THE EFFECTS OF ALGAL DENSITY

ON GROWTH OF HETEROTROPHIC MICROFLAGELLATES

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Submitted to the Massachusetts Institute of Technology/
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Joint Program in Oceanography and Oceanographic Engineering
in Partial Fulfillment of
the Requirements for the Degree of
Master of Science

at the

Massachusetts Institute of Technology
and the
Wood Hole Oceanographic Institution

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Signature of Author _____
Joint Program in Oceanography and Oceanographic Engineering
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August, 1988

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Joint Committee for Biological Oceanography

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ABSTRACT

The major role of heterotrophic nanoflagellates in the ocean is generally thought to be as grazers of bacteria, but they may also play an important role as grazers of photoautotrphs. The goal of the present study was to understand the basic growth kinetics of nanoflagellates feeding herbivorously. This was done using batch cultures in quasi steady-state growth.

Growth (increase in biomass) can involve changes in both cell numbers and cell size. Because fixed samples were examined, it was necessary to quantify the effects of fixation on the cell volume of heterotrophic protozoa before proceeding with the growth studies. Fixation resulted in cell shrinkage, and the degree of shrinkage varied with heterotrophic protozoan species and with algal prey species. It was hypothesized that egestion of food particles upon fixation was a major cause of shrinkage.

The growth rates of two heterotrophic nanoflagellates were determined to be hyperbolic functions of algal prey densities over a range of prey sizes. However, the specific response of the two species varied. Paraphysomonas imperforata appeared to respond primarily to prey cell numbers, and Strain HM-2 (unidentified species) responded most to available prey biomass (expressed as carbon or nitrogen). Minimum prey biomass for growth of both species feeding herbivorously was within the ranges reported for similar species feeding bactivorously. The growth kinetics suggest that heterotrophic nanoflagellates are adapted to heterogeneous distribution of prey within their environment.

The result of this study strongly suggests that previous studies of heterotrophic nanoflagellates based on the examination of fixed samples may have severely underestimated the role of theses taxa as herbivores. Herbivory by heterotrophic nanoflagellates may be much more important than previously thought.

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INTRODUCTION

Our view of the open ocean has been dramatically changed in recent years due to finding that the major activity in the open ocean often occurs within the microbial food web, presumably consisting of organisms smaller than 20 µm (Azam et al. 1983, Pomeroy 1974, Sherr et al. 1986). These small organisms, once assigned to the role of decomposers, are now believed to be the main mover of energy and materials because of the their high metabolic rates. It has been reported that 70-100% of the total respiration of the plankton occurs in the <10 µm size fraction of organisms (Pomeroy & Johnson 1966, Williams 1981). Small, solitary forms passing through a 10-20 µm mesh screen account for most of the biomass of phototrophic organisms in the open ocean (Malone 1980). Recently, autotrophs smaller than 2 um have been shown to be responsible for a large share of the total primary production (Li et al. 1982, Iturriaga and Mitchell 1986, Waterbury et al. 1979). In addition, bacteria have been shown to utilize as much as 50% of the primary production, assuming a carbon conversion efficiency of 50% (Fuhrman and Azam, 1982).

The organisms responsible for the great majority of the recycled nutrients in oceans are organisms smaller than 10 µm (Glibert 1982, Harrison 1980). Evidence has accumulated which indicates that small protozoa, such as heterotrophic nanoflagellates and aloricate ciliates, are the major regenerators in this system (Goldman et al. 1985, Wheeler and Kirchman 1986). At the same time, protozoa are considered to be

a trophic link between picoplankton and macrozooplankton (Sherr et al. 1986). Thus, knowledge about heterotrophic protozoa is very crucial to our understanding of oceanic ecosystems.

Even though it is relatively well confirmed that heterotrophic nanoflagellates are a major consumer of bacteria, there is an increasing interest in the possibility that heterotrophic nanoflagellates may also have a major role as consumers of photoautotrophs in the sea. This is quite likely because photosynthetic picoplankton, specifically cyanobacteria and pico-sized eukaryotic algae, are ubiquitous and contribute much of the primary production (Li et al. 1982, Iturriaga and Mitchell 1986, Waterbury et al. 1979). To date, there has been no attempt to quantify herbivory by heterotrophic nanoflagellates over a range of prey densities. The major aim of this research was to provide basic growth kinetics for nanoflagellates feeding herbivorously.

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Chapter 1: EFFECTS OF FIXATION ON CELL VOLUME OF MARINE PLANKTONIC PROTOZOA

Abstract

The effects of fixation on the cell volume of marine heterotrophic nanoflaglates and marine planktonic ciliates were investigated. Decreases in cell volume depend on a combination of the protozoan taxa and on the particular fixative. For a particular fixative and protozoan species, degree of shrinkagee is independent of physiological state.

The volume of fixed cells is approximately 20-55% lower than the cell volume of live organisms. For the heterotrophic microflagellates, the fixatives ranked, in order of decreasing effect on cell volume, as glutaraldehyde, formaldehyde, acid Lugol's solution and modified van der Veer solution. With the oligotrichous ciliates and a tintinnid ciliate, formaldehyde caused less shrinkage than glutaraldehyde or acid Lugol's solution. With the aldehyde fixatives, the microflagellates shrunk more than did the ciliated protozoans. Differential effects of fixation on cell volume may result in an underestimation of the biomass of certain protozoan taxa in natural samples.

Introduction

Protozoans are major consumers of bacterioplankton (15, 32, 37) and phytoplankton (11, 12, 21) in marine planktonic ecosystems. In addition to their role as grazers, heterotrophic protozoa, rather than bacteria, may be the major nutrient remineralizers in the open ocean (18, 46). The microbial food web is considered to be an important source of metabolic

activity in the open ocean (31, 34, 47), and thus data on the abundance and activity of heterotrophic protozoa are crucial to our understanding of the flow of energy and material in the ocean.

Heterotrophic microflagellates and ciliates usually dominate the protozoan biomass; because they overlap in size with phytoplankton and some micrometazoans, they cannot be separated from these groups by size fractionation. Estimates of the biomass of heterotrophic protozoa are usually based on cell counts and microscopic measurements of cell size. Carbon is widely used as a currency of biomass in order to compare different groups of organisms and/or to calculate the efficiency of energy transfer between trophic levels, although biovolume (4) and wet weight (40) are sometimes also reported. Cell volume to cell carbon conversion factors for protozoa have been reported for a few species of flagellates (7, 14, 24) and ciliates (16, 42), but conversion factors derived for phytoplankton (12, 29, 43) have also been used to estimate the biomass of heterotrophic protozoa.

Fixation can cause cells to shrink (6, 17, 18) or increase in volume (23) and thus may affect cell volumes. Some fixatives also affect retention of ingested food particles (38) and this, in turn, affects cell volume estimates for protozoans. Volume estimates of heterotrophic protozoa, from which biomass was calculated, have been based on live cells (39), Lugol's-fixed cells (24), glutaraldehyde-fixed cells (11) and formaldehyde-fixed cells (1, 2, 3, 5, 22, 35, 36). Although

volume:carbon conversion factors may vary with fixatives and taxa, values from the literature are often applied without consideration of sources of variation. Variability in the % of shrinkage or swelling could result in the overestimation or underestimation of biomass from fixed samples. Therefore, data on the effects of fixation on cell volume will be useful in accurately estimating the biomass of protozoa from field samples, and in estimating secondary production from field and laboratory data. The objectives of the present study are: (1) to compare the effects of commonly used fixatives on cell volume of marine phagotrophic protozoa, including heterotrophic flagellates and ciliates, (2) to determine if protozoan taxa vary in their response to fixation, and (3) to determine if physiological state (i.e. growth rate) affects cell volume change due to fixation.

Material and methods

Culture of flagellates and ciliates: Two species of heterotrophic flagellates, a chrysomonad, Paraphysomonas imperforata (Strain HM-1) and a Bodo-like species (Strain HM-2), and three species of ciliates, a tintinnid, Favella sp. (Strain JunFan), and two oligotrichous ciliates, a strictly heterotrophic species, Strobilidium spiralis (Strain Stro), and a mixotrophic species, Strombidium acutum (Strain Gpgr) (27) were used. Paraphysomonas imperforata and HM-2 were fed Isochrysis galbana (Strain Iso). Favella sp. was fed Heterocapsa triquetra (Strain A984), Strombilidium spiralis and Strombidium

acutum were fed a mixture of Heterocapsa pygmaea (Strain Gymno), Isochrysis glabana (Strain Iso), and Chroomonas salina (Strain 3C). The algae were grown in f/2 medium (19) without silicic acid. The heterotrophic flagellates were inoculated into 500 ml of the algal cultures in 1 liter flasks, and kept in the dark at 20°C. Favella sp., Strombilidium spiralis and Strombidium acutum were grown at 15°C; the protocols are found elsewhere (27, 42).

Sampling and fixation: Protozoa were preserved with commonly used fixatives: Acid Lugol's solution (44), glutaraldehyde (final concentration 2%) (10), and formaldehyde (final concentration 1%) (44). Recent studies have indicated that van der Veer fixative (45) is useful in preventing egestion by heterotrophic flagellates (38). D. A. Caron (personal communication) has found that a modified van der Veer fixative, final concentration glutaraldehyde 2% and tannic acid 2%, is as effective as the original formulation in preventing egestion by heterotrophic flagellates. This modified van der Veer fixative was included in this study, although it has not been commonly used to preserve protozoa. Since different concentrations of glutaraldehyde have been used to preserve heterotrophic flagellates, the effects of 1%, 2%, and 3% of glutaraldehyde on Paraphysomonas imperforata were compared. Cell volumes of fixed protozoa were compared to cell volumes of live organisms. Nickel sulfate (25) and Polyox (41) were used to reduce motility of protozoa in order to microscopically measure live cells. Working concentrations of slowing agents were determined for each

species of protozoa. Size measurements of live cells, in the presence and absence of slowing agents, were compared in order to detect the effects, if any, of these slowing agents on cell volume of live protozoa. Comparisons between live cells with and without nickel sulfate were made with a Coulter counter, but all other comparisons are based on microscopic measurements.

