ELEMENT STOICHIOMETRY OF *OCHROMONAS DANICA* AS A FUNCTION OF GROWTH RATE AND TEMPERATURE

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I thank my family, who never let me think I couldn't do it. My parents always told me I could do anything I wanted, so I did! Mostly, I want to thank my husband, his endless encouragement kept me going.

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ABSTRACT

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The microbial loop stresses the importance of flagellated protozoa as predators of bacteria and regenerators of nutrients, the outcome of which affects higher trophic levels. The element stoichiometry of flagellates has only been examined while feeding upon bacteria or phytoplankton. In this study, the element stoichiometry of the protozoan flagellate, *Ochromonas danica,* was determined while growing osmotrophically thus eliminating the potential errors due to element contributions of prey in food vacuoles.

The element and nucleic acid content of the flagellate were examined under a range of environmentally relevant temperatures and growth rates using chemostat cultures. Growth rate was the driving force of change for the element content of the flagellate, temperature and the interaction of temperature and growth rate played very small roles in variation. These results and results of previous studies of flagellates predict that flagellates may be weakly homeostatic.

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CHAPTER 1

INTRODUCTION

1.1 Microbial Loop

Aquatic ecologists have historically considered bacteria to be the base of the food web where they were assigned the role of 'decomposers'. In this role, they were believed to function as regenerators of critical nutrient elements (Odum 1956). Pomeroy (1974) questioned this belief arguing that the rapid growth rates of bacteria, coupled with their metabolic diversity, would result in nutrients becoming sequestered in their biomass rather than becoming re-cycled. Thus, while clearly being the primary vehicle by which nutrient elements associated with dissolved organic matter is moved into living tissue, bacteria could not be the primary means by which these nutrients are recycled and subsequently made available to higher trophic levels. Pomeroy further argued that predation upon bacteria by protists ultimately makes these essential elements available to higher trophic levels. Azam et al. (1983) later formalized this idea into what is known as the microbial loop. In this construct, Azam et al. (1983) incorporated the idea that protists serve as the primary consumers of bacteria but also recognized that protists regenerate those nutrients consumed in excess of their own metabolic demands.

Their ubiquity, abundance in all aquatic systems, and high rates of bacterivory place protists in a position of importance in aquatic trophic structure since these organisms regulate the flux of carbon (C), nitrogen (N), and phosphorus (P) through the food web (Azam et al. 1983; Sherr and Sherr 2002). Among the protists, the nanoflagellates are of particular interest. Their small size, and high rates of bacterivory and metabolism facilitate the movement of nutrients from microbial biomass (Sherr and Sherr 2002). Despite their importance in the microbial loop, little is known about how this group of protists reacts to changes in their environment.

1.1.1 Flagellate Protists

Phagotrophic protists range in size from heterotrophic picoflagellates that are < 2 µm to > 100 µm, to the larger ciliates and dinoflagellates (Sherr and Sherr 2002). Protists represent a diverse metabolic group of organisms whose members range from purely heterotrophic, through a range of mixotrophy, to purely autotrophic (Jones 1994). Phagotrophic protists are omnivorous, grazing on autotrophic and heterotrophic bacteria, phytoplankton, and other heterotrophic protists (Boenigk and Arndt 2002; Sherr and Sherr 2002). Mixotrophy is a particularly interesting form of metabolism as these organisms may not only use alternative sources of carbon for energy, but may also acquire nutrients (N, P, vitamins, and other essential biomolecules as phospholipids) both osmotrophically and phagotrophically.

1.1.2 Heterotrophs

Heterotrophic flagellates are important bacterivores in aquatic systems and their small size implies that they handle prey items individually. There are several modes of feeding but suspension and raptorial feeding are the two forms of interception feeding that are the most common. Suspension feeding occurs by the flagellate beating its flagella, creating a current where the prey are suspended, thus pulling them into contact with the flagellate. Raptorial feeding occurs when the flagellate actively seeks contact with prey (Leadbeater and Green 2000). Some heterotrophs have also been found to directly take up dissolved organic carbon (DOC). Growth varies with food type, concentration and temperature. Maximum growth rates are extremely variable and *in situ* measurements in lakes and oceans are usually much lower than those reported for laboratory cultures.

1.1.3 Mixotrophs

Ecologically, the ability to use substitutable resources makes trophic interactions more complex because mixotrophic organisms occupy two potential trophic levels and blur the line between producers and consumers. Clearance rates of mixotrophic nanoflagellates are similar to heterotrophic flagellates in many systems (Leadbeater and Green 2000). They have been reported eating pico-, nano-, and micro-plankton (Zhang and Watanabe 2001). There are

facultative osmotrophs as well and these organisms are primarily phototrophic or mixotrophic but can supplement phototrophic or phagotrophic growth with dissolved nutrients including carbon.

Heterokonts are the most dominant group in pelagic habitats with 20 - 50% of heterotrophic nanoflagellate biomass, mainly chrysomonads and bicosoecids (Boenigk and Arndt 2002), derived from this group. Mixotrophy seems to occur most commonly among the chrysophytes, and mixotrophic chrysomonads are important bacterivores in marine and freshwater ecosystems. They may contribute as much as 50% total bacterivory in coastal, estuarine, and fresh water systems (Flöder et al. 2006). In one system, phytoflagellate grazing constituted 55% of the total bacterivory of microflagellates, ciliates, rotifers, and crustaceans combined (Porter 1988).

1.2 Stoichiometry of elements in cells and organisms

Stoichiometry may be defined in a variety of ways, but, in general, it is the quantitative relationship between the elements that make up a substance, and between the elements and compounds that are involved in reactions. The principle underlying the concept of stoichiometry is simply the mass balance of multiple elements. In living systems, biochemical resources may be considered reactants, and consumer biomass and excretions as products. “Yield”, or the production of new cell biomass, would depend on the proportions of all the reactants, making the concept of stoichiometry as important in ecological settings as in chemical reactions (Sterner and Hessen 1994). In ecological settings, the rate limiting steps of a “reaction” (uptake of dissolved nutrient or flow of nutrients in a predator-prey interaction) would be expected to follow Liebig’s Law of the Minimum (Hessen et al. 2004). Thus, the element in shortest supply (in a prey item for example) relative to demand (by a consumer) would mediate the flow of material through the trophic structure (Hessen et al. 2004).

This concept may be best understood by considering the allocation of elements in the biomolecules of a cell. Ribonucleic acid (RNA) contains 10% P by mass and ribosomal RNA (rRNA) makes up 75-80% of total RNA in most cells (Vrede et al. 2004) but its concentration depends on growth rate. Proteins are nitrogen rich containing about 17.2% N (Elser et al. 1996).

Thus, depending on the nature of the resources, dissolved as in the case of a cell growing osmotrophically, or the cells themselves, as in the case of a predator-prey interactions, the flow of nutrients into biomass and of those excreted will depend upon the metabolic state of the interacting organisms.

1.2.1 Element Stoichiometry of Autotrophs and Mixotrophs

Autotrophs have high and flexible C-to-nutrient ratios. The C:N and C:P ratios of autotrophs decrease as growth rate increases (Sterner and Elser 2002). The shift of element ratios as a function of growth rate appears to stem from both the reallocation of resources due to changing growth status and from the ability of autotrophs to take up excess nutrients and store them internally (Hessen et al. 2004). These interacting factors affect the capacity to store excess nutrients. N and P can vary 10 times or more relative to C in algae (Sterner and Hessen 1994). Nitrogen limitation can also cause a build up of excess C, usually in the form of lipids or other products for several species of algae (Shifrin and Chisholm 1981).

The stoichiometry of mixotrophic organisms may not be readily predictable. The ability to retain or excrete mineral nutrients seems to depend on the organisms place in the spectrum of mixotrophy, if a mixotroph depends mostly on photosynthesis, it may have a greater tendency to store nutrients than a mixotroph that depends largely on heterotrophy. Mixotrophic protists show the potential to have maximal excretion similar to like-sized heterotrophs (Dolan 1997). Using a strain of *Ochromonas* sp., Rothhaupt (1997) found that the protist was able to store nutrients when growing photosynthetically; but when growing heterotrophically, the cells released nutrients.

1.2.2 Element Stoichiometry of Flagellates

It has been assumed that flagellates have a constant stoichiometry because of their similarities to zooplankton; thus, variations in prey quality will influence the conservation and excretion of nutrients (Goldman et al. 1987). In a study of *Paraphysomonas imperforata*, P and N cell quota (defined at the concentration of nutrient per cell or per cell volume) varied as a function of prey type and nutritional state during exponential growth (Goldman et al. 1985, Andersen et al. 1986). Goldman et al. (1985) hypothesized that the limiting element was conserved and the rest

excreted to maintain stoichiometric balance, very similar to metazoan-phytoplankton nutrient interactions. The C:N and C:P ratios are lower in bacteria than in protists, thus protists may be expected to maintain a balanced C:N:P ratio by excretion of nutrients consumed in excess of need. C:N:P ratios of flagellates are species specific with life histories (growth rates) explaining a large part of the variation (Eccleston-Parry and Leadbeater 1995). There are only a few studies that consider flagellate element ratios in relation to the changing ratios of their prey (Nakano 1994; Goldman et al. 1987). When available data are considered in log-log plots of consumer verses resource nutrient ratios, they suggest that phagotrophic flagellates may only have weak homeostasis (Nakano 1994; Goldman et al. 1987; Grover and Chrzanowski 2006). C:N ratios of flagellates range from 2.5 to 8.9 and C:P ratios vary from 18 to 233 (Nakano 1994; Goldman et al. 1987). Some of have argued that flagellates should have a variable stoichiometry; Grover (2003), using the well-studied genus *Paraphysomonas,* incorporated a variable stoichiometric ratio for flagellate predators in a mathematical model.

