MEASUREMENT OF THE CARBON AND NITROGEN DENSITIES OF THE MARINE HETEROTROPHIC FLAGELLATE *PARAPHYSOMONAS* SP.

A Thesis
Presented to
The Faculty of the School of Marine Science
The College of William and Mary in Virginia

In Partial Fulfillment
Of the Requirements for the Degree of
Master of Arts

by
Yongsik Sin
1992

Library

This thesis is submitted in partial fulfillment of
the requirements for the degree of

Master of Arts

Yongsik Sin

Approved, August 1992

Michael E. Sieracki, Ph.D.
Committee Co-Chairman/Advisor

Kenneth L. Webb, Ph.D.
Committee Co-Chairman/Advisor

Eugene M. Burreson, Ph.D.

Leonard W. Haas, Ph.D.

John M. Brubaker, Ph.D.

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I gratefully acknowledge the motivation and support of my major advisors, Dr. M. E. Sieracki, and Dr. K. L. Webb throughout the course of this research. They supplied the patience, excellent academic advice, and equipment necessary to complete this research. I am also indebted to my other committee members; Drs. E. M. Burreson, L. W. Haas, and J. M. Brubaker for their interest and helpful discussions. Their skillful editing made this thesis readable.

I wish to thank to Grace Battisto for the CHN analysis and Charlie Viles for assistance with image analysis. I owe many thanks to Dr. Cheol Mo and Kyeong Park who helped me in every aspect. I am also thankful to all my friends; Aaron Adams, Al Curry, Mary Rybitski, Nantarika Issarasak, and Padma Venkatraman for their constant encouragement and kindness during my stay in a foreign land. Finally, I wish to express my gratitude to my family for their love and support.

LIST OF TABLES

Table Page

LIST OF FIGURES

ABSTRACT

Cell carbon and nitrogen densities (fg μm^{-3}) and C:N ratio of heterotrophic (bacterivorous) flagellates *Paraphysomonas* sp. at different growth phases were studied in batch cultures. Daily image analysis using epifluorescence microscopy was carried out to determine growth phase of flagellates. At logarithmic and stationary growth phase, flagellate cultures were filtered through GF/F filters and CHN analysis was performed on the samples. Total C and N contents ($\mu g\ ml^{-1}$) of bacteria retained on GF/F filters were estimated from CHN analysis for bacterial C and N and image analysis for bacterial number and biovolume. Bacterial C and N densities in bacterial cultures were used to calculate total C and N contents of bacteria in flagellate cultures. The total C and N contents of bacteria were subtracted from total C and N contents of retained cells (bacteria and flagellates) on GF/F filters, which gave total C and N contents of the flagellates only. C and N densities of flagellates were determined by dividing the total flagellate biovolume ($\mu m^3\ ml^{-1}$) into the total flagellate C and N contents ($\mu g\ ml^{-1}$).

One tailed *t* test showed that there is a significant difference between C and N densities of *Paraphysomonas* sp. at logarithmic and stationary growth phase at significance level 0.05. Andersen & Fenchel (1985) reported that heterotrophic flagellates in nature had lower cell number within the range of 2×10^2 to $5 \times 10^3\ ml^{-1}$ but showed a similar growth curve to cells in culture. Therefore, to estimate biomass, I suggest using 430 C and 90 N fg μm^{-3} for growing cells similar to *Paraphysomonas* sp. and 280 C and 60 N fg μm^{-3} for stationary growth cells in nature. C:N ratio was not significantly different between logarithmic and stationary growth phase (5.97) but found to be similar to 5.87 of starved *Paraphysomonas imperforata* found by Goldman et al. (1987).

MEASUREMENT OF THE CARBON AND NITROGEN DENSITIES OF THE MARINE HETEROTROPHIC FLAGELLATE *PARAPHYSOMONAS* SP.

INTRODUCTION

Heterotrophic microflagellates are generally regarded as the most important bacterivorous protozoa in the marine microbial community in terms of abundance and activity (Sorokin 1977, Fenchel 1982 a,b,c,d, Sherr & Sherr 1983). The heterotrophic flagellates play a significant role in maintaining the bacteria in a young physiological state by grazing the microbes (Barsdate et al. 1977, McManus & Fuhrman 1988, Caron 1990). These microflagellates are also important as consumers of primary producers like small phytoplankton, including coccoid cyanobacteria, in marine ecosystems (Johnson et al. 1982, Goldman & Caron 1985, Goldman et al. 1987, Caron et al. 1985, Andersen et al. 1986, Campbell & Carpenter 1986, Parslow et al. 1986, Caron 1991). In addition to the role of the cycling of carbon, the protozoa are also considered important organisms (even more than bacteria) in the regeneration of the major nutrients in the marine environment (Pomeroy 1974, Sieburth 1976, Haas & Webb 1979, Fenchel 1986, Azam et al. 1983, Laacke et al. 1983, Goldman & Caron 1985, Stoecker & Evans 1985, Sherr et al. 1986, 1988, Caron 1991). There is a controversy, however, over the relative importance of this group in the flow of organic carbon, that is, the "link or sink" concept; link refers to their role as food source for higher level consumers (metazoa) in the marine food web, while sink is the term describing the loss of fixed carbon by means of respiratory activity (Ducklow et al. 1986, Sherr et al. 1987, Sherr & Sherr 1988). All these microbial processes are controlled by microbial community composition and biomass, that is, the kinds of microbes present and the abundance of the micro-organisms in the "microbial loop" (Azam et al. 1983).

In studies of microbial ecosystems, the biomass of flagellates is generally estimated by microscopy. After staining with fluorescent stains, the abundance and biovolumes of a representative subsample of microflagellates are calculated and a total biovolume is determined. Fixation is important in the process of microscopic measurement because it can have an effect on the apparent volume of heterotrophic flagellate cells (Borsheim & Bratbak 1987, Choi & Stoecker 1989). Cell carbon density (C per unit biovolume, fg carbon μm^{-3}) is used to convert total biovolumes of the microbes to biomass which is, in general, expressed in units of mass of carbon per volume of water.

In a discussion of the measurement of phytoplankton biomass, Sicko-Goad et al. (1977) mention several inherent problems of biovolume estimates including the following: (i) potential inaccuracy in measuring and computing volumes of microscopic organisms, complicated by irregular shapes of cells, (ii) different amounts of metabolizing cytoplasm found within each cell in a species and between each species, and (iii) the physiological state of the cell which may affect its cytoplasm composition.

Some of the problems of estimating microbial biomass may be overcome with current technology. A cooled CCD (Charge Coupled Device) camera used with image-analyzed microscopy can significantly improve the quality of plankton images in the process of image analysis (Sieracki and Viles 1990) using the biovolume algorithm developed and implemented on a color image analysis system

(Sieracki et al. 1989). Small flagellates are a good model organism because they generally have simple shapes. Potential inaccuracy in measuring and computing volumes of microscopic, complicated cells (Problem #i), therefore, may be negligible. Problem #ii for small flagellates is also negligible because the percentage of empty volume within cells is very low i.e., less than 3% of the cell volume (Sicko-Goad et al. 1977). This study attempts to clarify Problem #iii. The reported direct measurements of C density of marine heterotrophic flagellates was made by Borsheim & Bratbak (1987). They concluded that the carbon density of a living flagellate similar to *Monas* sp. was 100 fg C μm^{-3}. For preserved cells measured by epifluorescence microscopy, they suggested a carbon density of 220 fg C μm^{-3} because the fixative caused shrinkage of biovolume.

The main goals of this study include: (i) to determine cell carbon and nitrogen densities of heterotrophic flagellates (*Paraphysomonas* sp.), (ii) to determine whether physiological state (i.e., growth stage) causes changes of cell carbon and nitrogen densities of these flagellates, (iii) to determine C:N ratio of *Paraphysomonas* sp. and whether there is any change of the ratio with physiological state. In this study it is hypothesized that cell carbon and nitrogen densities are positively correlated with growth rates and that C:N ratio is negatively correlated with growth rates. That is, growing cells have more carbon and lower C:N ratio than stationary growth cells.

MATERIALS AND METHODS

Experimental design.

To determine cell C or N density of heterotrophic flagellates (*Paraphysomonas* sp.), it was thought to be necessary to remove bacteria which serve as food in a flagellate culture. However, it was very difficult to separate bacterial cells from flagellate cells. Total C or N content of bacteria in a flagellate culture, therefore, was estimated from CHN analysis and image analysis for bacterial biovolume. These values were subtracted from total C or N contents of cells contained in a flagellate culture (bacterial and flagellate cells), which gave total C or N contents of the flagellates. It was necessary to culture bacteria only because cell C or N content (C or N per cell, fg $cell^{-1}$) and density of bacteria was also needed to calculate total C or N content of bacteria in a flagellate culture. Eight experiments (Samples #1 to #8 in Table 1) were carried out to determine the cell C or N content and density of bacteria in a bacterium culture having almost the same mean cell biovolume as that of bacteria in a flagellate culture. To complete this study, seven flagellate cultures (Samples I to VII in Table 2) were also analyzed. Two experiments (Sample #7 and #8) on bacteria only and five experiments (Samples III to VII) on heterotrophic flagellates were carried out at Bigelow Laboratory in Maine, while the others were done at Virginia Institute of Marine Science in Virginia.

Instruments for image analysis and CHN analysis.

Epifluorescence microscopy and visual counting are the primary methods for measuring the biomass of picoplankton (0.2 - 2.0 µm) and nanoplankton (2.0 - 20 µm) cells. However those methods need considerable operator effort and are prone to operator error and inconsistency. Recent advances in imaging instrumentation have made more available some cameras with significantly developed quantitative characteristics, such as CCD (Charge-coupled devices) camera. CCDs are light-sensitive silicon chips which accumulate an electrical charge linearly proportional to the quantum of photons striking them (Hiraoka et al. 1987). The CCDs are cooled from room temperature to between -20 and -40°C to reduce noise. The major advantages of this camera, the linearity, geometric stability, high brightness resolution, high efficiency and low noise, produce a sharp and clear cell edge in the image, in contrast to a standard video camera (Seiracki and Viles 1990). It is, therefore, possible to estimate biovolume of microbes more accurately by using a cooled CCD camera for imaging fluorescing plankton cells.

Viles & Sieracki (1992) described the general scheme of the CCD-based image analysis system. The system counts particles and makes a variety of measurements of each individual particle including biovolume. Biovolume is obtained by the method of Sieracki et al. (1989). Every microscope image was reviewed by the operator to delete any non-organismic particles and to account for connected multiple cells. Multiple cells were counted but not included for sizing. Total biovolumes (μm^3 ml^{-1}) were obtained by multiplying mean cell biovolume (μm^3 $cell^{-1}$) by the cell abundance (cells ml^{-1}).

A Carlo Erba Strumentazione Carbon/Nitrogen Analyzer 1500 was used for particulate carbon and nitrogen analysis of most samples. Diagram in Figure 1 shows the procedure of this analysis. The original analytic method is based on the complete and instantaneous oxidation of the sample by flash combustion. The Perkin-Elmer Model 240B elemental analyzer was used for measuring two samples (Samples #3 and #5) of bacteria and one flagellate sample (Sample II).

Cultures of *Paraphysomonas* sp. and bacteria only.

Aged Sargasso seawater and Gulf of Maine seawater were passed through 0.2 μm pore size Nuclepore membrane filters to prepare culture medium. The seawater (35 and 30 psu respectively) was diluted to about 25 psu with deionized water. Yeast extract was added (concentration: 0.01%, wt/vol) to provide a food source for heterotrophic bacteria which are the prey of flagellates. Marine heterotrophic flagellates (*Paraphysomonas* sp.) collected from the North Atlantic Ocean and grown in enrichment culture with bacteria were inoculated into 200 ml of new autoclaved media. Experiments I and II were performed in duplicate. Daily image analysis was carried out with the Culture 1 to determine flagellate abundance and biovolume. On that day when the first culture indicated logarithmic growth phase, C and N analyses were carried out with some of the Culture 1 (65 ml). The remainder of Culture 1 was retained for subsequent image analysis to determine stationary growth phase. On those days when the first culture indicated stationary growth phase, samples were taken from Culture 2 for image analysis. These results were combined with those of Culture 1 to draw a composite growth curve; cell number and biovolume for the stationary date were derived from Culture 2, for late logarithmic and the other dates from Culture 1 in Figure 6a and 6b. C and N analyses were performed with the rest of Culture 2. I missed logarithmic growth phase in Experiment II. Experiments III to VII were carried out in triplicate. On those dates when the first culture indicated logarithmic or stationary growth phase, Cultures 2 and 3, respectively, were used for C and N analyses. Just prior to this analyses samples were taken from these flasks for image analysis to determine cell numbers and biovolumes of flagellates. These results were combined with those of Culture 1 to draw a composite growth curve (Figure 6c - 6g), thus cell abundances and biovolumes for the late logarithmic dates were derived from Flask 2, for stationary dates from Flask 3 while the remainder of the data were derived from Flask 1. Cell number and biovolume for the second stationary date of Experiment III (Figure 6c) were derived from Flask 1. Duplicate or triplicate culture flasks were prepared at the same time with the same conditions (temperature, volume of inoculated sample, medium volume).