The growth stage of a protozoan can influence the cell volume in both heterotrophic flagellates (13) and ciliates (20), and may, in turn, affect response to fixation. Protozoans were grown in batch culture and samples collected during various growth phases characterized by different size cells. At each sampling, three 5 ml replicate samples were prepared for each treatment (fixed or live samples of heterotrophic flagellates). A final volume of 10-25 ml was prepared for ciliates, depending on cell density in the cultures, which varied from <5 to 50 cells/ml. For the ciliates, a batch culture was sacrificed at each sampling in order to obtain sufficient cells. Samples from batch cultures representative of different growth stages were then compared.

Size measurements and shrinkage calculation: Sizes were measured using light microscopy, with X1000 magnification for flagellates and with X400 magnification for ciliates. Dimensions of 20 to 30 cells from each replicate sample were measured with a calibrated ocular micrometer, for ciliates and flagellates respectively. Diameters of flagellates and oligotrichous ciliates were measured. The volume of the protoplast of Favella sp. was calculated assuming that the shape of protoplasm is

either a cone or a paraboloid, depending on cell shape. Shrinkage (%) was calculated as (fixative-treated diameter/live diameter)^3 x 100 except for Favella sp. for which it was calculated as (fixative-treated volume/live volume) X 100.

Results & Discussion

Neither Polyox nor $NiSO_4$ influenced the cell volume of Paraphysomonas imperforata (Table 1) and thus these two agents are suitable for use in the microscopical determination of live cell dimensions. The Coulter counter was used to compare the volumes of untreated and $NiSO_4$ treated cells of Paraphysomonas imperforata, but it was not possible to make similar comparisons with the other cultures of protozoa because the cultures were not dense enough for efficient use with the Coulter counter. With $NiSO_4$, the cells could be concentrated before microscopical examination, and thus it was possible to measure cells from samples with low protozoan densities. The working concentrations of $NiSO_4$ were 0.025%, 0.0025%, 0.07%, 0.002% (W/V) for HM-2, Strobilidium spiralis, Strombidium acutum, and Favella sp. respectively.

Samples were taken at 5 times during the growth of the heterotrophic microflagellate cultures. The average volumes of live cells varied with growth stage (Fig.1 and 2), and were significantly different between samplings (tested by 1-way ANOVA; $P < 0.01$). Under the same fixation conditions, shrinkage, in terms of % of live volume, was similar for cells from different growth phases (i.e. sizes) (Table 2). However, there

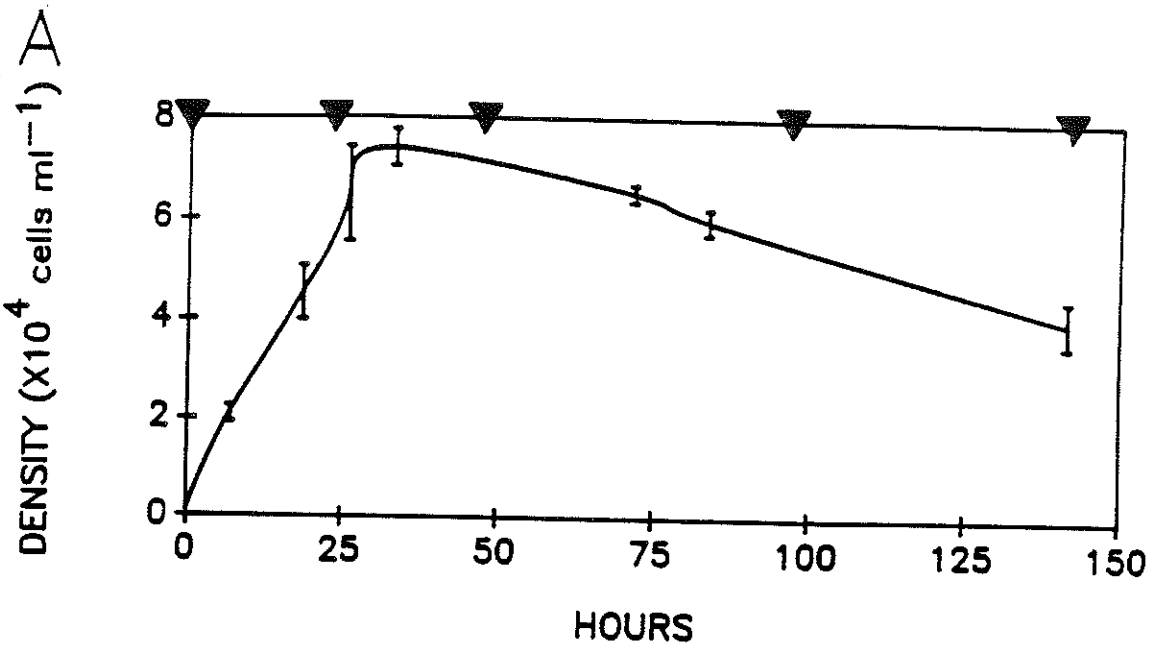
Table 1. Effects of slowing agents on live volume of Paraphysomonas imperforata. 1-way ANOVA tests were performed.

	Means ± S.E. (μm^3)	1-way ANOVA Test			
		Source of variation	df	Fs	P
Untreated	4656 ± 107	Treatment	1	<0.001	n.s.
vs.					
Nickel Sulfate	4639 ± 116	Error	12		
Nickel Sulfate	5760 ± 276	Treatment	1	0.116	n.s.
vs.					
Polyox	5680 ± 177	Error	12		

Table 2. Effects of live sizes on shrinkage of heterotrophic flagellates under each fixation: GT 2% = Glutaraldehyde 2% + Tannic acid 2%, LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 1%, 2%, 3% = Glutaraldehyde 1%, 2%, 3%. 1-way ANOVA tests were performed. Refer to Fig.-1 & 2 for data.

Flagellate species	Fixatives	Source of variation	df	Fs	P
Paraphysomonas imperforata	GT 2%	Sizes	4	0.2435	n.s.
		Error	10		
	LU	Sizes	4	1.9276	n.s.
		Error	10		
	GL 1%	Sizes	4	1.7704	n.s.
		Error	10		
	GL 2%	Sizes	4	3.8693	n.s.
		Error	10		
	GL 3%	Sizes	4	3.9152	n.s.
		Error	10		
HM-2	GT 2%	Sizes	4	4.0475	n.s.
		Error	10		
	LU	Sizes	4	2.8769	n.s.
		Error	10		
	FO 1%	Sizes	4	2.2878	n.s.
		Error	10		
	GL 2%	Sizes	4	3.1151	n.s.
		Error	10		

FIG. 1. A: Cell density change of Paraphysomonas imperforata after inoculation. Filled triangles indicate times at which samplings were taken for size measurements. B: % of live cell volume (average ± standard error) of P. imperforata with different fixatives at the times indicated in A. Average live volume for each sampling time is given below each set of bar graphs.