1.2.3 Element Stoichiometry of Bacteria

The details of heterotrophic bacterial stoichiometry are still being investigated. There is variation of element ratios due to several factors; resource ratios, nutrient limitation, growth rate, and temperature have all been shown to affect the stoichiometry of bacteria (Vrede et al. 2002; Chrzanowski and Grover 2008; Chrzanowski and Kyle 1996). Makino et al. (2003) used published literature on the element ratios of bacteria and the logarithmic homeostasis model of Sterner and Elser (2002) and found that pure cultures were more homeostatic than mixed communities. Sterner and Elser (2002) plotted the data from Chrzanowski and Kyle (1996) that included *Pseudomonas fluorescens* and several others from a literature study. The logarithmic homeostasis plot of Sterner and Elser (2002) revealed a slope of 0.28, representing some level of homeostasis. In a more recent study of *Pseudomonas fluorescens,* Chrzanowski and Grover (2008) varied the temperature and growth rate in continuous cultures. This illustrated the amount of variation of element stoichiometry when resource was held constant. Ultimately, the trend may

be homeostatic element ratios in bacteria but there appears to be a large variation due to environmental factors.

1.3 Factors affecting element stoichiometry

1.3.1 Temperature

Temperature plays a role in the functionality of all cells as it affects an enzymes optimal efficiency, growth and metabolic processes (Caron et al. 1986; Rhee and Gotham 1981). Organisms that are adapted to cold seem to have larger bodies than warm adapted organisms. Montagnes and Franklin (2001), working with eight diatom and two flagellate species, demonstrated that cell size decreased by ~ 4% of their mean volume per °C. For phytoplankton, cell quotas of nutrients do not seem to be associated with increasing cell size and C fixation remains constant through temperature variations, suggesting that change in cell size is most likely due to changes in water content (Rhee and Gotham 1981).

There are mixed results that may be species specific for temperature affects on element quotas for phytoplankton. Rhee and Gotham (1981) found an increase in cell quotas of N and P at lower temperatures in the phytoplankter, *Scenedemus* sp. during balanced and P-limited growth. Goldman (1979) found that P quotas were affected by temperature but C and N quotas were only slightly affected. Montagnes and Franklin (2001) conclude that C and N per cell volume are temperature invariant in diatoms exposed to environmentally relevant temperatures. When examining several species of phytoplankton, Thompson et al. (1992a) found no patterns for N or C quotas and temperature across the species examined. Woods et al. (2003) surveyed a variety organisms and found organisms or tissues exposed to cold had a 32% increase in N and a 49% increase in P compared to warm exposed tissues. They proposed four reasons why temperature may have an overall effect on the stoichiometry of an organism: temperature may have differential effects on the synthesis and degradation of macromolecules, it may have differential effects on growth and N and P accumulation, increasing the level of RNA and protein may be to offset the reduced rates of reactions, or the increase in protein and RNA may be to protect

against intracellular freezing. Organisms seem to respond to cold by increasing catalytic components and or increasing in size.

1.3.2 Growth rate

There appears to be a variety of ways for cells to allocate biomolecules when growing slowly, consequently, wide variations in element stoichiometries may be expected. There seems only to be a limited number of allocation patterns for cells growing at faster growth rates and therefore a narrower range of stoichiometries (Vrede et al. 2004). For phytoplankton, when temperature was held steady, the quota of limiting nutrients increased as growth rate increased (Rhee and Gotham 1981). Growth rate and rRNA content are positively related in single celled and multicellular organisms. This may be because all life shares similar protein synthesis rates (Vrede et al. 2004). The growth rate hypothesis states that as growth rate increases so does the demand for P because of the P rich ribosomal RNA required for increased growth. This leads to a variation of C:P and N:P ratios in many biota excluding vertebrates (Elser et al. 2003).

1.4 Integrating trophic dynamics with Stoichiometry

Understanding the patterns of element storage for flagellates is an important step in clarifying the role of these organisms in the microbial loop. The system appears to be quite dynamic with temperature, growth rate, nutrient limitation, and prey availability interacting to alter the outcome of nutrient regeneration and energy flow. A model organism that identifies those patterns could be used to predict ecosystem outcomes when factors influencing storage change. Measuring the change in biomass concentrations of C, N and P allows for determination of stoichiometric ratios that can be used to infer fitness, demands for rRNA, and life histories (Elser and others 2000); it will also allow for predictions of nutrient flow during predator-prey interactions and nutrient regeneration in ecosystems.

In this work, the stoichiometry of a mixotrophic nanoflagellate *Ochromonas danica* (Chrysophyceae) was assessed when growing at varying temperatures and growth rates in steady state chemostat cultures. This approach allowed for the creation of a matrix of temperature and growth rate from which the individual effects of growth rate and temperature, as

well as any interaction effect on the organism's stoichiometry on the growth of *O. danica* could be assessed. The strain of *O. danica* used in this study cannot grow and divide purely photosynthetically and it voraciously consumes bacteria. It can grow osmotrophically, directly taking up nutrients from the dissolved state (Andersson et al. 1989). The work described herein addressed the hypothesis that *Ochromonas danica* would maintain a constant ratio of elements (as C, N, and P) despite changes in temperature or growth rate.

CHAPTER 2

MATERIALS AND METHODS

2.1 Organism and Maintenance

Ochromonas danica (UTEX 1298) was maintained axenically in *Ochromonas* medium (OM; Starr 1978) at room temperature (25°C) and was transferred weekly to fresh OM.

2.2 Growth Rates at Various Temperatures

Optimal growth temperature was determined from batch cultures (OM, 70 mL) grown at 13 to 35°C. Samples were taken at irregular time intervals and cell density was measured spectrophotometrically at 540 nm. Growth rates were calculated as the slope of a regression line fitting the natural log of optical density to time.

2.3 Chemostats

Ochromonas danica was grown in OM in continuously-stirred 800-mL chemostats. Sixteen temperature-growth rate combinations were established; four dilution rates (D = 0.03, 0.06, 0.08, and 0.10 h^{-1}; hereafter, growth rate) and four temperatures (15°C, 20°C, 25°C, and 28°C). Triplicate chemostats were prepared for each temperature-growth rate combination.

Chemostats reached steady-state after three complete turnovers at a given temperature and dilution rate. Outflow was aseptically captured in pre-sterilized 1 or 2 liter bottles (Nalgene).

2.3.1 Dissolved Chemical Analysis

The nutrient composition of OM (see below) was determined for each medium reservoir feeding a chemostat. Medium from reservoirs was collected and stored in acid-washed bottles (Quorpak) at -20°C until analysis. Dissolved organic carbon (DOC) and total dissolved nitrogen (TDN) were determined using a Shimadzu TOC-V_{CSH} Analyzer. Ammonium (NH_4^+), total dissolved phosphorus (TDP) and soluble reactive phosphorus (SRP) were determined using the methods of Strickland and Parsons (1972). 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.

2.3.2 Determination of Cell abundance and Size

Ochromonas abundance was determined by direct epifluorescent microscopic enumeration (250X) of glutaraldehyde fixed cells (2% final concentration) using acridine orange as the fluorochrome (Francisco et al. 1973). Cell volume (V) was determined from the length (L) and width (W) of at least 100 cells assuming that the general shape of *Ochromonas* conformed to a spindle where $V = \pi ((W^2 \cdot L)/6)$ (Eccleston-Parry and Leadbeater 1995). Length and width of cells were determined from digital images captured at 250X (Olympus BH-2 coupled to an Olympus DP70 camera) and imaging software (Simple PCI).

2.3.3 Determination of Ochromonas Element Composition

The C, N and P content of *Ochromonas* was determined for cells grown at each growth rate-temperature combination. Cells were collected on pre-combusted glass-fiber filters (25 mm Whatman GF/F). Carbon and N were determined using a CHN analyzer (Perkin-Elmer series 2200). Phosphorus was determined using persulfate oxidation and subsequent analysis of soluble reactive phosphate (Strickland and Parsons 1972). Quotas of each element are reported as fmole $cell^{-1}$ or as fmole μm^{-3}. Element ratios are reported mole:mole.

2.3.4 Determination of RNA and DNA

Bulk DNA and RNA were extracted from cells collected on 0.2 µm polycarbonate filters. Filters were immediately immersed in 4 mL extraction buffer (200 mM Tris and 20 mM EDTA in 0.17% laurylsarcosyl) and frozen (-20°C). After thawing, bulk DNA and RNA were determined according to the methods of Gorokhova and Kyle (2002) using the fluorochrome RiboGreen© (Invitrogen) in conjunction with a Fugi FLA-3000g imager.

2.4 Data Analysis

Data was analyzed with a two way ANOVA and log transformed when necessary. Statistical analysis was performed with Sigma Plot (v10) and SigmaStat (v3.5)

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Growth rate of *Ochromonas danica* at Various Temperatures

Figure 3.1 depicts the growth rates achieved when *O. danica* was grown at various temperatures (batch cultures in OM). Maximum growth appears to occur at approximately 30°C, while μ_{min} appears to occur at ~8°C (Figure 3.1). The maximum growth rate for *O. danica* feeding osmotrophically was essentially the same as when *O. danica* was fed bacteria (Grover and Chrzanowski 2009).

