

## Prey Food Quality Affects Flagellate Ingestion Rates

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### Abstract

Flagellate feeding efficiency appears to depend on morphological characteristics of prey such as cell size and motility, as well as on other characteristics such as digestibility and cell surface characteristics. Bacteria of varying morphological characteristics (cell size) and mineral nutrient characteristics or food quality (as determined by the C:N:P ratio) were obtained by growing *Pseudomonas fluorescens* in chemostats at four dilution rates (0.03, 0.06, 0.10, and 0.13 h<sup>-1</sup>) and three temperatures (14°C, 20°C, and 28°C). Cells of a given food quality were heat-killed and used to grow the flagellate *Ochromonas danica*. Ingestion and digestion rates were determined by using fluorescently labeled bacteria of the same food quality as the bacteria supporting growth. Ingestion rates were affected by both food quality and cell size. Cells of high food quality (low carbon:element ratio) were ingested at higher rates than cells of low food quality. Multiple regression analysis indicated that cell size also influenced ingestion rate but to a much lesser extent than did food quality. Digestion rates were not correlated with either food quality or cell size. Results suggest that flagellates may adjust feeding efficiency based on the quality of food items available.

### Introduction

Considerable literature exists that characterize the role of flagellate nanozooplankton in planktonic systems. Predation by flagellates is a major mortality factor for aquatic bacteria [23, 48, 55] and it is clear that, in some systems, flagellates are capable of cropping as much as 100% of bacterial production [10, 45]. Given the abundance of bacteria in both marine and freshwaters, their potential for rapid growth, their ability to use dilute organic and inorganic nutrients, and their high assimilation efficien-

cies, flagellates must play a crucial role in transferring nutrients sequestered in bacterial biomass to consumers in higher trophic levels [2, 50].

Flagellate grazing can also influence the characteristics of bacterial communities and lead to changes in their structural and taxonomic composition [29, 32, 67]. Some of the characteristics of bacterial communities attributed to flagellate grazing include small average cell size [1, 15, 23, 49], high frequency of motile cells [23], and cell types of complex inedible morphologies [47, 59].

Flagellate feeding efficiency and/or prey selection appears to depend on prey morphology characteristics such as cell size and motility [23, 34, 42], as well as other features such as digestibility and cell surface characteristics [22, 30, 38, 43]. Nisbet [44] speculated that feeding selectivity may, in part, be receptor-mediated and regulated by “signal substances” on prey cell surfaces. Thus, nutritional and biochemical food quality may influence prey selection and ingestion [8].

Food quality has been shown to greatly influence the rate at which metazoan zooplankton exploit prey [12, 18, 62]. However, analogous studies on predator-prey food quality interactions in nanozooplankton communities are lacking. In this work, the mixotroph nanoflagellate, *Ochromonas danica* was fed bacteria (*Pseudomonas fluorescens*) of known, but varying food quality, where food quality was measured as the C:N:P ratio, an index of macronutrient composition that has proven useful in studies on metazoan zooplankton. Ingestion and digestion rates were determined for the protozoan during short-term feeding experiments. The relationships among food quality, ingestion, and digestion are discussed for this predator-prey couple.

### Materials and Methods

**Organisms and Analytical.** *P. fluorescens* (ATCC 3214) stock cultures were maintained on Standard Mineral Base (SMB) [65] supplemented with 10 mM glucose (SMBg). Bacteria-free *O. danica* (UTEX 1298) was maintained in

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**Table 1.** C:N:P of *Pseudomonas fluorescens* grown at different temperatures and dilution rates in chemostats

Dilution rate ( $h^{-1}$ )	Temperature					
	14°C		20°C		28°C	
	Before	After	Before	After	Before	After
0.03	91:16:1	84:18:1	51:8:1	90:18:1	44:7:1	88:18:1
0.06	65:11:1	82:17:1	70:12:1	59:13:1	66:11:1	74:18:1
0.10	68:12:1	101:21:1	61:10:1	101:21:1	72:11:1	121:26:1
0.13	56:11:1	139:26:1	72:13:1	92:19:1	61:12:1	107:21:1

Element ratios are before and after heat killing (mol:mol).