A
DENSITY (X10^4 cells ml^{-1})
8
6
4
2
0
0
25
50
75
100
125
150
HOURS

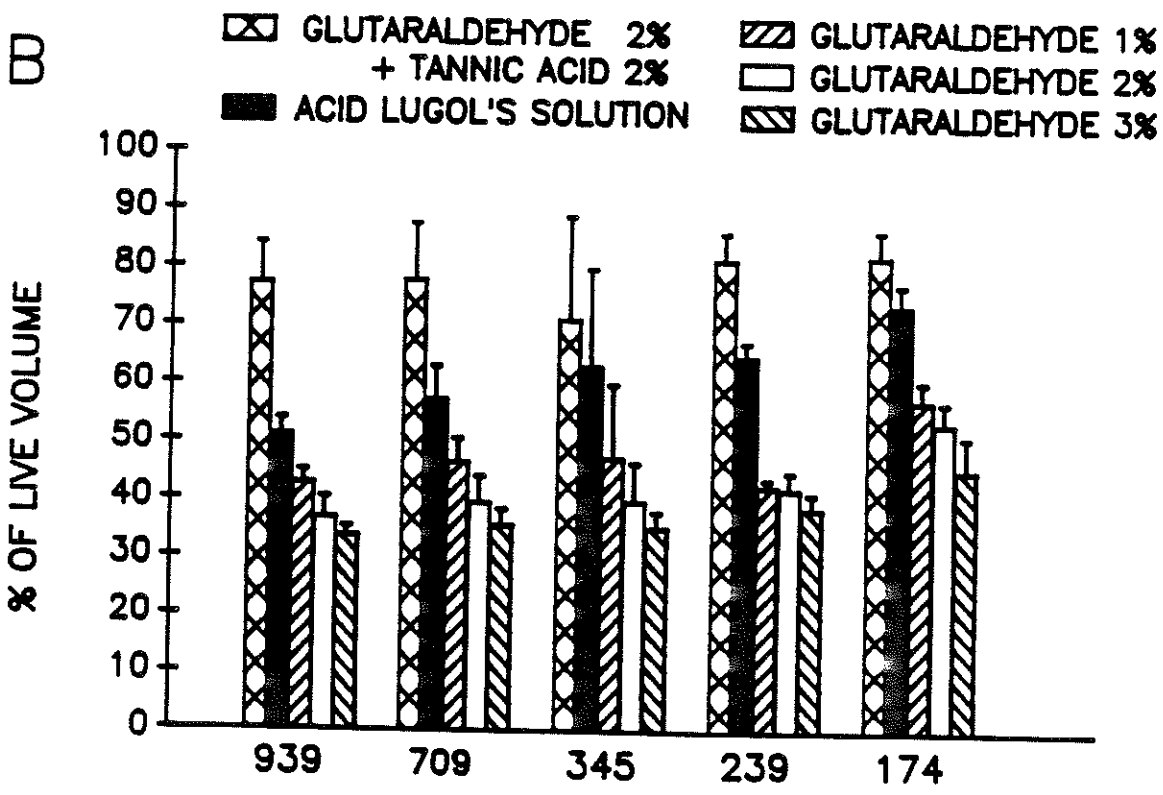
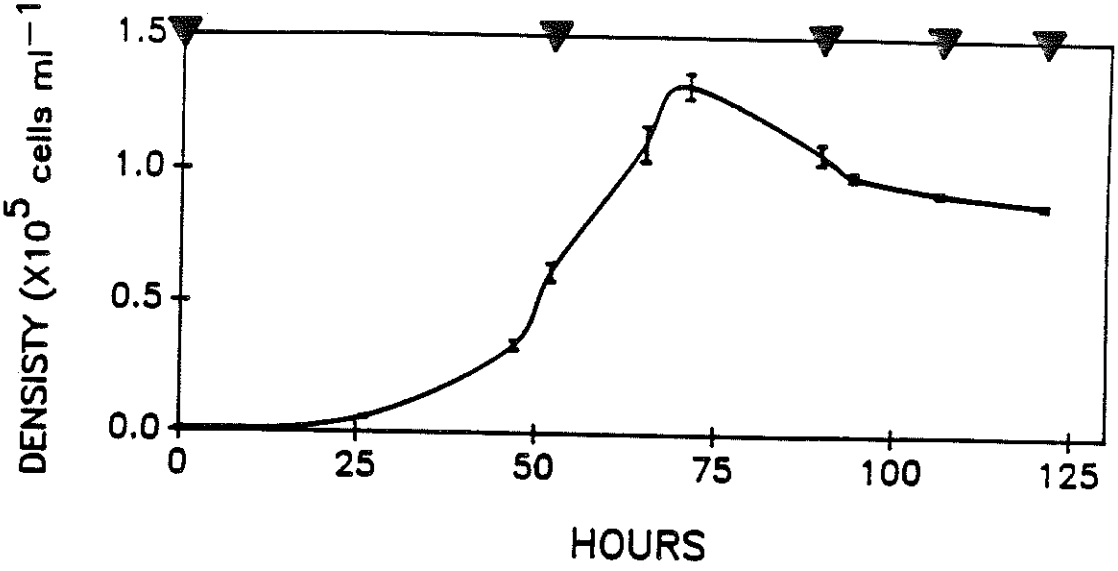
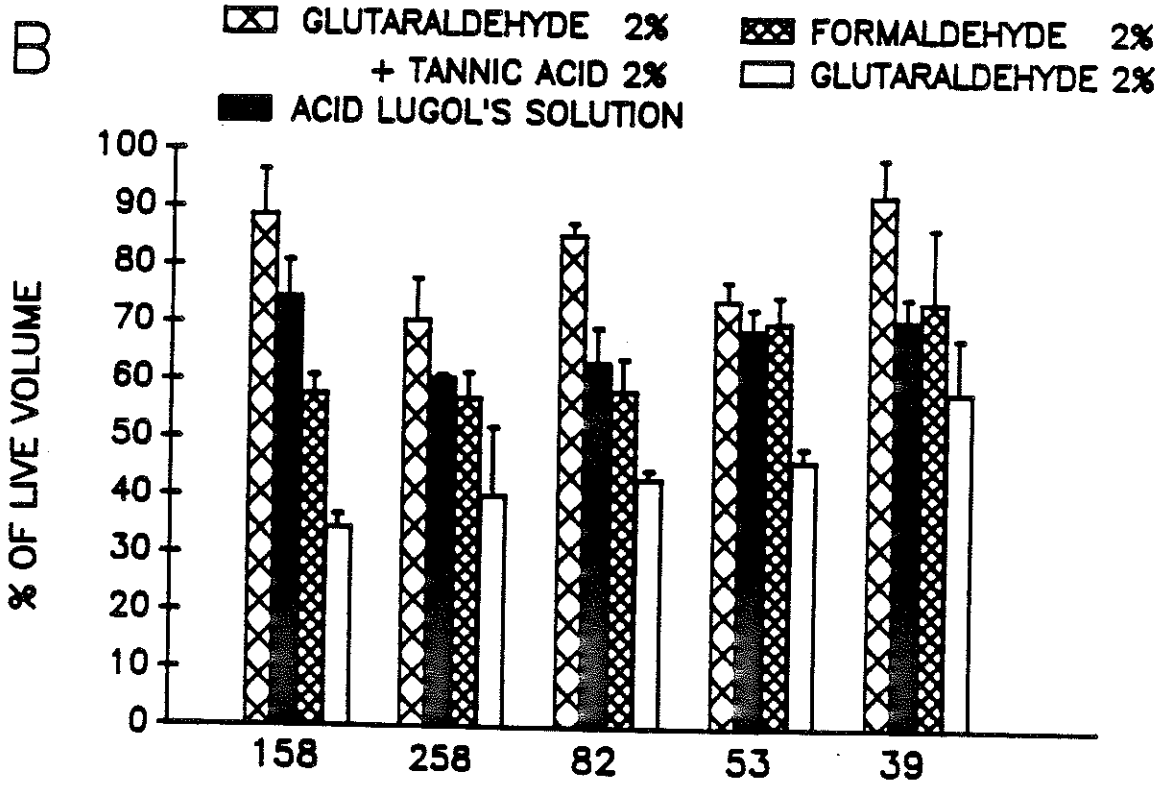

B
GLUTARALDEHYDE 2%
+ TANNIC ACID 2%
ACID LUGOL'S SOLUTION
GLUTARALDEHYDE 1%
GLUTARALDEHYDE 2%
GLUTARALDEHYDE 3%
% OF LIVE VOLUME
100
90
80
70
60
50
40
30
20
10
0
939
709
345
239
174

FIG. 2. A: Cell density change of HM-2 after inoculation. Filled triangles indicate times at which samplings were taken for size measurements. B: % of live cell volume (average ± standard error) of HM-2 with different fixatives at the times indicated in A. Average live volume for each sampling time is given below each set of bar graphs.

A


DENSISTY (X10^5 cells ml^{-1})
1.5
1.0
0.5
0.0
0
25
50
75
100
125
HOURS

B


GLUTARALDEHYDE 2%
+ TANNIC ACID 2%
ACID LUGOL'S SOLUTION
FORMALDEHYDE 2%
GLUTARALDEHYDE 2%
% OF LIVE VOLUME
100
90
80
70
60
50
40
30
20
10
0
158
258
82
53
39

were significant differences among fixatives on shrinkage of both species of heterotrophic microflagellate (Fig. 1 and 2; Table 4 and 5).

Glutaraldehyde caused the most shrinkage of the microflaglletes , yielding 38-47% and 45% of live volume for Paraphysomonas imperforata and HM-2, respectively (Table 3-5). The higher concentrations of glutaraldehyde caused more shrinkage of Paraphysomonas imperforata than did lower concentrations (Table 4). Of the fixatives tested, a mixture of glutaraldehyde and tannic acid (modified van der Veer fixative) caused the least change in cell volume; the average volume of fixed cells was 78% of the volume of live cells for Paraphysomonas imperforata and 82% of the volume of live cells for HM-2 (Table 3-5). Paraphysomonas imperforata and HM-2 fixed with acid Lugol's solution were 62% of live volume. HM-2 fixed with formaldehyde yielded 64% of live volume (Table 3).

One size of Strombidium acutum, two sizes of Favella sp. and three sizes of Strobilidium spiralis were obtained from the batch cultures in different growth phases (Fig. 3; tested by 1-way ANOVA; $P < 0.01$). As for the heterotrophic microflagellates, the percentage of shrinkage was independent of cell size (Table 6). However, there were significant differences among the effects of different fixatives on the percent shrinkage of each ciliate species (Fig. 3: Table 7-9). Formaldehyde had the least effect of three tested fixatives on cell volume (Table 3): Strobilidium spiralis, 80%; Strombidium acutum, 83%; and Favella sp., 87% of live volume. Glutaraldehyde

Table 3. Shrinkage of protozoa after fixation (% of cell live volume)

Fixatives: GT 2% = Glutaraldehyde 2% + Tannic acid 2%, LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 1%, 2%, 3% = Glutaraldehyde 1%, 2%, 3%. Data: Average (Standard error), n = No. of means.

SPECIES (n)	GT 2%	LU	FO 1%	GL 1%	GL 2%	GL 3%
Paraphysomonas imperforata (15)	77.93 (10.79)	61.96 (11.26)	-	47.16 (8.29)	42.22 (7.34)	37.82 (5.47)
HM-2 (15)	82.28 (10.33)	67.68 (6.93)	63.52 (10.17)	-	44.58 (10.62)	-
Favella sp. (10)	-	70.31 (7.69)	86.61 (6.22)	-	75.87 (10.85)	-
Strombidium acutum (3)	-	74.48 (2.18)	82.58 (0.49)	-	64.32 (2.42)	-
Strobilidium spiralis (9)	-	63.85 (3.56)	79.94 (3.71)	-	54.48 (10.32)	-

Table 4. Comparison among effects of fixatives on shrinkage of Paraphysomonas imperforata: 1-way ANOVA test & the Student-Newman-Keuls (SNK) tests were performed. Fixatives: Live = No fixative, GT 2% = Glutaraldehyde 2% + Tannnic acid 2%, LU = Acid Lugol's solution, GL 1%, 2%, 3% = Glutaraldehyde 1%, 2%, 3%. * : If Q value between means > Qc, two means are significantly different. Refer to Table 3 for data.