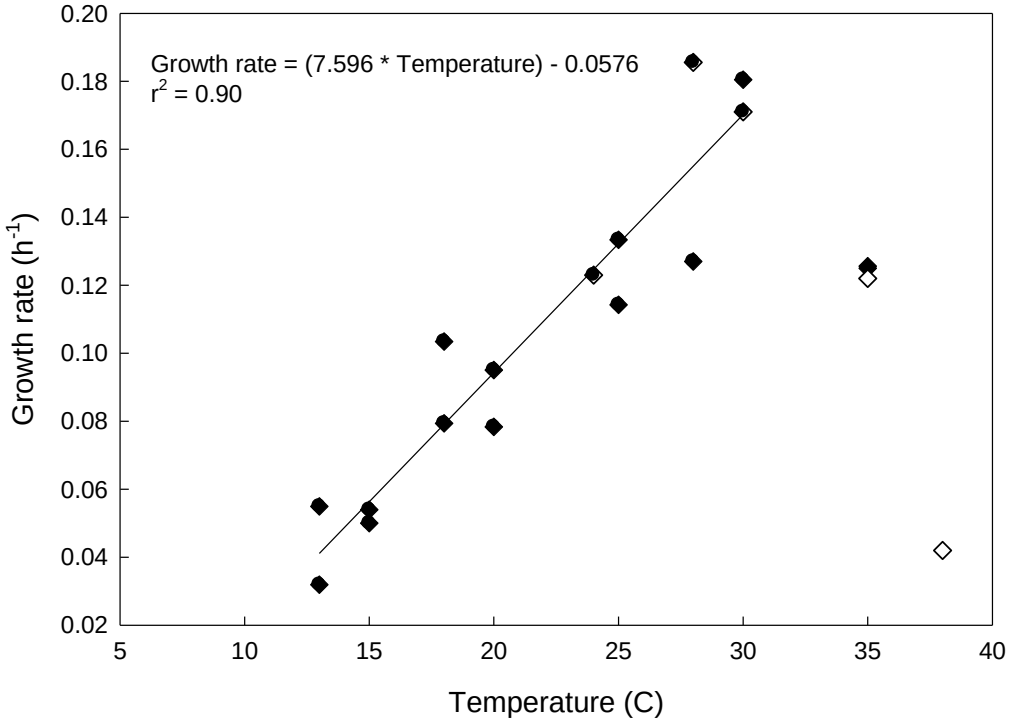

Figure 3.1 Growth rate of *Ochromonas danica* grown in Ochromonas medium at various temperatures. Closed diamonds are data from this study, open diamonds are data from previous unpublished data, solid line is regression.

3.2 Chemostats

3.2.1 Dissolved Chemical Analysis

Growth medium fed to the chemostats was made in 10 liter batches on 19 occasions. There was little variability in critical elements among the batches. Total dissolved carbon averaged 60.02 ± 3.89 mM, TN averaged 7.65 ± 0.51 mM with NH_4^+ making up 7% (0.54 ± 0.06 mM) of the total N, and TDP averaged 1.16 ± 0.10 mM with SRP making up 30% (0.34 ± 0.04 mM) of the TDP. The average C:N:P ratio of the medium was 52.4:6.7:1.

3.2.2 Limiting Nutrient

C, N, or P were not likely the limiting nutrients in the chemostats. The dissolved nutrients in the medium far exceeded the amount of particulate nutrients found in the cells. Table 3.1 presents the amount of elements in the dissolved state in the medium compared with the amount of particulate element in the cells and the surplus element remaining in the medium. Oxygen availability was also likely not limiting since the chemostats were mixed and aerated and growth rates were always below μ_{max}.

Table 3.1 Dissolved C, N, and P content of *Ochromonas* medium, particulate C, N, and P of *Ochromonas danica* grown at various temperature and growth rate combinations, and the surplus of elements remaining in the medium.

Growth Rate (h^{-1})	Temperature (ºC)	Dissolved Element (mM)			Particulate Element (mM)			Surplus in medium (mM)		
		C	N	P	C	N	P	C	N	P
0.03	15	62.1	7.9	1.2	12.2	1.4	0.2	49.9	6.5	1.0
	20	63.0	8.0	1.2	17.5	2.0	0.2	45.5	6.0	1.0
	25	63.0	8.0	1.2	15.6	1.8	0.2	47.4	6.2	1.0
	28	63.0	8.0	1.2	16.7	1.7	0.2	46.3	6.3	1.0
0.06	15	61.4	7.8	1.2	6.4	0.7	0.1	55.0	7.1	1.1
	20	56.3	7.3	1.2	12.7	1.3	0.1	43.6	6.0	1.1
	25	51.5	6.5	1.3	10.0	1.1	0.1	41.5	5.4	1.2
	28	51.5	6.5	1.3	5.7	0.7	0.1	45.8	5.8	1.2
0.08	15	60.3	7.8	1.0	3.3	0.4	0.0	57.0	7.4	1.0
	20	60.3	7.8	1.0	8.0	0.9	0.1	52.3	6.9	0.9
	25	60.3	7.8	1.0	9.6	1.0	0.1	50.7	6.8	0.9
	28	60.7	7.8	1.2	9.2	0.9	0.1	51.5	6.9	1.1
0.10	15	61.3	7.8	1.2	2.3	0.3	0.0	59.0	7.5	1.2
	20	61.3	7.8	1.2	3.7	0.5	0.0	57.6	7.3	1.2
	25	60.2	7.8	1.2	6.2	0.7	0.1	54.0	7.1	1.1
	28	60.2	7.8	1.2	6.3	0.7	0.1	53.9	7.1	1.1

3.2.3 Cell Abundance

Cells were more abundant in chemostat reactors when growth rates were slow than when growth rates were rapid (Figure 3.2, compare panel A to panel D). The concentration of flagellates within reactors varied from $1.42 \pm 0.08 \times 10^5$ cells mL^{-1} at the lowest temperature and fastest growth rate to $2.27 \pm 0.14 \times 10^6$ cells mL^{-1} at the lowest temperature and slowest growth rate (Figure 3.2 and Table 3.2).

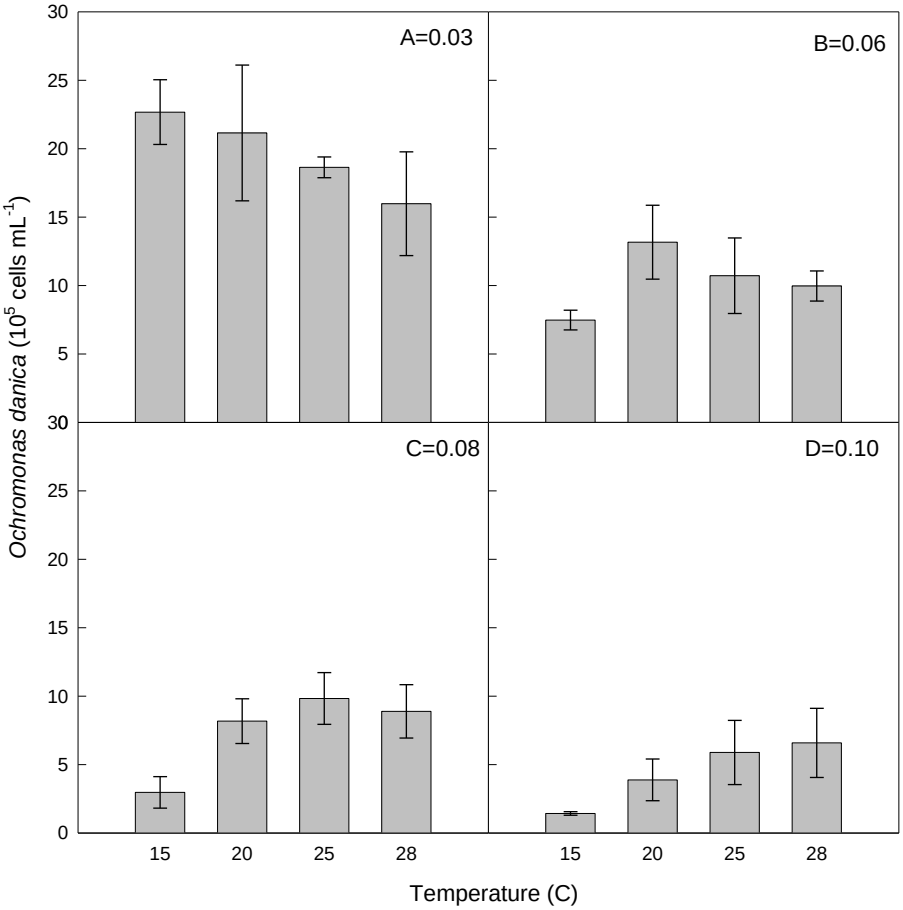

Figure 3.2 Concentration of *Ochromonas danica* (cells mL^{-1}) grown in chemostats. Panels (A-D) depict cell abundances at various growth rates. Mean values and standard errors are given in Table 3.2.

Table 3.2 Concentration of *Ochromonas danica* (10^5 cells mL^{-1}) growing in chemostats at different temperatures and growth rates. (Mean ± SE, n = 3 chemostats).

