

COMPARISON OF INGESTION AND DIGESTION RATES OF *OCHROMONAS*  
*DANICA* GRAZING ON *PSEUDOMONAS FLUORESCENS*  
OF VARYING FOOD QUALITY

by

STEPHEN PAUL SHANNON

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ABSTRACT

COMPARISON OF INGESTION AND DIGESTION RATES OF *OCHROMONAS*  
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OF VARYING FOOD QUALITY

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Stephen Paul Shannon, Ph.D.

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Supervising Professor: Thomas H. Chrzanowski, Ph.D.

Flagellate prey selection appears to depend on morphological characteristics of prey such as cell size and motility, as well as on physiochemical characteristics such as digestibility and cell surface characteristics. The bacterium, *Pseudomonas fluorescens*, was grown in chemostats at four dilution rates (0.03, 0.06, 0.10, 0.13 h<sup>-1</sup>) and three temperatures (14, 20, 28°C) to produce cells of varying morphological characteristics (cell size) and physiochemical characteristics or food quality (as determined by the C:N:P ratio). Heat-killed bacteria of a given food quality were prepared and used to grow the flagellate *Ochromonas danica*. Ingestion and digestion rates were determined 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 use chemosensory cues to preferentially select food items.

## TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	iii
ABSTRACT .....	iv
LIST OF ILLUSTRATIONS.....	viii
LIST OF TABLES.....	x
Chapter	
1. INTRODUCTION .....	1
2. MATERIALS AND METHODS .....	5
2.1 Stock Cultures.....	5
2.1.1 Organisms.....	5
2.1.2 Chemostats.....	6
2.2 Preparation of Cells for Feeding Experiments.....	7
2.2.1 Heat Killed Bacteria .....	8
2.2.2 Fluorescently Labeled Bacteria .....	9
2.2.3 Growth Kinetics of <i>Ochromonas</i> fed <i>Pseudomonas</i> .....	10
2.3 Experimental Design for Grazing Experiments.....	10
3. RESULTS AND DISCUSSION.....	13
3.1 Growth Kinetics.....	13

3.2 Preparation of Cells of Varying Food Quality.....	14
3.3 Grazing Experiments .....	19
REFERENCES .....	40
BIOGRAPHICAL INFORMATION.....	58

## LIST OF ILLUSTRATIONS

Figure	Page
2.1 <i>O. danica</i> with ingested FLB (green) and chloroplasts (red).....	12
3.1 Growth kinetics of <i>Ochromonas</i> growing on <i>Pseudomonas</i> .....	13
3.2 Comparison of distribution of HK and FLB cells.....	17
3.3 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 84:18:1 .....	21
3.4 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 82:17:1 .....	22
3.5 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 59:13:1 .....	23
3.6 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 74:18:1 .....	24
3.7 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 101:21:1 .....	25
3.8 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 101:21:1 .....	26
3.9 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 121:26:1 .....	27
3.10 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 139:26:1 .....	28
3.11 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 92:19:1 .....	29
3.12 Three separate feeding trials when <i>O. danica</i> was fed heat killed bacteria having a CNP = 107:21:1 .....	30



3.13	Comparison of ingestion rate and digestion rate constants with element ratios (C:N, N:P, C:P) .....	34
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## LIST OF TABLES

Table	Page
2.1 Chemostat preparation matrix .....	6
3.1 C:N:P of chemostat grown cells (mole:mole).....	14
3.2 C:N:P of heat killed cells (mole:mole).....	14
3.3 Comparison of the mean cell sizes and distribution of HK and FLB cell sizes of bacteria used in feeding studies.....	16
3.4 Characteristics of <i>Pseudomonas fluorescens</i> used a prey for <i>Ochromonas danica</i> and ingestion and digestion rates ( $\pm$ SE) of <i>O. danica</i> when fed <i>P. fluorescens</i> .....	31
3.5 Proportion of the variation in the rates at which <i>Ochromonas</i> <i>danica</i> ingested and digested <i>Pseudomonas fluorescens</i> of varying size and food quality. N=30 for each model. ....	35

## CHAPTER 1

### INTRODUCTION

Our understanding of the role of bacteria in ecosystem dynamics has evolved considerably since Lindeman (1942) first incorporated 'bacterial ooze' into the trophic dynamic model of Cedar Bog. Much of this evolution has occurred since the publication of two seminal studies on marine food webs. Pomeroy's (1974) integration of data on rates of carbon and energy flow through various components of the oceanic food web brought about the realization that a significant proportion of carbon and energy in the planktonic oceanic food web must flow through microorganisms. This paradigm represented a significant shift in the understanding of food webs in general since it not only added new pathways to existing food web structure, but also shifted the relative importance of carbon and energy flow from the classical phytoplankton-zooplankton food-web paradigm to these new microbial pathways. Azam et al. (1983) developed a similar concept by coupling data addressing bacterial abundance and growth rate to data suggesting high rates of predation upon bacteria by flagellate protozoa. Termed the 'Microbial-Loop', this food web paradigm suggests that bacterial assemblages bottle-neck a potentially large carbon and energy flow by sequestering carbon (and mineral nutrients) in bacterial biomass and that this bottle-neck is relieved by predation upon bacteria by flagellate nanozooplankton.

A considerable literature now exists characterizing the role of flagellates (both mixotrophic and heterotrophic) in planktonic systems and this body of work has fully embedded the concept of the microbial loop into aquatic trophic dynamics. While research into this pivotal area continues, separate lines of investigation have begun to describe some of the consequences of flagellate grazing on bacterial communities and have begun to elucidate some of the factors that regulate or control flagellate grazing on bacteria.

Flagellate grazing has been shown to influence the size distribution of bacterial communities (Andersson et al. 1986, Chrzanowski and Simek 1990, Simek et al. 1994, Kinner et al. 1998, Hahn and Hofle 1999, Pfandl et al. 2004) and size-selective grazing is widely believed to contribute to the small average-cell-size of bacterioplankton within these communities. These data also suggest that large bacterial morphotypes are an effective defense mechanism against flagellate predation. Moreover, flagellate predation pressure may cause bacterial assemblages to become dominated by motile cells (Gonzalez et al. 1993) or cell types having complex inedible morphologies, such as filaments and microcolonies. Thus, flagellate predation upon bacteria may ultimately lead to changes in the structural and taxonomic composition of bacterial communities (Jurgens and Matz 2002, Wu et al. 2004, Jezbera et al. 2005).

A variety of feeding strategies for flagellates have been characterized. They are typically grouped according to their contact with the substrate: free swimming, temporarily attached, or attached. Since most flagellates feed on small food items, attachment has been considered to be an important mechanism to increase contact

probability thus leading to an increase in ingestion rate (Fenchel 1986). Only a few flagellate species appear to be raptorial feeders and these usually occur in areas where bacteria are concentrated (Boenigk and Arndt 2002). Free-swimming flagellates seem to feed more extensively on larger food particles such as algae and other protists than do attached flagellates (Boenigk and Arndt 2000).

A hyperbolic relationship exists between flagellate growth rate and initial prey concentration (Eccleston-Parry and Leadbeater 1994, Zubkov and Sleight 1995). A critical minimum food concentration is required to enable flagellates to capture enough food items to sustain growth and support reproduction. Food selection appears to involve both active (ingestion) and passive components (contact probability and handling) (Jürgens and DeMott 1995, Boenigk et al. 2002).

The probability of prey being ingested by a flagellate has been shown to be correlated with both prey size (Fenchel 1987, Kiørboe and Titelman 1998) and prey motility (Monger and Landry 1992, González et al. 1993) since both factors increase encounter probability. Thus, individual feeding capacity is positively correlated with cell size, and larger, more motile species are generally ingested at higher rates than are small non-motile species (González et al. 1990, 1993; Chrzanowski and Simek 1990, Boenigk 2002).

Prey selection also depends on both morphological and physicochemical characteristics including digestibility and cell surface characteristics (González et al. 1990, 1993; Monger et al. 1999, John and Davidson 2001, Matz et al. 2002a, 2002b). Surface characteristics, such as hydrophobicity, have been shown to affect the contact

probability and ingestion of bacteria by phagocytes of mammalian immune systems (VanOss 1978, Absolom 1998). However, Matz and Jurgens (2001) have shown that neither hydrophobicity nor electrostatic cell surface properties affect uptake rates of suspended bacteria by raptorial feeding flagellates. Nisbet (1987) speculated that selection of items as prey may be, in part, receptor mediated and regulated by 'signal substances' such as phospholipids on the surface of the prey. Thus, food quality may influence ingestion rate and food selection (Boenigk et al. 2001c). In support of this hypothesis, *Paraphysomonas* was found to discriminate between two phytoplankton prey species of differing food quality (measured as the ratio of carbon to nitrogen). Contrary to this speculation, rates of ingestion of bacteria by *Spumella* and *Ochromonas* were not significantly affected by the presence of inert microbeads (Boenigk et al. 2002). Here, we further explore the feeding behavior of flagellates preying upon bacteria through an investigation of the relationship between ingestion and digestion of bacterial prey of varying food quality. Based on the forgoing discussion, the following hypotheses were developed.

Null hypothesis: Digestion times of *P. fluorescens* by *O. danica* will be equal regardless of food quality (nutrient-rich cells = C:P ratio of approximately 50:1; nutrient-poor = C:P ratio of approximately 150:1).

Alternate hypothesis: Digestion times for nutrient-rich cells is slower than digestion time for nutrient-poor cells.