1-way ANOVA	Source of variation	df	Fs	P
	Fixatives	5	250.44	<0.01
	Error	84		
* SNK Test Qc = 3.762 (α = 0.01)	Fixatives		Q value between adjacent means	
	Live vs. GT 2%		28.662	
	GT 2% vs. LU		20.740	
	LU vs. GL 1%		19.220	
	GL 1% vs. GL 2%		6.415	
	GL 2% vs. GL 3%		5.714	

Table 5. Comparison among effects of fixatives on shrinkage of HM-2: 1-way ANOVA test & the Student-Newman-Keuls (SNK) tests were performed. Fixatives: Live = No fixative, GT 2% = Glutaraldehyde 2% + Tannic acid 2%, LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 2% = Glutaraldehyde 2%. * : If Q value between means > Qc, two means are significantly different. Refer to Table 3 for data.

1-way ANOVA	Source of variation	df	Fs	P
	Fixatives	4	117.29	<0.01
	Error	70		
* SNK Test Qc = 3.762 (α = 0.01)	Fixatives		Q value between adjacent means	
	Live vs. GT 2%		17.453	
	GT 2% vs. LU		14.380	
	LU vs. FO 1%		4.048	
	FO 1% vs. GL 2%		18.704	

Fig. 3. % of live volume (average ± standard error) of different species and sizes of ciliates after fixation: A; Favella sp., B; Strombidium acutum, C; Strobilidium spiralis. Average live volume or dimensions are given below each set of bar graphs.

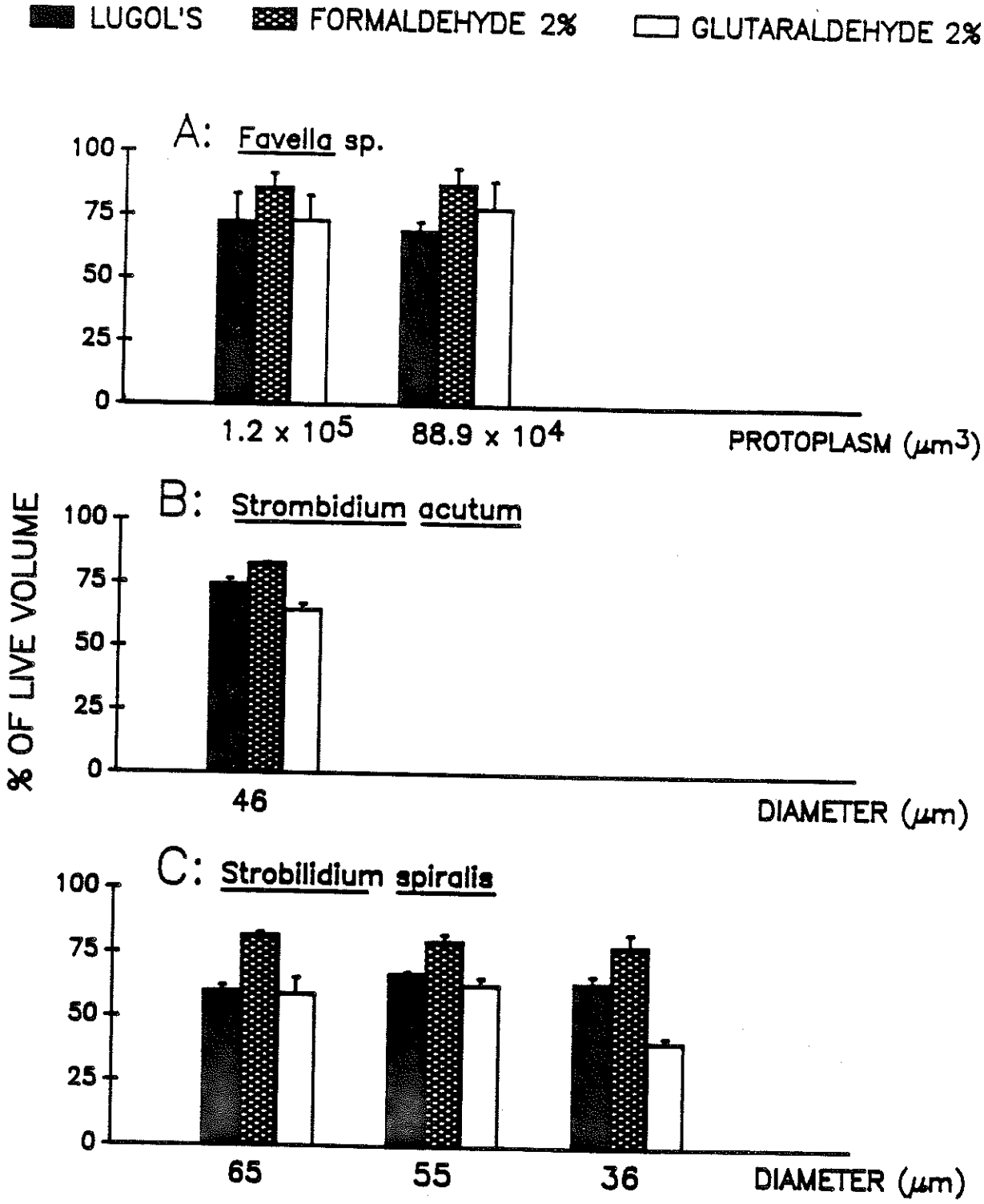

LUGOL'S
FORMALDEHYDE 2%
GLUTARALDEHYDE 2%
A: Favella sp.
100
75
50
25
0
1.2 x 10^5
88.9 x 10^4
PROTOPLASM (μm3)
% OF LIVE VOLUME
B: Strombidium acutum
100
75
50
25
0
46
DIAMETER (μm)
C: Strobilidium spiralis
100
75
50
25
0
65
55
36
DIAMETER (μm)

Table 6. Effects of live sizes on shrinkage of heterotrophic ciliates under each fixation: LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 2% = Glutaraldehyde 2%. 1-way ANOVA tests were performed. Refer to Fig. 3 for data.

ciliates species	Fixatives	Source of variation	df	Fs	P
Favella sp.	LU	Sizes	1	0.5480	n.s.
		Error	8		
	GL 1%	Sizes	1	0.1587	n.s.
		Error	8		
	GL 2%	Sizes	1	0.5623	n.s.
		Error	8		
Strobilidium spiralis	LU	Sizes	2	4.7830	n.s.
		Error	6		
	FO 1%	Sizes	2	0.4697	n.s.
		Error	6		
	GL 2%	Sizes	2	1.2344	n.s.
		Error	6		

Table 7. Comparison among effects of fixatives on shrinkage of Favella sp.: 1-way ANOVA test & the Student-Newman-Keuls (SNK) test were performed. Fixatives: Live = No fixative, LU = Acid Lugol's solution, FO 1% = Formalin 1%, GL 2% = Glutaraldehyde 2%. * : If Q value between means > Qc, two means are significantly different. Refer to Table 3 for data.

1-way ANOVA	Source of variation	df	Fs	P
	Fixatives	3	57.142	<0.01
	Error	32		
* SNK Test Qc = 3.956 (α = 0.01)	Fixatives		Q value between adjacent means	
	Live vs. FO 1%		7.194	
	FO 1% vs. GL 2%		5.770	
	GL 2% vs. LU		34.934	

Table 8. Comparison among effects of fixatives on shrinkage of Strombidium acutum: 1-way ANOVA test & the Student-Newman-Keuls (SNK) test were performed. Fixatives: Live = No fixative, LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 2% = Glutaraldehyde 2%. * : If Q value between means > Qc, two means are significantly different. Refer to Table 3 for data.

1-way ANOVA	Source of variation	df	Fs	P
	Fixatives	3	443.421	<0.01
	Error	8		
* SNK Test Qc = 4.746 (α = 0.01)	Fixatives		Q value between adjacent means	
	Live vs. FO 1%		38.310	
	FO 1% vs. LU		17.813	
	LU vs. GL 2%		22.344	

Table 9. Comparison among effects of fixatives on shrinkage of Strombilidium spiralis: 1-way ANOVA test & the Student-Newman-Keuls (SNK) test were performed. Fixatives: Live = No fixative, LU = Acid Lugol's solution, FO 1% = Formaldehyde 1%, GL 2% = Glutaraldehyde 2%. * : If Q value between means > Qc, two means are significantly different. Refer to Table 3. for data.

1-way ANOVA	Source of variation	df	Fs	P
	Fixatives	3	445.2913	<0.01
	Error	29		
* SNK Test Qc = 3.956 (α = 0.01)	Fixatives		Q value between adjacent means	
	Live vs. FO 1%		41.438	
	FO 1% vs. LU		33.237	
	LU vs. GL 2%		19.356	

caused the most shrinkage in the two naked ciliates; Strobilidium spiralis (54% of live volume), and Strombidium acutum (64% of live volume). With the loricate ciliate, Favella sp., acid Lugol's solution caused the most shrinkage (76% of live volume) (Table 3).

Changes in protozoan volume in response to fixation varied with species and fixatives, but responses of each species to a fixative were constant irrespective of its physiological state (Fig. 1 & 3). In this study, the effects of fixatives on the heterotrophic flagellates were, in order of increasing shrinkage: van der Veer fixative < Lugol's solution < formaldehyde < glutaraldehyde (Table 4-5); and for the oligotrichous ciliates: formaldehyde < Lugol's solution < glutaraldehyde (Table 8 & 9); and for the tintinnid: formaldehyde < glutaraldehyde < Lugol's solution (Table 7). Under the same fixation procedures, the flagellate volumes decreased in volume more than ciliates and among the ciliates the oligotrichous ciliate decreased in volume more than the tintinnid.