Growth Rate (h^{-1})	**Temperature (ºC)**			
	15	**20**	**25**	**28**
0.03	22.7 ± 1.4	21.1 ± 2.9	18.6 ± 0.4	16.0 ± 2.2
0.06	7.5 ± 0.4	13.2 ± 1.6	10.7 ± 1.6	10.0 ± 0.6
0.08	3.0 ± 0.7	8.2 ± 0.9	9.8 ± 1.1	8.9 ± 1.1
0.10	1.4 ± 0.1	3.9 ± 0.9	5.9 ± 1.4	6.6 ± 1.5

3.2.4 On Growth

The maximum growth rate at any temperature between approximately 8 and 30°C may be estimated using the regression line established for data presented in Figure 3.1. The growth rate at which chemostats were operated may then be examined as a fraction of the maximum potential growth rate (i.e. growth in batch culture; $\mu:\mu_{max}$). For example, when operated at a growth rate of 0.03 h^{-1}, *O. danica* grew at rates that ranged between 19 and 53% of μ_{max}. *Ochromonas danica* was growing at μ_{max} in chemostats operated at 15°C at 0.06 h^{-1}, and 20°C at 0.10 h^{-1}. Careful examination of these data suggests that chemostats operated at 15°C and growth rates above 0.06 h^{-1} could not be maintained for extended periods of time since $\mu > \mu_{max}$. This prediction is supported by the low cell abundance occurring in chemostats operated at 15°C and growth rates > 0.06 hr^{-1}. In the following analyses, all data depicted for chemostats operated at 15°C and growth rates > 0.06 h^{-1} should be considered cautiously as cells under these conditions are at the extreme limits of growth allowed by chemostat conditions. Consequently, data collected for cells grown at 15°C were excluded from all ANOVA seeking to characterize the interaction of growth rate and temperature on the element content of cells.

Table 3.3 $\mu:\mu_{max}$ ratio predicted for chemostats operated at various temperatures and growth rates.

Temperature	**$\mu:\mu_{max}$ at growth rates (hr^{-1}) of:**			
(°C)	**0.03**	**0.06**	**0.08**	**0.1**
15	0.53	1.06	1.42	1.78
20	0.32	0.64	0.85	1.06
25	0.23	0.45	0.60	0.76
28	0.19	0.39	0.52	0.64

ANOVA indicated that, with few exceptions, the source of variation explaining the changes in measured parameters (see Table 3.4) was growth rate. Temperature affected the cell volume and the interaction of temperature with growth rate had an effect on the C:N ratio. Temperature and the interaction of temperature with growth rate did not significantly affect any of the other variables.

Table 3.4 Outcomes of Two-way Analysis of variance. Data are from chemostats operated between 20 and 28°C at growth rates between 0.03 and 0.10 hr^{-1}. Values are the probability of an event occurring by chance (*p*). n.s. = not significant at $p > 0.05$.

Parameter	Source of Variation		
	Temperature	Growth Rate	Temperature × Growth Rate
Volume (μm^3)	0.040	< 0.001	n.s.
QC (fmol μm^{-3})	n.s.	0.007	n.s.
QN (fmol μm^{-3})	n.s.	0.001	n.s.
QP (fmol μm^{-3})	n.s.	n.s.	n.s.
QRNA (fg μm^{-3})	n.s.	< 0.001	n.s.
QDNA (fg μm^{-3})	n.s.	< 0.001	n.s.
C:N (mole:mole)	n.s.	0.014	0.004
C:P (mole:mole)	n.s.	n.s.	n.s.
N:P (mole:mole)	n.s.	n.s.	n.s.

3.2.5 Cell Volume

The average volume of *O. danica* in each group of replicate chemostats is presented in Figure 3.3 and the data given in Table 3.5. Cell volume varied with temperature (ANOVA, $p = 0.04$) and growth rate (ANOVA, $p < 0.001$) and ranged between 116.65 ± 5.82 and 202.84 ± 4.83 μm^3. The interaction of temperature a growth rate did not influence cell volume suggesting that cell size may be independently regulated by physiological factors (growth rate) and environmental factors (temperature). Cells growing at slow growth rates were larger than cells growing more rapidly (Figure 3.3 bottom panels). The distribution of cell size in the matrix created by the combination of growth rate and temperature is shown in the upper panel of Figure 3.3. In this figure, the contour mesh fitting the data points clearly reveals the decrease in mean cell size as functions of growth rate and temperature. This pattern is consistent for all chemostats (see above). The distribution of *O. danica* cell size as a function of growth rate and temperature is contrary to expectations. A survey of 61 aquatic ectotherms, revealed that all but six species were larger when grown at low temperature than when grown at warm temperatures (Atkinson, 1995). Thompson et al. (1992), working with batch cultures of phytoplankton, also found a smaller cell size at lower temperatures in six of eight species grown over a similar temperature range. Cells growing at a constant rate are generally larger when grown at low temperatures than when growing at warmer temperatures (Montagnes and Franklin 2001; Rhee and Gotham 1981).

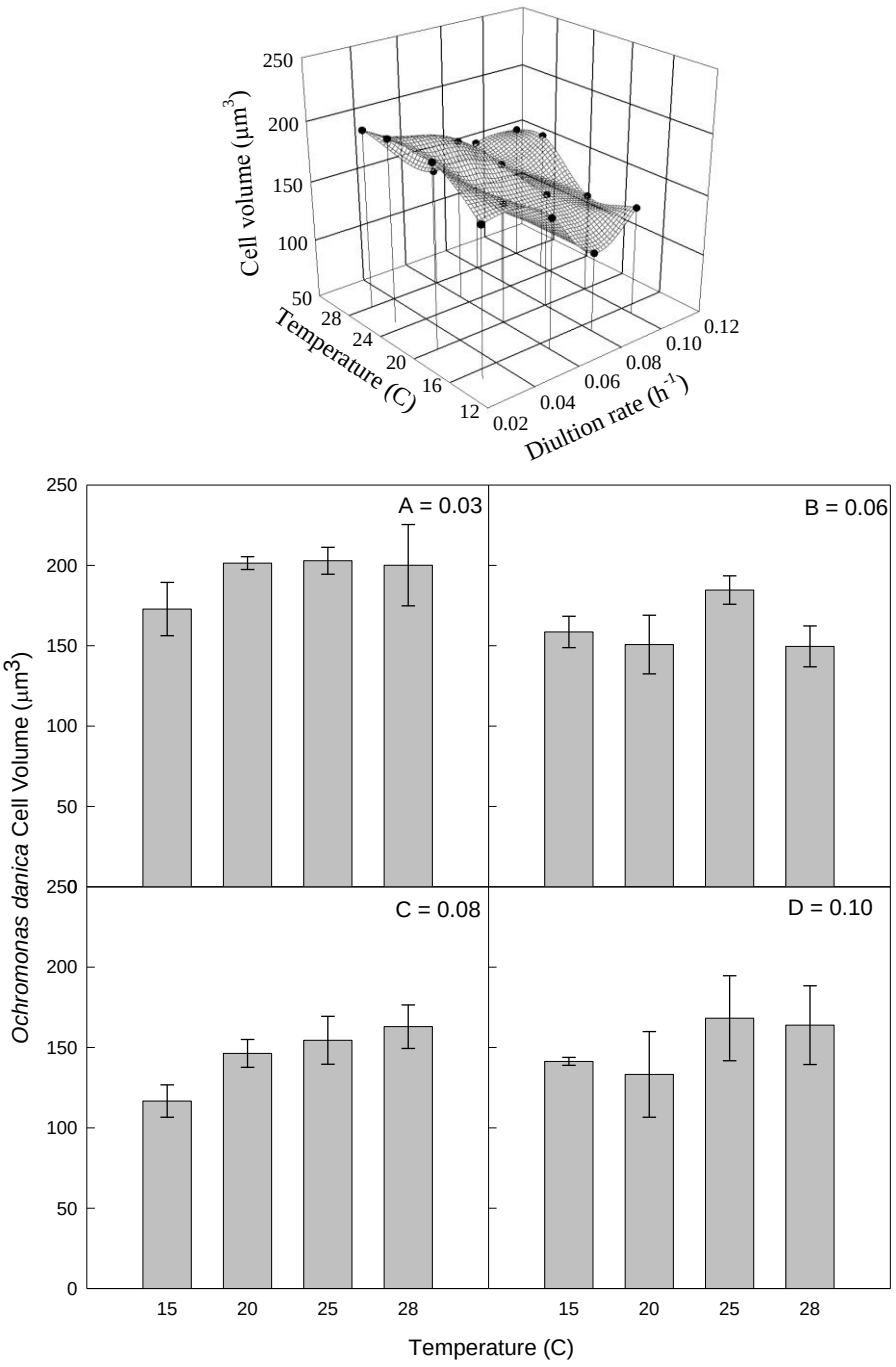

Figure 3.3 Cell Volume of *Ochromonas danica*. For 3-D plots, the contour mesh fits the mean for the replicate chemostats along the growth rate-temperature matrix and is shown as a solid line and closed circle. 2-D plots are split by growth rate. Mean values and standard errors are given in Table 3.5.

Table 3.5 Volume of *Ochromonas danica* (μm^3 $cell^{-1}$) growing in chemostats at different temperatures and growth rates. (Mean ± SE, n = 3 chemostats).

Growth Rate (h^{-1})	**Temperature (ºC)**			
	15	**20**	**25**	**28**
0.03	172.83 ± 9.57	201.41 ± 2.31	202.84 ± 4.83	200.08 ± 14.58
0.06	158.56 ± 5.62	150.74 ± 10.5	184.63 ± 5.09	149.58 ± 7.32
0.08	116.65 ± 5.82	146.32 ± 5.01	154.45 ± 8.63	162.92 ± 7.81
0.10	141.33 ± 1.41	133.19 ± 15.40	168.18 ± 15.28	163.91 ± 14.15

3.2.3 Ochromonas Element Composition—Cell Quotas

Figure 3.4 and 3.5 presents the element content of *Ochromonas* normalized to cell volume (quota). The C quota varied four-fold from 31.6 to 113.4 fmol μm^{-3}, whereas the N quota varied five-fold from 3.6 to 16.8 fmol μm^{-3}. P quota was the least variable of the three elements and varied two-fold from 0.09 to 0.19 fmol μm^{-3}. Cells grown rapidly at low temperatures had the highest cell quotas while those grown slowly at warmer temperatures had the lowest cell quotas. Included in these plots are the data collected for chemostats operated at the limits of *O. danica* growth. These data appear consistent with data collected from other growth rate – temperature combinations and suggest that element quotas increase at high growth rate. The relationship between growth rate and temperature was explored by removing the data collected at 15°C. The concentration of C and N in cells was significantly affected by growth rate (ANOVA, $p = 0.007$, $p = 0.001$, respectively); however neither temperature alone nor the interaction of growth rate and temperature could explain any of the variation in C or N concentration. The concentration of P in cells was not explained by growth rate, temperature or the interaction of temperature and growth rate.

