-Limited: $p < 0.0005$) following filtration.

A.

B.

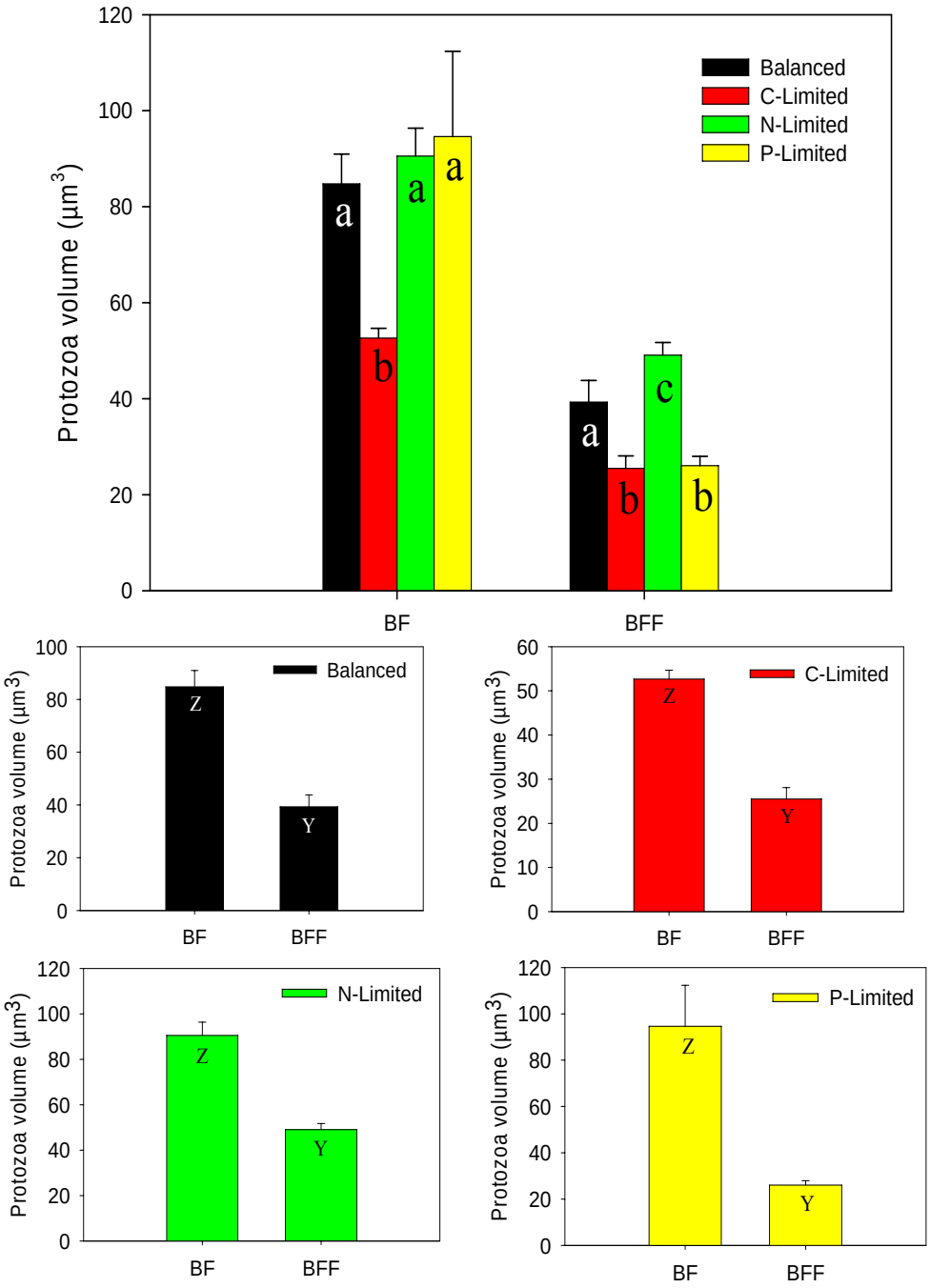

Figure 3.12 Volume of *Ochromonas danica* grown in each media and culture type. Mean values and standard errors are reported in Table 3.6. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

Cell size distribution graphs were constructed for further analysis using the entire flagellate population volume measurements in BF and BFF cultures types (Figure 3.13). A statistically significant shift in flagellate cell size distribution between culture types of all media (χ^2: p <0.05) appeared to result in a reduction in the number of larger cells in the distribution and increased the number of smaller cells, thus decreasing the average flagellate volume.

Prey type based on media had a significant effect on protozoa cell volume (ANOVA, $F_{3,15} = 151.96$, $p < 0.00001$), and suggested that protozoa and bacteria could be easily separated by filtration. However, *Ochromonas* proved to be highly plastic and only the largest of cells could be removed by filtration of BFF cultures (Figure 3.13). The effect of the filtration process can be seen when comparing flagellate mean cell size distributions of BF and BFF cultures in Figure 3.13. This result complicated efforts to determine the element content of *Ochromonas*.

Table 3.10 Comparison of *O.danica* mean cell sizes grazing on *P.fluorescens* in varying media type at a 1:6.6 dilution. S.E. = standard error and N = number of cells measured.

Medium	Mean Cell Size (μm^3) ± S.E. (N)	
	BF cultures	BFF cultures
Balanced	84.78 ± 6.17 (231)	39.30 ± 4.48 (53)
C-Limited	52.65 ± 2.03 (253)	25.51 ± 2.60 (157)
N-Limited	90.54 ± 5.80 (294)	49.09 ± 2.62 (204)
P-Limited	94.62 ± 17.73 (72)	26.06 ± 1.95 (90)

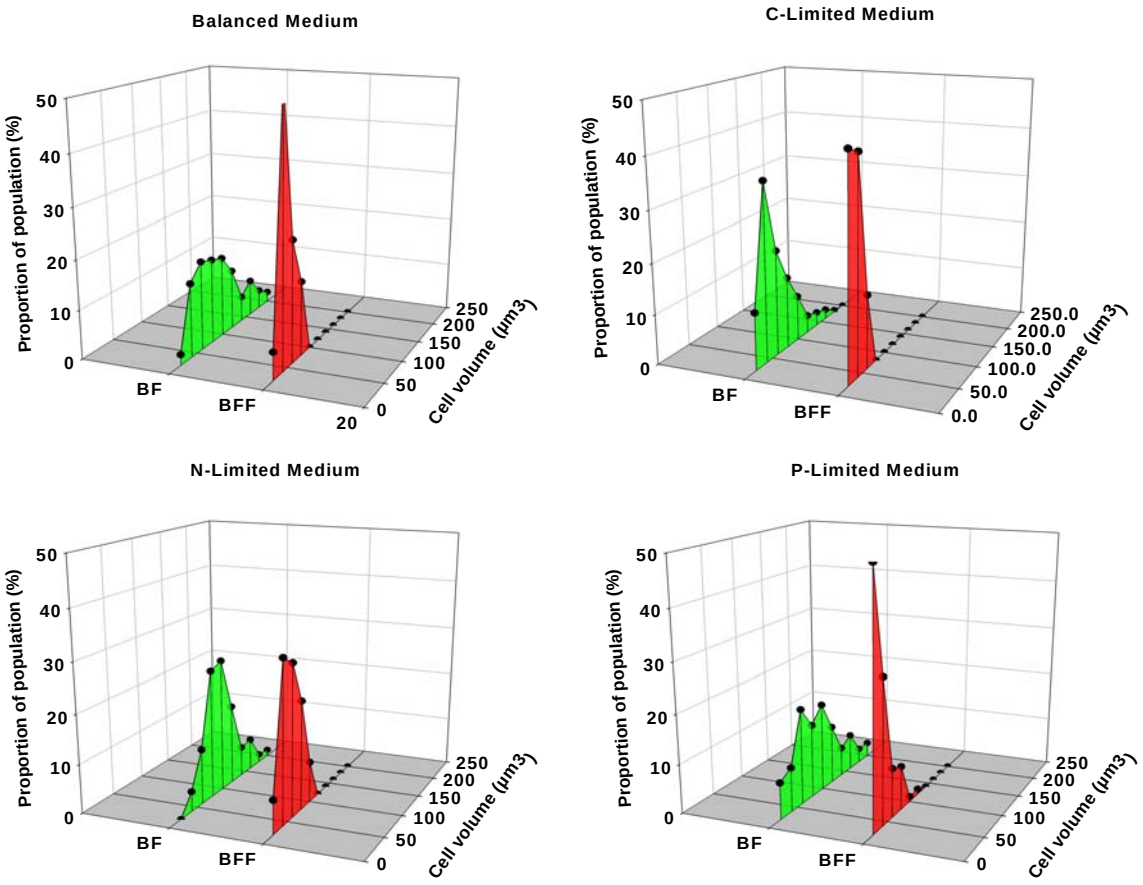

Figure 3.13 Comparison of protozoan volume (μm^3) distribution curves among media and culture types.