## CHAPTER 2

### MATERIALS AND METHODS

#### 2.1 Stock Cultures

##### *2.1.1 Organisms*

*Pseudomonas fluorescens* encompasses a group of common, nonpathogenic saprophytes that colonize soil, water, and plant surface environments. It secretes a soluble fluorescent pigment, fluorescein. It is Gram-negative and is mobile by means of multiple polar flagella. *P. fluorescens* has simple nutritional requirements and grows well in mineral salts media supplemented with any of a large number of carbon sources. *P. fluorescens* has been used in many protozoan grazing studies (Lawrence and Snyder 1998, Hahn and Hofle 1999, Hammer et al. 2001, Huws et al. 2005, Matz and Jurgens 2005, and many others). *P. fluorescens* (ATCC 3214) stock cultures were maintained on Standard Mineral Base (SMB) (White and Hegeman 1998) supplemented with 10 mM glucose (SMBg).

The nutrition of the Genus *Ochromonas* was first analyzed by Pringsheim (1952). The genus has been well studied and contains chrysoomonads typically having two unequal flagella and at least one chloroplast. *Ochromonas danica* is unusually plastic and capable of assuming a variety of shapes ranging from the normal tear-drop to spherical (Pringsheim 1952). *O. danica* is considered mixotrophic since some strains

are capable of growth in the dark on dissolved organic carbon (osmotrophic), by ingesting food (phagotrophic), or through photosynthesis (autotrophic) (Brown and Bouck 1973, Daley et al. 1973, Sibbald and Albright 1991). The strain used in this study, *O. danica* (UTEX 1298), was incapable of autotrophic growth and maintained in *Ochromonas* Medium (Starr 1978).

### 2.1.2 Chemostats

Chemostats were used to grow bacteria (described in detail in section 2.2) required for feeding experiments. Chrzanowski (unpublished data) has shown that *Pseudomonas fluorescens* of known C:N:P ratios can be produced by adjusting chemostat parameters. Specifically, adjustments in growth rate and temperature were utilized to produce cells of known C:N:P ratios. *Pseudomonas fluorescens* was grown in SMBg in continually stirred and aerated 800 mL chemostats (Applikon) at four dilution rates ( $d = 0.03, 0.06, 0.10, 0.13 \text{ h}^{-1}$ ) and three temperatures (14, 20, 28°C). Table 2.1 identifies experiments conducted at each dilution rate and temperature combination.

Table 2.1 Chemostat preparation matrix

Dilution Rate	Temperature (C)		
	14	20	28
0.03	Exp 2	Exp 4	Exp 7
0.06	Exp10	Exp 13	Exp 16
0.10	Exp 19	Exp 22	Exp 25
0.13	Exp 28	Exp 31	Exp 34

Reactors were assumed to be in steady-state after three complete turnovers at a given temperature and dilution rate. Outflow was aseptically captured in pre-sterilized 1-L bottles (Nalgene). Cell abundance was determined by direct epifluorescent



microscopic enumeration (1250X) of formaldehyde preserved (2% final concentration) samples using DAPI as the fluorochrome (Porter and Feig 1980). Bacteria were assumed to be represented as a cylinder with two half-spheres on each end. 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 in  $\mu\text{m}$ .

Length and width of individual cells was determined from digital images (Olympus DP70) and Simple PCI imaging software (Compix, Inc.).

The carbon (C) and nitrogen (N) content of cells was determined using a CHN analyzer (Perkin-Elmer series 2200 CHN Analyzer). The P content of cells was determined from persulfate digests and subsequent SRP analyses (Strickland and Parsons 1972). Ratios of elements are reported as mole:mole.

## 2.2 Preparation of Cells for Feeding Experiments

Cells harvested from chemostats (720 mL) were distributed into conical polypropylene centrifuge tubes (Nalgene) and pelleted (Sorvall RT6000B 5000 rpm, 15°C, 25 min). Supernatants were discarded, pellets combined into one tube, and 180 mL phosphate-buffered saline (PBS; 0.83% NaCl, 0.238%  $\text{K}_2\text{HPO}_4$ , 0.0467%  $\text{KH}_2\text{PO}_4$ ) was added resulting in a 4X concentration of original sample. The homogenized 4X sample was divided into two portions: 150 mL to prepare heat killed (HK) bacteria and 30 mL to prepare fluorescently labeled bacteria (FLB).

### 2.2.1 Heat Killed Bacteria

Preparations of heat-killed cells, instead of live cultures, have been routinely used as prey in laboratory grazing experiments (Pace and Bailiff 1987, Bloem et al. 1989, Sherr and Sherr 1993, Bratvold et al. 2000, Zhang and Watanabe 2001, Fu et al. 2003). Some advantages of using heat-killed stocks include: knowledge of food quality (as C:N:P ratios) prior to starting an experiment; prey cells are not able to grow, reproduce, exhibit defensive mechanisms, or produce toxic substances; and afford greater experimental control. Two key items were addressed when preparing heat-killed cells. The cell integrity must be maintained and the non-viability must be ensured. Inactivation methods involving steam, UV light, or chemicals can cause significant damage to the cell, altering the quality of the cell as a prey item. Consequently a gentle heat killing procedure was established (Sherr et al. 1987, Landry et al. 1991). It was critical to minimize the amount of time and temperature required to inactivate the population while still maintaining cell integrity.

The time-temperature relationship necessary to produce heat killed *P. fluorescens* was established by trial-and-error. Bacteria (4X concentrated from chemostats) were held for two hours at 70°C in a water bath and mixed every 30 minutes. Following heat killing, cells were pelleted, supernatant discarded, and resuspended in 150 mL SMB. Cell concentration, cell size, and element content were determined as described above.

### *2.2.2 Fluorescently Labeled Bacteria*

Fluorescently labeled bacteria (FLB) are commonly used to measure bacterivory in protozoan communities (Sherr et al. 1987, Bloem et al. 1989, Epstein and Roseel 2004). A widely cited reference for the preparation of the FLB is Sherr et al. (1987), in which the authors describe a mono-dispersed method and compare the FLB method with the use of microspheres. Other methods for measuring bacterivory rates include: radioactive labeling (Taylor and Sullivan 1984, Nygaard and Hessen 1990); fluorescent objects (Borsheim 1984, McManus and Fuhrman 1986, Wiedner and Vareschi 1995) and genetically marked minicells (Wikner et al. 1986, Wikner 1993). Many types of stains have been used to prepare FLBs. Sherr et al. (1987) used 5-([4,6-dichlorotriazin-2yl] amino) fluorescein (DTAF), Rodriguez et al. (1992) utilized 5 cyano-2,3 ditolyl tetrazolium chloride (CTC), and Alonso et al. (2000) preferred rhodamine isothiocyanate. The present studies were patterned after Sherr et al. (1987) and employ DTAF as the fluorescence agent.

Advantages of using FLB include: the ability to prepare FLB from natural populations or pure isolates; the capability to store and use when needed without a significant loss of fluorescence intensity; and an insignificant impact on the nutritional value to protozoan (i.e. FLB are not toxic and can be metabolized to support protozoan growth). There are some challenges as well, specifically in the preparation of the FLB. When utilizing DTAF as the fluorochrome several key parameters have to be balanced. The cells have to be stained well enough so that they can be observed inside the target organism while at the same time not affecting the phagotroph itself. Based on

preliminary experiments, retention of undissolved DTAF made it exceedingly difficult to differentiate cells within the food vacuole. Removing undissolved DTAF reduces the interference and improves observation and enumeration of FLB. If the FLB are not stained properly FLB may be indistinguishable from live bacteria or heat-killed bacteria. It is critical that FLB be clearly discernable so that accurate measures of bacterivory rates can be determined. Six mg of 5-([4,6-dichlorotriazin-2yl] amino) fluorescein (DTAF) was added to 30 mL of bacteria (4X concentrated suspended in PBS) and vortexed. The mixture was held for two hours at 60°C in a water bath and mixed every 30 minutes, after which, cells were pelleted, and washed (at least 3X) with 30 mL PBS. The pellet was resuspended in 30 mL SMB and stored refrigerated. Cell concentration was determined by direct microscopic enumeration.

### 2.2.3 Growth Kinetics of *Ochromonas* fed *Pseudomonas*

A concentration gradient ranging between  $5 \times 10^5$  and  $3 \times 10^8$  HK *Pseudomonas*  $\text{mL}^{-1}$  was prepared. Each culture (40 mL) in the gradient was inoculated with 1 mL of a stationary phase *Ochromonas* culture and incubated with gently shaking at 22°C. Two mL samples were taken daily for five to seven days and preserved in 2% ice-cold glutaraldehyde (2% final concentration, Sieracki et al. 1987). *Ochromonas* abundance was determined by direct microscopic enumeration (500X).

## 2.3 Experimental Design for Grazing Experiments

Each grazing experiment consisted of four stages: preconditioning, ingestion, digestion, and enumeration. During preconditioning, cultures were prepared using 20 mL of HK bacteria, 16 mL of SMB, and 12 mL of *O. danica* in stationary phase

(*Ochromonas* medium). Each set was grown using HK bacteria originating from one of the chemostat growth rate-temperature combinations.

The ingestion and digestion phase for each experiment occurred during the time *O. danica* was in exponential growth (two to four days after the start of preconditioning). A pulse of FLB were added to the cultures and used as tracers to monitor ingestion and digestion rates. FLBs were added to yield a final tracer concentration approximately 10% of the total bacteria available for ingestion. Experiments were run in triplicate for each of the grazing studies.

Once FLBs were added, 1 mL aliquots were removed from each replicate at 5, 20, 40 and 60 minutes and preserved in ice-cold glutaraldehyde. After 60 minutes, the FLB pulse was chased with unlabelled HK bacteria (same used in preconditioning) to dilute the concentration of FLB ten-fold. Samples were taken at 10, 25, 50, 75, and 100 minutes (post chase) and preserved in glutaraldehyde.