In order to culture bacteria without grazers, an old culture containing flagellates in addition to bacteria was filtered through a Whatman GF/A (1.6 μm) filter and the filtrate was transferred into new media (200 ml). The flasks of culture were incubated at room temperature on the desk in the laboratory or in an incubator (20°C) with the caps open to furnish O_2 for them. No flagellates were observed in the incubated cultures.

Cell counting and sizing by image analysis.

Samples were taken from flagellate cultures every day and image analysis was performed to determine the cell abundance and biovolume. The samples were preserved with glutaraldehyde solution of a final concentration of 0.3%. Fixative and stains were passed through 0.2 μm pore size Nuclepore membrane filters to remove unwanted materials contained in those solutions prior to their use. For image analysis of bacteria cells, 2 mls of preserved sample were sonicated for 10 seconds at 32 W to disperse the clumps of bacteria; bacteria began forming clumps when flagellates first appeared in the culture and such bright clumps of bacteria interfere with the analysis carried out with epifluorescence microscopy. For image analysis of flagellates, 2 mls of samples were stained with acridine orange solution (0.01%, wt/vol) and filtered through 0.2 μm black stained Nuclepore filters. Sonication was found to break up flagellate cells. The volume of sample taken was dependent on the cell abundance of the samples. Usually, 2 mls of sample were used for analysis of flagellates and 0.5 ml of sample were used for image analysis of bacteria. The filters were floated on distilled and prefiltered (0.2 μm) water in a small beaker for even filtration and the samples were diluted to three or four times (about ten times for the analysis of bacteria) the volume of the original samples with fresh culture medium containing no cells. This made cells disperse evenly on the filters. The isotonic culture medium was used for dilution to prevent the cells from disrupting. A vacuum pump was used for filtration. After making the slideglasses wet with breath, the filters were mounted on slides and covered with immersion oil and a coverglass. Two slides for each counting of flagellates and bacteria were made. The slide having the most even cell distribution was chosen for counts. One image analysis was done for each slide and the number of cells per sample analyzed ranged from 23 to 249 per slide.

For image analysis of the bacteria from bacteria culture, samples were taken and analyzed by image analysis after several days' incubation; Sample #1, #4, #5, #7 and #8 (Table 1) were cultured for three days and Sample #2 was incubated for five days, while the others were cultured for four days. The samples were not sonicated because bacteria alone did not form clumps during the incubation. The fixative and stains used for the flagellate sample were used for these samples. The sample (0.5 ml) was in general used for analysis of the bacteria. For even cell distribution, the samples were diluted to about ten times the volume of the original samples. The rest of the procedure was the same as that for the flagellate sample. The number of cells per sample analyzed ranged from 420 to 1179 per slide.

Counting was carried out with an image analyzed microscope connected with the cooled CCD camera system using either a 2 × (optovar) and 100 × oil immersion objective for counting bacteria cells or 1.25 × (optovar) and 63 × oil immersion objective for counting flagellate cells. The image calibration called "flat fielding" was needed before the image analysis of samples. For completing this calibration, a microscope field with no cells on it was found. This field was used for a representative of the background illumination. All cells or particles that were not of interest were deleted in the process of analysis. All cells that were poorly sized or were multiples (i. e., cells which are connected) were counted but not sized. A cell abundance (cells ml^{-1}), mean biovolume (μm^3 $cell^{-1}$), total biovolume (μm^3 ml^{-1}), and acridine orange fluorescence intensity (IOD) of bacteria and flagellates cells was obtained after this image analysis.

Cell carbon and nitrogen measurement.

1. Bacteria in a bacterium culture: The procedure for determining cell C or N content (fg $cell^{-1}$) and density (fg μm^{-3}) was shown in Figure 2. After three or four days of incubation, triplicate samples (65 ml) of the bacteria culture were filtered through precombusted (500°C for 5 hours) Whatman GF/F filters (0.7 µm). A vacuum pump was used for filtering the samples. Filters were wrapped

with aluminium foil which had been precombusted at 500°C for 5 hours and stored in a freezer until the experiment was done. After the experiment, the filters were dried completely in a desiccator over night because wet samples could decay. The desiccated filters were analyzed by the Virginia Institute of Marine Science nutrient center with a Carlo Erba Strumentazione Carbon/Nitrogen analyzer or with a Perkin-Elmer Model 240B elemental analyzer. Sample #3, #4 and #5 (Table 1) were analyzed by the Perkin-Elmer analyzer. Image analysis of the samples carried out before and after the GF/F filtration gave the abundance (cells ml^{-1}) of retained bacteria on the filters (Equation 1). Bacterial total C or N contents (μg ml^{-1}) were determined by CHN analysis. To correct for the dissolved organic matter on filters of enriched samples, blank filters were produced by refiltering the filtrate for CHN analysis. These blanks were subtracted from the C and N values of the samples; this method might capture particulates missed the first time, but very few cells passed through the GF/F filters in most case as shown in Table 1 and Table 2 and the cells would be missed again in the next filtration. Dividing the total C or N contents ($\mu g\ ml^{-1}$) by the retained bacteria abundance (cells ml^{-1}) yields cell C or N content of bacteria (fg $cell^{-1}$): Equation 2. Cell C or N density (fg μm^{-3}) of the bacteria is determined by dividing mean biovolume ($\mu m^3\ cell^{-1}$) into the cell C or N content (fg $cell^{-1}$): Equation 3.

$$N_B = N_{B1} - N_{B2} \qquad (1)$$

$$BC_{GFF} = C_B \div N_B \qquad (2)$$

$$BD_{GFF} = BC_{GFF} \div MV_B \qquad (3)$$

where, N_B : abundance (cells ml^{-1}) of GF/F retained bacteria

N_{B1} : abundance (cells ml^{-1}) of bacteria before filtering with GF/F

N_{B2} : abundance (cells ml^{-1}) of bacteria after filtering with GF/F

BC_{GFF} : cell C or N content (fg cell^{-1}) of GF/F retained bacteria

C_B : total C or N (μg ml^{-1}) of bacteria retained on GF/F

BD_{GFF} : cell C or N density (fg μm^{-3}) of GFF retained bacteria

MV_B : mean biovolume (μm^3 cell^{-1}) of the bacteria

Eight experiments were carried out to determine the cell C or N content (fg cell^{-1}) and density (fg μm^{-3}) of bacteria from a bacterium culture having almost the same mean cell biovolume as bacteria in a culture containing *Paraphysomonas* sp. as well as bacteria. C:N mass ratio of the bacteria from bacteria culture was determined from the bacterial total C contents and N contents (μg ml^{-1}).

2. Heterotrophic flagellates (*Paraphysomonas* sp.): Growth of microbes such as flagellates and bacteria is considered to represent cell division (nominal increase of cells) for a specific time instead of increase of biomass per cell. The rate of increase in cell abundance is exponentially proportional to the present population number. The growth rate of flagellates and bacteria is, therefore, an exponential function (Equation 4).

$$\text{Growth rate} = \ln (C \div C_o) \times t^{-1} \qquad (4)$$

where, C = abundance of cells after time t elapses

C_o = initial abundance of cells

t = specific incubation interval (h^{-1})

Based on daily analysis of flagellate abundance and biovolume, the growth phase of the flagellates was determined and a separate culture under identical conditions was sampled for CHN analysis. The cultures were filtered through precombusted Whatman GF/F filters (0.7 μm). From image analysis of samples before and after Whatman GF/F filtration, total biovolume (μm^3 ml^{-1}) of flagellates (V_f in Figure 3) and concentrations (cells ml^{-1}) of bacteria retained on the filters (N_b) were known: Equation 5. All flagellates cells were retained on the filters. As explained in Equation 6.1, total C or N content (μg ml^{-1}) of the bacteria (C_b) in the flagellate culture was determined by multiplying the abundance (cells ml^{-1}) of remaining bacteria (N_b) by cell C or N content per cell (BC_{GFF}) of bacteria samples (Table 1) having almost the same mean biovolume as that of bacteria grown in the culture to incubate grazers i.e., heterotrophic flagellates (*Paraphysomonas* sp.): I will call it the first method and use a "^{1st}" symbol to indicate results based on this method later on. As described in Equation 6.2, total C or N content (μg ml^{-1}) of the bacteria (C_b) in the flagellate culture was also determined by multiplying total biovolume (μm^3 ml^{-1}) of retained bacteria (V_b) by cell C or N density (fg μm^{-3}) of bacteria (BD_{GFF}): this is called the second method and the results based on this calculation are designated by a "^{2nd}" symbol. It is assumed that bacteria in bacteria culture and in the flagellate culture contain the same amount of carbon and nitrogen when they have the same mean biovolume, that is, the carbon and nitrogen density are the same. Subtracting calculated biomass (total C or N content, μg ml^{-1}) of retained bacteria (C_b) from total biomass (μg ml^{-1}) of retained flagellates and bacteria cells (C_{f+b}) measured by CHN analyzers (Carlo Erba Strumentazione) gave total C or N contents (μg ml^{-1}) of the flagellates (C_f) of interest only: Equation 7. Finally, the cell C or N density (fg μm^{-3}) of the heterotrophic flagellates was determined by dividing biomass (total C or N contents, μg ml^{-1}) of *Paraphysomonas* sp. (C_f) by total biovolume

(μm^3 ml^{-1}) of the flagellates (V_f); Equation 8. C:N mass ratio of *Paraphysomonas* sp. was determined from these total C or N contents.

$$N_b = N_{b1} - N_{b2} \quad (5)$$

$$C_b = N_b \times BC_{GFF} \quad (6.1)$$

$$C_b = V_b \times BD_{GFF} \quad (6.2)$$

$$C_f = C_{f+b} - C_b \quad (7)$$

$$D_f = C_f \div V_f \quad (8)$$

where, N_b : abundance (cells ml^{-1}) of GF/F retained bacteria

N_{b1} : abundance (cells ml^{-1}) of bacteria before filtering with GF/F

N_{b2} : abundance (cells ml^{-1}) of bacteria after filtering with GF/F

C_b : total C or N (μg ml^{-1}) of bacteria retained on GF/F

BC_{GFF} : cell C or N content (fg $cell^{-1}$) of GF/F retained bacteria determined from bacterium cultures.

V_b : total biovolume (μm^3 ml^{-1}) of retained bacteria

BD_{GFF} : cell C or N density (fg μm^{-3}) of GFF retained bacteria determined from bacterium cultures

C_f : total C or N (μg ml^{-1}) of *Paraphysomonas* sp. retained on GF/F

C_{f+b} : total C or N contents (μg ml^{-1}) of *Paraphysomonas* sp. and bacteria retained on GF/F

D_f : cell C or N density (fg μm^{-3}) of *Paraphysomonas* sp.

V_f : total biovolume (μm^3 ml^{-1}) of *Paraphysomonas* sp.

Statistical analysis.

Six C or N densities and C:N ratios of *Paraphysomonas* sp. at logarithmic and eight at stationary growth phase and the means in each physiological phase were obtained for performing statistical analysis. Unpaired *t* test statistics (one tailed) and one factor ANOVA analyses were used to test the significance of C or N density difference of the flagellates in the various physiological phases. The null hypothesis for *t* tests (one tailed) and ANOVA analyses was that cell C or N density and C:N ratio are the same in the logarithmic growth stage and in the stationary growth stage ($\mu_1 - \mu_2 = 0$ or $\mu_1 = \mu_2$). The alternative hypothesis for ANOVA analysis was that they differ from each other ($\mu_1 \neq \mu_2$). In the *t* tests (one tailed), the alternative hypothesis was that cell C or N density and C:N ratio in logarithmic growth stage (μ_1) are larger than the values in stationary growth stage(μ_2); $\mu_1 > \mu_2$, $\alpha = 0.05$.

RESULTS

Cell carbon content and cell nitrogen content of bacteria in bacterial culture.

Table 1 shows the results of experiments for incubation interval (day), bacterial abundance (cells ml^{-1}), mean biovolume (μm^3 $cell^{-1}$), cell carbon and nitrogen content (fg $cell^{-1}$), and cell carbon and nitrogen density (fg μm^{-3}) of bacteria. Total C and N contents of blank filters were averaged 5% of concentrations measured in CHN analysis for bacteria samples; the range was 1 to 18% for C and 1 to 12% for N. In general, samples measured by Perkin-Elmer Model 240B elemental analyzer showed high blank (11% and 8% of the total C and N contents respectively). The bacterial abundance of samples incubated for three days were usually higher (except Sample #8) than those of samples cultured for four days. The range of bacterial abundance was from 2.83×10^7 to 8.35×10^7 cells ml^{-1}. Mean biovolumes ranged from 0.109 to 0.570 μm^3. Mean cell volumes of Samples #1 through #6 were very small compared to mean volumes of Sample #7 and #8. The range of total carbon contents of culture was from 2.30 to 11.6 μg C ml^{-1}. Enriched culture of bacteria contained total nitrogen contents (μg N ml^{-1}) ranging from 0.665 to 3.34 μg N ml^{-1}. All cell carbon contents (fg C $cell^{-1}$) except that of Sample #3 were higher than 100 fg C $cell^{-1}$ but lower than 230 fg C $cell^{-1}$. Sample #6 showed the highest value, 226 fg C $cell^{-1}$, while Sample #3 showed the lowest value, 67 fg C $cell^{-1}$. Mean of cell C content was 161 fg C $cell^{-1}$, including Sample #3. Cell nitrogen contents ranged from 20 to 65 fg N cell^{-1} ($\overline{X} = 47$, $N = 8$). Cell carbon density and cell nitrogen density of five samples from Sample #1 to #6 (except Sample #2) were much higher than values of Sample #7 and #8 shown in Table 1. The highest values were 1390 fg C μm^{-3} for C density and 401 fg N μm^{-3} for N density, and the lowest values were 600 fg C μm^{-3} and 173 fg N μm^{-3}. Figure 4 shows cell C and N density and cell C and N content vs. cell size relationship. A typical biovolume distribution of acridine orange-stained bacteria is shown in Figure 5.