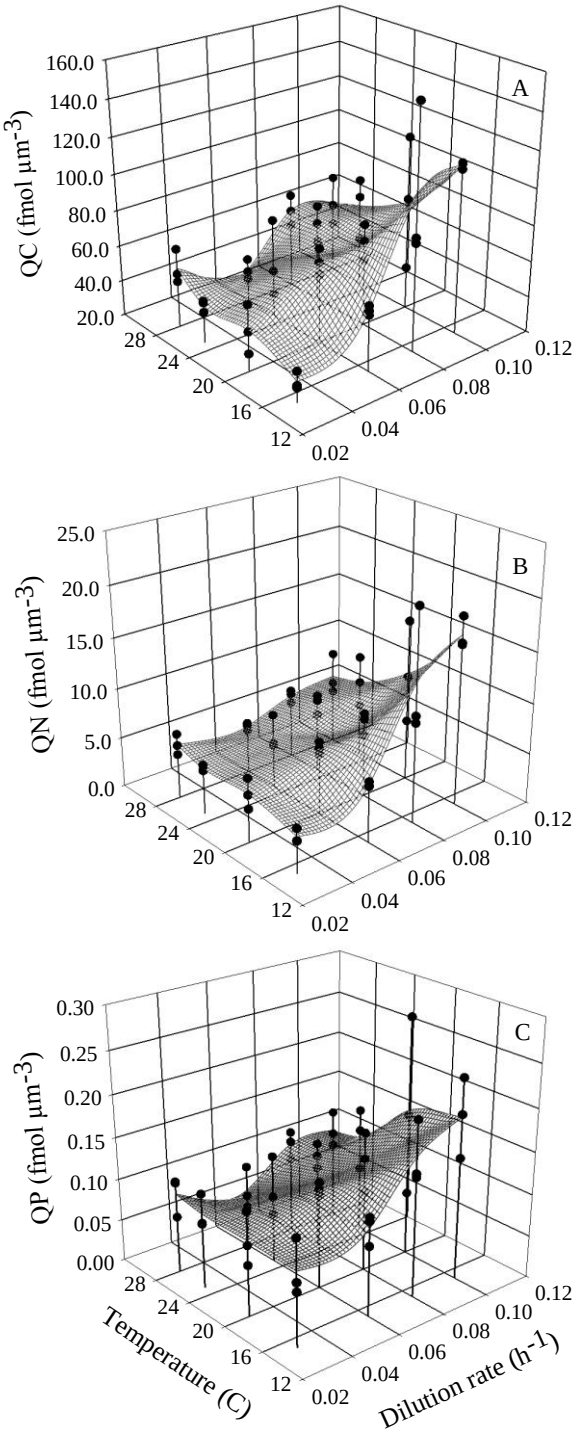

Figure 3.4 (A) Cell quota of carbon (QC), (B) nitrogen (QN), and (C) phosphorus (QP) for *Ochromonas danica* grown in chemostats at different growth rates and temperatures. Data from individual chemostats are shown as a solid line and closed circle. The contour mesh fits the mean for the replicate chemostats along the growth rate-temperature matrix. Mean values and standard errors are given in Table 3.6.

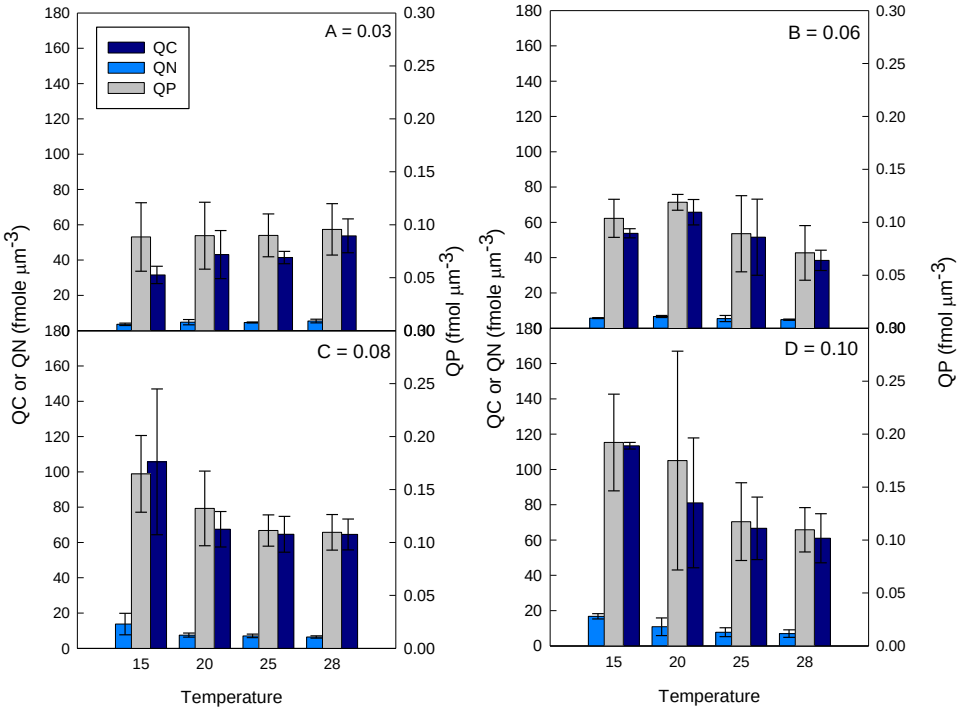

Figure 3.5 Cell quota of C, N, and P for *Ochromonas danica* grown in chemostats at different growth rates and temperatures.

Table 3.6 C, N, and P content of *Ochromonas danica* grown at different temperatures and growth rates in chemostats. (Mean ± SE, n = 3 chemostats)

Growth Rate	**Temperature**	**Element (pmol cell^{-1})**		
(h^{-1})	**(ºC)**	**C**	**N**	**P**
0.03	**15**	5.4 ± 0.2	0.6 ± 0.04	0.015 ± 0.002
	20	8.6 ± 1.5	1.0 ± 0.2	0.018 ± 0.003
	25	8.4 ± 0.3	0.9 ± 0.02	0.018 ± 0.002
	28	10.7 ± 0.8	1.1 ± 0.1	0.019 ± 0.003
0.06	**15**	8.5 ± 0.5	0.9 ± 0.04	0.017 ± 0.002
	20	10.0 ± 1.3	1.0 ± 0.1	0.018 ± 0.002
	25	9.4 ± 2.0	1.0 ± 0.2	0.016 ± 0.003
	28	5.7 ± 0.3	0.7 ± 0.01	0.010 ± 0.002
0.08	**15**	12.1 ± 2.2	1.6 ± 0.3	0.019 ± 0.002
	20	9.8 ± 0.6	1.1 ± 0.07	0.019 ± 0.002
	25	10.0 ± 1.0	1.1 ± 0.09	0.017 ± 0.002
	28	10.5 ± 0.6	1.0 ± 0.08	0.018 ± 0.002
0.10	**15**	16.0 ± 0.1	2.4 ± 0.1	0.027 ± 0.004
	20	10.0 ± 1.7	1.4 ± 0.2	0.022 ± 0.006
	25	10.9 ± 0.9	1.3 ± 0.2	0.019 ± 0.002
	28	9.8 ± 0.6	1.1 ± 0.1	0.018 ± 0.001

Table 3.7 C, N, and P content of *Ochromonas danica* grown at different temperatures and growth rates in chemostats. (Mean ± SE, n = 3 chemostats)

Growth Rate (h^{-1})	Temperature (ºC)	Element (fmol μm^{-3})		
		C	N	P
0.03	**15**	31.6 ± 2.8	3.6 ± 0.4	0.088 ± 0.019
	20	43.1 ± 7.9	4.8 ± 0.9	0.090 ± 0.018
	25	41.4 ± 2.0	4.6 ± 0.2	0.090 ± 0.012
	28	53.7 ± 5.6	5.5 ± 0.6	0.096 ± 0.014
0.06	**15**	53.8 ± 1.5	5.7 ± 0.1	0.10 ± 0.01
	20	65.8 ± 4.2	6.7 ± 0.3	0.12 ± 0.004
	25	51.6 ± 12.5	5.5 ± 1.0	0.089 ± 0.021
	28	38.5 ± 3.3	4.8 ± 0.2	0.071 ± 0.015
0.08	**15**	105.6 ± 23.9	13.8 ± 3.5	0.16 ± 0.02
	20	67.5 ± 5.8	7.5 ± 0.7	0.13 ± 0.02
	25	64.6 ± 5.9	7.0 ± 0.6	0.11 ± 0.01
	28	64.6 ± 5.0	6.4 ± 0.4	0.11 ± 0.01
0.10	**15**	113.4 ± 1.1	16.8 ± 0.9	0.19 ± 0.03
	20	81.1 ± 21.2	10.9 ± 2.9	0.18 ± 0.06
	25	66.6 ± 10.2	7.8 ± 1.5	0.12 ± 0.02
	28	61.0 ± 8.0	7.0 ± 1.2	0.11 ± 0.01

3.2.7 Ochromonas Element Composition—Element Ratios

Ratios of elements (C:N, C:P and N:P) are show in Figures 3.7, and 3.8 and given in Table 3.8. Temperature did not affect the ratios of elements within cells (Table 3.8). However, ANOVA indicates that the variation in the C:N ratio (Figure 3.7, panel A) could be explained by growth rate and that there was a significant interaction of growth rate and temperature. The C:N ratio ranged between 6.67 and 10.02 (ANOVA; growth rate, $p = 0.014$; temperature × growth rate, $p = 0.004$, 15°C data excluded from analysis). Cells growing at the limits of growth (15°C, $\mu > 0.06$) clearly reapportion elements (Figure 3.4 and 3.7). The low C:N ratio at the fastest growth rate and coldest temperatures may be the result of increased lipid stores within cells. At the highest growth rates (0.08 and 0.10 h^{-1}) and cold temperatures (20 and 15°C) lipid-like molecules were observed blebbing from cells (Figure 3.6) and a lipid-like film formed on the sides of the chemostats (personal observations).