Previous studies have used a variety of sizes and types of filters, numerous contrast staining techniques, and different filter sets for counting under epifluorescence (Pace and Bailiff 1987, Sieracki et al. 1987, Bloem et al. 1988, Sherr and Sherr 1993, Wikner 1993, Landry 1994, Isaksson et al. 1999, Bratvold et al. 2000, Zhang and Watanabe 2001, Ishii et al. 2002, Fu et al. 2003, Matz and Jurgens 2003, Epstein and Roseel 2004). In this study a direct observation method was used to enumerate ingested bacteria. A ten  $\mu$ L aliquot of each preserved sample was placed directly on a microscope slide, covered with a cover slip and the slides were placed in the refrigerator until counted, typically within two hours. FLB contained in food vacuoles in each of 50

*O. danica* cells were counted (500X). An example of FLB contained within food vacuoles of *O. danica* is shown in Figure 2.1.



Figure 2.1 *O. danica* with ingested FLB (green) and chloroplasts (red)

## CHAPTER 3

### RESULTS AND DISCUSSION

#### 3.1 Growth Kinetics

*O. danica* displayed characteristic growth kinetics when feeding on heat-killed *P. fluorescens*. Growth rate of *O. danica* remained constant above a prey concentration of  $5 \times 10^5 \text{ ml}^{-1}$ . Consequently, all subsequent experiments were conducted at prey densities sufficient to ensure that growth of *O. danica* were saturated.

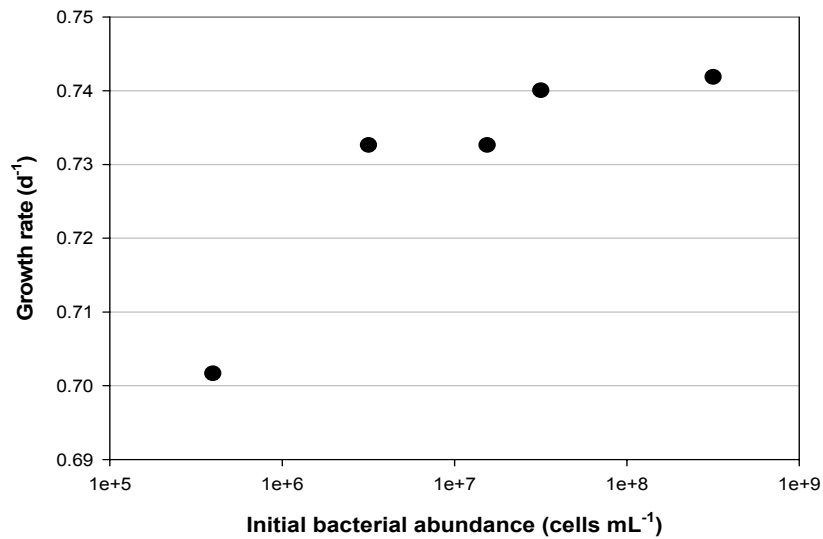


Figure 3.1 Growth kinetics of *Ochromonas* growing on *Pseudomonas*

### 3.2 Preparation of Cells of Varying Food Quality

The ratio of nutrient elements (N and P) relative to C has often been used as an index of food quality (Sterner 1993, Sterner and Smith 1993, Kilham et al. 1997, Albarino and Balseiro 2001, Rubenstein and Wikelski 2003, Park et al. 2003). Low values of the ratio (C:N or C:P) are indicative of a high quality food resource. The element stoichiometry of bacteria can vary depending on growth rate, temperature, and nutrient sources (Schaechter et al. 1958, Tempest and Hunter 1965). *P. fluorescens* cultures of known element composition were grown in chemostats by adjusting dilution rate and temperature. The element stoichiometry (C:N:P) of chemostat grown cells is given in Table 3.1.

Table 3.1 C:N:P of chemostat grown cells (mole:mole)

Dilution Rate (h <sup>-1</sup> )	Temperature (C)		
	14	20	28
0.03	91:16:1	51:8:1	44:7:1
0.06	65:11:1	70:12:1	66:11:1
0.10	68:12:1	61:10:1	72:11:1
0.13	56:11:1	72:13:1	61:12:1

Table 3.2 C:N:P of heat killed cells (mole:mole)

Dilution Rate (h <sup>-1</sup> )	Temperature (C)		
	14	20	28
0.03	84:18:1	90:18:1	88:18:1
0.06	82:17:1	59:13:1	74:18:1
0.10	101:21:1	101:21:1	121:26:1
0.13	139:26:1	92:19:1	107:21:1



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 3.2). Nevertheless, resulting cells for use as prey items were of distinct element composition. If food quality was considered as the ratio of C:P, then prey food quality spanned almost a two-fold 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 range in the ratio of elements was found when N and P were considered. N:P 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.

FLBs produced from the same HK bacteria fed to *O. danica* were used to trace the incorporation of HK cells by the protozoan. The mean cell size (Table 3.3) and size distribution (Figure 3.2) of both types of cells (HK and FKB) are shown below.

Table 3.3 Comparison of the mean cell sizes and distribution of HK and FLB cell sizes of bacteria used in feeding studies. S.E. = standard error, N = number of cells measured,  $P_m$  = probability that the mean cells sizes are different,  $\chi^2$  = Chi square,  $P_\chi$  = probability that the distribution of cells sizes are different.

Preparation	Mean cell size ( $\mu\text{m}^3$ ) $\pm$ S.E (N)		$P_m$	$\chi^2$	$P_\chi$
	HK	FLB			
2	0.1652 $\pm$ 0.0112 (172)	0.1161 $\pm$ 0.0091 (62)	0.012	12.72	0.079
10	0.0757 $\pm$ 0.0049 (116)	0.1509 $\pm$ 0.0089 (122)	<0.0001	33.02	0.001
13	0.1073 $\pm$ 0.0074 (100)	0.0990 $\pm$ 0.0041 (283)	0.317	1.88	0.966
16	0.1134 $\pm$ 0.0062 (199)	0.0653 $\pm$ 0.0029 (228)	<0.0001	29.94	0.001
19	0.1328 $\pm$ 0.0072 (123)	0.1523 $\pm$ 0.0054 (154)	0.028	13.64	0.058
22	0.0924 $\pm$ 0.0072 (104)	0.1105 $\pm$ 0.0051 (128)	0.038	8.790	0.117
25	0.1141 $\pm$ 0.0067 (126)	0.1001 $\pm$ 0.0044 (230)	0.084	0.000	1.000
28	0.1623 $\pm$ 0.0088 (240)	0.1159 $\pm$ 0.0095 (52)	0.017	13.434	0.062
31	0.0799 $\pm$ 0.0053 (117)	0.1266 $\pm$ 0.0054 (132)	<0.0001	0.000	1.000
34	0.1076 $\pm$ 0.0061 (148)	0.0963 $\pm$ 0.0046 (114)	0.163	17.273	0.008

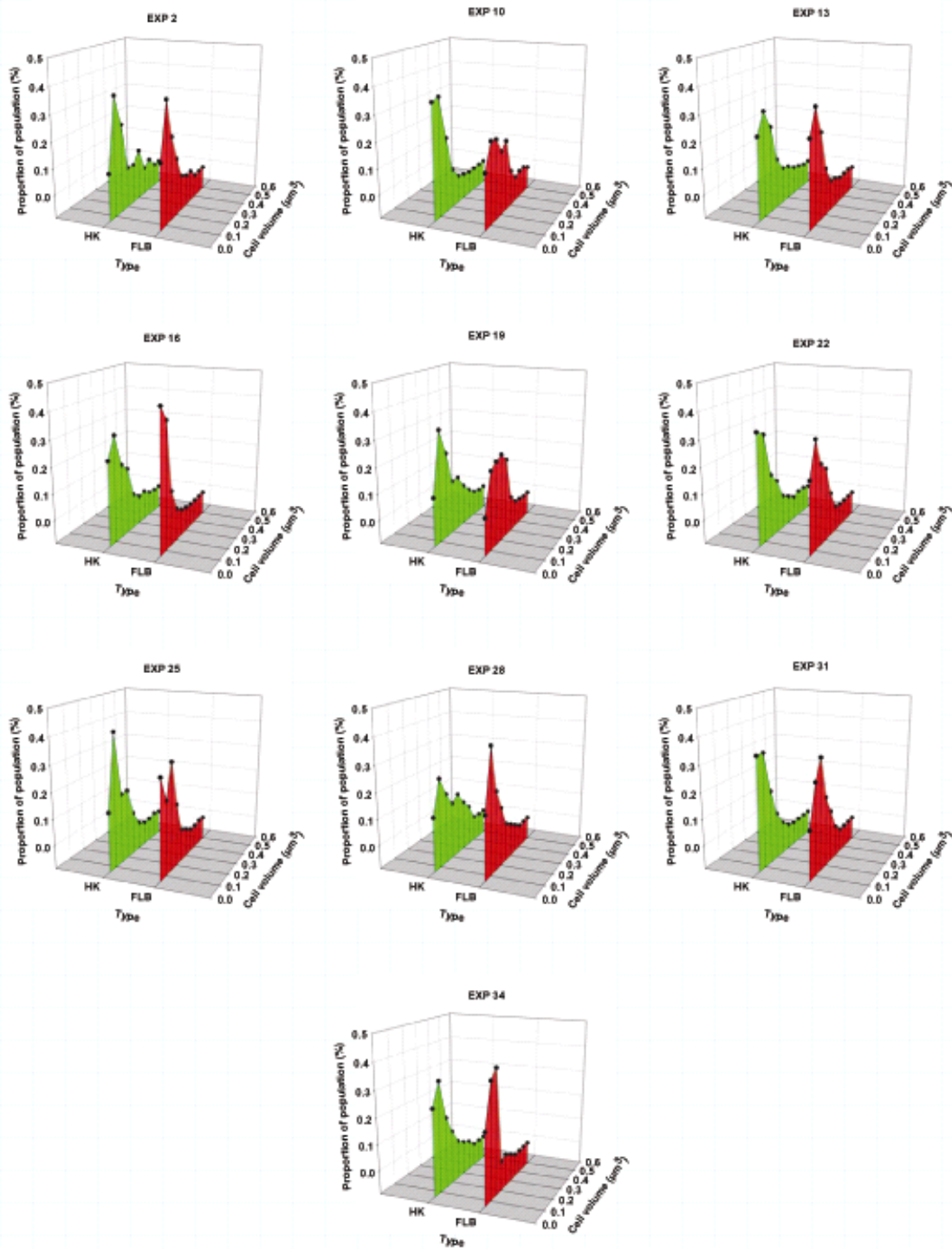


Figure 3.2 Comparison of distribution of HK and FLB cells

Mean cell size for HK ranged from  $0.0757 \mu\text{m}^3$  to  $0.1652 \mu\text{m}^3$  while the size for the FLB ranged from  $0.0653 \mu\text{m}^3$  to  $0.1523 \mu\text{m}^3$ . Table 3.3 and Figure 3.2 reveal size (as  $\mu\text{m}^3$ ) distribution differences between HK and FLB in three of the ten preparations while mean cell size differed in seven out of the ten preparations. One question that stems from these differences is: Did *Ochromonas* discriminate between HK and FLB in the grazing experiments? If so, then grazing rates could either be under- or over-estimated.