Growth curves and growth rates of *Paraphysomonas* sp.

Figure 6 shows the growth curves and biovolumes of flagellates for each experiment. Actual incubation time was not a day exactly as shown in Figure 6. I approximated the interval as a day for convenience in drawing curves. Exponential growth began after a lag of from 2 to 4 days, and continued for 2 to 4 days. In general, cell size was greatest during early growth and cells got smaller as the populations went into stationary phase, declining to between 20 and 40% of their volume during logarithmic growth. The representative size distributions of heterotrophic flagellates (*Paraphysomonas* sp.) is shown in Figure 7.

Table 2 shows the specific growth rate of the heterotrophic flagellates at different growth phase. Calculation of growth rates was based on a change of cell abundance over about 24 hours from a previous day to the time of interest. The specific growth rates at logarithmic growth phase were much higher than those at stationary growth phase (except that of Sample V). Averaged over all experiments, the mean specific growth rate was 1.3 d^{-1} at logarithmic growth phase and 0.3 d^{-1} at stationary growth phase.

Cell abundance and size, and carbon and nitrogen densities of *Paraphysomonas* sp.

Table 3 shows a part of the results of this study, bacterial abundance (cells ml^{-1}) before and after filtration, the difference (number of cells retained on GF/F filter) mean biovolume (μm^3 $cell^{-1}$), and total biovolumes (μm^3 ml^{-1}) of the heterotrophic flagellates and bacteria. Total C and N contents (μg ml^{-1}) of blank filters averaged 5% and 3% respectively of concentrations measured in CHN analysis; the range was 2 to 10% for C and 1 to 8% for N. Sample II measured by Perkin-Elmer Model 240B elemental analyzer showed relatively high blank (10% and 5% of the total C and N contents respectively). Bacterial concentrations ranged from 7.07×10^6 to 2.27×10^7 cells ml^{-1}, but most samples except #3 had concentrations higher than 1×10^7 cells ml^{-1}. The range of bacterial mean volume was 0.116 to 0.674 μm^3 $cell^{-1}$ and bacterial mean biovolumes of Sample I and II were ca. 1/4 compared to the other samples. The average of mean biovolume of bacteria of Sample I and II was 0.132 μm^3 $cell^{-1}$, whereas the average of bacterial mean biovolume of the Samples III through VII was 0.451 μm^3 $cell^{-1}$. The abundance of flagellates ranged from 5.14×10^4 to 3.78×10^5 cells ml^{-1} and the mean biovolume of flagellates ranged from 12.4 to 56.0 μm^3 $cell^{-1}$. There was no difference between the average cell biovolume of *Paraphysomonas* sp. of Sample I and II (16.0 μm^3 $cell^{-1}$) and that of the other samples (22.0 μm^3 $cell^{-1}$) as was seen in the mean bacterial biovolumes.

Cell C and N densities of *Paraphysomonas* sp. flagellates (μg ml^{-1}) are shown in Table 4. The bacterial C and N contents of Sample #6 (226 fg C $cell^{-1}$, 65 fg N $cell^{-1}$ in Table 1) were used in calculating bacterial total C and N contents of Sample I and II. To calculate total C and N contents of Sample III.2, III3 and

VII.2, cell C and N content (224 fg C cell^{-1} and 65 fg N cell^{-1}) of Sample #7 in Table 1 was used and cell C and N contents (198 fg C cell^{-3} and 60 fg N cell^{-1}) of Sample #8 was used for the other samples. Cell C and N content of Sample #3 was not used, considering total C and contents (2.30 µg C ml^{-1}) of bacteria was not enough for the Model 240B Elemental analyzer to carry out the analysis accurately. The result of Sample #6 was chosen instead of Sample #1, #2 and #4 because this result was from the most recent experiment. Cell C and N densities^{1st} (fg µm^{-3}) of *Paraphysomonas* sp. ranged from 159 to 769 fg C µm^{-3} and from 19 to 145 fg N µm^{-3} respectively. The average of cell C and N densities^{1st} of *Paraphysomonas* sp. was 420 fg C µm^{-3} (SE 61) and 80.2 fg N µm^{-3} (SE 14.8) respectively. Sample V.2 (stationary) showed total C and N contents (µg ml^{-1}) of the flagellates below zero and were not included in calculating mean values because those numbers were below zero.

The ranges of cell C and N densities^{2nd} were from 86 to 765 fg C µm^{-3} and from 23 to 140 fg N µm^{-3}. Sample V.2 showed negative numbers for cell C and N densities and Sample III.2 and III.3 showed densities below zero for cell N density of flagellates. The mean values of cell C and N densities^{2nd} of *Paraphysomonas* sp. were 441 fg C µm^{-3} (SE 62) and 97.1 fg N µm^{-3} (SE 11.7).

Carbon : Nitrogen ratio of *Paraphysomonas* sp. and the bacteria of bacterial culture.

Table 5 shows C:N mass ratio of the flagellates and bacteria of interest in this study. C:N mass ratio^{1st} of *Paraphysomonas* sp. in logarithmic growth phase ranged from 4.80 to 6.45 and C:N mass ratio^{1st} in stationary growth phase ranged from 2.36 to 11.1. The mean of all C:N mass ratios^{1st} of *Paraphysomonas* sp.

regardless of the growth stage was 5.97 (SE 0.74). C:N ratio of Sample V.2 was not included for computing the mean because total C and N contents were below zero.

C:N mass ratio^{2nd} in logarithmic growth phase ranged from 4.81 to 6.50 and C:N ratio in stationary growth phase ranged from 2.53 to 13.7. The mean of C:N mass ratio^{2nd} of *Paraphysomonas* sp. was 5.90 (SE 0.88). Since the total N contents of Sample III.2, III.3 and V.2 were below zero, C:N ratios of those samples were excluded in computing the mean value.

The mean of C:N ratio of bacteria in bacteria cultures was 3.44 (SE 0.03) and they showed very little variation (SE 0.03) as shown in Table 6. C:N ratio of the bacteria ranged from 3.32 to 3.57. C:N ratios of bacteria was lower than any C:N ratios of *Paraphysomonas* sp. at logarithmic growth rate. C:N ratios of the bacteria were also lower than the mean of C:N ratios of the flagellates in stationary growth phase (6.59) for C:N ratio^{1st} and 6.65 for C:N ratio^{2nd} in Table 6 and redfield C:N ratio (i.e., 5.68).

Comparison for cell C and N densities and C:N ratios of *Paraphysomonas* sp. and the first and second method in the two different physiological states.

Table 6 shows the mean, standard error, P value, the smallest level of significance at which null hypothesis would be rejected, of F-test (ANOVA) and *t* test . The mean values of cell C and N densities^{1st} of *Paraphysomonas* sp. were 608 fg C μm^{-3} (SE 50) and 116 fg N μm^{-3} (SE 7.5) during logarithmic growth state, and 259 fg C μm^{-3} (SE 53) and 49.6 fg N μm^{-3} (SE 11.7) during stationary growth state. The mean values of cell C and N densities^{2nd} of the flagellates of interest in this study were 606 fg C μm^{-3} (SE 55) and 115 fg N μm^{-3} (SE 9.0) during logarithmic growth state, and 300 fg C μm^{-3} (SE 69) and 75 fg N μm^{-3} (SE 20.5) during stationary growth state. The means of cell C and N densities^{1st} and cell C and N densities^{2nd} in logarithmic and stationary growth phase were 607 and 277 fg C μm^{-3} for C, and 116 and 60 fg N μm^{-3} for N. There was no significant difference between C and N densities^{1st} and C and N densities^{2nd} in either logarithmic or stationary growth phase ($P = 0.9791$ and 0.6859 for C density, $P = 0.9552$ and 0.2707 for N density). The P values of ANOVA and t test for cell C and N densities^{1st} were very small (0.0006 and 0.0003 for C density, 0.0008 and 0.0004 for N density). These statistical results suggest that there is a significant difference between cell C and N densities of the heterotrophic flagellates (*Paraphysomonas* sp.) during logarithmic growth and stationary growth phase and that the former (μ_1) is larger than the latter (μ_2). P values of F test and t test used for cell C and N densities^{2nd} of *Paraphysomonas* sp. were higher (0.0060, 0.0028 for C density, 0.0899, 0.0449 for N density) than those of cell C and N densities^{1st} but the null hypothesis for ANOVA analysis is rejected in favor of the alternative hypothesis at significance level 0.05. The null hypothesis for t test is also rejected at level 0.01.

The mean of C:N ratios^{1st} of *Paraphysomonas* sp. in logarithmic growth stage and in stationary stage were 5.24 (SE 0.26) and 6.59 (SE 1.37). Mean C:N ratios of the flagellates at logarithmic growth rate were very close to redfield C:N ratio (5.68). The mean of C:N ratios^{1st} of flagellates at stationary growth rate was 6.59, which was higher than the redfield ratio; 5.68. Mean C:N ratios^{2nd} of *Paraphysomonas* sp.in the logarithmic and stationary growth stage were 5.26 (SE

0.26) and 7.37 (SE 1.90). The means of C:N ratios^{1st} and C:N ratios^{2nd} in logarithmic and stationary phase were 5.25 and 6.62. There was no significant difference between C:N ratios of flagellates^{1st} and C:N ratios^{2nd} of the flagellates in logarithmic and stationary growth phase (P = 0.9536 and 0.7449). P values of ANOVA and *t* test for C:N ratios^{1st} of *Paraphysomonas* sp. were relatively high (0.3916 for ANOVA and 0.1957 for *t* test), and then the null hypothesis can not be rejected at level 0.1; the sample data do not indicate that the C:N ratios^{1st} are significantly different from each other in logarithmic and stationary growth state. P values of statistical test for C:N ratios^{2nd} were also quite high (0.7563 for ANOVA and 0.3781 for *t* test) and the null hypothesis can not be rejected at level 0.1. The average of C:N ratios of bacteria enriched for 3 days and 4 days was 3.42 and 3.48 respectively and statistical results showed that they are not different significantly.

If the regression (Figure 4) of these data is used to choose C or N content and C or N density that were used in determining C or N density of flagellates, we can compare the results with C or N density of flagellates determined already (Table 6). Statistical results in Table 8 support the result in Table 6 that there is a significant difference of C density of flagellates between logarithmic growth phase and stationary growth phase at level 0.01.

Regressions of result data

Figure 8 shows significant linear relationships between cell C or N content (pg cell^{-1}) and mean biovolume at significance level 0.05 (F-test). C or N content increased with flagellate cell size. Growth rates of *Paraphysomonas* sp. also were significantly correlated with mean biovolume ($p = 0.0007$, $r^2 = 0.25$). Cell size was larger at higher growth rate. However, growth rates of *Paraphysomonas* sp. were not significantly correlated with C or N density ($r^2 < 0.15$), and C:N ratio ($r^2 < 0.18$) at significance level 0.05. Relationship between cell C or N density and mean biovolume or AO fluorescence intensity were not significant at α level 0.05 ($r^2 < 0.11$). Relationships of cell biovolume vs C:N ratio ($r^2 < 0.001$) and AO fluorescence intensity vs C:N ratio ($r^2 = 0.3$) were not significant at level 0.05. The elemental composition of cell C density (fg C μm^{-3}) and N density (fg N μm^{-3}) was significantly correlated:

$C = 4.63\ N + 48.57$ (1st method) $\quad p = 0.0001,\ r^2 = 0.79$

$C = 3.6\ N + 150.38$ (2nd method) $\quad p = 0.0001,\ r^2 = 0.58$

DISCUSSION

The cell C and N densities of *Paraphysomonas* sp., 607 and 116 fg μm^{-3} respectively, including results from two different methods at logarithmic growth phase are much higher than the densities, 220 and 46 respectively, found by Borsheim & Bratbak (1987) for preserved cells similar to *Monas* sp. which were stained with DAPI. The C and N densities of flagellates at stationary growth phase found by them are even lower than mean cell C and N densities, 277 and 60 fg μm^{-3} respectively, at stationary growth phase. Borsheim and Bratbak also reported C and N densities of 100 and 20 for live flagellate cells with a similar cell structure as *Monas* sp.: live cell volumes were measured with the Coulter Counter, and phase contrast microscope. However, in estimating a biomass of the flagellates from biovolumes of the protozoa by using cell C or N density (cell C or N conversion factor, fg μm^{-3}) in marine ecosystems, a fixative is needed because epifluorescence microscopy is the primary methods for measuring the biovolume. The C density they recommended for preserved cells stained with DAPI (220 fg C μm^{-3}) may not be appropriate to use because the calculation of the density was based on the mean live biovolume measured by Coulter Counter and corrected for observed shrinkage percentage of live cells (44%) preserved with the fixative they tested. They reported the Coulter Counter results did not always agree with microscopy. Particulate organic carbon (0.669 μg C ml^{-1}) and particulate organic nitrogen (0.150 μg C ml^{-1}) of an enrichment culture were very low, whereas total

C content and N content (mean: 4.64 for C and 1.35 for N) of batch cultures used in this study are enough for CHN analyzer to measure the contents accurately .