3.2.3 Ochromonas Element Content as a Function of Cells

The element content (QC, QN and QP) of *Ochromonas* (fmoles $cell^{-1}$) feeding on bacteria was determined using Equation 2a and QC and QP varied depending on the medium type (ANOVA: QC: $F_{3,8} = 21.56$, $p < 0.0005$; QP: $F_{3,8} = 56.24$, $p < 0.00005$; Figure 3.14 and Table 3.11).

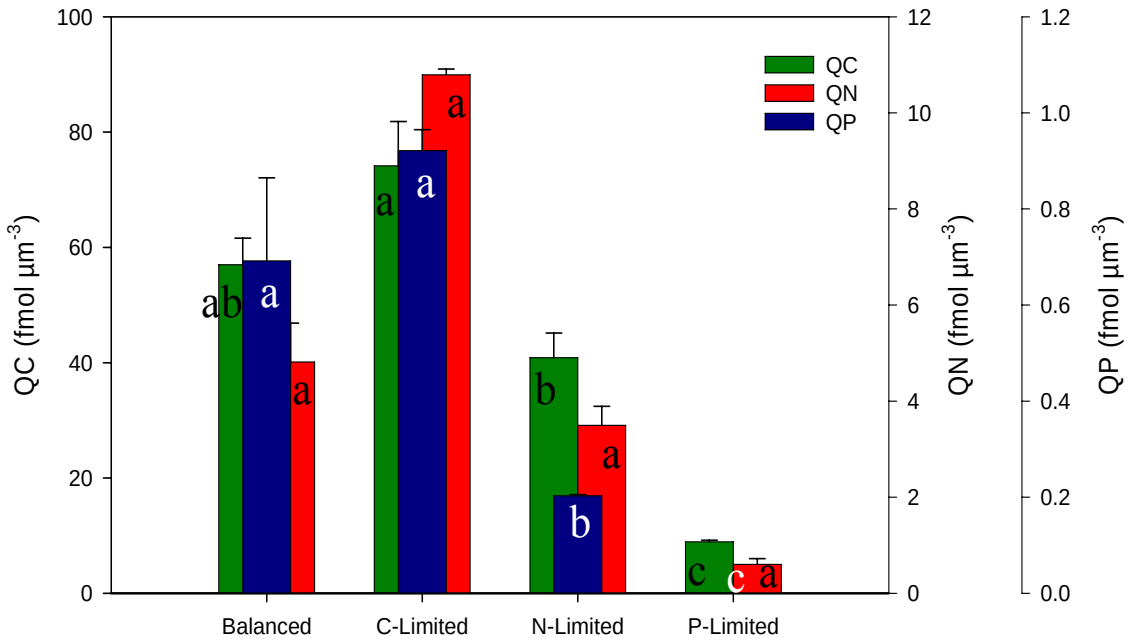

Figure 3.14 Cellular quotas of carbon (QC), nitrogen (QN), and phosphorus (QP) for *O. danica* feeding on bacteria grown in different media types reported as fmoles cell^{-1}. Mean values and standard errors reported in Table 3.11. Error bars represent SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Ochromonas grazing on Balanced, C-Limited and N-Limited bacteria contained the equivalent amounts carbon compared concentrations obtained for flagellate cells feeding on P-Limited bacteria (Tukey's post hoc: Balanced: p <0.005; C-Limited: p <0.0008; N-Limited: p <0.005). Phosphorus concentrations of *Ochromonas* grazing on Balanced and C-Limited bacteria were similar (Tukey's post hoc: p = 0.7) and found to be greater than when feeding on N-Limited and P-Limited bacteria. *Ochromonas* had intermediate QP concentrations when grazing on N-Limited. QP values were significantly lower for the flagellate when grazing on bacteria grown in P-Limited medium compared to all other media types (Tukey's post hoc: Balanced: p <0.0003; C-Limited: p <0.0003; N-Limited: p <0.0004). Bacteria grown in C-Limited medium produced *Ochromonas* with the highest nitrogen concentrations, however this value was not statistically different from

those obtained in all other media types (Tukey's post hoc: Balanced: p = 0.21; N-Limited: p = 0.79; P-Limited: p = 0.11). Overall, C concentrations ranged four-fold among media types, N concentration ranged approximately eight-fold, while P concentrations ranged ninety-eight fold.

Table 3.11 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Ochromonas danica* (fmol cell^{-1}) feeding on bacteria grown in different media types. (Mean ± SE, n = 3 triplicate batch cultures)

Medium	Element (fmol cell^{-1})		
	C	N	P
Balanced	3,591.1 ± 749.4	211.0 ± 123.8	43.25 ± 23.12
C-Limited	3,922.8 ± 353.6	574.1 ± 55.50	49.12 ± 3.216
N-Limited	3,467.7 ± 476.79	276.0 ± 37.80	14.34 ± 1.853
P-Limited	877.4 ± 119.23	73.75 ± 24.19	0.4715 ± 0.1065

It is conceivable that an interaction between flagellate and bacteria may result in unpredictable changes in element composition of predator and/or prey largely due to re-growth of prey on regenerated nutrients. Consequently, filtration was used to separate the two organisms and an assessment of predator element quota was made accounting for separation efficiency. The separation efficiency for bacteria is a measure of how good the filter was at removing bacteria from the BFF cultures. Theoretically bacterial separation efficiency should approach a value of zero since the filters used had pore sizes of 3.0 µm and removal of bacterial cells from the cultures was not intended. Flagellate separation efficiency was a measure of how good the filter was at removing the flagellates from the BFF cultures. In these cultures removal of the flagellate population was intended and theoretically should have approached a value of one.

Using this approach, *Ochromonas* element quotas (QC, QN and QP) varied depending on the media in which bacteria cells were grown (ANOVA: QC: $F_{3,8} = 9.77$; $p < 0.007$; QN: $F_{3,8} = 19.59$; $p < 0.0009$; QP: $F_{3,8} = 18.84$; $p < 0.002$; Figure 3.15 and Table 3.12). *Ochromonas* acquired high levels for QC, QN and QP while growing on nutritionally Balanced bacteria and C-Limited bacteria; however there were no statistical differences between C, N and P quotas (Tukey's post hoc: QC: $p = 0.95$; QN $p = 1.00$; QP: $p = 0.58$). The low element concentrations for protozoan C and N were found for *Ochromonas* grazing upon N-Limited and P-Limited bacteria, which were also not statistically different from each other (Tukey's post hoc: QC: $p = 0.29$; QN $p = 0.33$). The lowest P quota was measured for *Ochromonas* feeding on P-Limited bacteria, which was statistically different from P quotas found in all other media types (Tukey's post hoc: Balanced: $p < 0.002$; C-Limited: $p < 0.008$; N-Limited: $p < 0.05$). Overall, there was a ten fold difference between the highest and lowest carbon concentration values for *Ochromonas*, an eleven fold difference for nitrogen concentration values and almost a two-hundred fold difference between phosphorus concentration values. It seems that carbon and nitrogen content are more tightly regulated in *Ochromonas* stoichiometry as compared to phosphorus content.