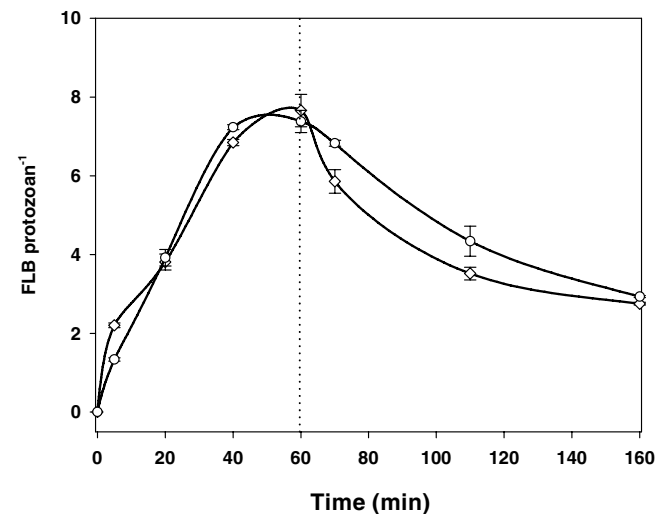
*Ochromonas* Medium [61]. Attempts to grow this strain as an exclusive phototroph have not been successful.

*P. fluorescens* was grown in SMBg in continually stirred and aerated 800 mL chemostats (Applikon) at four dilution rates ( $d=0.03, 0.06, 0.10,$  and  $0.13 h^{-1}$ ) and three temperatures (14°C, 20°C, and 28°C). Reactors were assumed to be in steady state after three complete turnovers at a given temperature and dilution rate. Outflow was aseptically captured into presterilized 1-L bottles (Nalgene). Cells harvested from chemostats (720 mL) were distributed into conical polypropylene centrifuge tubes (Nalgene) and pelleted (Sorvall RT6000B refrigerated centrifuge, Sorvall Instruments; 5000 rpm, 15°C, 25 min). Pellets were combined into 180 mL phosphate-buffered saline (PBS) resulting in a 4× concentration of original sample. The homogenized 4× sample was subsequently divided into two portions: 150 mL to prepare heat-killed (HK) bacteria [53] and 30 mL to prepare fluorescently labeled bacteria (FLB) [57], according to the modifications of Chrzanowski and Šimek [15].

Bacterial abundance was determined by direct epifluorescent microscopic enumeration (1250×) of formaldehyde-preserved (2% final concentration) samples using DAPI as the fluorochrome [51]. Cell volume ( $V$ ) was determined from length and width of at least 100 cells according to the formula:  $V = [\pi((0.5W^2)(L - W)) + ((4/3)\pi)(0.5W^3)]$ , where  $W$  is the maximum cell width and  $L$  is the maximum cell length ( $\mu m$ ). Length and width of individual cells was determined from digital images (Olympus DP70) and Simple PCI imaging software (Compix, Inc., Brandywine, PA, USA). Bacteria contained in 2 mL were collected on precombusted glass-fiber filters (Whatman GF/F) for element analysis. Two sets of triplicate filters were prepared for each culture condition; one set for carbon (C) and nitrogen (N) analysis and one set for particulate phosphorus (P) analysis. The C and N content of cells was determined using a CHN analyzer (PerkinElmer series 2200 CHN Analyzer). The P content of cells was determined from persulfate digests and subsequent analysis of soluble reactive P [63]. Ratios of elements are reported as mol:mol.

**Grazing Experiments.** Culture conditions have been found to influence flagellate grazing [8, 52, 66]. Bacteria used in grazing experiments were of varying element composition or food quality (Table 1). Consequently, *O. danica* was preconditioned to grow (50 mL cultures) on HK *P. fluorescens* of a given food quality (range  $1.2 \times 10^8$  to  $4.6 \times 10^8$  bacteria  $mL^{-1}$ ) immediately prior to assessing ingestion and digestion rates of that food item. Three feeding trials were conducted for 10 of the 12 food qualities at prey densities (range  $1.0 \times 10^8$  to  $4.2 \times 10^8$  bacteria  $mL^{-1}$ ) sufficient to ensure that *O. danica* ingestion and growth rates were saturated [68].