Fenchel (1982b) reported carbon density of heterotrophic flagellates at stationary growth phase as 180 fg C μm^{-3} (mean biovolume: 100 μm^3 $cell^{-1}$) for *Ochromonas* and 300 (mean biovolume: 25 μm^3 $cell^{-1}$) for *Pleuromonas jaculans*, fixed with formaldehyde (final concentration of 4%) and stained with acridine orange (0.01%) for epifluorescence microscopy. These numbers are similar to the C density of cells at stationary growth phase found in this study (277 fg C μm^{-3}) but are much lower than the density at logarithmic growth phase (607 fg C μm^{-3}). The C density (220 fg C μm^{-3}) found by Borsheim & Bratbak (1987) is similar to the C density (180 and 300 fg C μm^{-3}) of heterotrophic flagellates of stationary growth found by Fenchel (1982b), although the species in their studies were different from one another. My C density results for stationary growth phase heterotrophic flagellates are in substantial agreement with the published results of both Borsheim & Bratbak (1987) and of Fenchel (1982b). The present results, however, indicate that actively growing cells have a higher C and N density than stationary cells. To the extent that cells in nature are actively growing rather than senescent, using the literature values for C density from Fenchel (1982b) or Borsheim & Bratbak (1987) will underestimate the biomass by 50 - 60%, i.e., the actual biomass should be approximately twice that estimated.

Ultrastructural observations on heterotrophic flagellates of stationary cultures were done by Schuster et al. (1968) and Fenchel (1982c). In their studies, *Ochromonas* sp., which has a similar structure to that of *Paraphysomonas* sp., showed striking features such as an increase in lipid and an distinct leucosin vacuole after logarithmic growth phase. In addition, starvation immediately caused a decrease to 2 to 5% in respiratory rate (metabolic rate) and an autophagy of cell organelles, particularly mitochondria, which forms autophagous vacuoles. Therefore, a significant difference between C density of *Paraphysomonas* sp. of logarithmic growth and C density of cells of post growth in this study would support the observations on loss of organic material mentioned above.

Statistical results on C:N ratios (Table 6) showed a insignificant difference between logarithmic and stationary growth phase. The mean C:N ratio (5.97) is similar to 5.88 of stationary growth cells found by Goldman et al. (1987) despite the lack of statistical significance. I hypothesized that growing cells would have a lower C:N ratio than stationary growth cells because stationary phase cells have a lower respiration rate, greater lipid (hydrocarbon), smaller or fewer vacuoles containing nitrogen rich particles (Schuster et al. 1968, Fenchel 1982c), and an increase of regeneration efficiencies of nitrogen (Goldman et al. 1985) which leads to a relative increase of carbon within a cell. Fenchel (1982c) reported that growing cells (*Ochromonas* sp.) possess the vacuoles filled with food bacteria; I found the same things in the process of image analysis of my cultures. Rates of NH_4^+ excretion of growing cells (*Paraphysomonas imperforta*) were higher than those of stationary cells when growth of the prey was not nutrient-limited, but regeneration efficiencies of nitrogen i.e., (nitrogen ingestion rates $\times$ nitrogen excretion rates^{-1}) $\times$ 100, were highest in the stationary growth phase (Goldman et al. 1985), which may make C:N ratio increase in stationary phase.

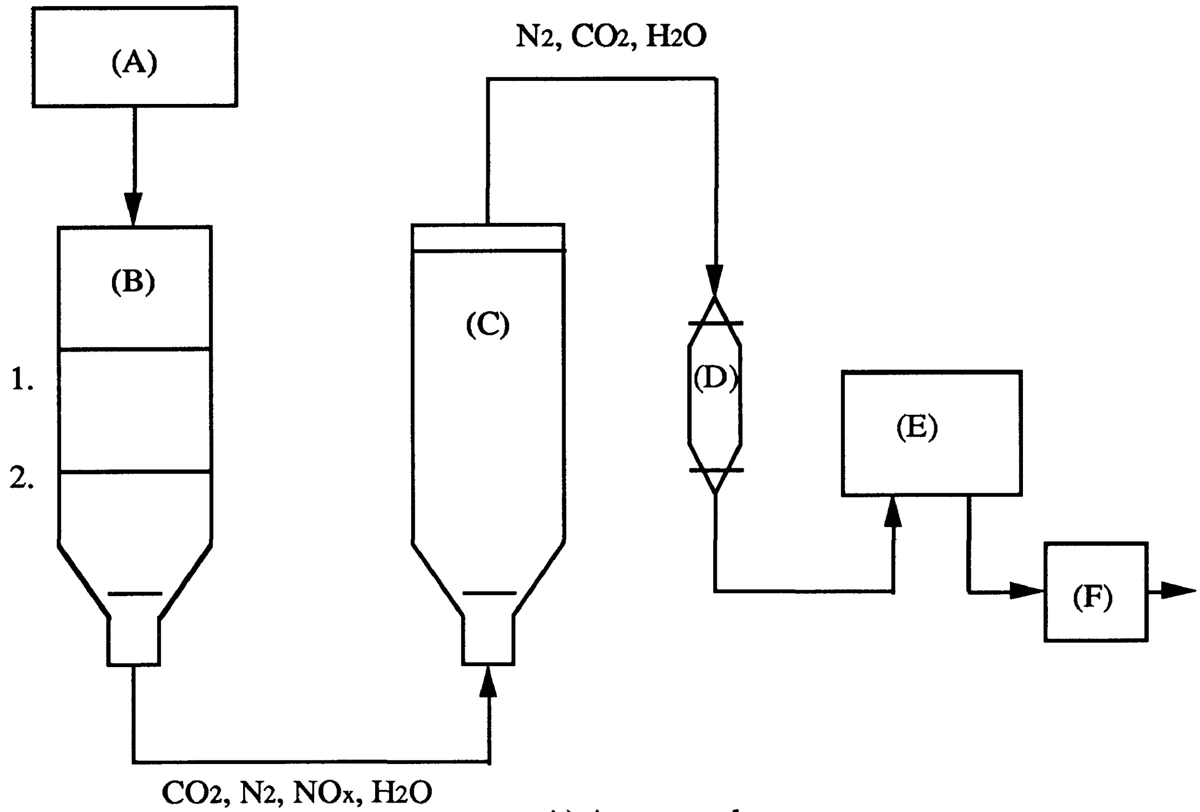
Table 7 shows a comparative compilation of bacterial cell C or N content and bacterial C or N density from the different literature sources. The mean of bacterial C and N densities (fg μm^{-3}) of this study, 841 and 244 respectively, are higher than the average densities of 560 and 104 respectively, reported by Bratbak (1985) and the average densities of 380 and 110 presented by Lee & Fuhrman (1987). However, the mean cell C and N densities (Sample #7 and #8) obtained from the experiments carried out in Maine was 413 and 122 which is lower than the density found by Bratbak (1985) and higher than the densities found by Lee & Fuhrman (1987). The mean of values selected for estimating the biomass of bacteria grown in the flagellate batches were 216 and 63 for cell C and N content and 740 and 215 for cell C and N densities. Most of the low cell C and N content and density (Table 7) were of natural bacteria not enriched with nutrients or bacteria grown in medium similar to the natural environment. Lee & Fuhrman (1987) reported that C and N content decreased with increased cell size for a very restricted range of cell sizes from 0.036 to 0.073 μm^3 $cell^{-1}$. My results (Figure 4a and 4b) and those of Simon and Azam (1989), from a broader range of cell sizes from 0.1 to 0.6, support the more reasonable view that bacterial C and N contents increase with increasing cell size. On the other hand, bacterial C and N densities decrease with increasing cell size (Figure 4c and 4d). With the anticipated increased usage of image analysis or flow cytometry to estimate microbial biomass in the future, it is reasonable to propose that a refinement of the relationship between cell size and C or N content can be used to estimate the C and N content of each individual cell as it is measured. This approach should improve the accuracy of microbial biomass determinations.

A C:N weight ratio of marine bacteria (3.44) found in this study was the same as that found by Heldal et al. (1985) with *Escherichia coli* , but lower than the 3.7 ratio reported by Lee & Fuhrman (1987) with marine bacteria and the 3.9

ratio found by Bratbak (1985) with *Pseudomonas putida* and mixed estuarine bacteria. The C:N ratio of bacteria was much lower than C:N ratio (5.97) of flagellates. These results suggest that bacteria are nitrogen-rich organisms compared to other plankton and support the idea that bacterivorous flagellates play a more important role in nitrogen regeneration than bacteria that may compete with primary producers for the dissolved inorganic nutrients (Wheeler & Kirchman 1986) to maintain a unique stoichiometry, i.e., low C:N ratio.

In Figure 6, I considered the samples as "logarithmic growth phase" when cells grew and had relatively large cell size and the samples as "stationary growth phase" when cells did not grow at all and had small cell size. The growth phase, characterized by rapid growth after 3 or 5 days of incubation, was considered as an early logarithmic growth phase and the growth phase following the early logarithmic growth phase as a late logarithmic growth phase. I could not measure cell biovolume of flagellates accurately at early logarithmic growth phase because bacterial cells stuck to flagellate cells and interfered with image analysis. Cell abundance of flagellates was also very low during early logarithmic growth phase, which would result in a very low total C of flagellates compared to total C of bacteria. My method (described in Materials & Methods) of determining C density of flagellates, therefore, would result in unacceptable variation. Samples VI and VII (Figure 6f and 6g) are characterized by a rapid decrease of biovolume to about a half of that of cells measured the previous day. Fenchel (1982c) reported that at the beginning of starvation, all studied species undergo one or two additional divisions. Therefore, the points (day 5 in both samples) may be considered as just starting points of post logarithmic phase growth. However, I regarded the points as logarithmic growth phase in considering the difference (Table 6) between C or N density of growing flagellate cells and that of starved cells in this study.

In conclusion, biomass of heterotrophic flagellates may be underestimated if the cell C or N density reported by some authors thus far is used without taking into account the growth stage because it was demonstrated that there is a significant difference of cell density in cells of different physiological states. It was reported that heterotrophic flagellates in nature had lower cell abundance within the range of 2×10^2 to 5×10^3 ml^{-1} but showed a similar growth curve to that of cells in culture (Andersen & Fenchel 1985), with cycle periods of 10-20 days (Fenchel 1982d, Sorokin 1977). I, therefore, suggest 430 and 90 fg μm^{-3} - average of C and N density^{1st} and C and N density^{2nd} - for growing cells similar to *Paraphysomonas* sp. and 280 and 60 fg μm $cell^{-1}$ for stationary growth cells in nature.



A) Auto sampler
B) Combustion Tube
  1. 120 mm Chromic Oxide
  2. 50 mm Cobaltous Cobaltic Oxide
C) Reduction Tube filled with Cuprin
D) Water Trap filled with Magnesium Perchlorate
E) GC Column Oven with 3M Porpack QS Packed Column
F) Thermal Conductivity Detector

Figure 1. Carrier gas flow diagram showing basic method of CHN analysis (From Carlo Erba Strumentazione Carbon/Nitrogen Analyzer 1500 instruction manual 1991).

