PREY DENSITY-DEPENDANT PROTOZOAN BACTERIVORY

by

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ABSTRACT OF THE DISSERTATION

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The initial ingestion rates of the marine, bacterivorous ciliates *E. vannus*, *E. plicatum*, and *Cyclidium* sp. were measured as a function of prey concentration. A method was developed using bacteria engineered to express green fluorescent protein (GFP-bacteria) as a naturally cultured tracer to directly measure ingestion rates at bacterial concentrations ranging from 5×10^4 to 10^{10} bacteria ml^{-1}. Ingestion rates increased with prey density ranging from 10^3-10^5 bacteria $ciliate^{-1}$ h^{-1}, and maximum ingestion rates were observed at a prey density of 10^9 bacteria ml^{-1}. Ingestion rate data for all three grazers was best modeled by a Type III functional response equation. In lieu of these results combined with a re-analysis of previous reports of ciliate ingestion, it was concluded that in nature, a traditional Type II response is probably rare and unstable.

Next, using GFP-bacteria as prey, ingestion rates, food vacuole formation rates, and the maximum number of food vacuoles were measured over time for the same ciliate species at a low (10^6-10^7 bacteria ml^{-1}) and a high (10^9 bacteria ml^{-1}) prey concentration. The time required to reach steady state maximum food vacuole capacity and digestion

rate were subsequently estimated. Food vacuole content and ingestion rates increased with prey concentration. Digestion rate decreased with increasing food vacuole content, refuting the idea that digestion is a rate constant. Estimates of ingestion rate and digestion efficiency suggested that ciliates are capable of exerting substantial top-down control on bacteria at typical water column concentrations.

The ingestion rates of each ciliate were next measured as a function of benthic bacterial concentration, by culturing GFP-bacteria directly in sediment microcosms to densities ranging 10^8-10^{10} bacteria per milliliter of pore water (ml^{-1} PW). Ingestion rates increased continually with sediment bacterial concentration, ranging from 10^2-10^5 bacteria $ciliate^{-1}$ h^{-1}. A decrease in ingestion rates below 2×10^8 bacteria ml^{-1} PW suggests a possible lower prey concentration threshold for benthic bacterivory. Benthic ingestion rates measured at a typical sediment bacterial concentration (10^9 bacteria ml^{-1} PW) were two times higher for both species of *Euplotes*, and 27 times higher for *Cyclidium* sp., than those measured for these species when grazing on suspended prey.

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CHAPTER I

Introduction

Protozoan Bacterivory

Bacterivores are organisms that consume bacteria. Seminal papers by Pomeroy (1974) and Azam et al. (1983) revealed that high growth capability and nearly constant concentrations of marine bacteria meant that there were important sources of removal that were maintaining the standing stock. Azam et al. (1983) formally defined the concept of the microbial loop, acknowledging that the microbial food web has the potential to significantly impact the cycling of energy, carbon and nutrients. The subsequent extensive research has since made it well established that protozoa are major consumers of bacteria in the marine environment. The ecological significance of protozoan bacterivores is a direct result of their high reproductive rates, combined with the ability to concentrate small food particles from a dilute medium. This gives protozoa the capability to rapidly respond to and exploit changes in resources, such as bacterial stock.

Protozoa do not only act as consumers. Protozoan bacterivory has also been shown to stimulate bacterial activity in many habitats, accelerating rates of organic matter cycling (Fenchel 1970; Fenchel and Jorgensen 1977; Kreuzer et al. 2006; Murase et al. 2006; Wright et al. 1995). This is counter intuitive, as grazing is responsible for limiting bacterial populations. The most common explanation for this phenomenon is that protozoa recycle nutrients, such as nitrogen and phosphorous, that would otherwise be depleted by bacterial production (Caron 1991; Caron and Goldman 1990; Caron et al. 1985; Gude 1985; Hondeveld et al. 1999; Suzuki et al. 1996). Recent work has shown

that protozoa may actively graze senescent cells from the population leaving the fast growing bacteria in higher abundance (Gruber et al., in press). This could possibly remove some of the competition for nutrients, causing higher activity for the bacterial population as a whole. In addition to increasing bacterial activity, bacterivores selectively graze prey based on attributes such as cell size (Epstein and Shiaris 1992; Fenchel 1980a; Gonzalez et al. 1990) and cell surface properties (Gurijala and Alexander 1990; Tso and Taghon 1999). Selective grazing has been recognized for having a number of influences on prokaryotic diversity, altering the genotypic, phenotypic and metabolic composition of the bacterial community (Jurgens and Matz 2002; Jurgens et al. 1999; Kinner et al. 2002; Matz and Jurgens 2003).