Since all of the cells used in each experiment originated from the same source and were treated in the same way (i.e. heat killing or staining), it is hard to accept that such great differences in mean cell volume could be found. One potential source of error lies with the fluorochrome and how the software and investigator processes the samples. DTAF is a general stain (targets multiple structures in cells) and used for FLB preparation while DAPI, used for the staining HK cells is a more structure-specific fluorochrome (targets DNA). Thus, different fluorescent signatures result (lightness, flare) that effect edge detection when attempting to measure cells either directly or by electronic means. Further, for reasons that are not entirely understood, the effectiveness and evenness of staining varies from preparation to preparation and from day to day introducing uncontrolled variability in estimate of cell size.

Despite the limitations outlined above, when the cell volumes are converted to an equivalent sphere diameter (ESD) where  $\text{ESD} = \sqrt[3]{4/3\pi R^3}$ , the differences in cell size really suggest only a slight shift in the size of the bacteria. For example, the worst case was Experiment 10 with almost 100% difference in volume between FLB and HK, but

the ESD differences were minor (0.53 versus 0.66  $\mu\text{m}$ ). When utilizing the ESD concept and applying it to other studies, it was clear that the range of cells used in the feeding experiments were in the ranges typically grazed by *Ochromonas* (Chrzanowski and Simek 1990, Gonzalez et al. 1996, Hahn and Hofle 1999, Pfandl et al. 2004).

These conclusions are reinforced when the distribution of cell sizes are considered. Cells were arbitrarily grouped into 0.1  $\mu\text{m}^3$  divisional categories. Using these categories, the size distribution of FLB and HK cells differed in only three experiments (see Table 3.3). It is likely that these differences would disappear if less stringent size categories were used.

### 3.3 Grazing Experiments

The impact of pre-culture conditions on feeding rate has been considered and found to influence grazing rate (Sin et al. 1998, Wilhelm et al. 1998, Boenigk et al. 2001c, Sanders et al. 2001). Consequently, during the preconditioning phase of each experiment *O. danica* was allowed to adjust feeding behavior to, and grow exclusively on, HK bacteria.

Grazing studies followed a pulse-chase design using FLBs as tracers of HK incorporation. Abundance of HK bacteria ranged between  $2 \times 10^8$  and  $1 \times 10^9 \text{ mL}^{-1}$  while FLB concentrations ranged between  $1 \times 10^8$  and  $3 \times 10^8 \text{ mL}^{-1}$ . The concentrations of total prey items should have been sufficient to saturate ingestion rate and growth rate (Grover and Chrzanowski, submitted). The final concentration of FLB in the grazing studies ranged from 4% to 19% of total bacteria, well within the range reported in previous studies (Sherr et al. 1987, Bloem et al. 1989, Ooms-Wilms 1991, Hondeveld et

al. 1992, Sherr and Sherr 1993, Keller et al. 1994, Boenigk et al. 2001a, Soto-Rodriguez et al. 2003, Epstein and Roseel 2004).

The incorporation and loss of FLB in each pulse-chase grazing experiment are shown in Figures 3.3 through 3.12. In each case FLB were rapidly incorporated into food vacuoles. *Ochromonas* cells typically contained between 5 and 10 FLB after 60 minutes. Following dilution of FLB with additional HK bacteria, FLB were gradually lost from the vacuoles. Dolan and Simek (1998) characterized uptake of FLB as a linear process while digestion (or loss) of FLB appeared to be logarithmic. Consequently, FLB uptake rate was determined from the slope of a regression line fitting FLB protozoan<sup>-1</sup> to time. Total ingestion of bacteria was determined by normalizing FLB uptake to the proportion of FLB in the population of HK bacteria supporting growth (McManus and Okubo 1991). Digestion rate constants were determined from the slope of a regression line fitting Ln (FLB) to time after dilution (Dolan and Simek 1998). Table 3.4 shows ingestion rates and the digestion rate constants for each of the replicates within each feeding trial.

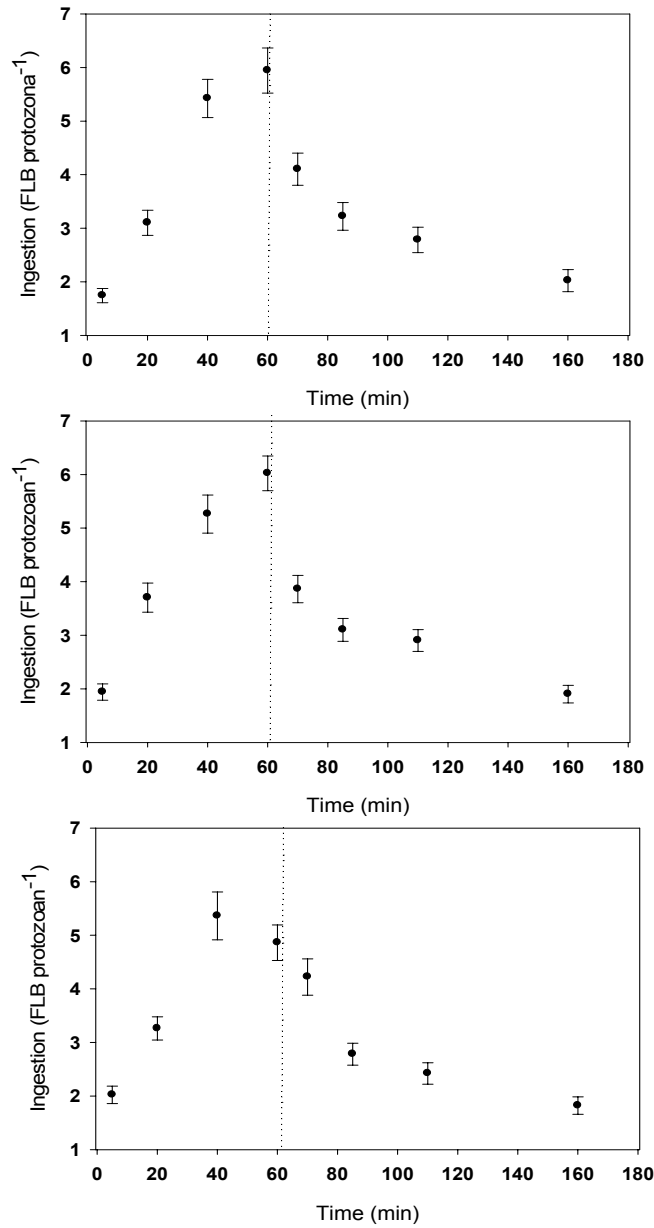


Figure 3.3 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 84:18:1. Each point represents the mean FLB occurring in 50 protozoa. Bars are  $\pm 1$  S.E.. At time 0, a pulse of FLB was added to the HK bacteria. The dotted line indicates the time when FLB were chased with an addition of HK unstained bacteria.