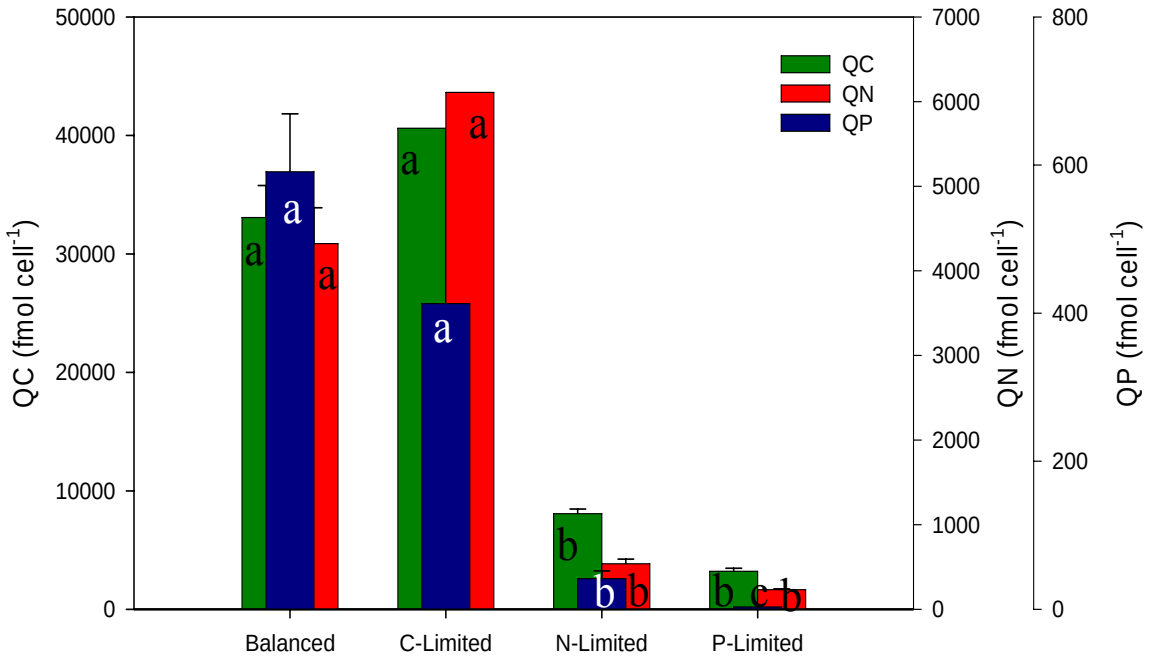

Figure 3.15 Cellular quotas of carbon (QC), nitrogen (QN), and phosphorus (QP) for *O. danica* feeding on bacteria grown in different media types reported as fmoles cell^{-1} while accounting for separation efficiency. Mean values and standard errors reported in Table 3.6. Error bars represent SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Table 3.12 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Ochromonas danica* (fmol cell^{-1}) feeding on bacteria grown in different media types while accounting for separation efficiency. (Mean ± SE, n = 3 triplicate batch cultures)
(* Indicates mean values of duplicate batch cultures and their range values)

Medium	Element (fmol cell^{-1})		
	C	N	P
Balanced	33,064.4 ± 2,694.5	4,320.02 ± 424.12	591.08 ± 78.07
C-Limited	*40,611.3 (63,900)	*6,106.1 (9,273)	*412.8 (702)
N-Limited	8,069.18 ± 392.36	536.06 ± 57.57	41.33 ± 10.52
P-Limited	3,200.03 ± 263.45	231.16 ± 9.628	*3.17 (2.44)

3.2.4 Ochromonas Element Content as a Function of Volume

Ochromonas cell quota (fmoles µm^{-3}) calculated from Equation 4 considers the average cell size of both predator and prey (Figure 3.16 and Table 3.13). Using this approach, *Ochromonas* element quotas (QC, QN and QP) varied depending on the media in which bacteria cells were grown (ANOVA: QC: $F_{3,8} = 126.00$; $p < 0.00001$; QN: $F_{3,8} = 66.08$; $p < 0.00001$; QP: $F_{3,8} = 120.06$; $p < 0.00002$).

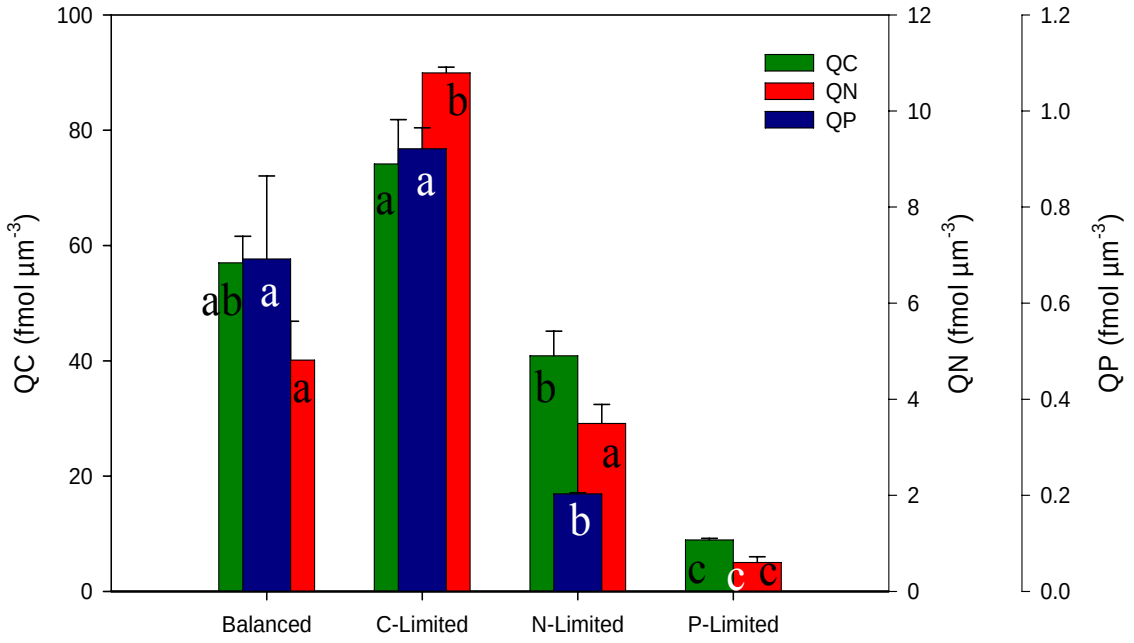

Figure 3.16 Cellular quotas of carbon (QC), nitrogen (QN), and phosphorus (QP) for *O. danica* feeding on bacteria grown in different media types reported as fmoles µm^{-3}. Mean values and standard errors reported in Table 3.7. Error bars represent SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

The flagellate C quota when feeding on Balanced prey was statistically similar to those found when *Ochromonas* was grazing on C-Limited and N-Limited bacteria (Tukey's post hoc: C-Limited: $p = 0.23$; N-Limited: $p = 0.09$). However, the carbon quota for *Ochromonas* grazing on C-Limited bacteria is statistically different from the carbon quota measured when feeding on N-Limited bacteria (Tukey's post hoc: $p < 0.006$). The

lowest C quota was measured for *Ochromonas* when grazing on P-Limited bacteria compared to all other media types (Tukey's post hoc: Balanced: $p < 0.003$; C-Limited: $p < 0.003$; N-Limited: $p < 0.003$). The greatest nitrogen concentration for the flagellate was measured when grazing on C-Limited bacteria and was statistically different from all other media types (Tukey's post hoc: Balanced: $p < 0.02$; N-Limited: $p < 0.003$; P-Limited: $p < 0.0003$). N quotas for *Ochromonas* were intermediate and not statistically different when growing on Balanced and N-Limited bacteria (Tukey's post hoc: $p = 0.45$). The lowest N quota for the flagellate was statistically different from all other media types and was found when grazing on *P. fluorescens* grown in P-Limited medium (Tukey's post hoc: Balanced: $p < 0.0003$; C-Limited: $p < 0.0003$; N-Limited: $p < 0.0004$). As for P quotas, high values were found when the flagellate was grazing on Balanced and C-Limited bacteria and these values were not statistically different from each other (Tukey's post hoc: $p = 0.53$). Intermediate P concentrations were found when *Ochromonas* was feeding on N-Limited bacteria (Tukey's post hoc: Balanced: $p < 0.02$; C-Limited: $p < 0.004$; P-Limited: $p < 0.0003$). The lowest P concentration was found when *Ochromonas* grazing on *P. fluorescens* grown in P-Limited media (Tukey's post hoc: Balanced: $p < 0.0002$; C-Limited: $p < 0.0002$; N-Limited: $p < 0.0003$). Overall, C quotas ranged eight-fold, N quotas ranged eighteen-fold and P quotas ranged one-hundred eighty-fold. Once again, it seems that C and N content are more tightly regulated in the flagellate stoichiometry compared to that of P content.

Table 3.13 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Ochromonas danica* (fmol μm^{-3}) feeding on bacteria grown in different media types. (Mean ± SE, n = 3 triplicate batch cultures) (* Indicates mean values of duplicate batch cultures and their range values)

Medium	Element (fmol μm^{-3})		
	C	N	P
Balanced	57.00 ± 4.51	4.814 ± 0.8112	0.6927 ± 0.1728
C-Limited	74.13 ± 7.693	10.79 ± 1.121	0.9216 ± 0.0435
N-Limited	40.86 ± 4.282	3.395 ± 0.3970	0.2030 ± 0.0023
P-Limited	8.933 ± 0.2942	0.6025 ± 0.1205	*0.0046 (0.0927)

Ochromonas element quotas, accounting for both average cell size and separation efficiency are given in Figure 3.17 and Table 3.14. Using this approach, *Ochromonas* element quotas (QC, QN and QP) varied depending on the media in which bacteria cells were grown (ANOVA: QC: $F_{3,8} = 11.34$; $p < 0.005$; QN: $F_{3,8} = 22.57$; $p < 0.0006$; QP: $F_{3,8} = 20.40$; $p < 0.002$). *Ochromonas* grazing on C-Limited bacteria contained the absolute highest C, N and P concentrations; however due to high variability associated with C-Limited medium, these element quotas were not statistically different from flagellate quotas found grazing on prey grown in Balanced medium (Tukey's post hoc: QC: $p = 0.98$; QN: $p = 0.84$; QP: $p = 0.87$). Lowest C, N and P concentrations, were found when *Ochromonas* was grown on prey grown in P-Limited medium; however these element quotas were also not statistically different from flagellate quotas found grazing on bacteria in N-Limited medium (Tukey's post hoc: QC: $p = 0.86$; QN: $p = 0.91$; QP: $p = 0.07$). Among media types, there was over a two-hundred fold range in phosphorus

quota, carbon content for *Ochromonas* ranged seven fold, while nitrogen content was thirteen times greater in Balanced than found in P-Limited medium.