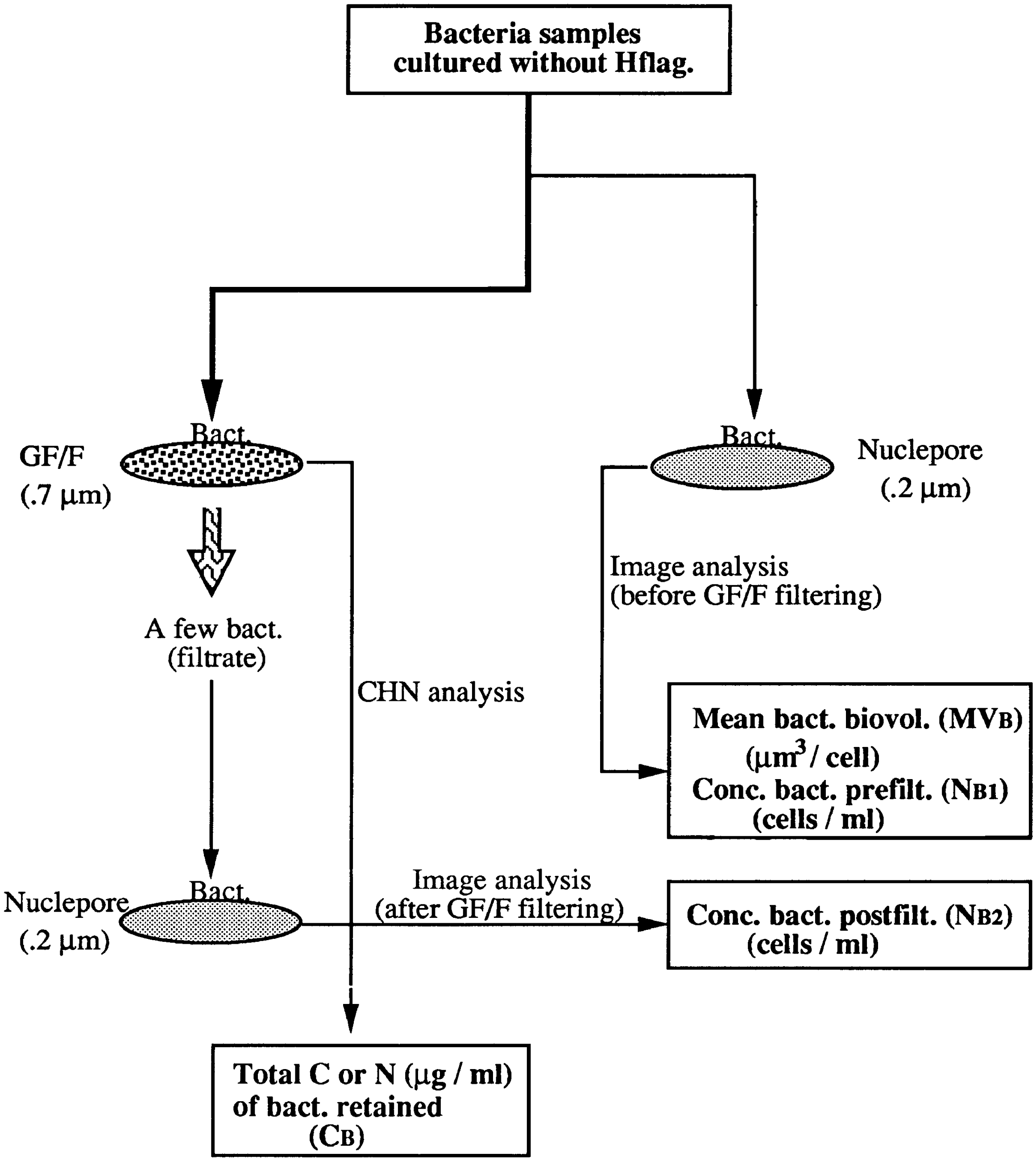


Figure 2. Flow diagram showing the procedure for determining cell C or N content (fg cell^{-1}) and density (fg µm^{-3}) of bacteria from a bacterial culture. A small fraction of bacteria prefiltered were passed through GF/F filter: Hflag. = heterotrophic flagellates, bact. = bacteria, biovol. = biovolume, conc. = concentration, prefilt. = prefiltered through GF/F, and postfilt. = postfiltered through GF/F.

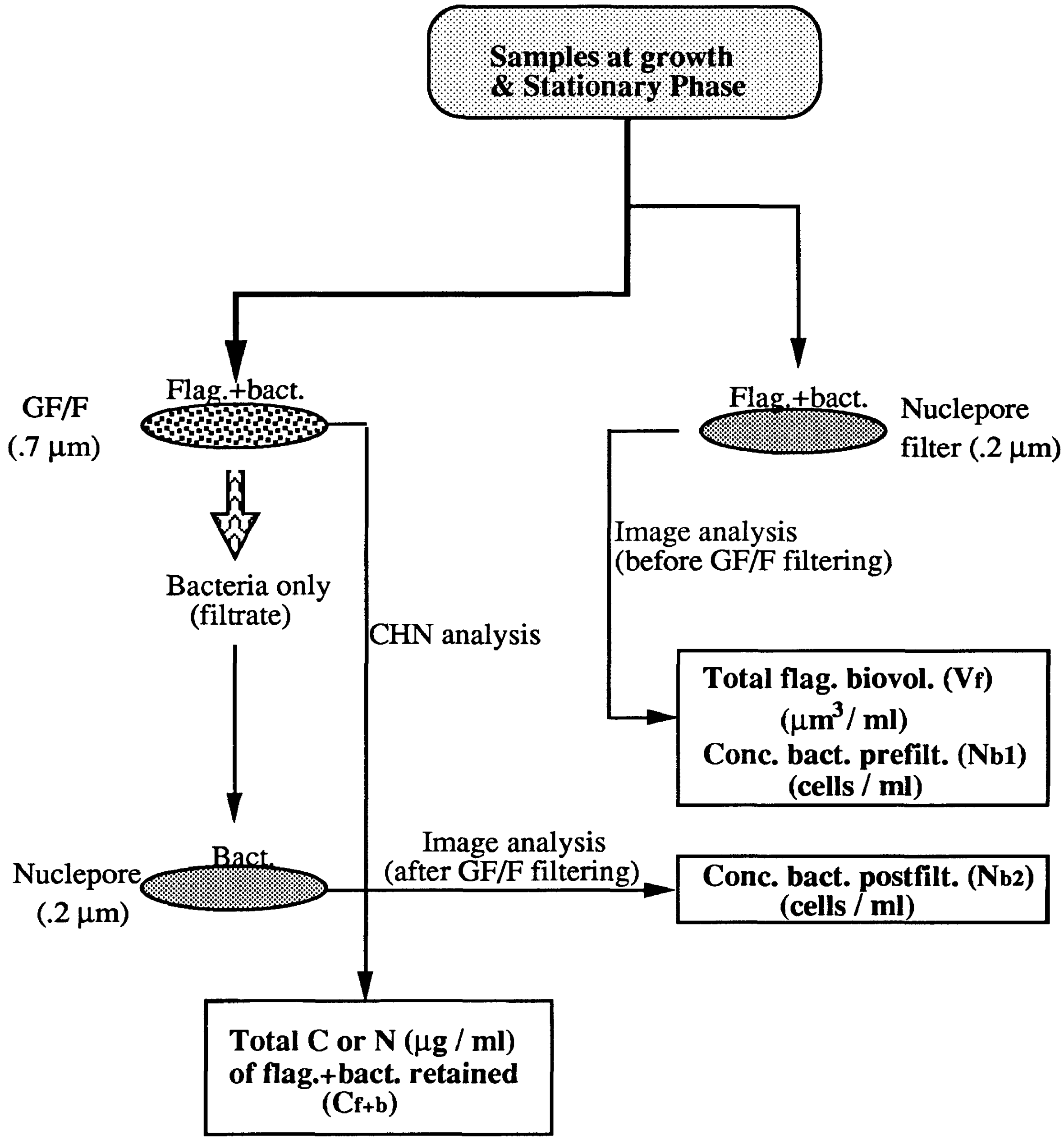


Figure 3. Flow diagram showing the procedure for determining of cell C or N density (fg μm^{-3}) of heterotrophic flagellates (*Paraphysomonas* sp.) in culture with bacteria. All flagellate cells remained on GF/F filter, while a small fraction of bacteria were passed through the GF/F filter: Flag.+bact. = flagellates and bacteria retained on GF/F filter, biovol. = biovolume, conc. = concentration, prefilt. = prefiltered through GF/F, postfilt. = postfiltered through GF/F.

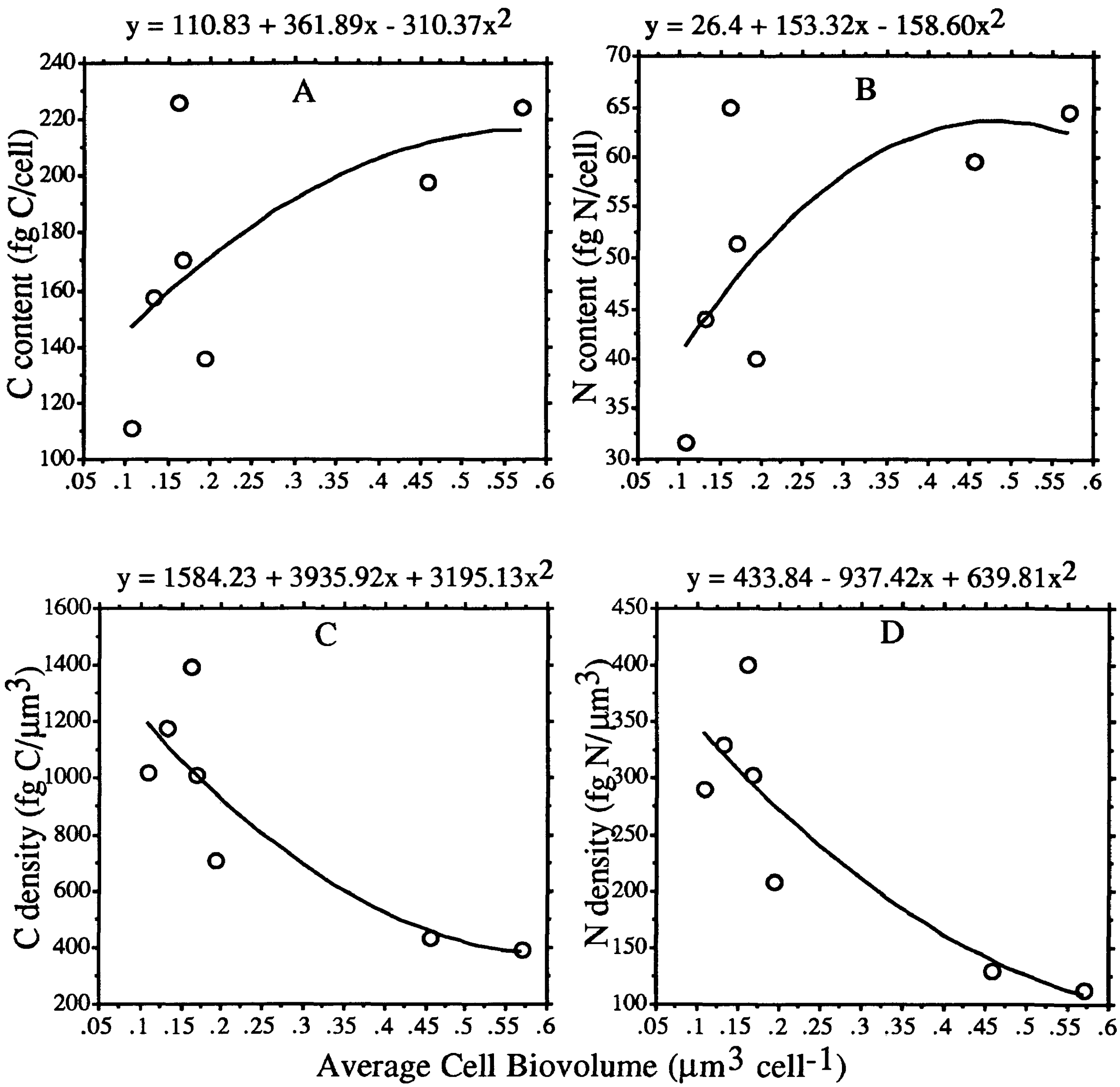


Figure 4. Polynomial regressions of bacterial C and N densities and bacterial C and N contents vs. average cell biovolume of bacteria.