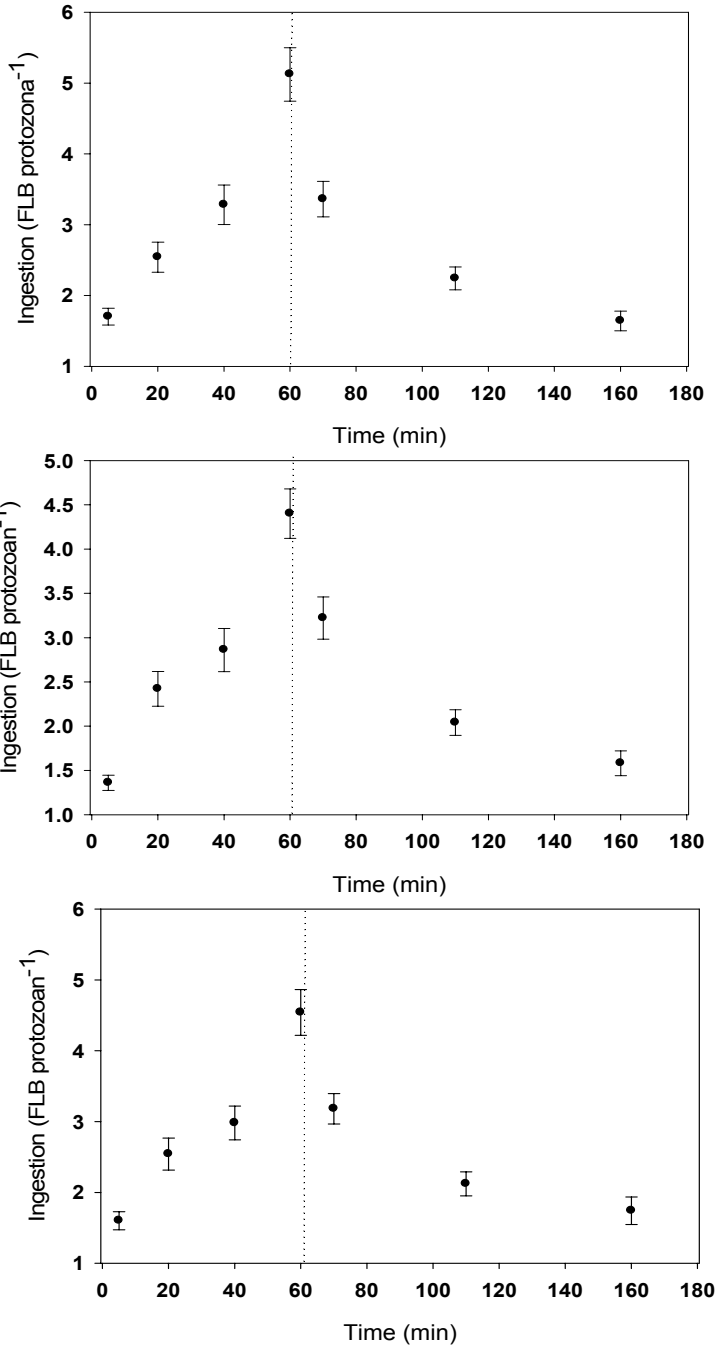


Figure 3.4 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 82:17:1. Refer to Figure 3.3 for a detailed description of the graphical elements.



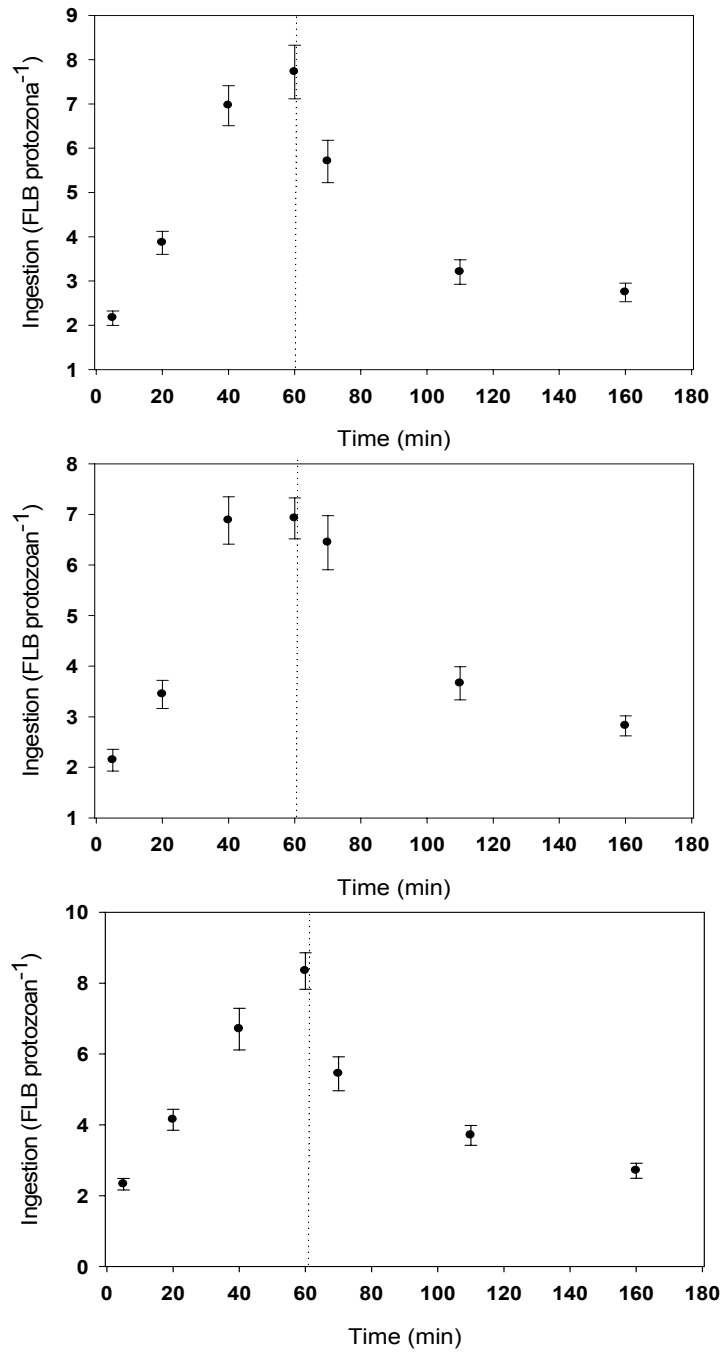


Figure 3.5 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 59:13:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

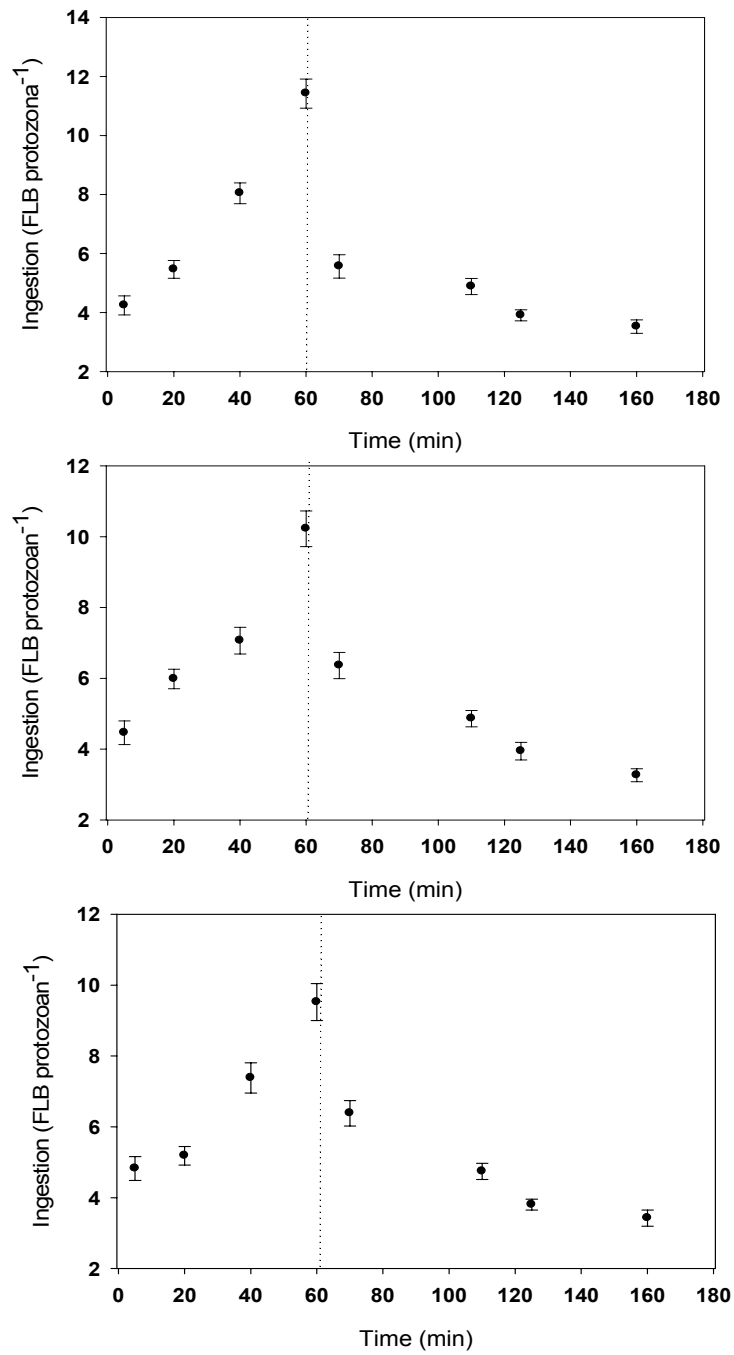


Figure 3.6 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 74:18:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