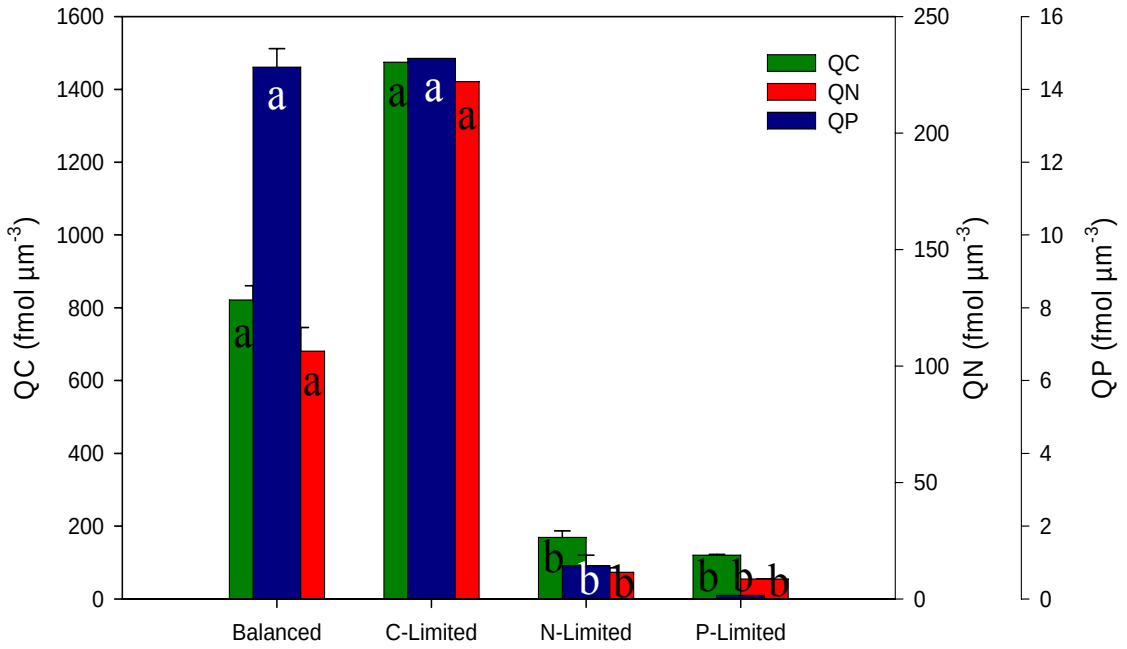

Figure 3.17 Cellular quotas of carbon (QC), nitrogen (QN), and phosphorus (QP) for *O. danica* feeding on bacteria grown in different media types reported as fmoles μm^{-3} while accounting for separation efficiency. Mean values and standard errors reported in Table 3.8. Error bars represent SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

Table 3.14 Carbon (C), Nitrogen (N), and Phosphorus (P) content of *Ochromonas danica* (fmol μm^{-3}) feeding on bacteria grown in different media types while accounting for separation efficiency. (Mean ± SE, n = 3 triplicate batch cultures)
(* Indicates mean values of duplicate batch cultures and their range values)

Medium	Element (fmol μm^{-3})		
	C	N	P
Balanced	821.2 ± 39.23	106.3 ± 10.26	14.61 ± 0.5105
C-Limited	*1,474.4 (2,194.57)	*222.1 (316.25)	*14.85 (24.34)
N-Limited	168.7 ± 18.08	11.42 ± 1.874	0.9116 ± 0.2875
P-Limited	119.8 ± 2.616	8.541 ± 0.7237	*0.1003 (0.0927)

It seems no matter the approach taken for calculations of *Ochromonas* quota measurements, carbon and nitrogen contents are more tightly regulated in flagellate stoichiometry than phosphorus content; *Ochromonas* grazing on *Pseudomonas* grown in Balanced and C-Limited media will have higher C, N and P concentrations than found when *Ochromonas* is grazing on N-Limited and P-Limited bacteria.

After reviewing normal probability plots and predicted verses residual plots, calculations attempting to account for separation efficiency introduced properties that resulted in increased variability and reduction of statistical power. Consequently, elemental ratios normalized to the average *Ochromonas* cell size will be reported (Figure 3.18 and Table 3.15).

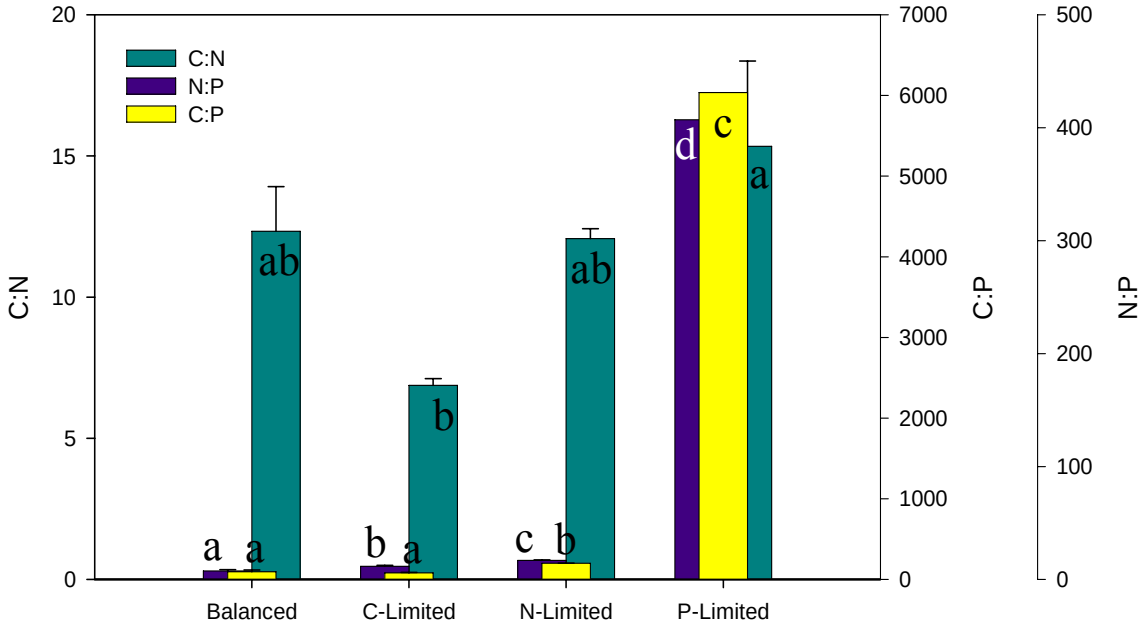

Figure 3.18 Elemental ratios for *O. danica* feeding on bacteria grown in different media types reported as mole:mole. Ratio calculations based on protozoan cells volume. Mean values and standard errors reported in Table 3.15. Error bars represent SE. Different lowercase letters indicate statistically significant differences among media types. Mean comparisons based on Tukey's HSD.

ANOVA revealed that *Ochromonas* ratios (C:N, C:P and N:P) varied depending on media type in which *P. fluorescens* were grown (C:N: $F_{3,8} = 4.18$; $p < 0.05$; C:P: $F_{3,8} = 17{,}256.64$; $p < 0.00001$; NP: $F_{3,8} = 12{,}427.37$; $p < 0.00001$). The only statistical difference found among C:N ratios of flagellate cells was when feeding upon C-Limited or upon P-Limited bacteria (Tukey's post hoc: $p < 0.04$). The C:N ratios of *Ochromonas* ranged approximately two fold.

C:P ratios of *Ochromonas* feeding upon bacteria of different nutritional value were variable with ratios ranging from a low in C-Limited and Balanced media, which were not statistically different (Tukey's post hoc: $p = 0.90$), to a high in P-Limited media. The high C:P ratio in P-Limited medium was statistically greater than C:P ratios found in all other media types (Tukey's post hoc: Balanced: $p < 0.0003$; C-Limited: $p < 0.0003$; N-Limited: $p < 0.0003$).