Ingestion and digestion of a bacteria of a given food quality was assessed during the time *O. danica* was in exponential growth (2–4 days after the start of preconditioning). FLB were used as tracers to monitor ingestion and digestion rates. FLB (1–1.5 mL) were added to



**Figure 1.** Data from two experiments showing accumulation of fluorescently labeled bacteria (FLB) into food vacuoles and the loss of FLB following dilution of the culture (indicated by dotted line) with additional heat-killed bacteria. Bars represent standard error,  $N=50$ .

12 mL of preconditioning culture to yield a final concentration of approximately 10% of the total HK bacteria. Following FLB addition, 1-mL aliquots were removed from each replicate at 5, 20, 40 and 60 min and preserved in ice-cold glutaraldehyde (2% final concentration) to prevent egestion of FLB [58]. After 60 min (post-FLB addition), 2 mL was removed from each replicate and diluted with a mixture of medium and bacteria (10 mL SMB and 8 mL HK bacteria) of the same food quality and concentration used in preconditioning thereby reducing the total concentration of FLB tenfold. Samples were taken at various time intervals up to 100 min after dilution and preserved in ice-cold glutaraldehyde.

As soon as possible after preservation, a 10- $\mu$ L aliquot of each preserved sample was placed directly on a microscope slide and covered with a cover slip. The slides were placed in a refrigerator and processed within 2 h. FLB contained in food vacuoles in each of 50 *O. danica* cells were counted.

The rate at which bacteria were ingested by *Ochromonas* was determined from the slope of a regression line fitting FLB protozoan<sup>-1</sup> to time and subsequently normal-

izing FLB uptake to their proportion of the population of HK bacteria supporting growth. Visual inspection of plots of FLB loss over time suggested an exponential decline similar to that reported by Dolan and Simek [19] (Fig. 1). Therefore, digestion rate constants (DRC) were determined from the slope of a regression line fitting the natural log of FLB protozoan<sup>-1</sup> to time after dilution. The half-life of bacteria contained in food vacuoles ( $T_{d1/2}$ ) was determined from DRC according to the formula:  $T_{d1/2} = \ln(0.5)(DRC^{-1})$ .

## Results and Discussion

There are few data characterizing the element stoichiometry of nanoflagellates largely due to the difficulties in separating elements associated with prey from elements associated with the flagellates. In protozoa, C and N appear to be maintained in a constant ratio [30], whereas considerably less is known about the C:P ratio. We have used element ratios as an index of food quality, assuming that nanozooplankton will have stoichiometric constraints similar to those of macrozooplankton (but see [24]).

**Table 2.** Characteristics of heat-killed *Pseudomonas fluorescens* used a prey for *Ochromonas danica* and ingestion and digestion rates ( $\pm$ SE) of *O. danica* when fed *P. fluorescens*