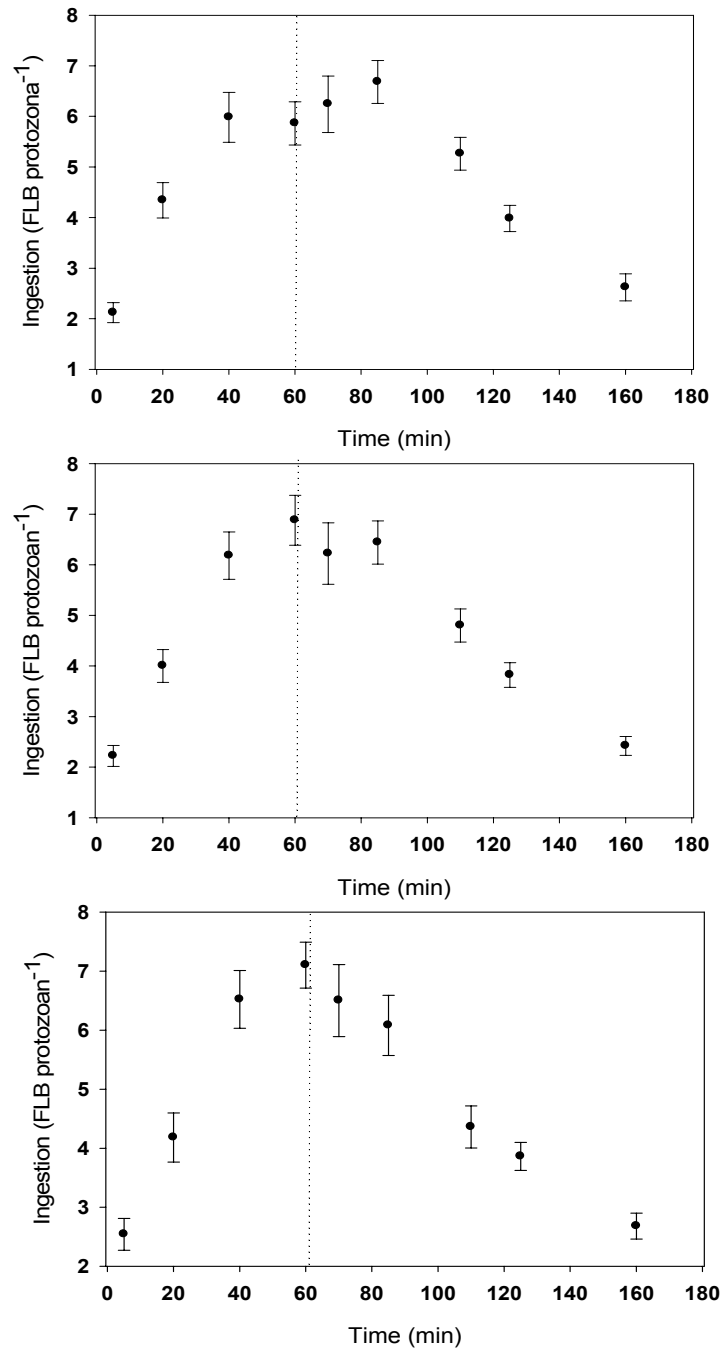


Figure 3.7 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 101:21:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

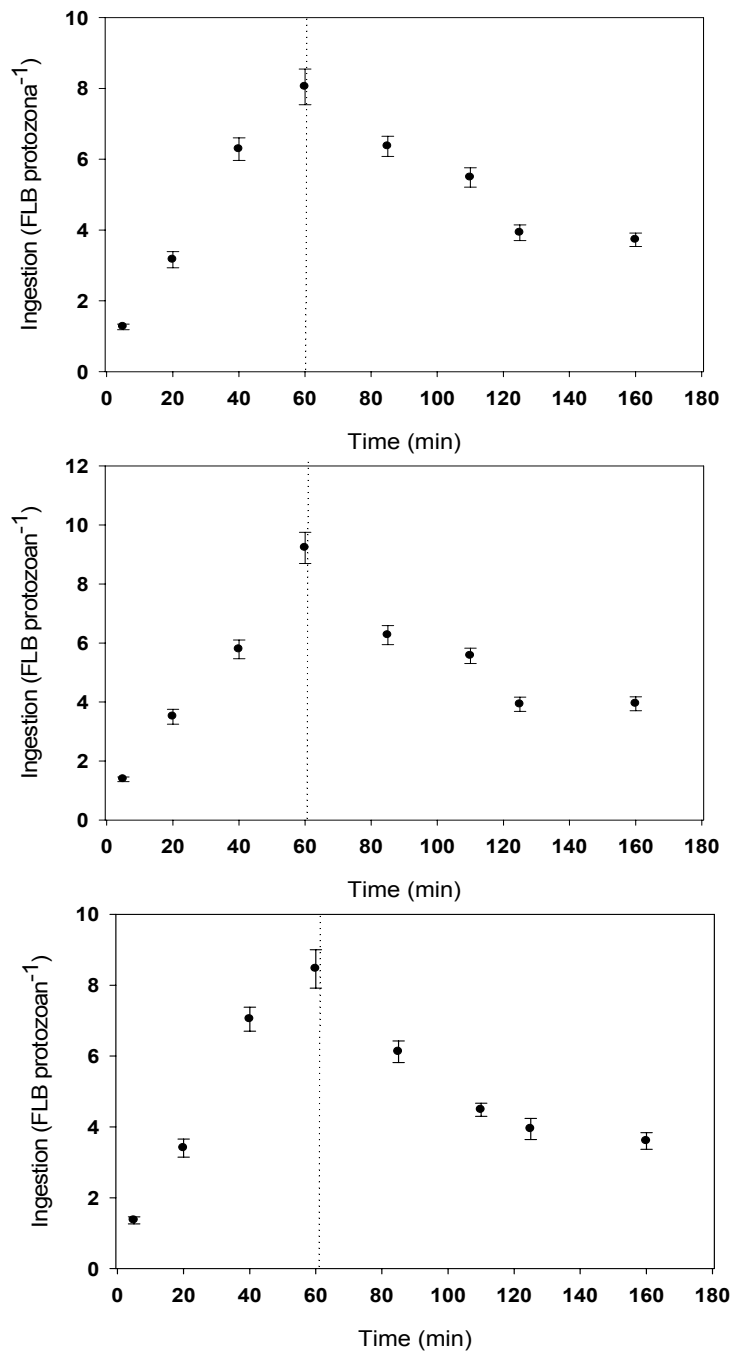


Figure 3.8 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 101:21:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

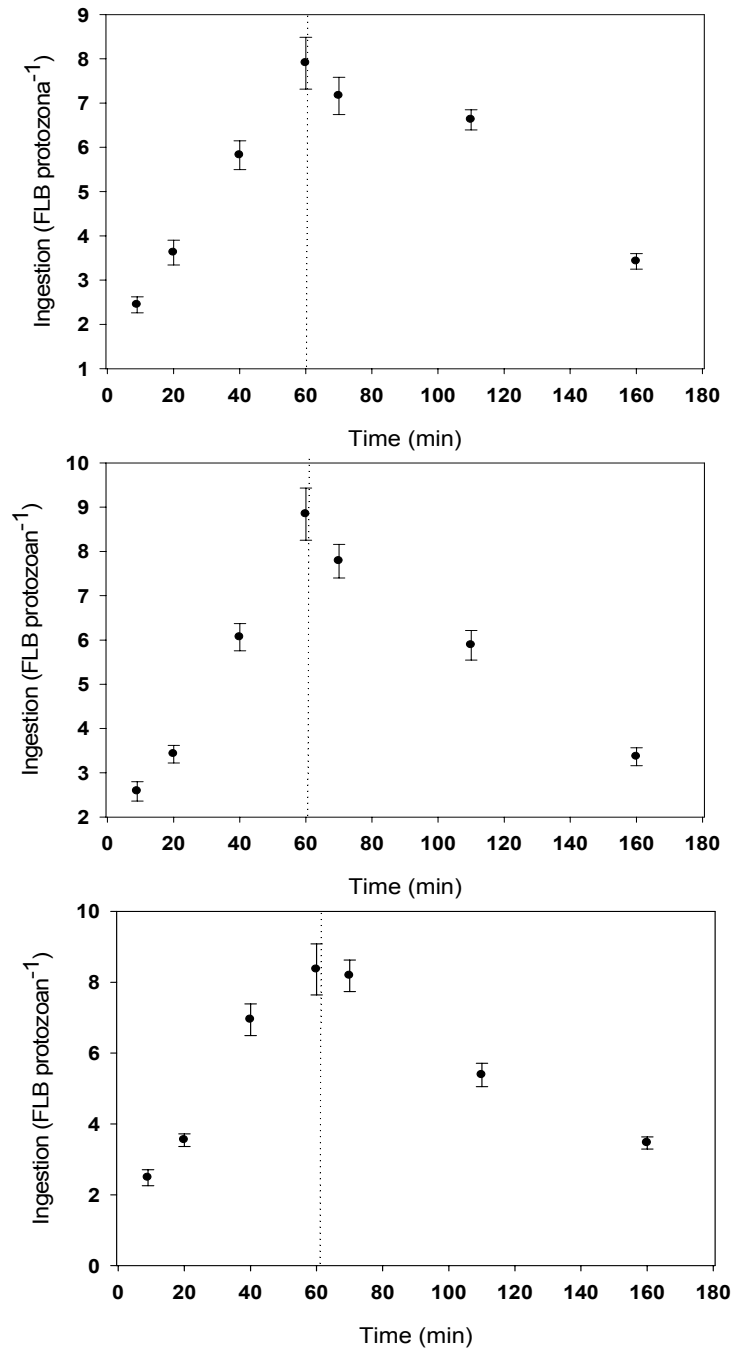


Figure 3.9 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 121:26:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