The greatest N:P ratio of *Ochromonas* was found when grazing on P-Limited medium, which was statistically different from all other media types (Tukey's post hoc: Balanced: $p < 0.0003$; C-Limited: $p < 0.0003$; N-Limited: $p < 0.0003$). The smallest N:P ratio was also statistically different from all media types (Tukey's post hoc: C-Limited: $p < 0.005$; N-Limited: $p < 0.002$; P-Limited: $p < 0.0003$) and was found when the protozoa was feeding on bacteria grown in Balanced medium.

There is considerable uncertainty associated with the C:P and N:P ratio of *Ochromonas* feeding on P-Limited bacteria, as measures of P content approached the limits of analytical resolution. These ratios were estimated based on only a single measure of P.

Table 3.15 Element ratios of *Ochromonas danica* (mole:mole) feeding on bacteria grown in different media types. Ratio calculations based on protozoa cell volume. (Mean ± SE, n = 3 triplicate batch cultures) († Indicative of only one phosphorus measure to calculate ratio value)

Medium	Ratio (mole:mole)		
	C:N	C:P	N:P
Balanced	12.33 ± 1.58	94.20 ± 23.61	7.45 ± 1.13
C-Limited	6.87 ± 0.24	80.34 ± 6.87	11.67 ± 0.81
N-Limited	12.07 ± 0.35	201.60 ± 1.61	16.73 ± 0.49
P-Limited	15.34 ± 3.02	†6035.81	†407.09

By knowing the element content of both predator and prey, it becomes possible to determine the minimum predatory effort *Ochromonas* must make to acquire enough elements from its prey to satisfy its stoichiometric demand. Compare element content of *Ochromonas* in Table 3.13 to that of *Pseudomonas* in Table 3.7. Based on the element composition of the flagellate and that of the bacterial prey, the minimum number of cells consumed to obtain the flagellate content for each element is provided in Table 3.16.

Table 3.16 Minimum and actual number of bacterial cells consumed by a flagellate to meet their Nitrogen and Phosphorus demand.

Medium	Minimum number of cells		Actual
	N	P	
Balanced	71.7	172	197.4
C-Limited	247	267	1110
N-Limited	98.6	40	173.7
P-Limited	67.7	8.7	136.4

It appears that *Ochromona*s feeding on bacteria grown in C-Limited media had to consume eight times as many cells to reach their nutrient stoichiometry, compared to

what would have to be consumed when feeding on prey in P-Limited media. As for the small, P-Limited bacteria, *Ochromonas* would only consume a fraction of a bacterial cell to reach its low stoichiometric demand (Table 3.13) and ultimately this prey would provide the best growth rate (Figure 3.8 and Table 3.9). It is evident that bacteria grown in Balanced medium were not 'balanced' in terms of flagellate demands. On the other hand, since bacteria grown in P-Limited and N-Limited media did provide higher growth rates for the flagellate, prey resource stoichiometry is assumed to be closer to the demands of *Ochromonas*.

3.2.5 Grazing Experiment Dissolved Nutrients

The experimental design allowed us to consider the N and P recycled by *Ochromonas* when it was feeding upon prey of varying nutritional quality. Regeneration of C was not considered since the experimental design did not account for respiratory losses.

Dissolved nitrogen in all media is represented in Figure 3.19 and Figure 3.20. At the beginning of the experiment, Balanced medium contained 909.09 μM N as dissolved ammonium (NH_4Cl), C-Limited contained 3030.30 μM N, N-Limited 378.79 μM N and P-Limited had 757.58 μM N. Ammonium (NH_4^+) is the preferred form of nitrogen for bacteria and values in all media types for BO cultures were low at stationary phase (Figure 3.19A). For each medium, greater that 97 percent of NH_4^+ supplied was incorporated into bacterial biomass. Clearly, predation upon bacteria by *Ochromonas* (BF and BFF cultures) has the potential to regenerate NH_4^+ back into the medium.

Recycled nutrients is estimated by the difference in dissolved nutrients in cultures where the predator is present (BF and BFF cultures) compared to cultures of bacteria

A.

B.

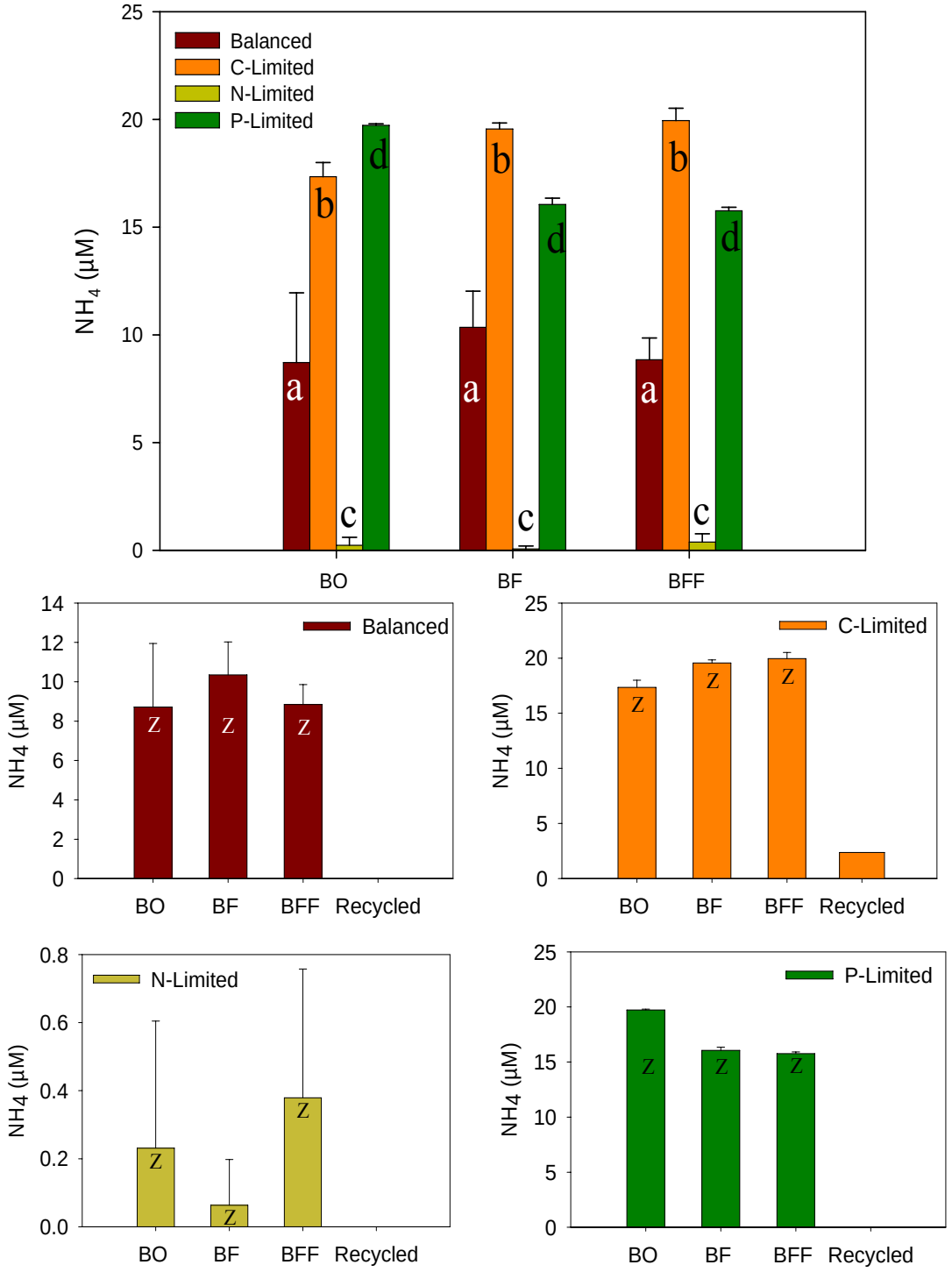

Figure 3.19 Dissolved Ammonium and Recycled nitrogen (µM) in the three culture conditions in all media types. Mean values and standard errors are reported. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

only. Despite the potential for NH_4^+ regeneration, regeneration was only detected when feeding on C-Limited prey and at exceedingly low levels, approximately 2 μM.

Large amounts of dissolved organic nitrogen (DON) were found in all three culture types (BO, BF and BFF; Figure 3.20A). *Ochromonas* regenerated the greatest amount of DON, approximately 520 μM when feeding on *Pseudomonas* grown in N-Limited medium. *Ochromonas* grazing on C-Limited bacteria recycled 340 μM, and when grazing on P-Limited bacteria 40 μM. Recycled DON was not found when *Ochromonas* was feeding on nutritionally Balanced prey.