Sieracki et al. (38) reported that particle retention by a phagotrophic chrysomonad flagellate was affected by the fixation method. Van der Veer's solution (2% acrolein, 2% glutaraldehyde, 1% tannic acid) resulted in better particle retention by the flagellate than glutaraldehyde and formaldehyde. Shrinkage of flagellates by fixatives showed the same trends as particle retention. Modified van der Veer's solution (2% glutaraldehyde, 2% tannic acid) caused the least shrinkage. We hypothesize that

particle egestion may be a major cause of shrinkage in phagotrophic flagellates as result of fixation. Autotrophic flagellates, which do not generally ingest particles, show less shrinkage than phagotrophic flagellates upon fixation. For example, Chlorophyte, Prymnesiophyte, and Prasinophyte flagellates are reported to shrink to 69%, 60%, and 85%, respectively, of their live volume after fixation with 2.5% glutaraldehyde (6), whereas, in this study, heterotrophic forms fixed with 2% or 3% glutaraldehyde shrank to 37-42% of their live volume.

It has been reported that naked ciliate species in closely related taxa can display quite different cell volume changes upon fixation; for example, Lohmanniella spiralis shrinks and Strombidium reticulatum swells (23). These differences may be due to difference in the concentration of fixatives, differences among species or strains, or perhaps to difference in salinity or prey size. Under low salinity (low osmotic pressure) condition, dead cells may shrink less than under high salinity conditions or possibly increase in size due to the influx of water into dead cells. Another possible explanation is that the size of the prey particles influences shrinkage. Particle egestion by phagotrophic microflagellates is inversely correlated with size of prey particle (in prep.); The smaller prey particle a heterotrophic microflagellate has been feeding on, the less it shrinks when fixed. The prey species used in this study were bigger than the prey species used by Jonsson (23) to feed ciliates.

The biovolume of phagotrophic protozoa is routinely estimated from size measurements of fixed cells and then is usually converted into a biomass currency, such as carbon or wet weight. Conversion factors for live phytoplankton (29, 43) have often been used for formaldehyde-fixed protozoa (22, 23, 30, 36), for Lugol's-fixed protozoa (26), and live protozoa (39). Conversion factors for live protozoa (24) also have been used for formaldehyde-fixed protozoa (35). Conversion factors based on the cell composition of live protozoa have been used for formaldehyde-fixed protozoa (2, 3, 5, 36). These estimates have generally underestimated the real biomass of heterotrophic protozoa because fixatives usually cause shrinkage of heterotrophic protozoa. Conversion factors from live protozoa (7, 16, 24) and from cell composition of live protozoa (5, 36), 0.04-0.10 gC/ml, are in the same range as conversion factors for live phytoplankton, but conversion factors for fixed protozoa are higher, 0.18-0.22 gC/ml (7, 14, 42). The size of ingested prey is another factor that appears to influence shrinkage; conversion factors derived from the study of bactivorous flagellates may result in an underestimation of the biomass of natural populations which probably ingest both bacteria and larger prey. Herbivorous and carnivorous protozoa should tend to shrink more than bactivorous species under same fixation procedures because of particle egestion. The biomass of heterotrophic protozoa in natural assemblages has probably been underestimated, by 20-55%, in investigations in which cell volumes were based on fixed samples but biomass conversions were

based on live cells or on non-phagotrophic taxa.

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Chapter 2: EFFECTS OF ALGAL PREY DENSITY ON GROWTH OF MARINE HETEROTROPHIC NANOFLAGELLATES

Abstract

Although most heterotrophic nanoflagellates are bactivores, many species can also graze small phytoplankton. The growth of two heterotrophic nanoflagellates was investigated as a function of algal prey density, and determined to be a hyperbolic function of this parameter. Comparison of the growth kinetics of the two species with various size algae as prey suggests that the response of heterotrophic nanoflagellates to prey density differs: Paraphysomonas imperforata was dependent on prey cell density, and HM-2 (unidentified species) was more dependent on the concentration of chemical constituents such as carbon and nitrogen. Rapid increases in growth rate and low half saturation constant (Ks) compared to minimum prey densities for growth indicate that heterotrophic nanoflagellates are adapted to heterogeneous prey densities and can respond rapidly to increased food availability.

Even though cell volume is a indicator of growth rate of heterotrophic nanoflagellates, it should not be applied at all: the actual relationship is specific to both predator and prey species, and sometimes particle egestion after fixation can result in an unreliable relationship between size and growth rate. The growth rate studies showed that heterotrophic nanoflagellates responded both to increases in prey density and in prey size by increasing their cell size. This plasticity in feeding behavior and growth response suggests that many heterotrophic nanoflagellates may be opportunistic in nature, utilizing food particles ranging in size from bacteria to

nanoplanktonic cells.

Introduction

Sheldon et al. (1972) hypothesized that phagotrophic planktonic organisms tend to graze prey particles one order of magnitude smaller than themselves. Based on this size principle, heterotrophic nanoflagellates should be the optimum grazers of bacteria-sized organisms, and microzooplankton (20-200µm) should be the optimum grazers of nanoplankton (2-20µm) in the oceans (Azam et al. 1983). The role of heterotrophic nanoflagellates as the major grazers of bacterioplankton has been supported by laboratory studies (Fenchel 1982a, Haas 1979, Andersson et al. 1986, Sherr et al. 1983), as well by field studies (Fenchel 1982b, Andersen & Sorensen 1986).

Phagotrophic nanoflagellates may also play a role as consumers of photoautotrophs in the sea. Photosynthetic picoplankton (0.2-2µm), including cyanobacteria and eukaryotic algae, are ubiquitous and contribute a major portion of the primary production (Iturriaga & Mitchell 1986), sometimes up to 80% (Li et al. 1983). Laboratory studies have shown that heterotrophic nanoflagellates graze cyanobacteria and pico-sized algae (Johnson et al. 1982, Parslow et al. 1986). Some heterotrophic nanoflagellates can graze directly on phototrophic & heterotrophic nanoflagellates, including cells larger than themselves (Linley et al. 1983, Suttle et al. 1986, Goldman & Caron 1985). Phagotrophic nanoflagellates graze larger bacteria preferentially to smaller ones (Andersen et al. 1986), and

nano-sized autotrophs to bacteria (Goldman et al. 1985). Heterotrophic nanoflagellates may, in general, preferentially graze on photoautotrophs rather than on bacteria because of the larger size of the latter, as long as the autotrophs are within size range of prey that the nanoflagellates can ingest, and the density of autotrophs is sufficient enough to be grazed by heterotrophic nanoflagellates.

In nature, many heterotrophic nanoflagellates probably consume a mixture of bacterioplankton and small phytoplankton. To date, most studies of grazing by heterotrophic nanoflagellates have focused on bactivory, and no data were available on the effects of algal cell size and algal density on the growth of heterotrophic nanoflagellates. The major objective of the present study was to investigate the effects of algal prey density on growth rates of heterotrophic nanoflagellates. Growth of two species of heterotrophic nanoflagellates when fed three algal prey were documented. The results were compared between predator species (nanoflagellates) and amongst prey species (algae). The importance of herbivory by heterotrophic nanoflagellates is discussed in terms of algal prey density.

Material and Methods

Two heterotrophic nanoflagellates, *Paraphysomonas imperforata* (Strain HM-1) and strain HM-2 were used in this study. HM-1 was obtained from Joel C. Goldman and is known to consume bacteria and algae (Goldman & Caron 1985). Strain HM-2, which is not identified yet, was isolated from an intertidal

pool in Provincetown Massachusetts, USA. Preliminary experiments demonstrated that Paraphysomonas imperforata could be grown on bacteria-free cultures of Dunaliella tertiolecta (Strain Dun), Isochrysis galbana (Strain Iso), Phaeodactylum tricornutum (Strain TFX-1), and Chlorella capsulata (Strain Fla E) and that HM-2 could be grown on bacteria-free cultures of Dunaliella tertiolecta (Strain Dun), Isochrysis galbana (Strain Iso), and Thalassiosira pseudonana (Strain 3H). HM-2 could also be grown on pure cultures of bacteria. For the experiments, Dunaliella tertiolecta (Strain Dun) and Isochrysis galbana (Strain Iso) were chosen because they support the growth of both heterotrophic nanoflagellates. However, none of the other algae supported the growth of both heterotrophic nanoflagellates. Phaeodactylum tricornutum (Strain TFX-1) and Thalassiosira pseudonana (Strain 3H), for the experiments with HM-1 and HM-2 respectively, were chosen in order to obtain a range of prey algal size as well as prey taxa (Table 1). All phytoplankton species were obtained from the culture collection of R.R.L. Guillard. All species were made and kept bacteria-free.

Algal prey species were grown in 2.8 liter flasks containing 2.0 liter of f/2 medium (Guillard 1975). The algae were grown at 20°C, at an irradiance of 200 uE/m²/sec under a 14hr/10hr (light/dark) cycle. Different algal densities were prepared by diluting exponentially growing batch cultures of algae with sterile sea water. The heterotrophic nanoflagellates used for grazing experiments were reared in the dark under the same

Table 1. Taxa, dimensions and carbon & nitrogen content of algal prey species: Volumes were calculated on assumption that I. galbana is a sphere, D. tertiolecta is a ellipsoid, T. pseudonana is a cylinder, and P. tricornutum is a ellipsoid. *: Length of frustule for P. tricornutum. Dimensions based on measurement of 20 cells. Chemical constituents were based on triplicate samples. Data: mean (S.D).