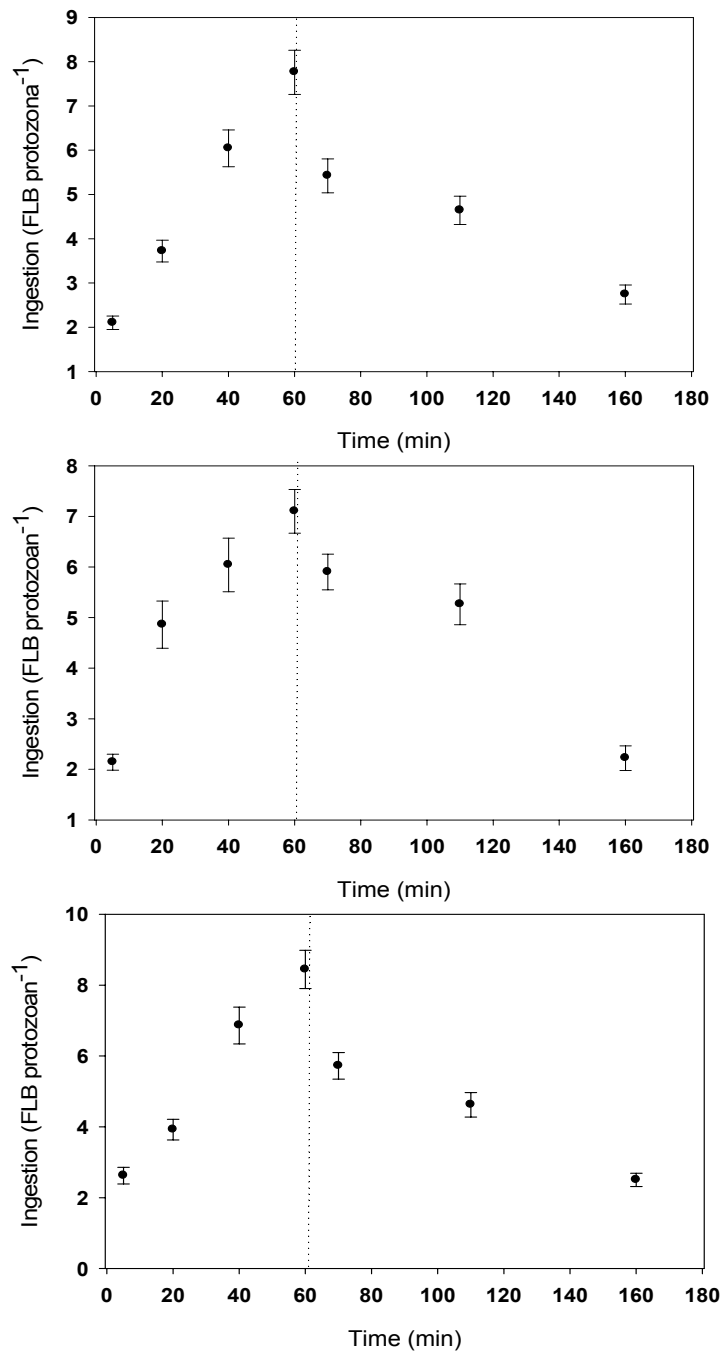


Figure 3.10 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 139:26:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

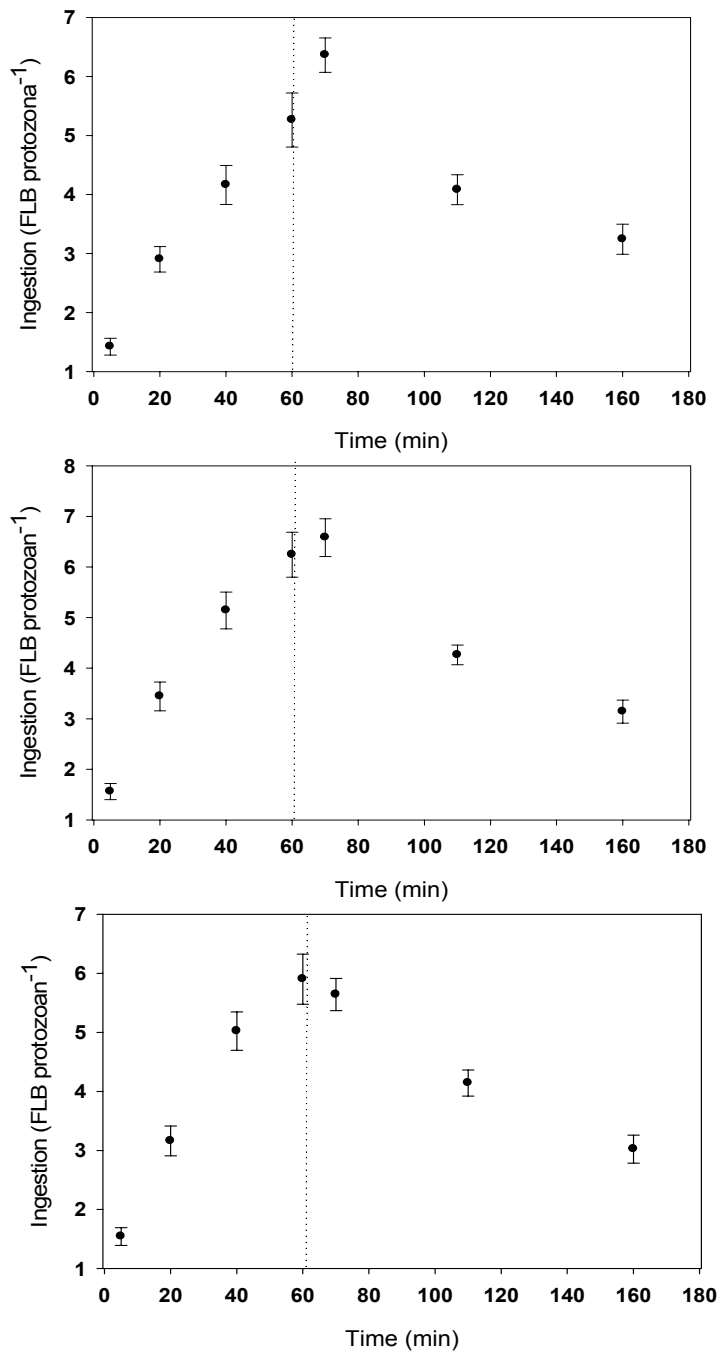


Figure 3.11 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 92:19:1. Refer to Figure 3.3 for a detailed description of the graphical elements.

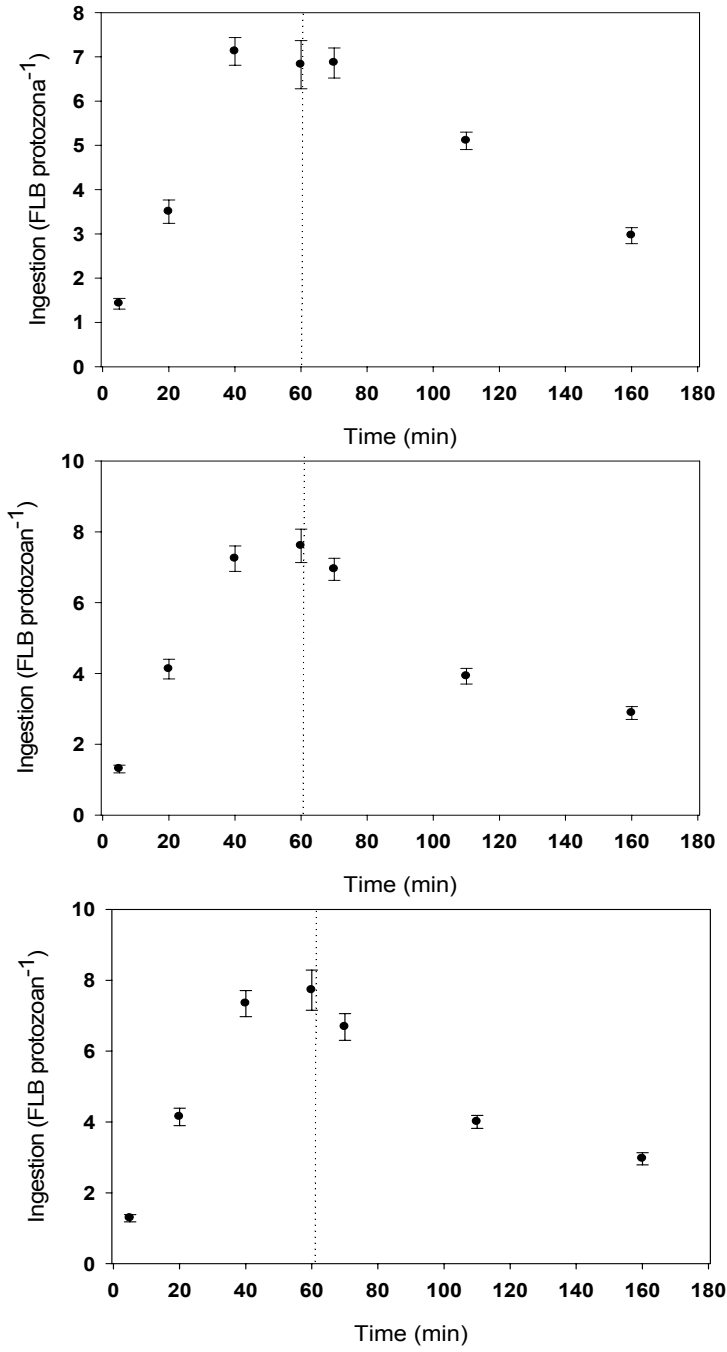


Figure 3.12 Three separate feeding trials when *O. danica* was fed heat killed bacteria having a CNP = 107:21:1. Refer to Figure 3.3 for a detailed description of the graphical elements.



Table 3.4 Characteristics of *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	0.42 $\pm$ 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 effects of prey food quality on ingestion and digestion were analyzed in two ways: simple regression of a food quality determinant (C:P, C:N, or N:P) upon ingestion rate or digestion rate constant, and through step-wise multiple regression where prey cell size was considered in addition to food quality determinants. In each case thirty separate measurements were used consisting of three independent samples from ten separate feeding experiments.

Ingestion rates ranged from 2.25 bacteria protozoan<sup>-1</sup> min<sup>-1</sup> when *Ochromonas* was fed high quality prey (CNP = 59:13:1) to 0.5 bacteria protozoan<sup>-1</sup> min<sup>-1</sup> when fed poor food quality prey (CNP = 139:26:1). Overall, ingestion rates were similar to rates reported previously (Sherr et al. 1987, Sherr et al. 1988, González et al. 1990, Holen and Boraas 1991, Sherr and Sherr 1993, Dolan and Simek 1998, Boenigk and Arndt 2000, Boenigk et al. 2001b, Boenigk et al. 2002).