<i>P. fluorescens</i>		<i>O. danica</i>		
Cell size ( $\mu\text{m}^3$ )	C:N:P	Ingestion rate (cell protozoan <sup>-1</sup> min <sup>-1</sup> )	Digestion rate constant (min <sup>-1</sup> )	Half-life of bacteria in food vacuole (min)
0.1624 $\pm$ 0.0088	139:26:1	0.59 $\pm$ 0.01	-0.0092 $\pm$ 0.0018	76
0.1624 $\pm$ 0.0088	139:26:1	0.29 $\pm$ 0.18	-0.0108 $\pm$ 0.0024	64
0.1624 $\pm$ 0.0088	139:26:1	0.58 $\pm$ 0.09	-0.0109 $\pm$ 0.0019	64
0.1141 $\pm$ 0.0067	121:26:1	0.77 $\pm$ 0.01	-0.0079 $\pm$ 0.0019	88
0.1141 $\pm$ 0.0067	121:26:1	0.88 $\pm$ 0.10	-0.0094 $\pm$ 0.0007	74
0.1141 $\pm$ 0.0067	121:26:1	1.04 $\pm$ 0.13	-0.0091 $\pm$ 0.0005	76
0.1076 $\pm$ 0.0061	107:21:1	1.52 $\pm$ 0.11	-0.0086 $\pm$ 0.0010	81
0.1076 $\pm$ 0.0061	107:21:1	1.57 $\pm$ 0.08	-0.0100 $\pm$ 0.0014	69
0.1076 $\pm$ 0.0061	107:21:1	1.60 $\pm$ 0.08	-0.0096 $\pm$ 0.0013	72
0.1327 $\pm$ 0.0072	101:21:1	0.74 $\pm$ 0.13	-0.0083 $\pm$ 0.0018	83
0.1327 $\pm$ 0.0072	101:21:1	0.76 $\pm$ 0.02	-0.0100 $\pm$ 0.0013	69
0.1327 $\pm$ 0.0072	101:21:1	0.77 $\pm$ 0.01	-0.0095 $\pm$ 0.0007	73
0.0924 $\pm$ 0.0072	101:21:1	1.08 $\pm$ 0.06	-0.0081 $\pm$ 0.0009	86
0.0924 $\pm$ 0.0072	101:21:1	0.94 $\pm$ 0.06	-0.0087 $\pm$ 0.0015	80
0.0924 $\pm$ 0.0072	101:21:1	1.22 $\pm$ 0.10	-0.0086 $\pm$ 0.0013	81
0.0789 $\pm$ 0.0053	92:19:1	0.32 $\pm$ 0.05	-0.0060 $\pm$ 0.0017	115
0.0789 $\pm$ 0.0053	92:19:1	0.54 $\pm$ 0.06	-0.0075 $\pm$ 0.0010	92
0.0789 $\pm$ 0.0053	92:19:1	0.53 $\pm$ 0.02	-0.0068 $\pm$ 0.0020	101
0.1652 $\pm$ 0.0112	84:18:1	2.62 $\pm$ 0.18	-0.0095 $\pm$ 0.0021	73
0.1652 $\pm$ 0.0112	84:18:1	2.33 $\pm$ 0.27	-0.0097 $\pm$ 0.0023	71
0.1652 $\pm$ 0.0112	84:18:1	2.39 $\pm$ 0.16	-0.0095 $\pm$ 0.0020	73
0.0753 $\pm$ 0.0049	82:17:1	1.19 $\pm$ 0.14	-0.0103 $\pm$ 0.0024	67
0.0753 $\pm$ 0.0049	82:17:1	1.37 $\pm$ 0.36	-0.0096 $\pm$ 0.0021	71
0.0753 $\pm$ 0.0049	82:17:1	1.33 $\pm$ 0.30	-0.0088 $\pm$ 0.0024	79
0.1134 $\pm$ 0.0062	74:18:1	2.93 $\pm$ 0.36	-0.0095 $\pm$ 0.0031	73
0.1134 $\pm$ 0.0062	74:18:1	1.96 $\pm$ 0.36	-0.0100 $\pm$ 0.0019	69
0.1134 $\pm$ 0.0062	74:18:1	2.39 $\pm$ 0.65	-0.0094 $\pm$ 0.0017	74
0.1073 $\pm$ 0.0075	59:13:1	2.32 $\pm$ 0.20	-0.0100 $\pm$ 0.0027	69
0.1073 $\pm$ 0.0075	59:13:1	2.31 $\pm$ 0.40	-0.0094 $\pm$ 0.0015	74
0.1073 $\pm$ 0.0075	59:13:1	2.10 $\pm$ 0.03	-0.0102 $\pm$ 0.0024	68