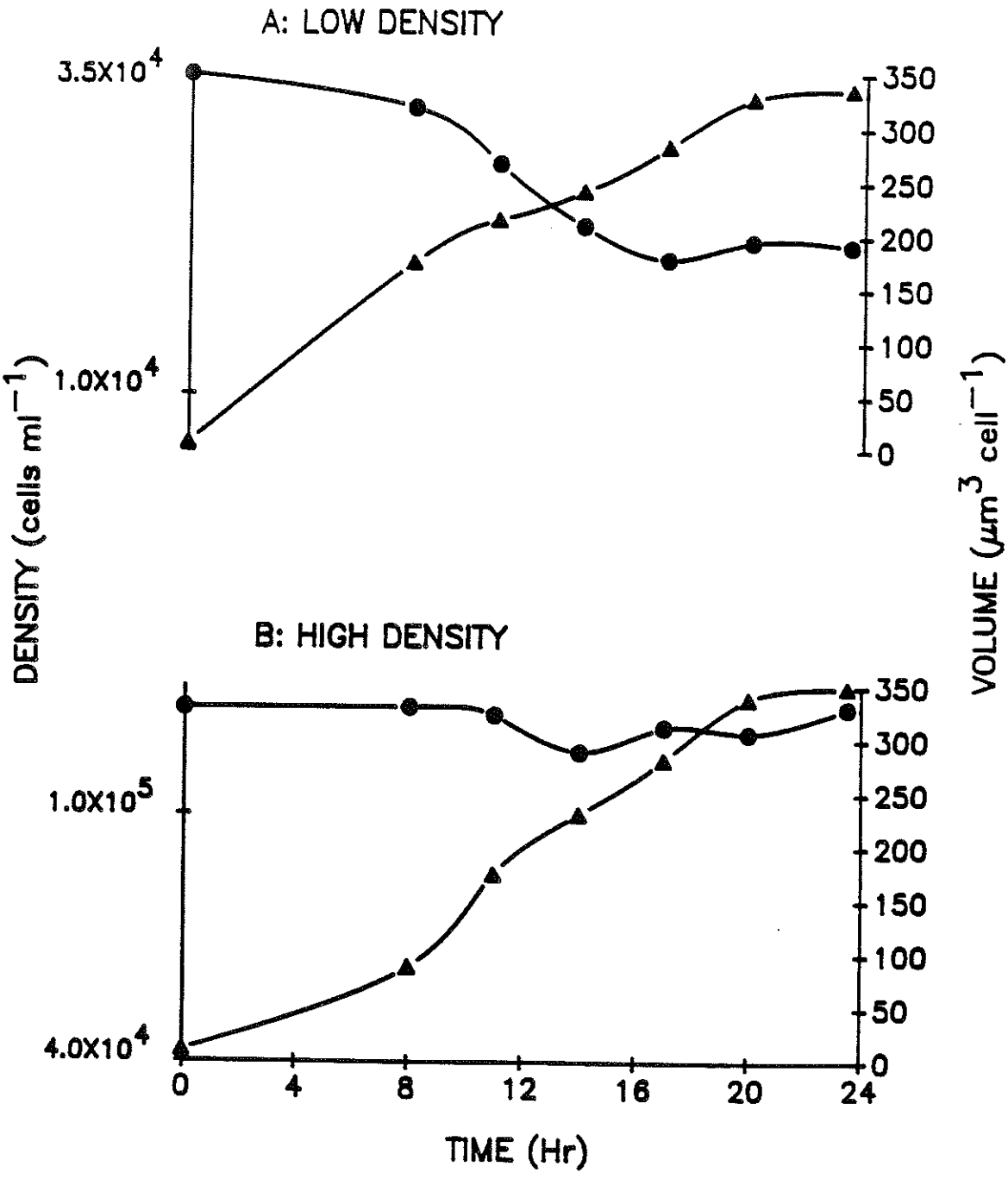
Algal prey species	Length (µm)	Diameter (µm)	Volume (μm^3)	Carbon (pgC/cell)	Nitrogen (pgN/cell)
I. galbana (Prymnesiophyte)	4.37 (0.41)		45 (14)	13.1 (0.09)	3.30 (0.03)
D. tertiolecta (Chlorophyte)	9.21 (0.72)	5.54 (0.69)	299 (80)	31.3 (0.58)	5.74 (0.18)
T. pseudonana (Diatom)	6.30 (0.46)	5.07 (0.73)	130 (45)	10.4 (0.34)	0.52 (0.01)
P. tricornutum (Diatom)	10.02 (2.14) *32.29 (2.86)	3.56 (0.33)	133 (38)	16.8 (0.34)	3.30 (0.02)

conditions of temperature and prey species as in the actual grazing experiments, and thus were well acclimated prior to the experiments. Experiments were done in 200 ml flasks containing 150 ml of culture. The flasks were incubated in the dark at 20°C on a table shaker. Initial densities of predator species were kept as low as possible in order to minimize changes in prey density during the grazing experiments. Initial cell densities of the predator species ranged from 1/20 to 1/50 of algal cell density.

After inoculation, prey density, predator density, and predator size were monitored at 4-6 hour intervals over 1 to 3 days. 3-10 ml of sample were withdrawn and fixed with acid Lugol's solution; in the treatments with lower algal and predator densities, larger volumes were collected and fixed and then the samples were concentrated by centrifugation (g=2000, 5 mins). Cell counts were made with a hemocytometer, or a Palmer-Malony slide, depending on cell density (Guillard 1973). Sizes of the heterotrophic nanoflagellates were measured by microscopy using a calibrated ocular micrometer, and volumes were calculated assuming that microflagellates were spheres. In each sample, 25 cells were measured.

The stock cultures of nanoflagellates were grown under non-food limitation. Acclimation to the new prey densities was accessed by monitoring changes in the cell volume of the nanoflagellates. The cell volume of protozoa changes according to their physiological state (Goldman and Caron 1985, Fenchel 1982, Hamilton and Preslan 1969). For example, average cell

Fig. 1. Acclimation of P. imperforata to new prey densities: P. imperforata at maximum growth rate were transferred into (a) $3.5X10^4$, (b) $8.43X10^5$ of P. tricornutum. Volume (filled circle) and cell density (filled triangle) of P. imperforata were monitored after inoculation. Cell volumes were not corrected for shrinkage.


A: LOW DENSITY
3.5X10^4
1.0X10^4
350
300
250
200
150
100
50
0
DENSITY (cells ml^-1)
VOLUME (μm^3 cell^-1)
B: HIGH DENSITY
1.0X10^5
4.0X10^4
0
4
8
12
16
20
24
TIME (Hr)

volume (μm^3 $cell^{-1}$) of P. imperforata decreased after transfer to a lower prey density and became stable only after the division rate had stabilized. (Fig. 1). The duration of acclimation was inversely related to the new prey density; the lower the prey density, the longer the acclimation time. Specific growth rates (day^{-1}) of heterotrophic nanoflagellates were calculated from the linear portion of semi-log plots of cell density of nanoflagellates after cell size was stable (i.e. acclimated growth). Cell volumes of heterotrophic nanoflagellates were estimated from acid Lugol's solution fixed samples. Although this fixative caused heterotrophic nanoflagellates to shrink, the shrinkage effect is usually constant irrespective of growth stage (Choi & Stoecker in prep.). However, after fixation, HM-2 fed D. tertiolecta shrunk to a similar size irrespective of live size, in this case acclimation was assumed to have occurred after 24 hrs based on the acclimation time of HM-2 fed other prey species.

Using the Gauss-Newton method of SAS statistical program (SAS institute Inc., Cary, USA), a functional relationship between prey densities and predator growth rates was fitted in the form of a hyperbolic function:

$$G = G_{max} \times (P - P_{min}) / (Ks + (P - P_{min}))$$

where G = growth rate (day^{-1}); G_{max} = maximum growth rate (day^{-1}); P = prey density (algal cells/ml); P_{min} = minimum prey density for growth (algal cells/ml); Ks = half saturation constant (algal cells/ml). Both Ks and P_{min} were also expressed in carbon and nitrogen based on conversion factors

which were determined from CHN analysis (Table 1) and cell counts of the algal prey species.

Results

The growth rates of the heterotrophic nanoflagellates were statistically fitted to hyperbolic functions of algal prey density (Fig. 2 & 3, Table 2). Table 2 summarizes the growth kinetics of the heterotrophic nanoflagellates. Even though the growth rates of P. imperforata are different depending on the algal prey species, the half saturation constant (Ks) and the minimum prey density for growth (P_{min}) expressed as cell densities are comparable (Table 3). But the Ks and P_{min} values expressed in carbon and nitrogen are different among treatments (prey species). The growth rates of HM-2 are similar on different algal species (Table 3). Ks and P_{min} values for HM-2 grown on different algal prey were not similar when compared by prey cell density but were similar in terms of carbon or nitrogen (Table 3). Growth rates of both heterotrophic nanoflagellates increased very rapidly as algal prey density increased (Fig. 2 & 3, Table 3): Ks values for P. imperforata are less than 3 times of P_{min}. This rapid increase in growth rates was more pronounced for HM-2; Ks values are less than 1½ times of P_{min}.

The cell volume (based on samples fixed with acid Lugol's solution) of the heterotrophic nanoflagellates was a linear function of the growth rates of the heterotrophic nanoflagellates, except when HM-2 was grown on D. tertiolecta

Table 2. Statistical analysis of non-linear regression: Unbiased R^2 values were calculated and F-tests were performed for the hyperbolic functions shown in Fig. 2 & 3.

Heterotrophic nanoflagellates	Algal prey species	Unbiased R^2	F-test P
P. imperforata	P. tricornutum	0.9832	$<10^{-6}$
	I. galbana	0.9783	$<10^{-6}$
	D. tertiolecta	0.9664	$<10^{-7}$
HM-2	T. pseudonana	0.9649	$<10^{-6}$
	I. galbana	0.9945	$<10^{-6}$
	D. tertiolecta	0.9216	$<10^{-6}$

Table 3. Parameters of growth kinetics of heterotrophic nanoflagellates: μmax, Pmin and Ks were obtained by Gauss-Newton method. Pmin and Ks were expressed as carbon and nitrogen as well as prey density. μmax: maximum gorwth rate (/day); D: prey density (cells/ml); C: prey biomass in carbon (μgC/liter); N: prey biomass in nitrogen (μgN/liter).