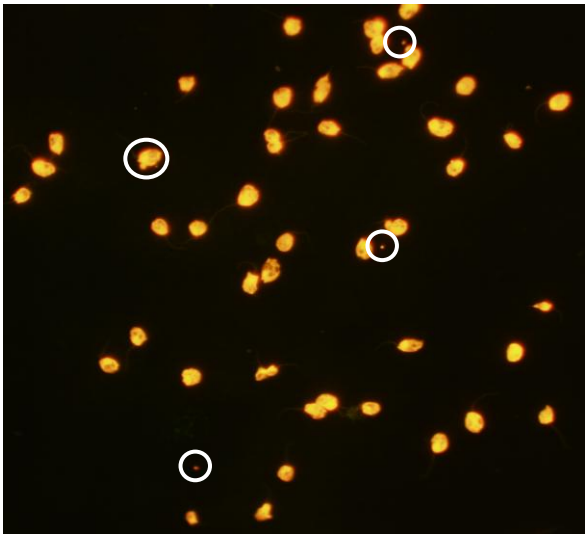

Figure 3.6 Image of *Ochromonas danica* from chemostat. Circles show lipid droplets.

The C:P ratio varied from 73 to 119 (Figure 3.7B and Table 3.8); however, apart from the low C:P ratio obtained for cells grown at the slowest growth rate and lowest temperature (15°C, 0.03 hr^{-1}) the C:P ratio remained stable, varying only between 92 and 119.

Similar to the C:P ratio, the N:P ratio varied little over much of the temperature-growth rate matrix, however; there was considerable variability in the N:P ratios driven by growth at low temperatures (Fig. 3.7C). Compare the N:P ratio of cells grown at 15°C to the N:P ratio of cells grown at all other temperature and growth rates. The ratio for cells grown at 15°C varied from 8.3 to 17.6 over the span of growth rates while the N:P ratio for all other combinations of temperature and growth rate only ranged between 10.4 and 13.5.

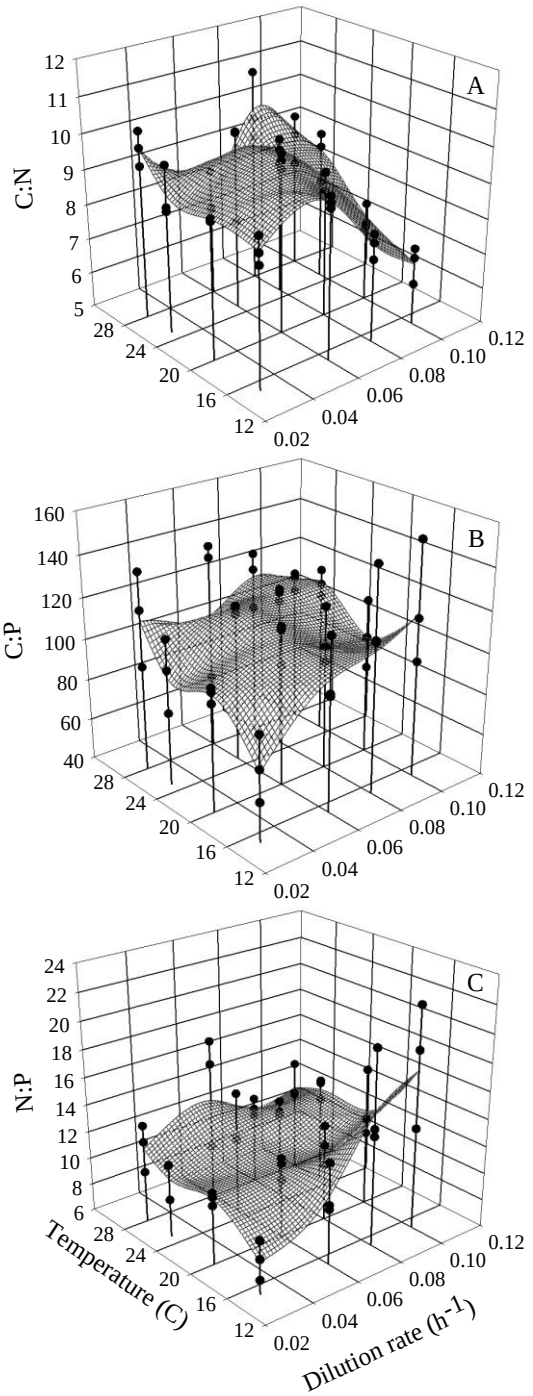

Figure 3.7 Element ratios of *Ochromonas danica* grown in chemostats at different growth rates and temperatures. Data from individual chemostats are shown as a solid line and closed circle. The contour mesh fits the mean for the replicate chemostats along the growth rate-temperature matrix. Mean values and standard errors are given in Table 3.8. Ratios are mole: mole.

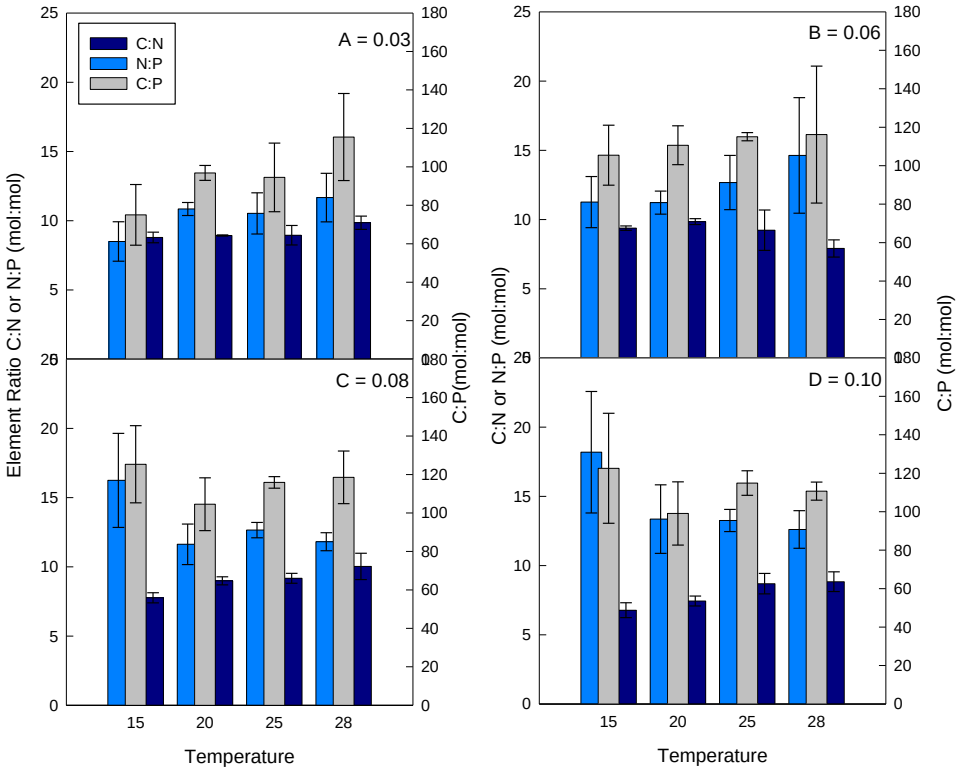

Figure 3.8 Element ratios of *Ochromonas danica* grown in chemostats at different growth rate and temperatures.