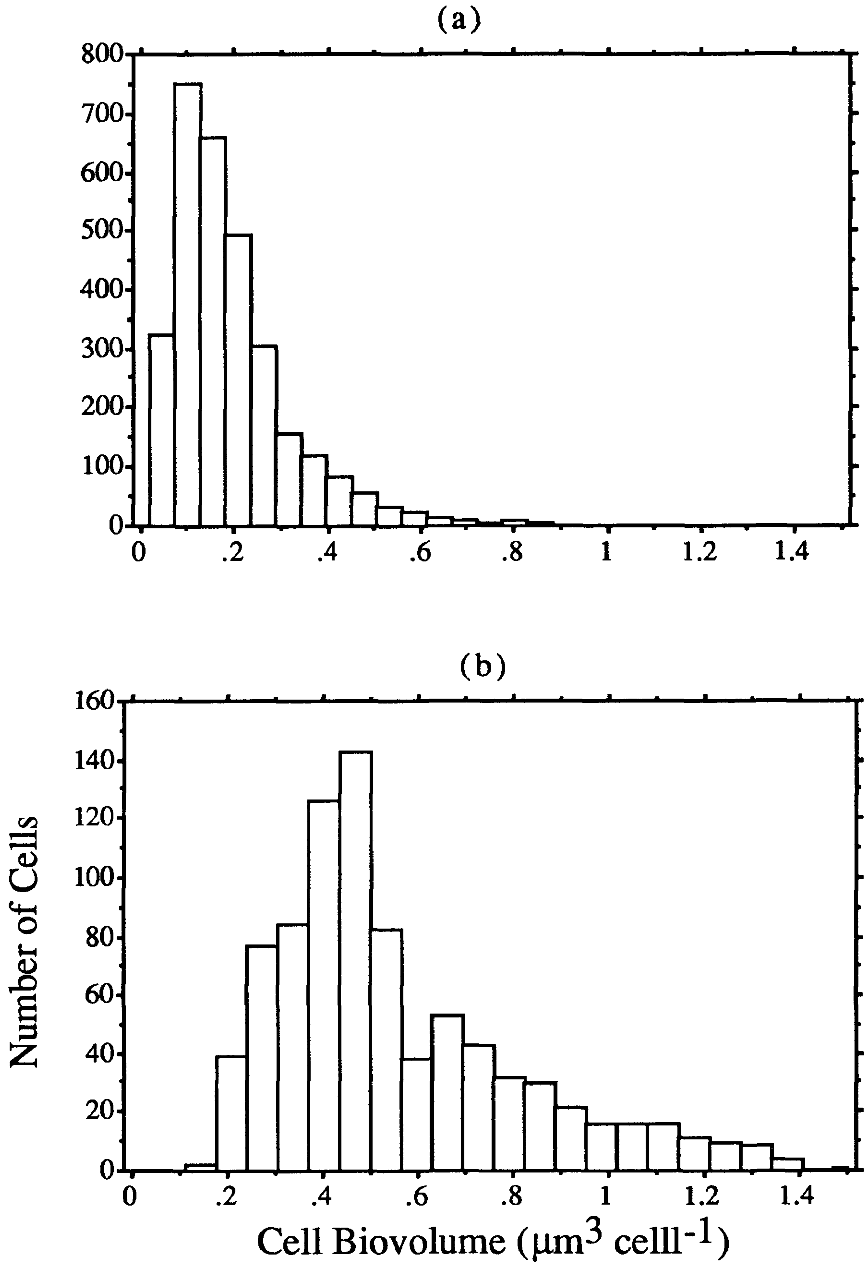
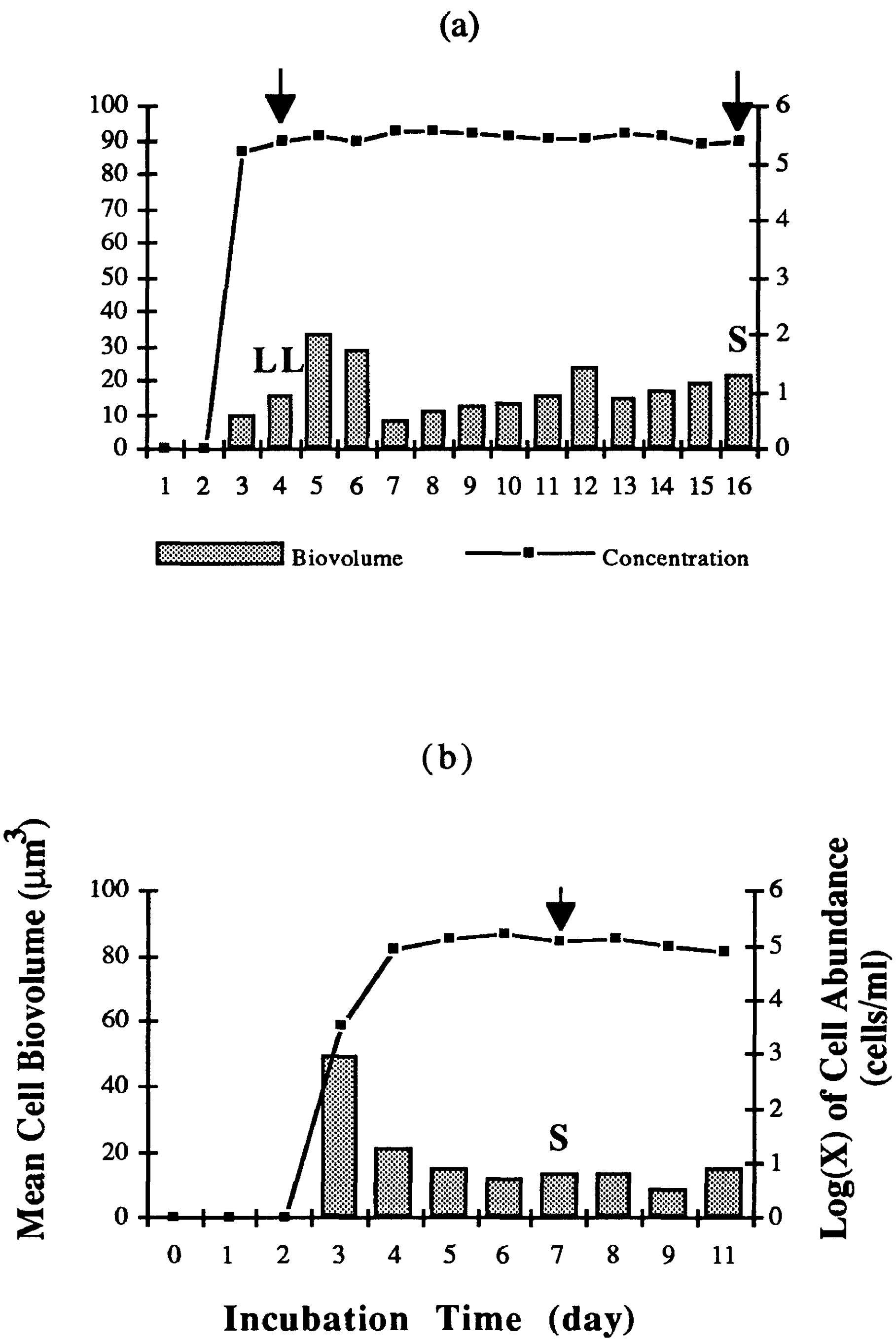
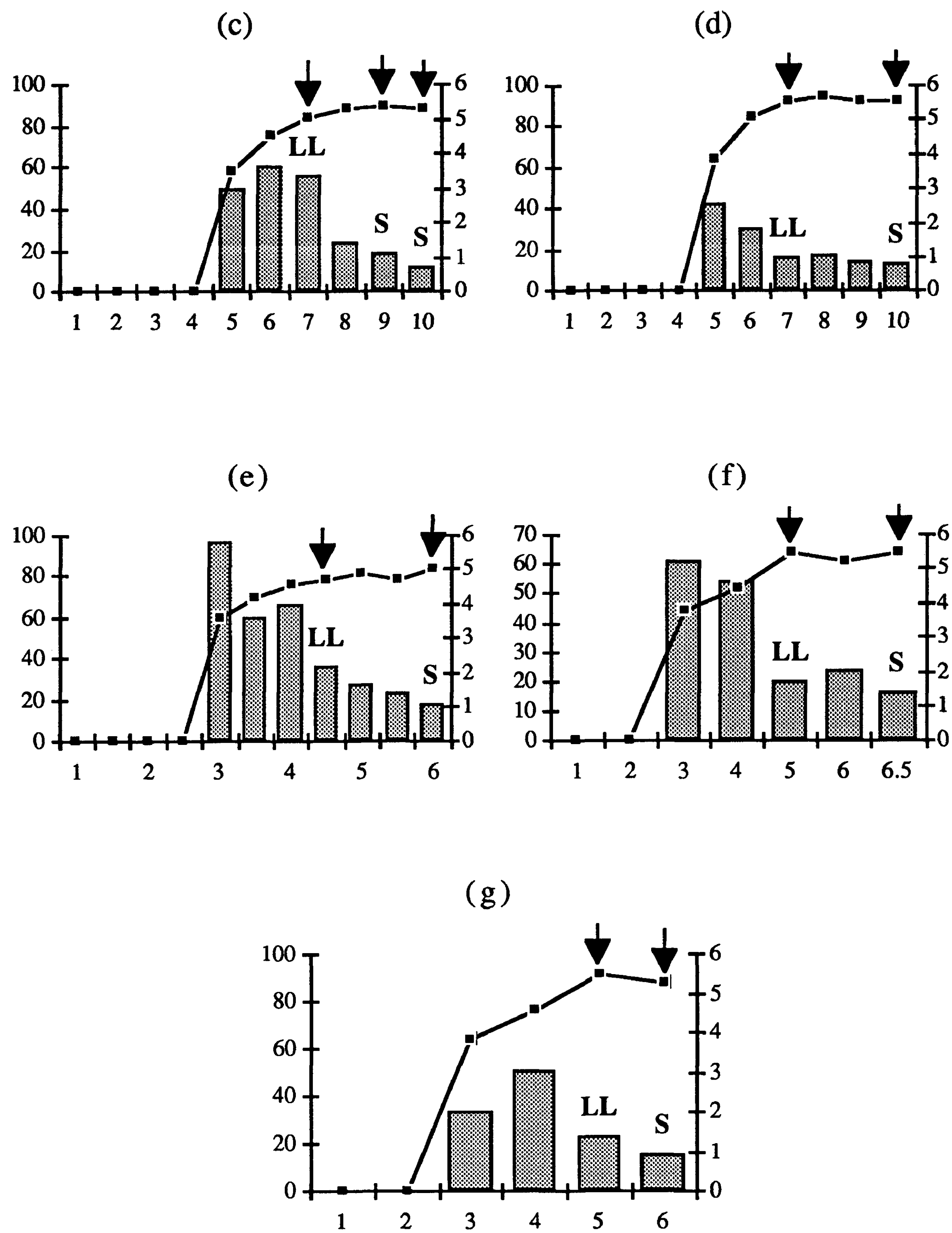


Figure 5. Typical biovolume distributions of acridine orange-stained heterotrophic bacteria with a low mean biovolume (0.2 μm^3 $cell^{-1}$; a) and a high biovolume (0.57 μm^3 $cell^{-1}$; b)

Figure 6. Growth curves and biovolumes of the heterotrophic flagellates (*Paraphysomonas* sp.) during the incubation. Growth rates of these flagellates were determined from these concentrations shown below. Arrows show when samples were taken, at late logarithmic (LL) growth phase and at stationary (S) growth phase (a; Sample I, b; Sample II, c; Sample III, d; Sample IV, e; Sample V, f; Sample VI, g; Sample VII).




(c)
100
80
60
40
20
0
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
LL
S
S
(d)
100
80
60
40
20
0
6
5
4
3
2
1
0
1 2 3 4 5 6 7 8 9 10
LL
S
(e)
100
80
60
40
20
0
6
5
4
3
2
1
0
1 2 3 4 5 6
LL
S
(f)
70
60
50
40
30
20
10
0
6
5
4
3
2
1
0
1 2 3 4 5 6 6.5
LL
S
(g)
100
80
60
40
20
0
6
5
4
3
2
1
0
1 2 3 4 5 6
LL
S

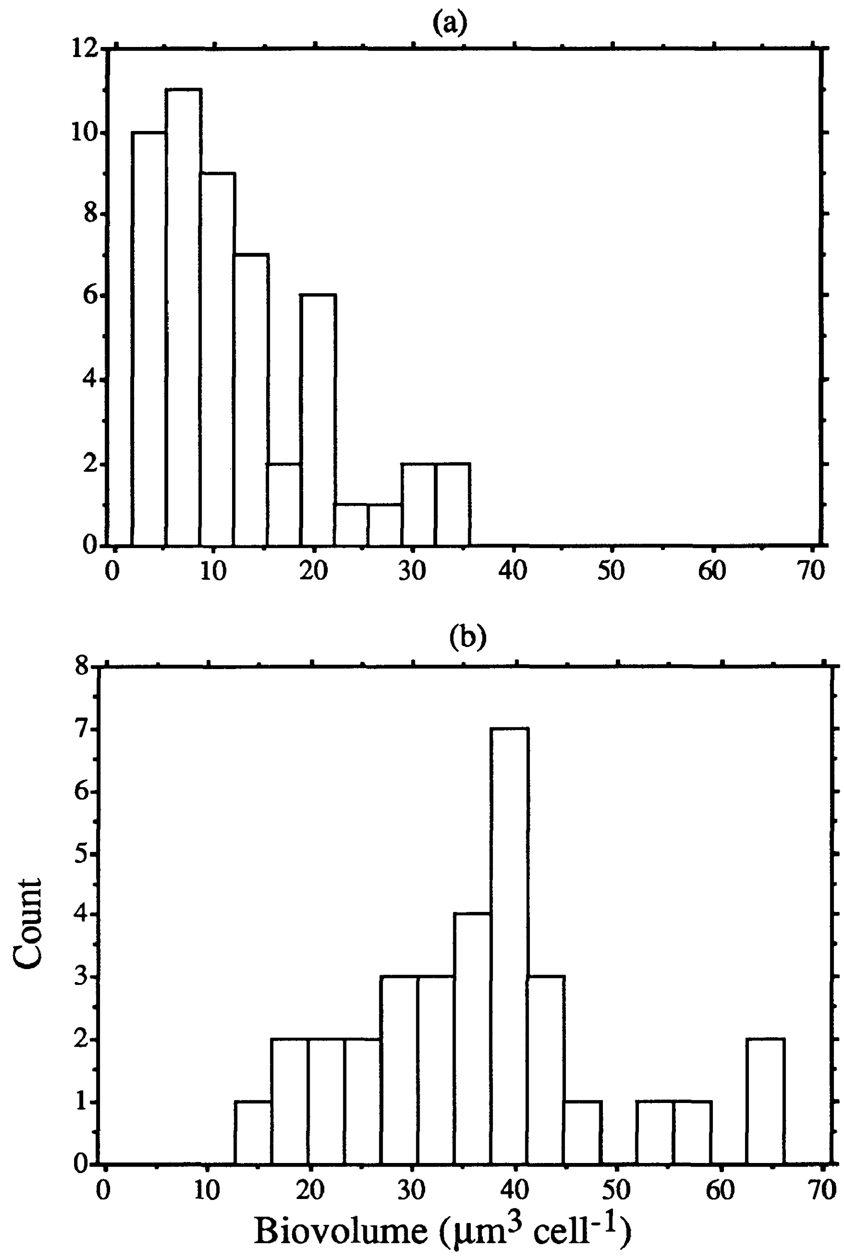


Figure 7. Typical biovolume distributions of acridine orange-stained heterotrophic flagellates (*Paraphysomonas* sp.) with the lowest mean biovolume (12.39 μm^3 $cell^{-1}$; a) and the high biovolume (35.86 μm^3 $cell^{-1}$; b).