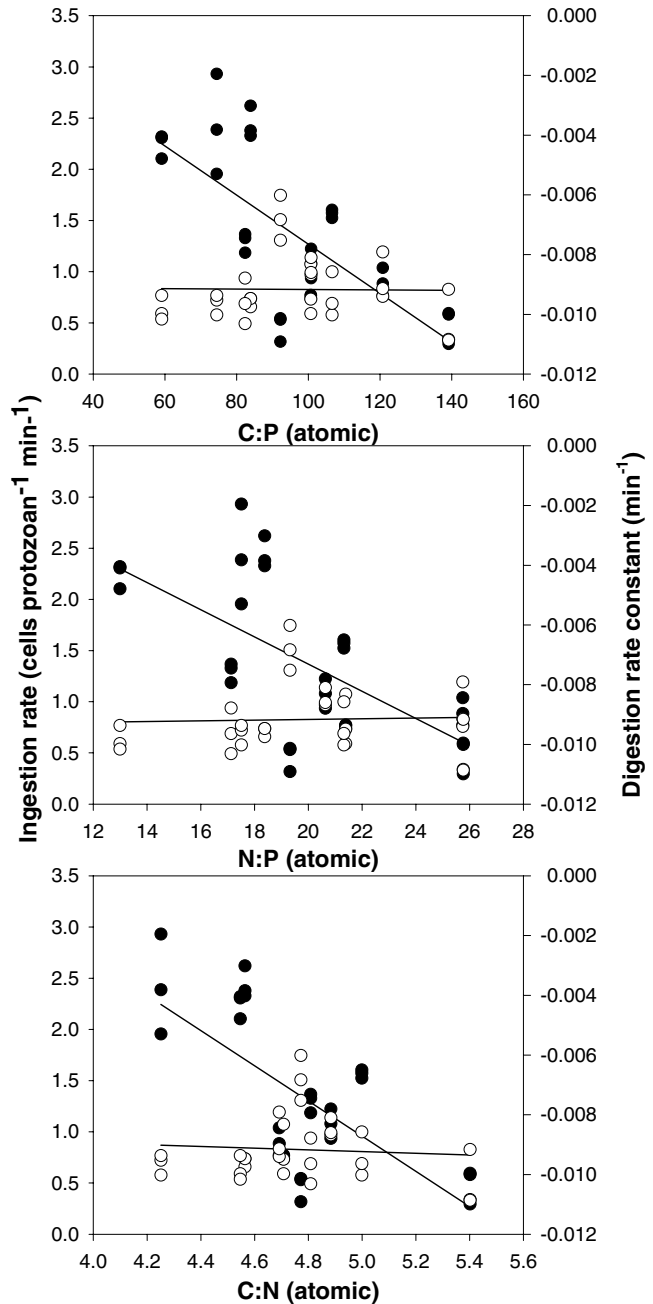
The element stoichiometry of bacteria can vary depending on growth rate, temperature, and nutrient sources [54, 64]. We grew *P. fluorescens* of varying element composition in chemostats by adjusting the dilution rate and temperature (Table 1) but without altering medium composition. Heat killing cells resulted in a significant loss of C, N, and P and, as a consequence, C and N were enriched relative to P (Table 1). Nevertheless, resulting HK cells were of distinct element composition. If the metric of food quality is considered as the ratio of C:P, then prey food quality spanned almost a twofold range; the best food quality cells had C:P ratios of 59:1 and the worst food quality cells had C:P ratios of 139:1. A similar twofold range in food quality is found when food quality is considered as N:P. N:P ratio of prey cells ranged between 13:1 and 26:1. When compared to both C:P and N:P, C:N ratios varied little and ranged between 4.3 and 5.4.

Average volumes of HK bacteria ranged between 0.0753 and 0.1652  $\mu\text{m}^3$  (Table 2). Although there was considerable scatter in the data, C:P and N:P ratios were weakly correlated to average cell volume (C:P:  $R^2=0.13$ ,  $N=30$ ,  $P=0.051$ ; N:P:  $R^2=0.13$ ,  $N=30$ ,  $P=0.055$ ). The C:N ratio was not correlated to cell volume.

**Grazing Experiments.** Grazing studies followed a pulse-chase design using FLB as tracers of HK incorporation. Abundance of HK bacteria used as prey in the various experiments ranged between  $1.0 \times 10^8$  and  $4.2 \times 10^8$  cells  $\text{mL}^{-1}$ , whereas FLB concentrations ranged between  $1.3 \times 10^7$  and  $2.5 \times 10^7$  cells  $\text{mL}^{-1}$ . The final concentration of FLB in grazing studies ranged from 4% to 19%, well within the range reported in previous studies [4, 6, 33, 57].

Two examples of typical feeding experiments are shown in Fig. 1. Table 2 shows ingestion rates and the DRC obtained for each feeding trial. Ingestion rates ranged from approximately 2 bacteria protozoan $^{-1}$  min $^{-1}$  when *Ochromonas* was fed high-quality prey (C:N:P = 59:13:1) to approximately 0.5 bacteria protozoan $^{-1}$  min $^{-1}$  when fed poor food quality prey (C:N:P = 139:26:1). Overall, ingestion rates were within the range of rates reported previously [5, 9, 19, 22, 28, 57].