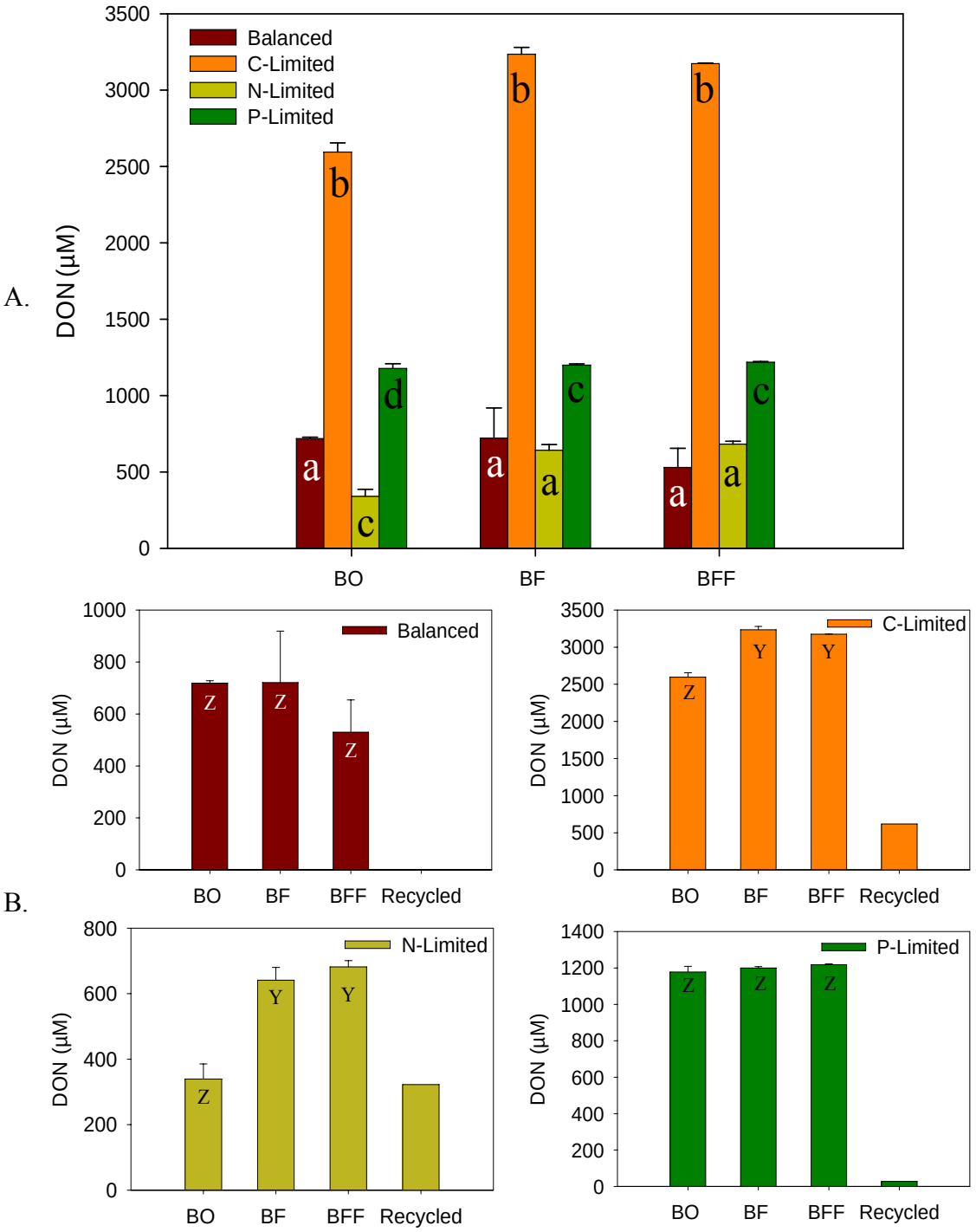

Figure 3.20 Dissolved Organic Nitrogen and Recycled nitrogen (µM) in the three culture conditions in all media types. Mean values and standard errors are reported. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

Dissolved phosphorus measurements in all media and culture types are shown in Figure 3.21 and Figure 3.22. At the beginning of the experiment, Balanced and N-Limited media contained 75.75 μM P as dissolved potassium phosphate (KH_2PO_4), C-Limited medium contained 303.03 μM and P-Limited medium had the lowest concentration at 15.15 μM. Orthophosphate (PO_4^{3-}) or soluble reactive phosphorus (SRP), the preferred form of phosphate of bacteria and algae (Bentzen 1992), represents <25% of the total dissolved phosphate pool in marine surface waters (Karl and Yanagi 1997). The greatest amount of recycled SRP was found when the flagellate was feeding on bacteria grown in C-Limited medium, 19.20 μM (Figure 3.21B). *Ochromonas* feeding on all nutritional types of bacteria resulted in <5 μM of regeneration of SRP.

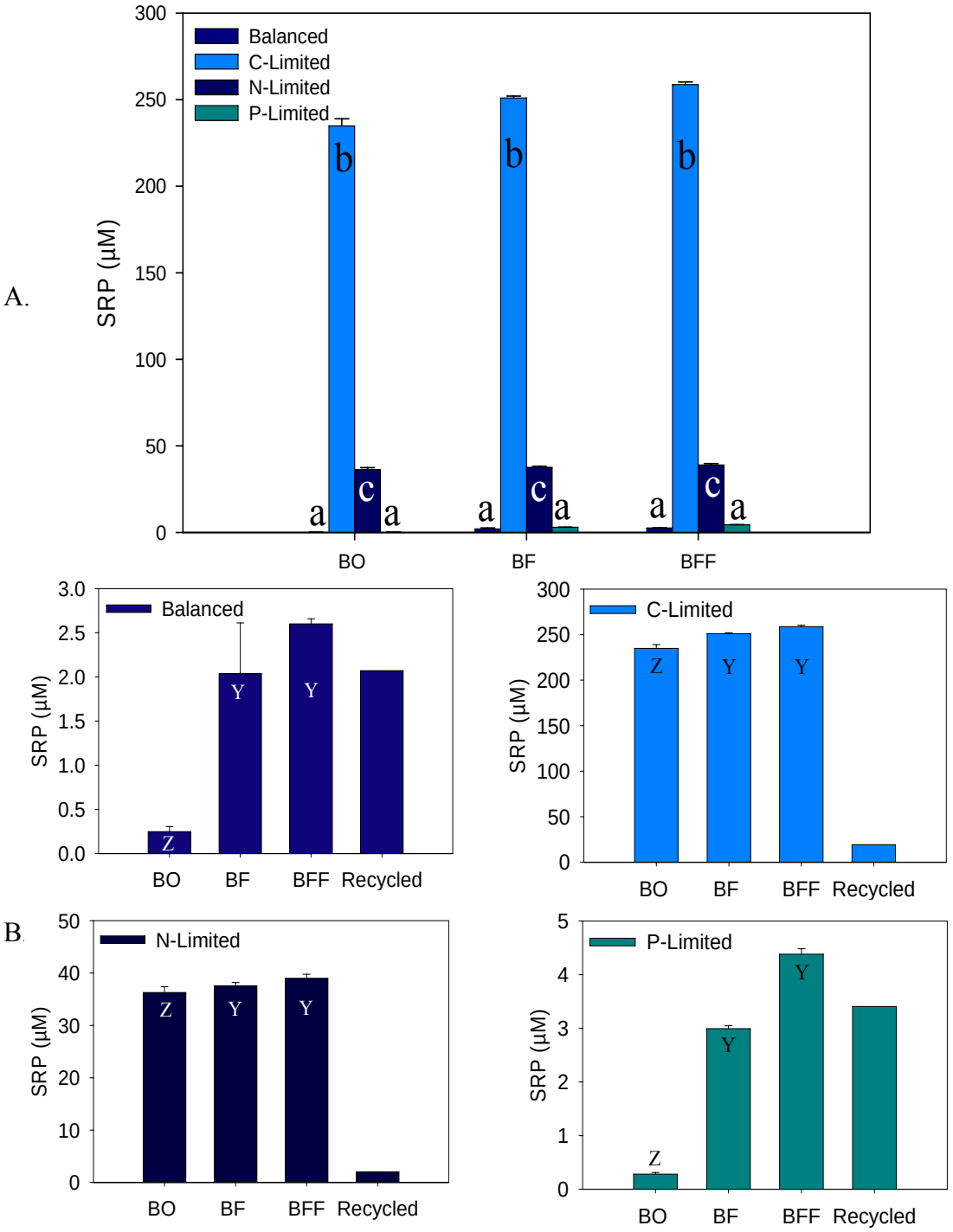

Figure 3.21 Soluble Reactive Phosphorus and Recycled Phosphorus (µM) in the three culture conditions in all media types. Mean values and standard errors are reported. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

Bacteria grown in C-Limited medium resulted in the greatest amount (57 μM; Figure 3.22B) of regenerated TOP by the flagellate. Ochromonas feeding on bacteria grown in N-Limited medium recycled 30 μM, while feeding on Balanced bacteria 10 μM were recycled. A meager amount of 4μM of TOP was recycled when the flagellate was grazing on P-Limited bacteria.