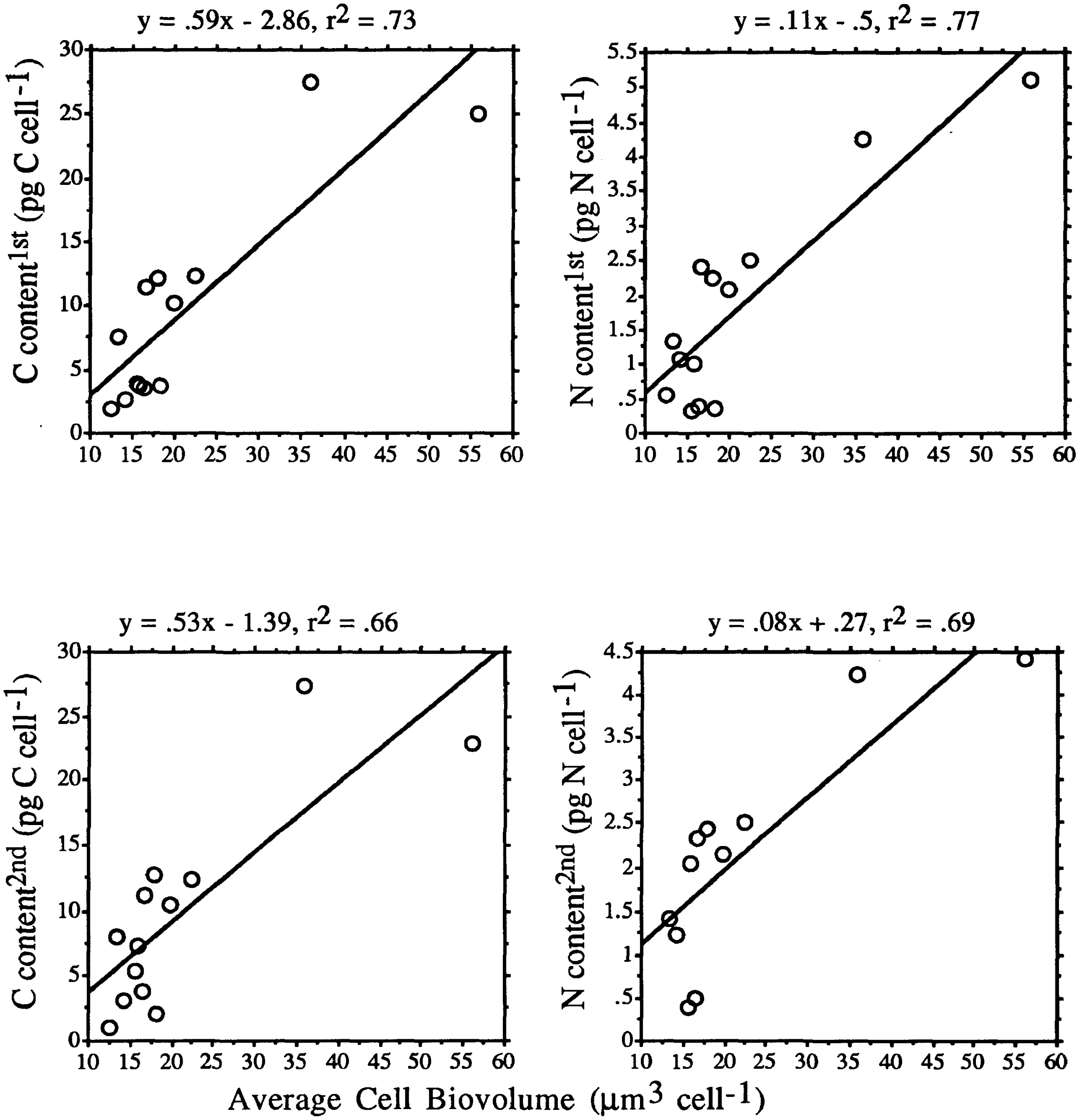


Figure 8. Regression of relationships between C and N per cell (cell C and N content) and mean biovolume of *Paraphysomonas* sp.

Table 1. Abundance, carbon and nitrogen content, mean biovolumes and cell carbon and nitrogen density of bacteria enriched from the bacteria culture containing no heterotrophic flagellates. Abundance and mean biovolumes were determined from image analysis and total C or N were estimated from CHN analysis. Cell C or N content of the bacteria were calculated by dividing the bacterial concentration into the total C or N contents. Dividing cell C or N content by mean biovolume yielded cell C or N density.

Sample #	Incubation Interval (day)	Bacterial Abundance			Mean Biovolume	Cell Content[d]		Cell Density[d]	
		Before filter[a] (cells ml^{-1})	After filter[b] (cells ml^{-1})	Difference[c] (cells ml^{-1})	(μm^3 $cell^{-1}$)	Carbon (fg C $cell^{-1}$)	Nitrogen (fg N $cell^{-1}$)	Carbon (fg C μm^{-3})	Nitrogen (fg N μm^{-3})
1	3	62193322	596249	61597073	0.169	**170**	**51**	**1009**	**304**
2	5	38450395	1418957	37031438	0.109	**111**	**32**	**1018**	**290**
3	4	34497244	252150	34245094	0.112	**67.2**	**19**	**600**	**173**
4	3	63724076	958257	62765819	0.133	**157**	**44**	**1179**	**331**
5	3	83515764	93795	83421969	0.193	**135**	**40**	**702**	**207**
6	4	51598442	189295	51409147	0.162	**226**	**65**	**1393**	**401**
7	3	46870890	51858	46819032	0.570	**224**	**65**	**393**	**113**
8	3	28251970	32670	28219300	0.458	**198**	**60**	**432**	**130**

[a] = Abundances of bacteria before passing through GF/F filters.
[b] = Abundances of bacteria after passing though GF/F filters.
[c] = Abundances of bacteria retained on GF/F filters after filtration.
[d] = Represent the mean of triplicate samples from each culture.

Table 2. Specific hourly growth rates, specific daily growth rates of the heterotrophic flagellates (*Paraphysomonas* sp.) from enrichment. " Logarithm-to-base e ($\log_e{}^x$)" was used in the calculation for specific growth rate.

Sample	Growth Phase	Specific Growth Rate (per hour)	(per day)
I	Late log	0.02	0.4
	Stationary	0.002	0.1
II	Stationary	-0.01	-0.3
III	Late log	0.03	0.8
	Stationary	0.004	0.1
	Stationary	-0.01	-0.2
IV	Late log	0.05	1.2
	Stationary	0.01	0.2
V	Late log	0.03	0.7
	Stationary	0.06	1.3
VI	Late log	0.10	2.4
	Stationary	0.08	2.0
VII	Late log	0.09	2.1
	Stationary	-0.02	-0.5

Table 3. Abundances and biovolumes (mean and total) of the bacteria grown in the flagellates culture and of the heterotrophic flagellates (*Paraphysomonas* sp.) feeding on those bacteria at each growth rate. These results were determined by image analysis.

Sample	Growth Phase	Bacterial Abundance (cells ml^{-1})			Bacterial Biovolume		*Paraphysomonas* sp.		
		Before Filter ($\times 10^7$)	After Filter ($\times 10^4$)	Difference ($\times 10^7$)	Mean (μm^3 $cell^{-1}$)	Total ($\times 10^6$ μm^3 ml^{-1})	Abundance ($\times 10^5$ cells ml^{-1})	Mean Biovol. (μm^3 $cell^{-1}$)	Total Biovol. ($\times 10^6$ μm^3 ml^{-1})
I	Late Log	0.707	49.0	0.658	0.145	0.954	2.51	17.92	4.50
	Stationary	1.41	7.25	1.40	0.116	1.63	2.51	15.83	3.97
II	Stationary	1.82	4.43	1.81	0.156	2.83	2.72	14.19	3.86
III	Late Log	2.27	14.0	2.25	0.484	10.9	1.09	55.94	6.11
	Stationary	0.808	2.33	0.806	0.674	5.44	2.29	18.27	4.19
	Stationary	0.689	4.56	0.685	0.635	4.35	1.94	12.39	2.41
IV	Late Log	1.22	4.44	1.22	0.473	5.76	3.31	16.62	5.49
	Stationary	1.28	7.05	1.28	0.439	5.60	3.78	13.44	5.07
V	Late Log	1.84	4.36	1.83	0.459	8.40	0.514	35.86	1.84
	Stationary	1.89	4.98	1.88	0.385	7.25	1.02	17.60	1.80
VI	Late Log	1.81	2.58	1.81	0.449	8.13	3.15	19.88	6.27
	Stationary	1.11	3.86	1.10	0.435	4.79	3.18	16.41	5.22
VII	Late Log	1.22	3.61	1.22	0.455	5.53	3.19	22.47	7.17
	Stationary	1.49	4.23	1.49	0.518	7.70	1.99	15.49	3.08

Table 4. Cell C and N densities of *Paraphysomonas* sp. at each different growth rate. Total C or N content of cells retained on GF/F filters (Flagellates & Bacteria) were measured by Carlo Erba Strumentazione Carbon/Nitrogen Analyzer 1500 of nutrient center at Virginia Institute of Marine Science. Total C or N content of bacteria were calculated by using cell C or N content of the bacteria grown in pure bacteria culture; the first method, and by using cell C or N density of bacteria; the second method.

Sample	Growth Phase	Cell density[1st]		Cell density[2nd]	
		Carbon (fg C μm^{-3})	Nitrogen (fg N μm^{-3})	Carbon (fg C μm^{-3})	Nitrogen (fg N μm^{-3})
I.1	Late Log	675	125	710	135
I.2	Stationary	235	64	461	129
II	Stationary	181	77	221	87
III.1	Late Log	449	91	407	79
III.2	Stationary	198	19	119	-35
III.3	Stationary	159	44	86	-47
IV.1	Late Log	690	145	676	140
IV.2	Stationary	571	99	592	105
V.1	Late Log	769	119	765	118
V.2	Stationary	-641	-273	-311	-174
VI.1	Late Log	518	104	530	107
VI.2	Stationary	213	24	234	30
VII.1	Late Log	546	111	548	112
VII.2	Stationary	253	21	351	26
		420 (61.4)[a]	80.2 (14.8)	441 (61.5)	97.1 (11.7)

[1st, 2nd] = Cell C or N density resulted from the calculation based on the first method (1st) and on the second method (2nd).

[a] = average (standard error) of cell C or N density without considering growth phase.

Table 5. C:N mass ratios of *Paraphysomonas* sp. from enriched culture and the bacteria enriched in pure bacteria culture. C:N mass ratios were determined from total C and N contents ($\mu g\ ml^{-1}$).

Sample	Growth Phase	C:N Ratio 1st	C:N Ratio 2nd
I	Late Log	5.41	5.26
	Stationary	3.72	3.66
II	Stationary	2.36	2.53
III	Late Log	4.89	5.17
	Stationary	10.38	-9.09
	Stationary	3.45	-1.86
IV	Late Log	4.80	4.81
	Stationary	5.80	5.64
V	Late Log	6.45	6.50
	Stationary	2.35*	1.79*
VI	Late Log	5.00	4.93
	Stationary	9.25	7.77
VII	Late Log	4.90	4.91
	Stationary	11.14	13.67
		5.97 (0.74)[a]	5.90 (0.88)
bact.#1	3 days[b]	3.32	-
bact.#2	5 days	3.51	-
bact.#3	4 days	3.46	-
bact.#4	3 days	3.57	-
bact.#5	3 days	3.39	-
bact.#6	4 days	3.47	-
bact.#7	3 days	3.48	-
bact.#8	3 days	3.32	-
		3.44 (0.032)[c]	-

* = invalid values because of the C and N contents below zero.