It is difficult to display the relationship between food quality and ingestion rate without separating the overall measure of quality (C:N:P) into components (C:P, C:N, or N:P). The effects of prey food quality on ingestion and digestion were analyzed by simple regressions of ingestion rate or DRC upon a food quality determinant (C:P, C:N, or N:P). In each regression, 30 separate measurements were used (three independent trials from each of 10 separate feeding conditions). There was a significant correlation between ingestion and each metric of food quality: C:P,  $R^2=0.49$ ,  $P<0.0001$ ; C:N,  $R^2=0.44$ ,  $P<0.0001$ ; N:P,  $R^2=0.43$ ,  $P<0.0001$  (Fig. 2). It is somewhat arbitrary to consider separate indexes of food quality in this manner when *Ochromonas* feeds upon bacterial cells



**Figure 2.** Ingestion rate (closed circles) and digestion rate constants (open circles) for *Ochromonas danica* as a function of the element ratio of prey (*Pseudomonas fluorescens*). Each point is the rate for a single trial. Error estimates are not shown, but given in Table 2.

represented by a complete C:N:P ratio; however, the data clearly indicate that bacteria of high food quality were ingested at more rapid rates than cells of poor food quality. The experimental design did not permit an identification of a particular element that promoted grazing; ingestion rates were high if P was abundant relative to C (low C:P), if N was abundant relative to C (low C:N),

**Table 3.** Proportion of the variation in the rates at which *Ochromonas danica* ingested *Pseudomonas fluorescens* explained by the variation in prey size and food quality

Dependent variable	Independent variables	Total variation explained (%)	Model probability	Variation partitioned between independent variables (%)	Partitioned probability
Ingestion rate	Cell size	53	<0.001	9	0.029
	C:N			44	<0.001
	Cell size	68	<0.001	19	<0.001
	C:P			49	<0.001
	N:P			43	<0.001

N = 30 (three trials for each of 10 food qualities) for each model.

or if P was abundant relative to N (low N:P). Thus, the similarity among the coefficients of determination probably reflects the composite measure of food quality (C:N:P) rather than variability due to an individual element.

Bacterial cell size is a function of growth rate: rapidly growing cells are larger than slowly growing cells. Bacteria used as prey were generated in chemostats where dilution (growth) rate was manipulated to generate cells of differing food quality; consequently, the bacteria also varied in size (Table 2). Prey selection by protozoa has been linked to size distribution of the prey items [1, 15, 26, 49]. The combination of variation in cell size and variation in the element composition of the bacteria could potentially confound interpretations of grazing experiments. To examine the relative effect of size and food quality on ingestion rate, stepwise multiple regression analysis was used to assess the importance of each factor. In the multiple regression models (Table 3), both cell size and food quality were important determinants of ingestion rate, but food quality accounted for a much greater proportion, approximately 45%, of the overall variation in ingestion rate. Variation in size of prey items accounted for 9–23% of the overall variation in ingestion rate.

The DRC (see Table 2) varied little and averaged  $0.00916 \pm 0.0002 \text{ min}^{-1}$ . In contrast to the strong correlations observed between ingestion rates and element ratios, no correlation was found between the DRC and the element ratio of the prey. The half-life of bacteria in a food vacuole averaged 77 min and was similar to the half-life of vacuole contents reported for *Bodo* sp. [19]. Digestion rates appear to be independent of food concentration [8, 9, 19, 23], and are also more likely a factor of the physiology of the protozoan than of the prey. Digestion rate is likely affected by accessibility of digestive enzymes [8], accumulation efficiencies [69], metabolic rate [7], or growth state [23, 56].

Many aspects of the impact of flagellate predators on bacterial communities have been examined previously. Much of this research focuses on the outcomes of the predator–prey interaction, for example, the size of cells

remaining in the community that are more or less resistant to grazing, the development of a prey community dominated by grazer resistant morphologies [38], or the taxonomic classes of cells more or less subject to grazing [29]. Often alluded to, but less frequently investigated, are those features of the prey items that make them susceptible to predation [39]. Those features that have been considered—capsules, surface hydrophobicity and charge—have been, to a large extent, found to be unimportant regulators of predation [23, 36, 38, 39].