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, and N:P. There was a significant correlation between ingestion and each index of food quality: C:P,  $R^2 = 0.49$ ,  $P < 0.0001$ ; C:N,  $R^2 = 0.44$ ,  $P < 0.0001$ , N:P,  $R^2 = 0.43$ ,  $P < 0.0001$  (Figure 3.13). *Ochromonas* feeds upon individual bacterial cells, each represented by a C:N:P ratio so it is somewhat arbitrary to consider separate indexes of food quality; however, the data clearly indicate that bacteria of high food quality were ingested at more rapid rates than cells of poor food quality. The similarity among the coefficients

of determination probably reflect the composite measure of food quality (C:N:P) rather than variability due to an individual element.

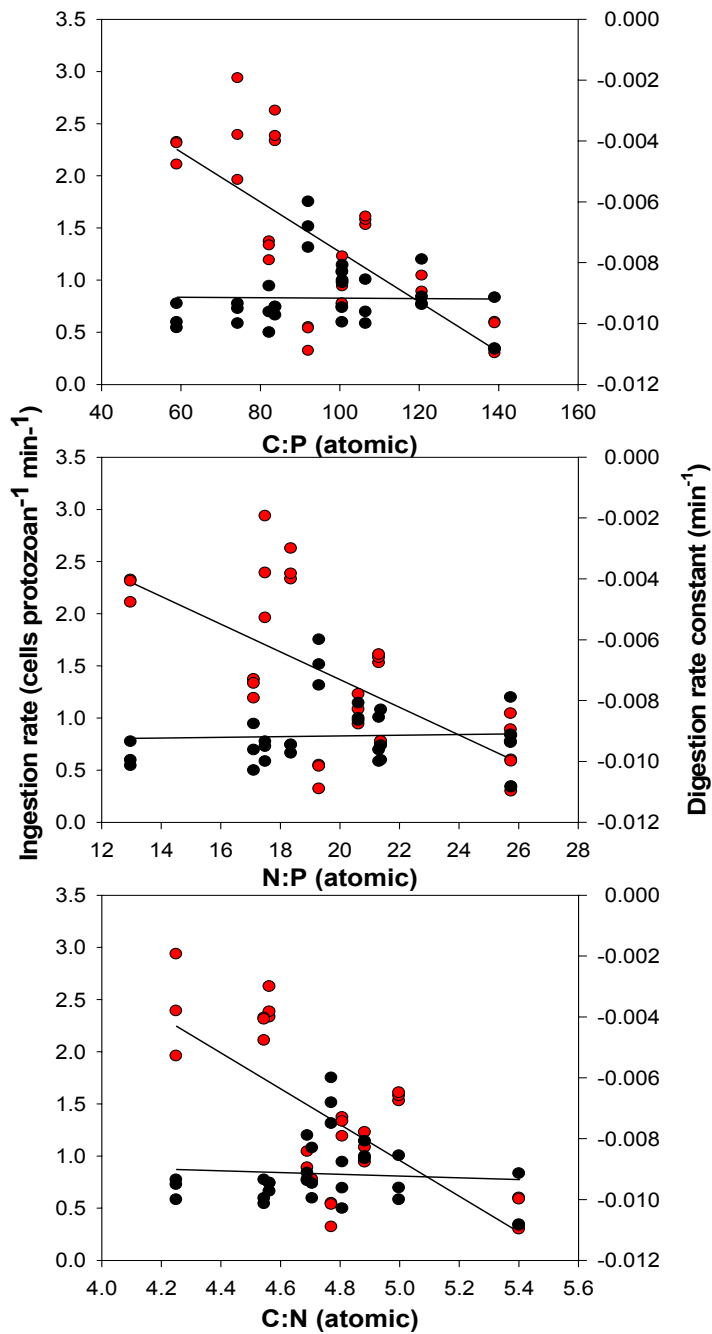


Figure 3.13 Comparison of ingestion rate and digestion rate constants with element ratios (C:N, N:P, C:P). Each point is the plot of an element ratio (C:P, N:P, or C:N of the heat killed bacteria) against either the ingestion rate or digestion rate constant from each individual experiment. S.E. for each point are not shown here but are presented in Table 3.4.

Prey selection by protozoa has been linked to size distribution of the prey items (Andersson et al. 1986, Chrzanowski and Simek 1990, Simek et al. 1994, Kinner et al. 1998, Hahn and Hofle 1999, Pfandl et al. 2004). Bacterial cell size is a function of growth rate: rapidly growing cells are larger than slowly growing cells. Since bacteria used as prey were generated in chemostats where growth rate was manipulated to generate cells of differing food quality prey items also varied in size. To examine the relative effect of size and food quality, stepwise multiple regression analysis was used to assess the importance of each factor.

Table 3.5 Proportion of the variation in the rates at which *Ochromonas danica* ingested and digested *Pseudomonas fluorescens* of varying size and food quality. N=30 for each model.

Dependent variable	Independent variables	Total variation explained (%)	Model Probability	Variation partitioned between independent variables (%)	Partitioned Probability
Ingestion rate	Cell size C:N	53	<0.001	9	0.029
				44	<0.001
	Cell size C:P	68	<0.001	19	<0.001
				49	<0.001
	Cell size N:P	66	<0.001	23	<0.001
				43	<0.001

In the multiple regression model, both cell size and food quality were important determinants of grazing but food quality explained a much greater proportion of the overall variation in ingestion rate.

The DRC (see Table 3.4) 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 digestion rate constant 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. (Dolan and Šimek 1998). Digestion rates appear to be independent of food concentration (González et al. 1990, Dolan and Šimek 1998, Boenigk et al. 2001a, Boenigk et al. 2002) and also are more likely a factor of the physiology of the protozoan than of the prey. Digestion rate is likely affected by: accessibility of digestive enzymes (Boenigk et al. 2001c), accumulation efficiencies (Zubkov et al. 2001), metabolic rate (Boenigk et al. 2001b), or growth state (Sherr et al. 1992, Gonzalez et al. 1993).

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 (Matz et al. 2002a), or the taxonomic classes of cells more-or-less subject to grazing (Jezbera et al. 2005). Often alluded to, but less frequently investigated, are those features of the prey items that make them susceptible to predation (Matz and Jürgens 2003). Those features that have been considered, capsules, motility, surface hydrophobicity and charge, have been, to a large extent, found to be unimportant regulators of predation (Gonzalez et al. 1993, Matz and Jürgens 2001, Matz et al. 2002b, Matz and Jürgens 2003).

Clearly, some flagellates can discriminate between high quality and poor quality prey items. When ample food is available, flagellates will discriminate between low quality food, such as microbeads, and high quality food, such as bacteria, preferring to feed upon the bacteria (Jürgens and DeMott 1995). 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 (Boenigk et al. 2001c). All bacteria are not of equivalent quality as prey. Bacteria grown under P-limitation were captured by flagellates with high frequency, yet most were egested immediately (Matz and Jürgens 2003). 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 (Šimek et al. 1990, Sherr et al. 1992, Pernthaler et al. 1996, Beardsley et al. 2003, Cottrell and Kirchman 2004, Jezbera et al 2005). 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 is a function of growth rate: rapidly growing cells are larger than are slowly growing cells; and RNA, protein, and to a lesser extent DNA, all increase as growth rate increases (Herbert 1976, Bremer and Dennis 1987). Basic microbial physiology and evolving theory in ecological stoichiometry predicts that rapidly growing cells are characterized by low C:P and N:P ratios

(Chrzanowski et al. 1996). 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 be simply a consequence of a different selection cue, the element composition of cells (see discussion in Wu et al. 2004). Multiple regression analysis (this work) lends support for this position. One of the potential sources 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 (Landry et al. 1991). Thus it 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 while element composition of cells has suggested some potential links to metabolic processes, it is unlikely that a given mineral element itself accounts for prey selection (see above). Further, since heat killed cells were used in this work, capsules and surface characteristics of cells were likely altered compared to that of live cells. Cytoplasmic signals could also be disregarded since heating and washing of cells probably greatly diminished their potential influence. However, recent findings relating to digestion processes seems 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 (Matz et al. 2002a). Bacterial cells with low C:N content, which may be indicative of high protein



content, also appear to be preferentially ingested (John and Davidson 2001, this study). Zubkov et al. (2001) 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 (Zwart and Darbyshire 1992) suggests a flagellate metabolism based largely on protein degradation.

Food quality has long been associated with predator prey-selection through optimal foraging theory (MacArthur and Pianka 1966). 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 (McNaughton 1990, but see Cebrián et al. 1998), ratios of essential elements (Sterner and Smith 1993, Elser et al. 2000, Güsewell 2004) or growth rate (Cowles et al. 1988, Butler et al 1989, Cebrián and Duarte 1994, Elser et al. 2003). Only recently have similar concepts been reported for flagellate nanozooplankton of microbial food webs (John and Davidson 2001, this study). While we cannot assert that food selectivity based on food quality is a concept that fully extends to all flagellate nanozooplankton, it certainly appears to be a feature that is not uncommon.

This particular work has highlighted some aspects of the interaction between protozoa and bacteria. Future studies that might flow out of this work include: investigating the affects of preconditioning on grazing rates, utilization of live cultures instead of heat killed stocks to better understand the ecological significance of this predator-prey model, and evaluating the regeneration of nutrients by varying the food quality.

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## BIOGRAPHICAL INFORMATION

S. Paul Shannon is currently employed for Alcon Research, LTD in Fort Worth, Texas. His current job responsibilities in the Microbiology Department include managing the preservative effectiveness testing group, overseeing the development and implementation of computer applications, coordinating validation and compliance activities, and serving on project teams. Prior to coming to Alcon in 1995, Paul was Laboratory Supervisor for a medical device contract laboratory for four years. Overall, Paul has over 15 years experience in medical, academic and pharmaceutical areas with widely developed technical, management, and computer skills.

Paul has a B.S. from Texas A&M University, a M.B.A. from The University of Texas at Arlington, and a Ph.D. in Quantitative Biology from The University of Texas at Arlington.