Heterotrophic nanoflagellates	Algal prey species	μmax	Pmin			Ks		
			D	C	N	D	C	N
P. imperforata	P. tricornutum	1.69	1.78X104	299	59	5.00X104	841	165
	I. galbana	2.19	2.54X104	334	40	7.09X104	931	113
	D. tertiolecta	1.84	2.95X104	922	169	4.31X104	1347	247
HM-2	T. pseudonana	2.49	1.06X105	1107	162	1.57X105	1638	239
	I. galbana	2.28	1.60X105	2020	245	1.59X105	2096	254
	D. tertiolecta	2.67	5.32X104	1666	305	5.85X104	1833	336

Fig. 2. Growth rates of P. imperforata as function of algal prey density with three algal species as prey: (a) P. tricornutum, (b) I. galbana and (c) D. tertiolecta.

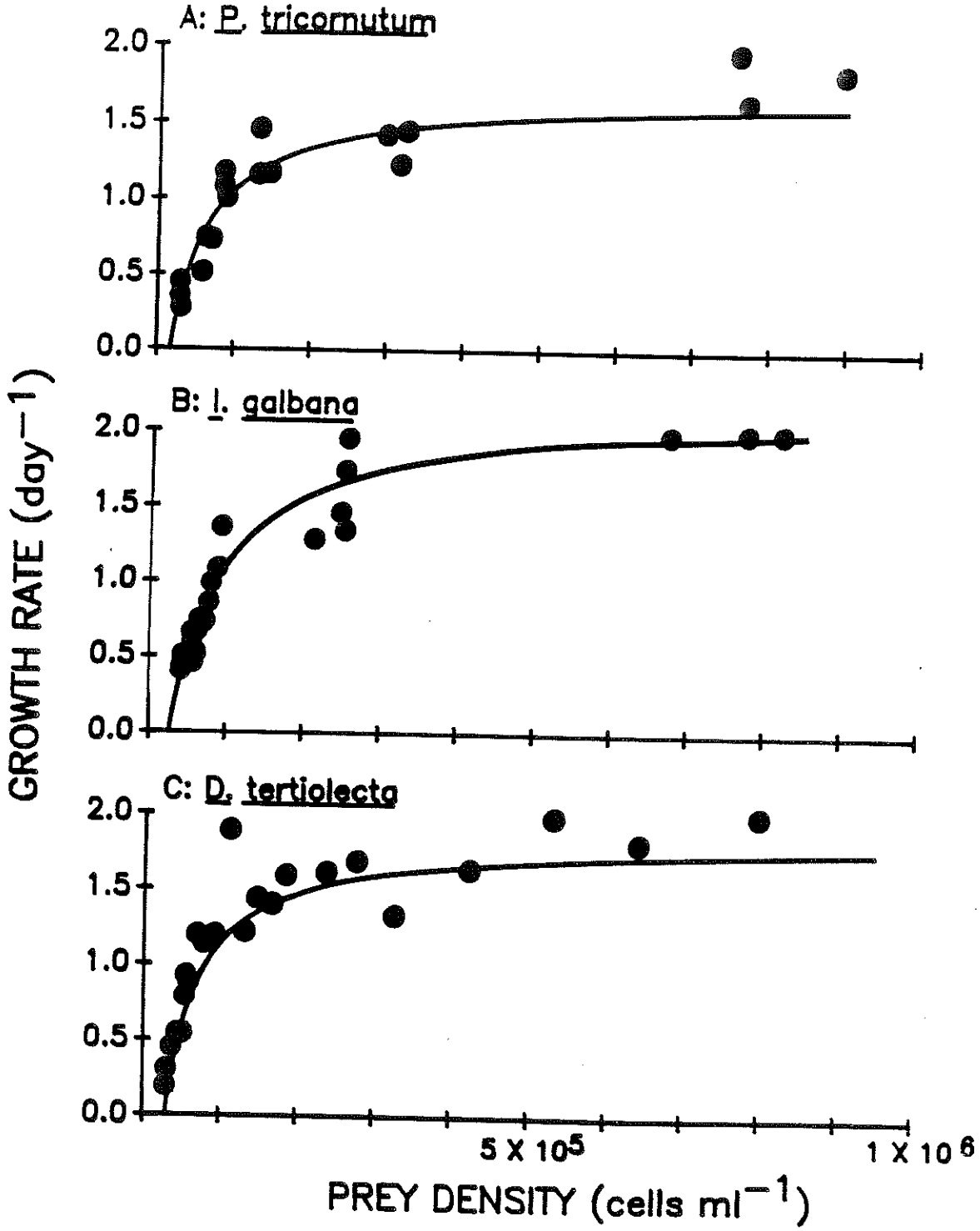
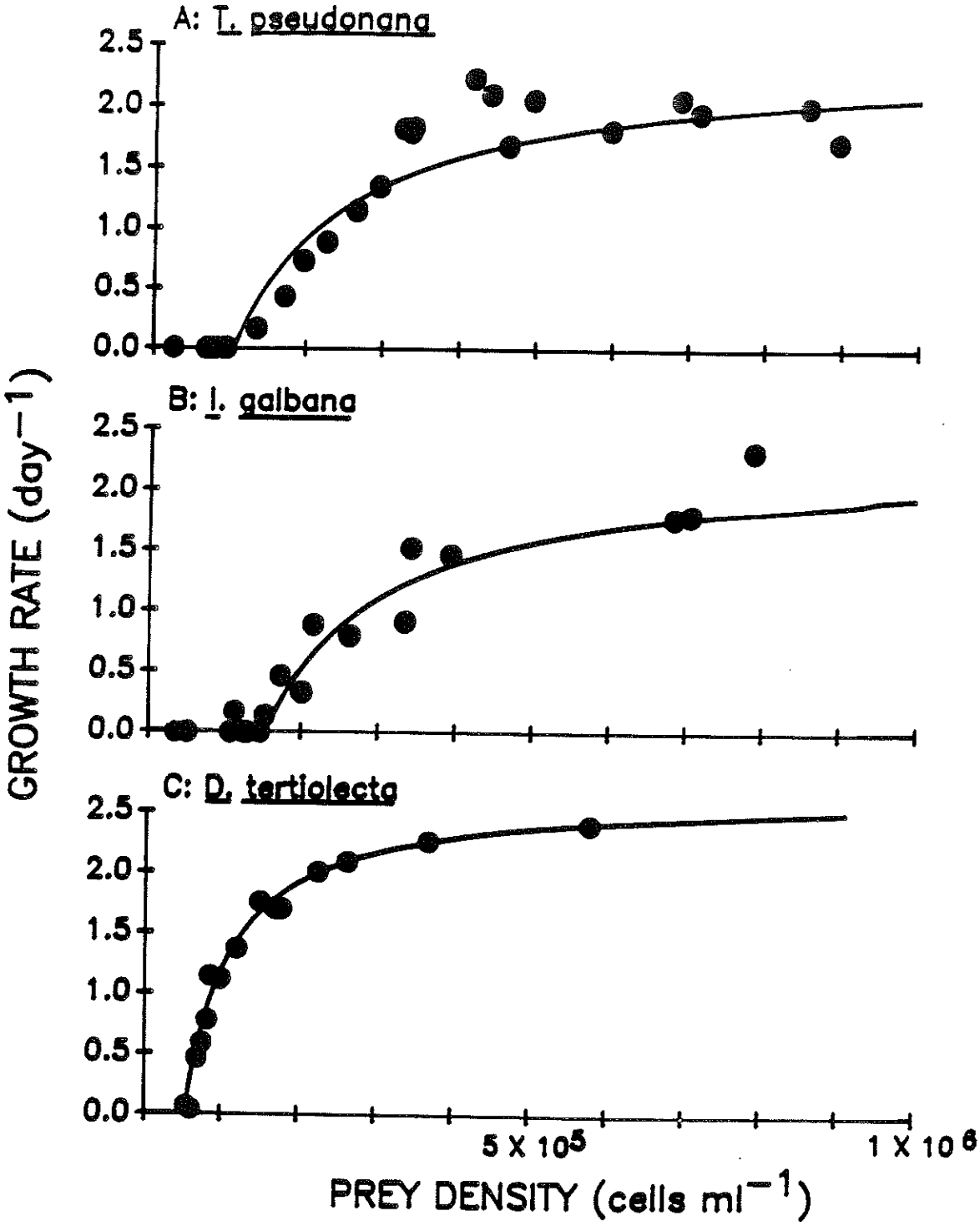

A: P. tricornutum
B: I. galbana
C: D. tertiolecta
2.0
1.5
1.0
0.5
0.0
GROWTH RATE (day⁻¹)
5 X 10⁵
1 X 10⁶
PREY DENSITY (cells ml⁻¹)

Fig. 3. Growth rates of HM-2 as function of algal prey density with three algal species as prey: (a) T. pseudonana, (b) I. galbana and (c) D. tertiolecta.


A: T. pseudonana
B: I. galbana
C: D. tertiolecta
GROWTH RATE (day⁻¹)
2.5
2.0
1.5
1.0
0.5
0.0
5 X 10⁵
1 X 10⁶
PREY DENSITY (cells ml⁻¹)

(Fig. 4) However, the relationship between cell size of P. imperforata or HM-2 and growth rate depended on the prey species (fig. 4). Oddly, apparent cell size of the heterotrophic nanoflagellates was generally smaller with the bigger algal prey (Fig. 4; Table 1). This is quite opposite to the observations made on live cells that the bigger algal prey species produce bigger heterotrophic nanoflagellate cells.