Clearly, some flagellates can discriminate between high-quality and poor-quality prey items. John and Davidson [30] found that *Paraphysomonas vestita* ingested low C:N algal cells (N-replete) at higher rates than high C:N cells (N-deplete). When ample food is available, flagellates will discriminate between low-quality (as microbeads) and high-quality food (as bacteria), preferring to feed upon the bacteria [31]. In situations where microbeads were found to be ingested at rates similar to those at which bacteria were ingested, the beads were also rapidly egested [8]. All bacteria are not of equivalent quality as prey. Bacteria grown under P limitation were captured by flagellates with high frequency, yet most were immediately egested [39]. These studies, as well as the work reported herein, suggest that other features of bacterial cells, apart from the well-studied aspect of cell size, may supply important cues to predators.

Several lines of evidence are beginning to converge, reinforcing the notion that flagellates may be selecting the rapidly growing members of the bacterial community [3, 17, 29, 46, 56, 60]. Part of the selection process, as revealed in this work, may be associated with the element composition of prey items. Bacterial cell size and macromolecular composition are functions of growth rate: rapidly growing cells are larger than slowly growing cells; and RNA, protein, and to a lesser extent DNA, all increase as growth rate increases [11, 27]. Basic microbial physiology and evolving theory in ecological stoichiometry predicts that rapidly growing cells are characterized by low C:P and N:P ratios [16]. The low ratios are brought about by



an increase in P-rich macromolecules, largely RNA, required to meet the demands of protein synthesis. Therefore, selection based on size may simply be a consequence of a different selection cue, the element composition of cells (see discussion in [67]). Multiple regression analysis (this article) lends support to this position; however, it is important to realize that in the experimental design of this work, *Ochromonas* was not given a choice among prey types. Clearly, high-quality prey items were ingested at higher efficiencies than low-quality prey items, but it remains to be determined if *Ochromonas* would select high-quality items when presented a mixture of high- and low-quality prey. In addition, a potential source of error in this experimental design is the use of HK bacteria. Flagellates have been shown to discriminate between live and HK bacteria of the same strain [35]. Thus it also remains to be determined if prey selection would be similar if live bacteria of varying quality were used as prey.

Features of the bacterial cells acting as cues to feeding or ingestion also remain unknown. Clearly, cell size is important in the overall process and although element composition of cells has suggested some potential links to metabolic processes, it seems unlikely that a given mineral element itself accounts for ingestion efficiency (see above). Moreover, as heat-killed cells were used in this work, capsules and surface characteristics of cells were likely removed or altered compared to that of live cells. Soluble cytoplasmic signals could also be disregarded because heating and washing of cells probably greatly diminished their potential influence. However, recent findings relating to digestion processes seem to suggest that some aspect of the protein content of prey may be important in regulating flagellate predation. Microbeads are generally a poor food surrogate in grazing studies; however, microbeads coated with protein (bovine serum albumin) seem to be ingested at higher rates than uncoated microbeads [37]. Bacterial cells with low C:N content, which may be indicative of high protein content, also appear to be preferentially ingested ([30], this study). Zubkov *et al.* [69] have shown that bacterial proteins appear to be digested by flagellates more easily than other bacterial macromolecules and this finding, when coupled with data indicating high N regeneration by flagellates [70], suggests a flagellate metabolism largely based on protein degradation.

Food quality has long been associated with predator prey selection through optimal foraging theory [40]. In terrestrial and aquatic food webs, predators have been found to exploit resources (prey) having the greatest food quality whether the metric of food quality is mineral content ([41], but see [14]), ratios of essential elements [20, 25, 62], or growth rate [12, 13, 18, 21]. Only recently have similar concepts been reported for flagellate nanozooplankton of microbial food webs ([30], this study). Although we cannot assert that differential

ingestion rates based on food quality is a concept that fully extends to all flagellate nanozooplankton, it certainly appears to be a feature that warrants further study.

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