Table 3.8 Element ratios in *Ochromonas danica* grown at different temperatures and growth rates in chemostats. (Mean ± SE, n = 3 chemostats)

Growth Rate	**Temperature**	**Ratio (mol : mol)**		
(h^{-1})	**(ºC)**	**C:N**	**C:P**	**N:P**
0.03	**15**	8.79 ± 0.22	73.21 ± 9.11	8.33 ± 0.82
	20	8.93 ± 0.02	96.73 ± 2.24	10.84 ± 0.27
	25	8.95 ± 0.40	92.95 ± 10.30	10.38 ± 0.86
	28	9.86 ± 0.28	113.44 ± 13.07	11.51 ± 1.01
0.06	**15**	9.38 ± 0.09	103.47 ± 9.02	11.03 ± 1.07
	20	9.84 ± 0.12	109.78 ± 5.84	11.15 ± 0.48
	25	9.46 ± 0.84	115.57 ± 1.23	12.21 ± 1.13
	28	7.88 ± 0.36	106.12 ± 20.56	13.46 ± 2.41
0.08	**15**	7.79 ± 0.21	108.90 ± 11.59	15.60 ± 1.96
	20	9.01 ± 0.17	103.04 ± 7.92	11.43 ± 0.84
	25	9.16 ± 0.21	115.74 ± 1.74	12.64 ± 0.32
	28	10.02 ± 0.55	118.65 ± 7.88	11.84 ± 0.37
0.10	**15**	6.77 ± 0.31	119.11 ± 16.51	17.59 ± 2.53
	20	7.49 ± 0.21	98.28 ± 9.50	13.12 ± 1.43
	25	8.82 ± 0.43	115.62 ± 3.69	13.10 ± 0.46
	28	8.94 ± 0.41	110.16 ± 2.72	12.32 ± 0.78

3.2.8 RNA and DNA Content

Figures 3.9 and 3.10 present the nucleic acid content of *Ochromonas danica.* DNA and RNA concentrations were below detection for many of the samples collected from chemostats operated at 15°C and $\mu > 0.06$ hr^{-1}. In Figure 3.9, the dramatic increase in both DNA and RNA at 15°C represents projections of nucleic acid content based on available data. Clearly, there was an increase in nucleic acid concentration as growth rate increased (ANOVA; growth rate, $p < 0.001$). There is a slight difference in the distribution of DNA and RNA when they are considered separately. There is a shift in the relative contribution of RNA to the total pool of nucleic acids. At the 0.03 h^{-1} growth, RNA was approximately 92% of the total nucleic acid pool, at 0.06 h^{-1} it was 86%, and at 0.08 h^{-1}, RNA was approximately 78% of the total pool of nucleic acids. These data suggest that there is a greater DNA content in rapidly growing cells rather than in slowly growing cells. This is opposite the pattern found in bacteria where at rapid growth rates, RNA makes up a larger proportion of total nucleic acid than at slower growth rates (Chrzanowski and Grover 2008).

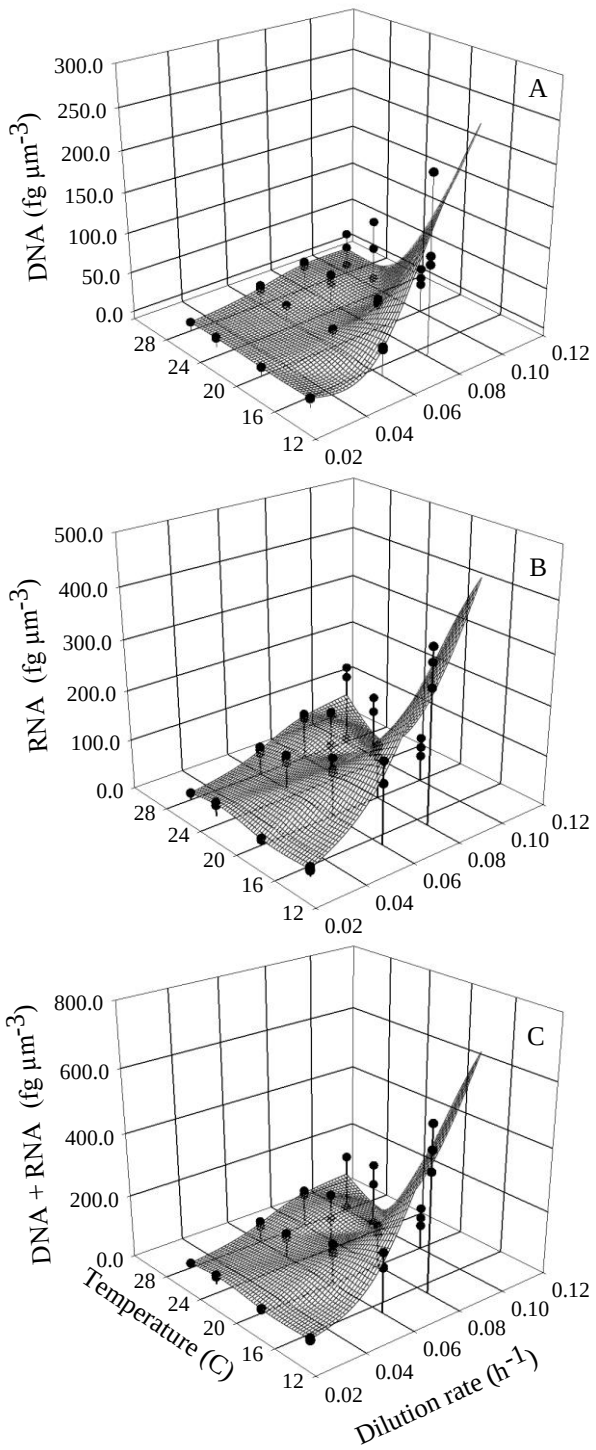

Figure 3.9 (A) Cell quota of DNA, (B) RNA, and (C) total nucleic acids for *Ochromonas danica* grown in chemostats at different growth rates and temperatures. Data from individual chemostats are shown as a solid line and closed circle. The contour mesh fits the mean for the replicate chemostats along the growth rate-temperature matrix. The mesh shows projections at 0.10 h^{-1}. Mean values and standard errors are given in Table 3.9.

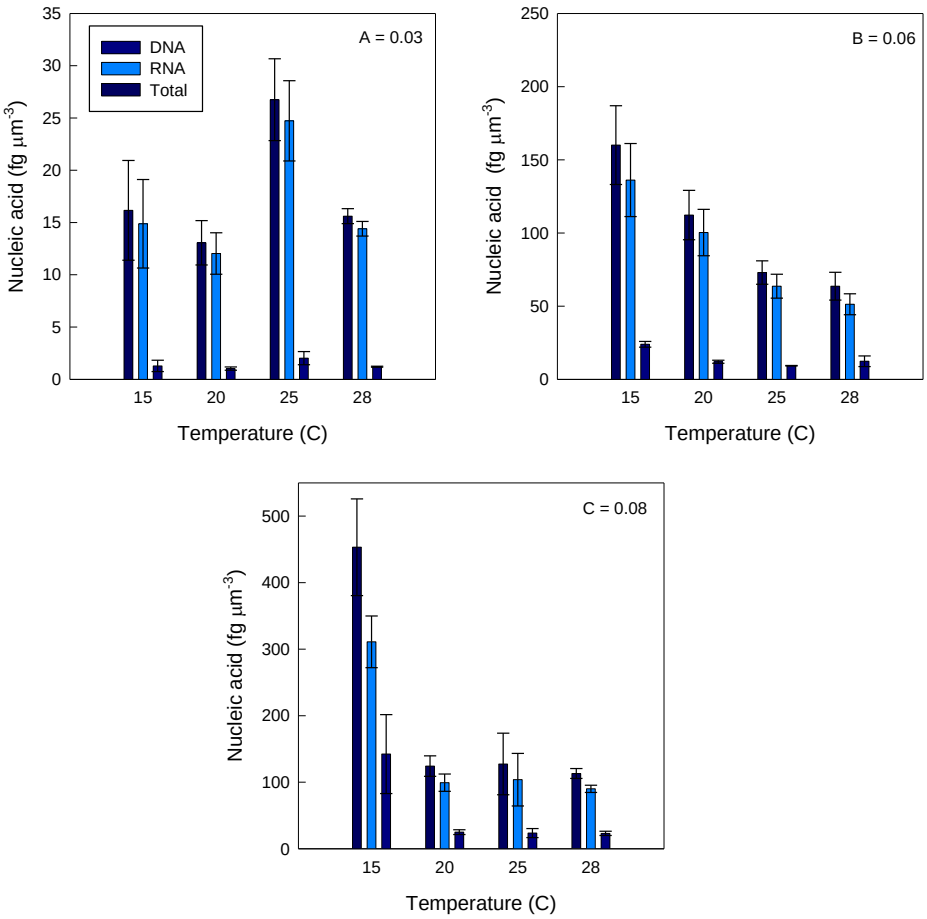

Figure 3.10 Cell quotas of DNA and RNA for *Ochromonas danica* grown in chemostats at different growth rates and temperatures.

Table 3.9 Nucleic acid content of *Ochromonas danica* grown at different temperatures and growth rates in chemostats. (Mean ± SE, n=3 chemostats)

Growth Rate	**Temperature**	**Nucleic acid (fg µm^{-3})**		
(h^{-1})	**(ºC)**	**DNA**	**RNA**	**Total**
0.03	**15**	1.3 ± 0.3	14.9 ± 2.5	16.2 ± 2.8
	20	1.0 ± 0.1	12.0 ± 1.2	13.1 ± 1.2
	25	2.0 ± 0.4	24.7 ± 2.2	26.7 ± 2.3
	28	1.2 ± 0.03	14.4 ± 0.4	15.6 ± 0.4
0.06	**15**	23.9 ± 1.1	136.0 ± 14.4	160.1 ± 15.5
	20	12.0 ± 0.6	100.0 ± 9.1	112.2 ± 9.7
	25	9.3 ± 0.1	63.6 ± 4.7	72.9 ± 4.6
	28	12.4 ± 2.1	51.3 ± 4.1	63.7 ± 5.5
0.08	**15**	142 ± 34.3	311.0 ± 22.4	453.2 ± 41.9
	20	24.9 ± 2.1	99.3 ± 7.5	124.2 ± 8.9
	25	23.6 ± 3.9	104.0 ± 22.8	127.4 ± 26.7
	28	23.1 ± 1.87	90.0 ± 3.1	113.1 ± 4.3

3.3 Conclusions and Impacts

3.3.1 Cell Size vs. Nutrient Content

Ochromonas ranged between 100 and 200 μm^3 with the largest cells associated with the slowest growth rates. Cells growing slowly are significantly larger than the cells growing rapidly but have low quotas of C and N. It seems likely that this result is due to increased water content in larger, slow growing cells compared to the faster growing smaller cells. While this size range is similar to that reported by Fenchel (1982), he found that cells growing exponentially were larger than starved cells. Variation in flagellate cell size has been linked to physiological state. The cell size variation of *Paraphysomonas* was found at the onset of stationary phase and related to the respiration rate of the organism (Caron et al. 1986). This pattern was also reported by Fenchel (1982) who found starved *Ochromonas* to produce small cells with respiration rates only 2-5% of growing cells.