Discussion

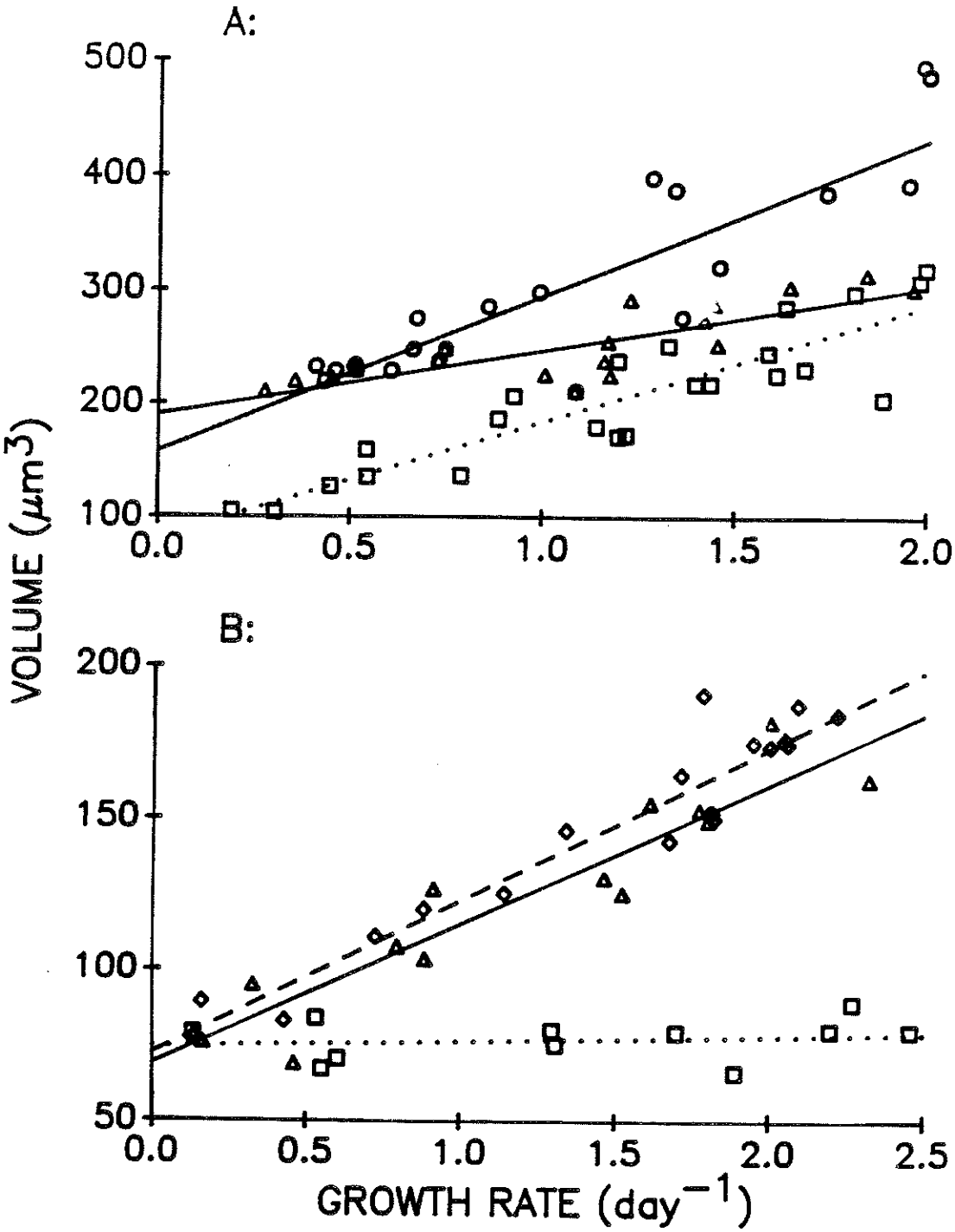
The data show that nano-sized phytoplankton can support the growth of heterotrophic nanoflagellates, and that growth rate is a hyperbolic function of prey density. Similar relationships between growth of heterotrophic protozoa and prey density have been reported for bactivorous nanoflagellates (Fenchel 1982a, Rivers et al. 1985), bactivorous ciliates (Rivers et al. 1985), herbivorous nanoflagellates (Parslow et al. 1986), and herbivorous ciliates (Jonsson 1986). P. imperforata grown on three different algal prey species have similar minimum algal prey densities (P_{min}) and half saturation constants (Ks) for growth, when prey density is expressed in cell numbers, while minimum algal prey densities (P_{min}) and half saturation constants (Ks) for HM-2 grown on three different algal species are similar when expressed in cell chemical constituents such as carbon or nitrogen. This implies that the physiological responses to different prey species varies among different species of heterotrophic nanoflagellates. HM-2 may need comparable amount of energy for minimum growth irrespective of

prey species. But species like P. imperforata may vary the metabolic energy needed for minimum growth according to the prey species.

Even though our data leave no doubt that heterotrophic nanoflagellates are capable of grazing nano-sized autophototrophs, there still remains the question of the importance of herbivory in comparison with bactivory by heterotrophic nanoflagellates. Circumstantial evidence in favor of herbivory includes: (1) heterotrophic nanoflagellates exclusively graze on smaller diatoms in mixtures of bacteria and small diatoms (Goldman et al. 1985), (2) heterotrophic nanoflagellates prefer bigger bacteria to smaller ones (Andersseon et al. 1986). 3) heterotrophic nanoflagellates graze nano-sized diatoms in nature (Suttle et al. 1986, Canter and Lund 1968). The major difficulty in quantifying the importance in nature of herbivory by heterotrophic nanoflagellates lies in the similarity in size of predator and prey; size fractionation can not be used to separate these two functional groups.

Minimum algal prey densities (P_{min}) and half saturation constants (Ks) for growth of nanoflagellates were 2-5X10^4 & 4-7X10^4 cells/ml for P. imperforata and 0.5-1.5X10^3 & 0.6-1.6X10^3 for HM-2. However, in the open ocean, densities of phototrophic nanoflagellates seldom exceed 5X10^3 cells/ml (Caron 1983, Olson et al. 1985, Davis et al. 1985), although in coastal areas densities of nanophytoplankton of over 10^4 cells/ml sometimes occur (Davis and Sieburth 1982). Minimum prey densities for bactivorous nanoflagellates which have been

Fig. 4. Cell volume as a function of growth rate: (a) P. imperforata grazing P. tricornutum (circle & dash-dotted line), I. galbana (triangle, solid line) and D. tertiolecta (square & dotted line), (b) HM-2 grazing T. pseudonana (diamond & dashed line), I. galbana (triangle, solid line) and D. tertiolecta (square & dotted line). R^2 values for regression lines were: (a) P. imperforata grazing P. tricornutum (0.68), I. galbana (0.80) and D. tertiolecta (0.80), (b) HM-2 grazing T. pseudonana (0.90), I. galbana (0.89) and D. tertiolecta (not-applied). Cell volumes were not corrected for shrinkage.


A:
500
400
300
200
100
0.0
0.5
1.0
1.5
2.0
B:
200
150
100
50
0.0
0.5
1.0
1.5
2.0
2.5
VOLUME (μm^3)
GROWTH RATE (day^{-1})

reported are 0.5-2.0X10^6 cells/ml (Fenchel 1982a) and 0.9-18.7X10^6 cells/ml (Rivers et al. 1985), which are equivalent to 50-1870 µgC/liter using a conversion factor 10^{-10} mgC/cell (Fenchel 1982a). These minimum prey densities (expressed as carbon) for bactivorous nanoflagellates are in the same range as our value for herbivorous nanoflagellates (300-2000 µgC/liter). Average cell densities of bacteria and nano-sized phototrophs in the open ocean are in the range of 10^3 cells/ml and 10^5 cells/ml, which are one order less than the minimum prey densities for growth of heterotrophic nanoflagellates (Sieburth 1984). This raises the question of "How do heterotrophic nanoflagellates find enough food in the open ocean?" One possibility is that heterotrophic nanoflagellates derive much of their nutrition from grazing on bacteria and algae associated with marine aggregates (Caron et al. 1986). The growth kinetics of heterotrophic nanoflagellates suggest that they are adapted to take advantage of patches of higher then average prey density, such as marine aggregates can provide in open ocean (Caron et al. 1986). However, the possibility that heterotrophic nanoflagellates grow in the open ocean without taking advantage of marine aggregates can not be completely ruled out because (1) some species of heterotrophic nanoflagellates can not take advantage of the higher prey densities on aggregates (Caron 1987, Sibbald and Albright 1988), (2) the biomass of combined prey, such as pico-sized autotrophs & heterotrophs and nano-sized autotrophs & heterotrophs in the water column may be sufficient for growth of heterotrophic

nanoflagellates, and (3) the nanoflagellates we used in this study are from a coastal area and may not have the same growth kinetics as species that flourish in the open ocean.

For individual protozoan species there is a linear relationship between cell volume and growth rate (Fenchel 1982a, Jonsson 1986, Parslow et al. 1986). In general, our data show the same relationship. However, cell size depends on prey type as well as growth rate: different algal prey species yield different sizes of heterotrophic nanoflagellates at the same growth rates. This may be due to variations in size among prey species and to the effects of fixation on cell size of heterotrophic nanoflagellates. Fixation with acid Lugol's solution causes heterotrophic flagellates to shrink, and these shrinkage effects are most severe when the heterotrophic nanoflagellates have ingested relatively large-sized prey (Observation; data are not shown). It seems that a major cause of shrinkage in phagotrophic protozoa is egestion of ingested food particles (Sieracki et al. 1987; Choi and Stoecker in prep.). Linear relationships between growth rates and volumes are not always evident with fixed samples, as shown in the case of HM-2 fed D. tertiolecta. In nature, if heterotrophic nanoflagellates graze on relatively large nanophytoplankters, herbivory may be severely underestimated based on examination of fixed specimens due to particle egestion.

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