[a, c] = the average (standard error) of C:N ratios of flagellates (a) and bacteria in bacteria culture (c).

[b] = incubation time of bacteria grown in bacteria culture.

Table 6. Mean and standard error of cell C and N density and C:N ratio of *Paraphysomonas* sp. and statistical results on comparison of the densities and C:N Ratios at each growth phase. One factor ANOVA and unpaired *t* test (one tailed) were employed for the comparison.

***Paraphysomonas* sp.**		Growth Phase	Count	Mean	Std. Error[a]	Prob[b]	Prob[c]
Cell Density[1st]	C	Late Log	6	607.94	49.84		
		Stationary	7	258.64	53.4	**.0006**	**.0003**
	N	Late Log	6	115.88	7.51		
		Stationary	7	49.64	11.72	**.0008**	**.0008**
C:N Ratio[1st]	Mass	Late Log	6	5.24	0.26		
		Stationary	7	6.59	1.37	**.3916**	**.1957**
Cell Density[2nd]	C	Late Log	6	605.95	54.64		
		Stationary	7	300.02	68.86	**.0060**	**.0028**
	N	Late Log	6	115.2	8.99		
		Stationary	5	75.33	20.48	**.0899**	**.0449**
C:N Ratio[2nd]	Mass	Late Log	6	5.26	0.26		
		Stationary	5	6.65	1.97	**.4586**	**.3781**
Bacteria							
C:N Ratio	Mass	**	8	3.44	0.032		
		3 days	5	3.42	0.05		
		4 days[d]	3	3.48	0.02	**.3665**	**.1833**

[a]Std. Error = standard Error.
[b, c] Prob = P value (the smallest level of significance at which null hypothesis would be rejected) of ANOVA and *t* test respectively.
[d]4 days = included samples incubated for 4 or 5 days.
- = the same number as the one just above.
** = included all samples into the calculation irrespective of incubation time.

Table 7. Method of carbon measurement, species name studied, size range, cell C or N content, and C or N density of marine bacteria from different sources.

Carbon Measurement	Species	Size Range (μm^3 $cell^{-1}$)	C (or N) Content (fg $cell^{-1}$)	C (or N) Density (fg μm^{-3})	Reference
Wet Oxidation with Chromic-sulfuric acid	*Pseudomonas* sp. (2 species)	0.6	100	166	Fenchel (1982b)
CHN analysis of bacteria on GF/F filters	*Pseudomonas putida*	0.11-0.71	110 (20)[a]-310 (60)	240 (50)-930 (170)	Bratbak (1985)
CHN analysis of bacteria on GF/F filters	Mixed estuarine bacteria	0.08-0.34	30-80	310-730	Bjornsen (1986)
CHN analysis of bacteria on GF/F filters	Marine bacteria	0.036-0.073	15 (4)-25 (9)	210 (70)-570 (200)	Lee & Fuhrman (1987)
HPLC[b] of hydrolyzed amino acids	Marine bacteria	0.026-0.400	10.0-50.0	130-400	Simon & Azam (1989)
CHN analysis of bacteria on GF/F filters	Marine bacteria	0.109-0.570	110 (30)-230 (70)	400 (110)-1400 (400)	This study (1992)

[a] = C and N content [C (N)].

[b] = High Performance Liquid Chromatography.

Table 8. Mean, standard error of cell C or N density and C:N ratio of *Paraphysomonas* sp. determined from predicted values of bacterial C or N content and C or N density and statistical results on comparison of the densities and C:N ratios at each growth phase. The unpaired *t* test (one tailed) was employed for the comparison.

***Paraphysomonas* sp.**		Growth Stage	Count	Mean	Std. Error	t Value	P
Cell Density[1st]	C	Late Log	6	577	56		
(fg μm^{-3})		Stationary	7	347	60.7	2.74	**.0096**
	N	Late Log	6	109	12.4		
		Stationary	6	74.4	28.4	1.12	**.1454**
C:N Ratio[1st]	Mass	Late Log	6	5.39	0.41		
		Stationary	6	15.3	8.84	-1.12	**.1443**
	Mole	Late Log	6	6.29	0.48		
		Stationary	6	17.9	10.3		
Cell Density[2nd]	C	Late Log	6	593	56		
(fg μm^{-3})		Stationary	7	336	72	2.76	**.0093**
	N	Late Log	6	108	12.7		
		Stationary	6	73.3	29.3	1.07	**.1538**
C:N Ratio[2nd]	Mass	Late Log	6	5.69	0.57		
		Stationary	6	12.2	5.27	-1.23	**.1226**
	Mole	Late Log	6	6.64	0.66		
		Stationary	6	14.3	6.14	-	-

LITERATURE CITED

Andersen, O. K., Goldman, J. C., Caron, D. A., and Dennett, M. R. (1986) Nutrient cycling in a microflagellate food chain: III. Phosphorus dynamics. Mar. Ecol. Prog. Ser. 31:47-55

Azam, F., Fenchel, T., Field, J. G., Gray, J. S., Meyer-Reil, L. A., and Thingstad, F. (1983) The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. 10:257-263

Barsdate, R. J., Fenchel, T., and Prentki, R. T. (1977) Phosphorus cycle of model ecosystems: significance for decomposer food chains and effect of bacterial grazers. Oikos. 25:239-251

Bjφrnsen, P. K. (1986) Automatic determination of bacterioplankton biomass by image analysis. Appl. Environ. Microbiol. 51:1199-1204

Bφrsheim, K. Y. and Bratbak, G. (1987) Cell volume to cell carbon conversion factors for a bacterivorous *Monas* sp. enriched from seawater. Mar. Ecol. Prog. Ser. 36:171-175

Bratbak, G. (1985) Bacterial biovolume and biomass estimation. Appl. Environ. Microbiol. 49:1488-1493

Campbell, L., and Carpenter, E. J. (1986) Estimating the grazing pressure of heterotrophic nanoplankton on *Synechococcus* sp. using the sea water dilution and selective inhibitor techniques. Mar. Ecol. Prog. Ser. 33:121-129

Caron, D. A. (1990) Growth of two species of bacterivorous nanoflagellates in batch and continuous culture, and implications for their planktonic existence. Mar. Microb. Food webs. 4:143-159.

Caron, D. A. (1991) Evolving role of protozoa in aquatic nutrient cycles. In: P. C. Ried, C. M. Turley, and P. H. Burkill. (ed.) Protozoa and Their Role in Marine Processes. NATO ASI, Springer-Verlag Berlin Heidelberg. G 25:387-415

Caron, D. A., Goldman, J. C., Andersen, O. K., and Dennett, M. R. (1985) Nutrient cycling in a microflagellate food chain: II. Population dynamics and carbon cycling. Mar. Ecol. Prog. Ser. 24:243-254

Ducklow, H. W., D. A. Purdie, P. J. Leb. Williams, and J. M. Davis (1986) Bacterioplankton: A sink for carbon in a coastal marine plankton community. Science 232:865-867

Fenchel, T. (1982a) Ecology of heterotrophic microflagellates. I. Some important forms and their functional morphology. Mar. Ecol. Prog. Ser. 8:211-223

Fenchel, T. (1982b) Ecology of heterotrophic microflagellates. II. Bioenergetics and growth. Mar. Ecol. Prog. Ser. 8:225-231

Fenchel, T. (1982c) Ecology of heterotrophic microflagellates. III. Adaptations to heterogeneous environments. Mar. Ecol. Prog. Ser. 9:25-33

Fenchel, T. (1982d) Ecology of heterotrophic microflagellates. IV. Quantitative occurrence and importance as bacterial consumers. Mar. Ecol. Prog. Ser. 9:35-42

Fenchel, T. (1986) The ecology of heterotrophic microflagellages. Adv. Microb. Ecol. 9:57-97

Goldman, J. C., and Caron, D. A. (1985) Experimental studies on an omnivorous microflagellate: Implications for grazing and nutrient regeneration in the marine microbial food chain. Deep-Sea Res. 32:899-915

Goldman, J. C., Caron, D. A., Andersen, O. K., and Dennett, M. R. (1985) Nutrient cycling in a microflagellate food chain: I. Nitrogen dynamics. Mar. Ecol. Prog. Ser. 24:231-242

Goldman, J. C., Caron, D. A., and Dennett, M. R. (1987) Nutrient cycling in a microflagellate food chain: IV. Phytoplankton-microflagellate interactions. Mar. Ecol. Prog. Ser. 38:75-87

Haas, L. W., and Webb, K. L. (1979) Nutritional mode of several non-pigmented microflagellates from the York River Estuary, Virginia. J. Exp. Mar. Biol. Ecol. 39:125-134

Heldal, M., S. Norland, and O. Tumyr. (1985) X-ray microanalytic method for measurement of dry matter and elemental content of individual bacteria. Appl. Environ. Microbiol. 50:1251-1257

Hiraoka, Y., J. W. Sedat, and D. A. Agard (1987) The use of a charge-coupled device for quantitative optical microscopy of biological structures. Science 238:36-41

Johnson, P. W., Xu, H., and Sieburth, J. McN. (1982) The utilization of chroococcoid cyanobacteria by marine protozooplankters but not by calanoid copepods. Ann. Inst. Oceanogr., Paris. 58(S):297-308

Laacke, M., Dahle, A. B., Eberlein, K., and Rein, K. (1983) A modelling approach to the interplay of carbohydrates, bacteria and non-pigmented flagellates in a controlled ecosystem experiment with *skeletonema costatum*. Mar. Ecol. Prog. Ser. 14:71-79

Lee, S., and J. A. Fuhrman. (1987) Relationships between biovolume and biomass of naturally derived marine bacterioplankton. Appl. Environ. Microbiol. 53:1298-1303

McManus, G. B., Fuhrman, J. A. (1988) Control of marine bacterioplankton populations: measurement and significance of grazing. Hydrobiologia 159:51-62

Parslow, J. S., Doucette, G.J., Taylor, F. J. R., and Harrison, P. J. (1986) Feeding by the zooflagellate *Pseudobodo* sp. on the picoplanktonic *prasinomonad Micromonas pusilla*. Mar. Ecol. Prog. Ser. 29:237-246

Pomeroy, L. R. (1974) The ocean's food web, a changing paradigm. Bioscience 24:499-504

Schuster, F. L., Hershenov, B., and Aaronson, S. (1968) Ultrastructural observations on aging of stationary cultures and feeding in *Ochromonas*. J. Protozool. 15(2):335-346

Sherr, B. F., and Sherr, E. B. (1983) Enumeration of heterotrophic microzooplankton by fluorescence microscopy. Estuar. Coast Shelf Sci. 16:1-7

Sherr, B. F., and Sherr, E. B. (1988) Role of microbes in pelagic food webs: A revised concept. Limnol. Oceanogr. 33(5):1225-1227

Sherr, E. B., Sherr, B. F., and Albright, L. J. (1987) Bacteria: Link or sink? Science 235:88-89

Sherr, B. F., Sherr, E. B., and Hopkinson. C. S. (1988) Trophic interactions within pelagic microbial communities: Indications of feedback regulation of carbon flow. Hydrobiologia 159:19-26

Sherr, E. B., Sherr, B. F., and Paffenhofer, G-A. (1986) Phagotrophic protozoa as food for metazoans: a "missing" trophic link in marine pelagic food webs?. Mar. Microb. Food Webs 1:61-80

Sicko-Goad, L., Stoermer, E. F. and Ladewski, B. G. (1977) A morphometric method for correcting phytoplankton cell volume estimates. Protoplasma 93:147-163

Sieracki, M. E., and C. L. Viles (1990) Color image-analyzed fluorescence microscopy: A new tool for marine microbial ecology. Oceanography 3:30-36

Sieracki, M. E., C. L. Viles and K. W. Webb (1989) Algorithm to estimate cell biovolume using image analyzed microscopy. Cytometry 10:551-557

Sieburth, J. McN. (1976) Bacterial substrates and productivity in marine ecosystems. Ann. Rev. Ecol. Syst. 7:259-285

Simon, M., and F. Azam. (1989) Protein content and protein synthesis rates of planktonic marine bacteria. Mar. Ecol. Progr. Ser. 51:201-213

Sorokin, Y. I. (1977) The heterotrophic phase of plankton succession in the Japan Sea. Mar. Biol. 41:107-117

Stoecker, D. K., and Evans, F. T. (1985) Effects of protozoan herbivory and carnivory in a microplankton food web. Mar. Ecol. Prog. Ser. 25:159-167

Viles, Charles L., and Michael E. Sieracki (1992) Measurement of marine picoplankton cell size by using a cooled, charge-coupled device camera with image-analyzed fluorescence microscopy. Appl. Environ. Microbiol. 58:584-592

Wheeler, P.A., Kirchman, D.L. (1986) Utilization of inorganic and organic nitrgen by bacteria in marine systems. Limnol. Oceanogr. 31:998-1009

VITA

YONGSIK SIN

Born in Korea, 25 December 1966. Earned B.S. in Oceanography from Chonnam National University in 1989. Entered masters program in College of William and Mary, School of Marine Science in 1990.