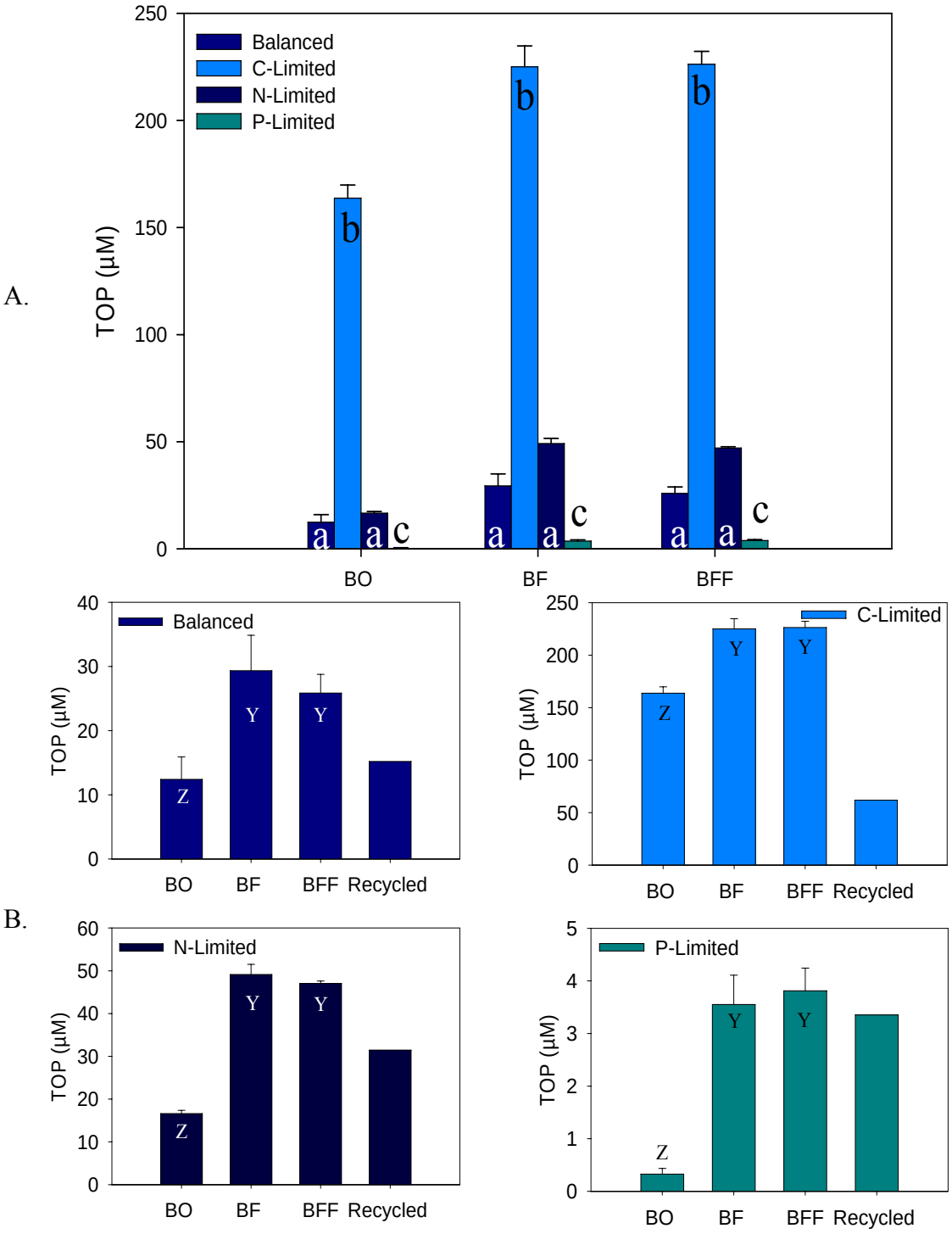

Figure 3.22 Total Organic Phosphorus and Recycled Phosphorus (µM) in the three culture conditions in all media types. Mean values and standard errors are reported. Error bars represent the SE. Different lowercase letters indicate statistically significant differences within culture types (A), while uppercase letters indicate statistically significant differences within media types (B). Mean comparisons based on Tukey's HSD.

3.2.6 Conclusion and Ecological Impact

A comparison of flagellate element ratios (C:N, C:P and N:P) to those of their prey is presented in Figure 3.23, also included are element ratios found for other heterotrophic flagellates in previous studies (Goldman et al. 1987, Nakano 1994). Ratios calculated from P-Limited medium were excluded since phosphorus values were at detection limits. Goldman et al. (1987) using a diatom and a chlorophyte as the prey for the predator *Paraphysomonas* found that the predator ratios of elements in the predator varied as a linear function of element ratios of the prey. Similarly, Nakano working with an unknown flagellate preying on bacteria found that the element ratios of the predator varied only slightly as a function of the element ratios of the prey. In Figure 3.23, similar data from this study (measured from the raw BF cultures, which do not consider the undigested bacteria within the protozoan food vacuole) are plotted in comparison with the data collected by Goldman et al. (1987) and Nakano (1994). These studies do not separate prey from the predator. It seems that regardless of prey type, phytoplankton or bacteria, flagellate element ratios still vary as a linear function of prey element ratios. Our current work allows comparison of the predator element ratios under different nutrient conditions free of element ratios of contaminating prey, these data are presented in Figure 3.24. Nevertheless, this makes little difference in our conclusion; predator element ratios vary as a linear function of prey element ratios.

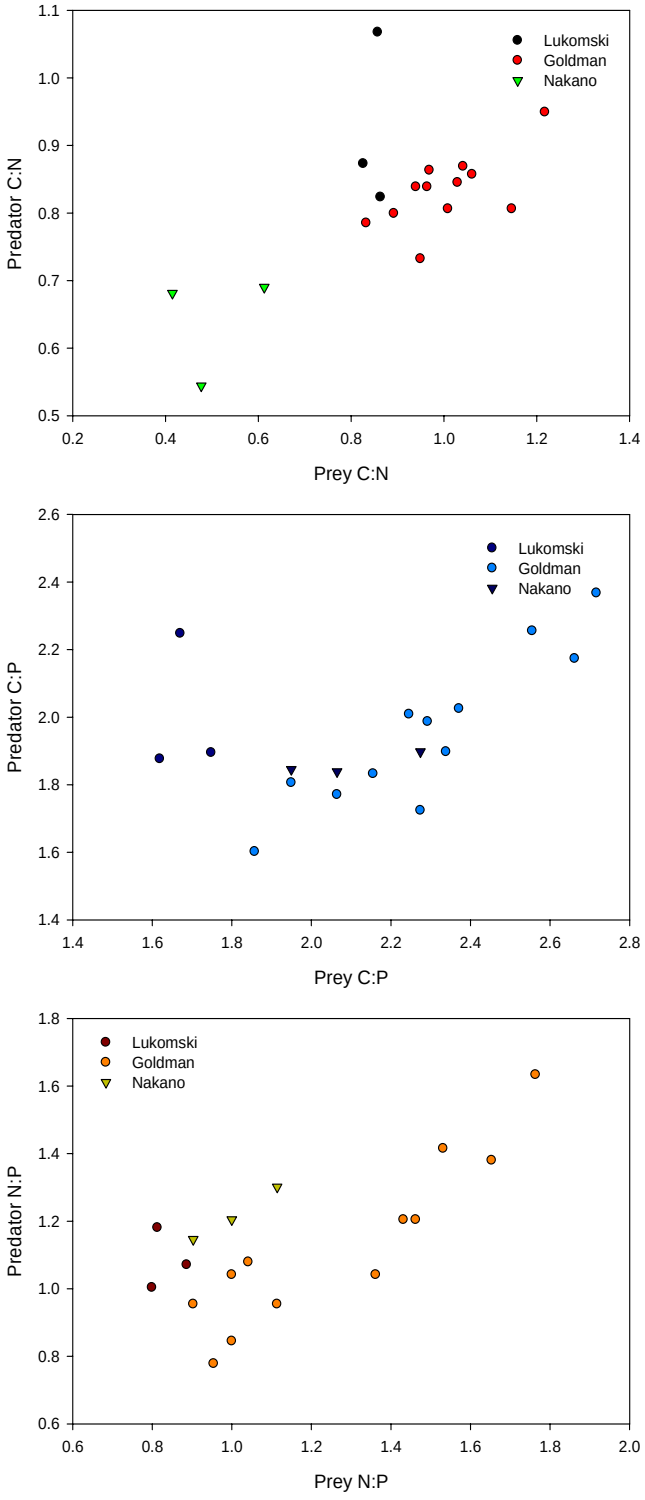

Figure 3.23 Relationship between log(predator elemental ratio) and log(supply elemental ratio) for flagellate strains [*Ochromonas danica*, present study; *Paraphysomonas imperforata*, Goldman et al. 1987; *Spumella* or *Paraphysomonas*, Nakano 1994].