3.3.2 Growth Rate vs. Temperature

Cell volume was the only feature of cells affected by temperature alone. In contrast, growth rate had a significant effect on all of the variables except P. These results indicate that variations in its element content are largely a consequence of changing chemical environment (expressed as growth rate) and less a consequence of physical environment (expressed as temperature). The physiological changes necessary to alter growth rate have more influence on the stoichiometry than the changes to cope with the temperature variation. The lack of change from P is interesting because it is contrary to the growth rate hypothesis that states an increased growth rate will be accompanied by an increase in RNA and subsequently P (Elser et al. 2003). This might be explained if P was limiting in the chemostat but there was ample excess P in the medium compared to the amount of particulate P in the cells.

The physiological response to changing growth rate and the reallocation of resources maybe reflected in the formation of lipid - like materials. At low temperatures, lipid - like globules appeared in the reactors and appeared to bleb from cells (Figure 3.6). We know of no other report of such lipid - like material formed by heterotrophic flagellates. *Ochromonas danica* was shown to

secrete lipids in a dissolved state but the growth conditions were not varied to see if the secretion levels changed (Billmire and Aaronson 1976). There are reports of lipid rearrangements due to changing growth conditions in other chrysophytes as well (Sriharan et al. 1990). While the experimental design did not address synthesis of specific biochemical compounds, it is clear that there is the need for further study into the relationship among temperature, growth rate and lipid synthesis.

3.3.3 Impacts on Aquatic Systems

Knowing the element stoichiometry of organisms, and how the organisms respond due to changing environmental factors, help elucidate the dynamic inner workings of predator - prey interactions, the affect the environment has on them, and the affects they have on the environment. Sterner and Elser (2002) developed a model to precisely describe an organism's level of homeostasis, or its ability to maintain a constant element stoichiometry. When comparing an organism's stoichiometry to its resource stoichiometry, slopes between zero (strict homeostasis) and one (no homeostasis) represent different strengths of homeostasis. The particular level of homeostasis of an organism will have several affects on the pools and fluxes of energy and matter in the ecosystem.

Metazoan zooplankton have a strictly homeostatic composition that is genus specific. The genus- specific nature of the element ratios explain the differences in recycled nutrients into the system and the different autotrophic communities found in their presence (Andersen and Hessen 1991; Elser et al. 1996; Hessen et al. 2004). Flagellates also seem to have genus- specific element ratios influenced by different life histories (Eccleston-Parry and Leadbeater 1995). There has been an assumption of strict homeostasis for flagellates in previous studies because regeneration of nutrients change along with changing prey nutrient status and higher levels systems have been found to work this way. Past studies on the stoichiometry of flagellates have used bacteria or phytoplankton as prey (Nakano 1994; Goldman et al. 1987). Prey can remain undigested in the food vacuole for ~75 minutes (Shannon et al. 2007). The presence of these unassimilated prey items in the food vacuole will alter the measurements of elements for the

flagellate. Using organic media for the experiment eliminated the added complexity of variable prey nutrient status and ingestion and digestion of prey items, allowing for a true measurement of flagellate stoichiometry and other variables to be easily studied. The next best thing will be discovering a defined media that will support the growth of this organism so the resource nutrient ratios can be manipulated.

Grover and Chrzanowski (2006) integrated the data from Goldman et al. (1987) and Nakano (1994) creating homeostasis plots for flagellates; plotting the element ratios of the flagellates verses element ratios of prey items. Figure 3.11 illustrates those homeostasis plots for nanoflagellate element ratios and the data from this study is included. Given that prey items were not used in this study, the element ratios of the media were used for the resource stoichiometry. In the figure, the black line illustrates the regression line of the data of the two previous studies and the red line shows a regression of all data from the three studies together. The dotted line is the 1:1 line, representing a complete non-homeostatic nature of flagellate stoichiometry; if prey ratios change then the predator's ratios change accordingly. If the slope of the regression line is zero, it represents strict homeostasis in the consumer and regardless of what the resource ratio is; the predator element ratio will be maintained. The regression lines show something in between those two extremes, predicting that flagellates have a weak homeostasis. Adding in the data from this study only slightly adjusts the slopes of the regression lines. The knowledge of weak homeostasis will change how nutrient regeneration is predicted at this trophic level of predator-prey interactions. It may convey some storage flexibility when food is scarce making flagellates more competitive in limiting environments.

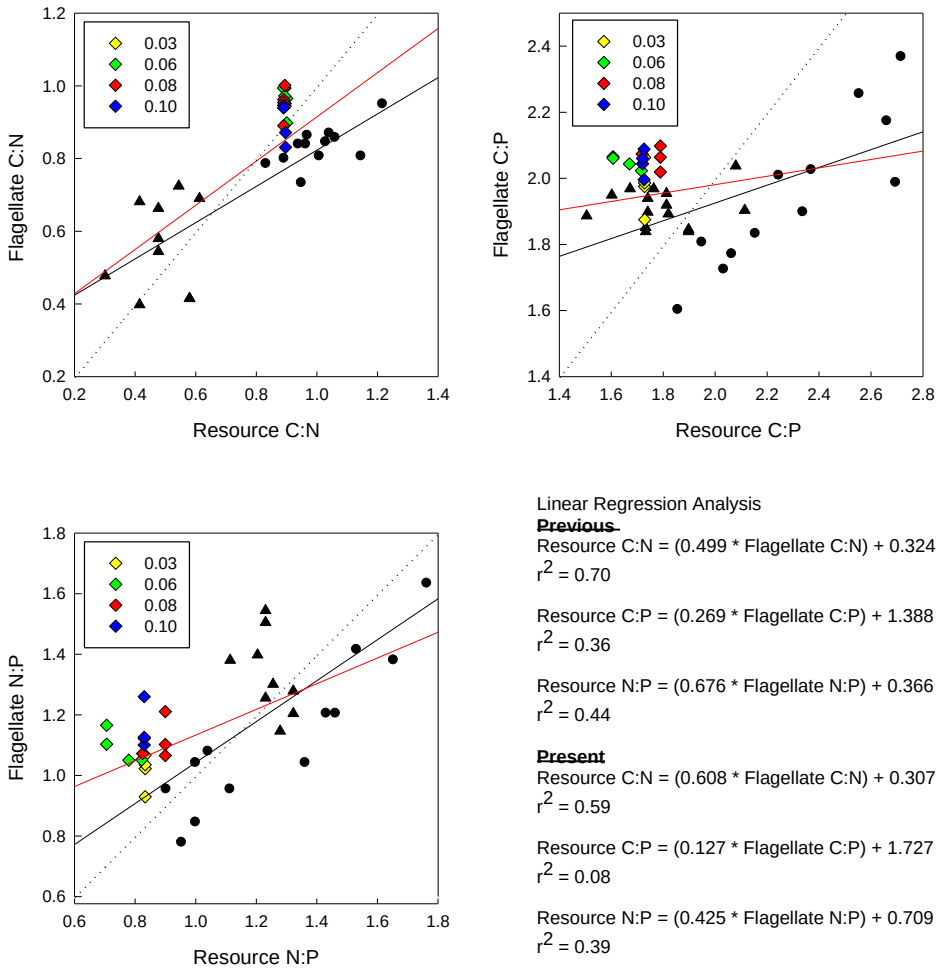

Figure 3.11 Stoichiometric homeostasis plots for flagellates fed prey items of varying element stoichiometry. Dotted line is 1:1 line. Circles—data from Goldman et al. (1987); triangles—data from Nakano (1994); diamonds—data from this study. Redrawn from Grover and Chrzanowski (2006).

Most of the work on nanoflagellate stoichiometry has been done on the strictly heterotrophic, *Paraphysomonas* sp (Caron et al. 1985, 1986; Andersen et al. 1986; Goldman et al. 1985, 1987; Eccleston-Parry and Leadbeater 1995). There is ample evidence to say that mixotrophs are major bacterivores in aquatic systems but studies addressing nutrient

regeneration and cell stoichiometry of the mixotrophs are few (Rothhaupt 1997, Caron et al. 1990). Mixotrophs that are mainly phagotrophic may have similar effects to heterotrophs on the flow and regeneration of nutrients and have similar weakly homeostatic element ratios.

A similar experiment has already been done with a prey for this organism. A matrix of growth rates and temperatures were used to see the changes in bacterial stoichiometry (Chrzanowski and Grover 2008). The outcome of the present study and the sister study on these two model organisms can be used together to model the nanoflagellate-bacteria predator-prey relationship at several different environmental settings. Regenerated nutrients should be a manifestation of the environmental temperatures, resources of the bacteria, their growth rate, and the element ratio and growth rate of the flagellate (Chrzanowski and Kyle 1996).

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BIOGRAPHICAL INFORMATION

Savannah Simonds received her Bachelor of Science in Microbiology and Biology from the University of Texas at Arlington in 2003. During her undergraduate degree she had Dr. Chrzanowski in several upper level microbiology classes and entrenched herself into his lab. She spent the following two years in Thomas Chrzanowski's lab working with *Ochromonas danica.* Savannah looked at the element ratio of *O. danica* in varying environmental conditions using chemostat studies. She taught microbiology labs for most of her time at UTA. Her research interests are microbial ecology and nutrient storage and dynamics in ecosystems. With the completion of this project, she earned a Master of Science in Biology in the summer of 2009. She plans to go into industry and hopes to work her way to researching clean biofuel options.