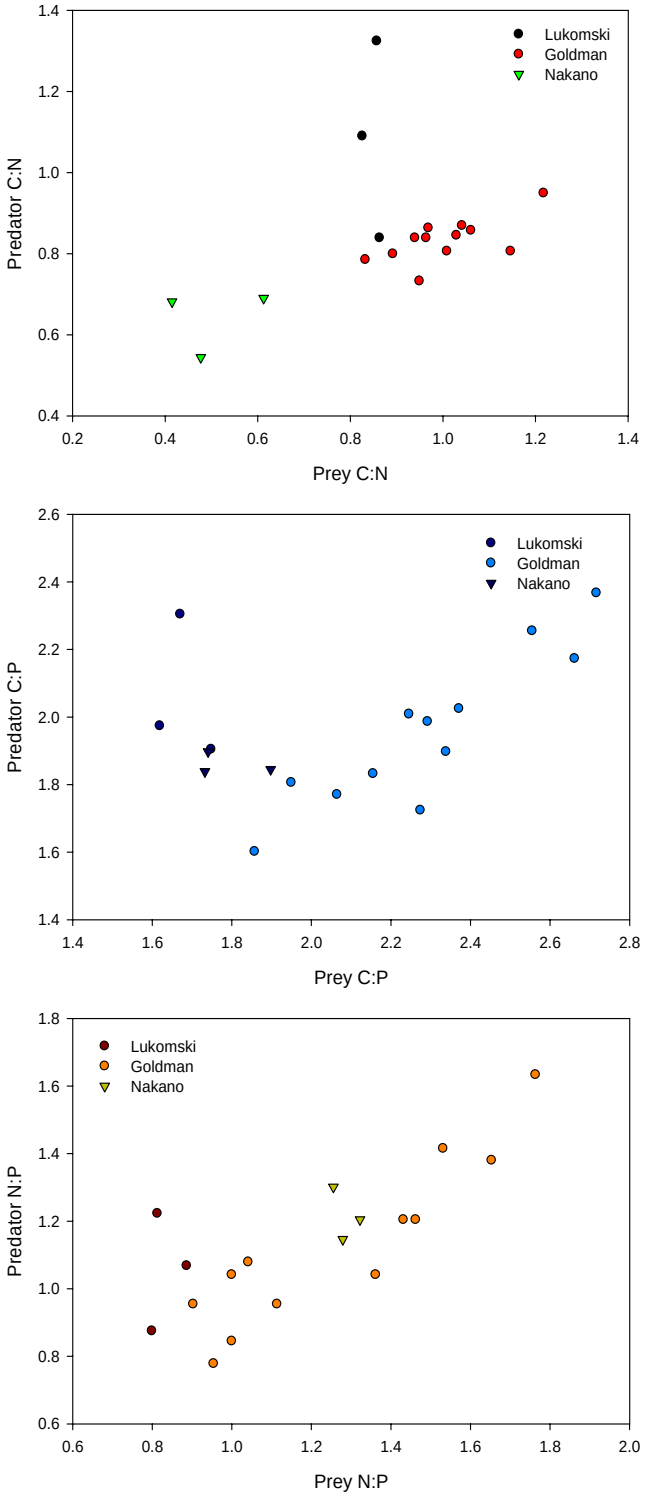

Figure 3.24 Relationship between log(predator elemental ratio) and log(supply elemental ratio) for flagellate strains present study accounting for undigested bacteria in food vacuole [*Ochromonas danica*, present study; *Paraphysomonas imperforata*, Goldman et al. 1987; *Spumella* or *Paraphysomonas*, Nakano 1994].

P. fluorescens grown in Balanced medium resulted in cells of small volume and high C, N and P concentrations. *Ochromonas* feeding on Balanced bacteria grew the slowest, had high concentrations of C, N and P and were large in volume. These protozoan cells also recycled <15μM of P and no N.

Bacteria grown in C-Limited medium resulted in large cells that had low concentrations of C, N and P. *Ochromonas* grazing on these bacteria cells had an intermediate growth rate, had high concentrations of C, N and P and were small sizes cells. These flagellates also recycled >75 μM of P and >520μM of N.

Pseudomonas grown in N-Limited medium resulted in cells of intermediate size and C, N and P quotas. The protozoa feeding on N-Limited bacteria had a rapid growth rate, low levels of C, N and P quotas, and were large in cell volume. *Ochromonas* grazing on these bacteria recycled >30 μM of P and 340 μM of N.

Bacteria grown in P-Limited medium produced small cells of intermediate C, N and P concentrations. The flagellate grazing on this Pseudomonas resulted in the fastest growth rate, low levels of C, N and P content, and were large in cell volume. *Ochromonas* recycled only a minute amount of P (<9 μM) and N (40 μM).

Based on data presented here, both bacteria and heterotrophic flagellates are found to be flexible not only in their nutrient composition, but also in their cellular volume depending on resource availability. This deviation from homeostasis has great implications in microbial food webs. Changes in nutrient composition can reflect storage ability; which provide cells with a competitive fitness advantage when resources are scarce or during periods of starvation, for both predator and prey as seen in computational models (Grover 1991). Another explanation in fluctuations in nutrient

composition could be due to differences in flagellate assimilation efficiency; which is considered a component of prey food quality. Nutrients released by predators are affected by differences in nutrient retention.

Recycled nutrients are a potential resource for bacterial growth, thus effecting trophic dynamics. Nutrient retention by bacterial predators reflects the flux of organic matter and energy transfer to predator consumers (movement to higher trophic levels). This study has revealed that *Ochromonas* is capable of maintaining high growth rates on bacteria containing meager phosphorus and nitrogen supplies, *Ochromonas*, a representative heterotrophic protozoan, provided insight on the role of bacterial consumers in microbial food webs. The largest amount of recycled nutrients (N and P) occurred when *Ochromonas* was feeding on C-Limited prey. However, the highest growth rates of *Ochromonas* occurred when growing on P-limited. It is interesting to speculate how this may influence events occurring at higher trophic levels; for example, response to prey quantity as opposed to prey quality when *Ochromonas* consumers, larger protozoan or metazoan, feed on the resulting large pool of P-Limited *Ochromonas*.

Predictions of recycled nutrients, made by comparing bacterial prey N:P (Table 3.8) ratios to that of the flagellate predator N:P (Table 3.15), were evaluated with the calculated amount of recycled N and P (Figures 3.19-22B). The flagellate was predicted to excrete a N:P ratio of 5:1 when grazing on bacteria in Balanced medium; an equivalent amount of nitrogen and phosphorus when feeding on C-Limited bacteria. In N-Limited medium, the flagellate was predicted to excrete two moles of phosphorus in order to obtain its nitrogen demand and to excrete twenty moles of phosphorus when grazing on P-Limited bacteria to obtain its nitrogen demand. The actual N:P ratios calculated from

the measured dissolved N and P when the protozoan was grazing on Balanced bacteria was 16:1; C- and N-Limited bacteria resulted in a ratio of 7:1; and P-Limited bacteria resulted in a ratio of 186:1.

Even though the predicted recycled ratios are not empirically the similar to those calculated with the dissolved data, a trend exists among Balanced, C-Limited and N-Limited values. The larger N:P ratio was predicted for Balanced medium compared to that of C- and N-Limited media, according to the calculated dissolved this trend holds true. As for predictions of recycled nutrients within P-Limited medium, this trend did not match with the conclusions of the calculated since a great amount of nitrogen was actual excreted by the protozoa instead of phosphorus, as predicted. Not much confidence was held in the P-Limited medium data set compared to the other three media since all phosphorus concentration measurements were at detection limits, replication of this medium data set is necessary.

Future studies include investigating protozoan stoichiometry feeding on live prey with complete cell separation via a cell sorter to address the question of nutrient recycling, the synergistic effect of temperature and prey stoichiometry on protozoan elemental content, and discovery of mechanisms used by *Ochromonas* to select bacterial prey.

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BIOGRAPHICAL INFORMATION

Natalie Lukomski was born in McKinney, Texas to Don and Teresa Hanna. She graduated from McKinney High School in 2000. She spent the next few years taking courses at Collin County Community College where she earned her Associate of Science. Natalie then transferred to The University of Texas at Arlington where she earned a Bachelor of Science in Biology and a Bachelor of Science in Microbiology in May of 2003. She remained at UTA, under the tutelage of Dr. Chrzanowski, to research predator-prey dynamics within aquatic microbial systems. Natalie's plans involve pursuing a career in secondary education and enjoying scenic desert vacations with her husband.