Protozoan bacterivores consist primarily of heterotrophic flagellates and ciliates. Some consider heterotrophic flagellates to be the chief consumers of bacteria in aquatic ecosystems (Sherr and Sherr 2002; Strom 2000). Early research predicted that the role of ciliates was negligible, assuming that they were unable to consume particles less than a few microns in diameter or attain appreciable clearance rates (Fenchel 1980b; Fenchel 1980c). However, evidence has refuted this idea showing that ciliates can represent > 50% of the micro-grazer biomass (Sherr et al. 1986), thus having the capability of accounting for a major portion of overall bacterivory (Sherr and Sherr 1987).

Benthic Bacterivory

It has been assumed that a microbial loop exists in benthic ecosystems as it does in the water column because the correct variables (organic matter, bacteria, and protozoa) are present (Azam et al. 1983; Fenchel 1987; Patterson et al. 1989). The dynamics of the

benthic microbial loop is likely to be quite different from the pelagic environment. The benthic environment is more complex and both the quantity of organic matter and microbial abundances can be orders of magnitude higher than in the water column. It is probable that the efficiency of the benthic microbial food web has a large impact on the fate of sediment organic matter, determining the quantity that is recycled, transferred up the food chain, or removed through burial (Patterson et al. 1989). Investigations to support and quantify these concepts for benthic environments are limited and much needed.

Estimates for benthic protozoan ingestion rates have been varied and generally low (Alongi 1986; Kemp 1988; Konigs and Cleven 2007; Tso and Taghon 1999). In some studies this has led to the conclusion that the impact that protozoan bacterivory has on controlling benthic bacterial biomass and production is negligible (Epstein 1997; Kemp 1990; Wieltschnig et al. 2003). This is due in part to the complexity of studying a benthic environment. The sediments differ from the water column in that prey exist both free in the pore water, as well as attached to particle surfaces. There are few studies that have addressed the effect that this dichotomy in sediment bacterial populations has on grazing rates of protozoa (Albright et al. 1987; Starink et al. 1994b). Studies measuring benthic protozoan ingestion have primarily used monodispersed fluorescently labeled bacteria (FLB). This method only presents suspended prey to the grazers and FLB can be selectively grazed by protozoa, possibly underestimating benthic ingestion rates.

Measuring Bacterivory

A useful way to gauge the ecological significance of a predator-prey interaction is to measure the density-dependant control a predator has over the prey population, or its functional response (Solomon 1949). This gives information about the limitations of the predator, what prevents extreme reduction of the prey population, and what the conditions are for the prey to escape the predator for population recovery. Thus, measuring a predator's consumption rate as a function of prey concentration allows for predictions of behavior, and has important implications for how the predator-prey relationship impacts ecosystem functioning (i.e. ecosystem stability, prey population control, and biomass turnover). The different types of functional responses and their meaning are discussed in Chapter II. It is well known that ingestion rates of water column bacterivores are positively correlated with prey concentration (Fenchel 1980c; Jonsson 1986; Jürgens and Simek 2000; Wilks and Sleigh 1998). However, measurements are usually made over relatively narrow prey range (10^6-10^7 bacteria ml). This range does not encompass the higher prey concentrations found in areas or times of high productivity (i.e. estuaries and sediments) (Santos et al. 2007; Schmidt et al. 1998), where control of bacterial stock is likely to be important. Furthermore, if the ingestion rate data do not encompass the entire prey range that the grazer is capable of consuming, the confidence that the model is accurately describing the predator's functional response is reduced. The effects of prey concentration on benthic bacterivory have yet to be quantitatively reported.

Several methods have been used to directly estimate protozoan ingestion rates. An earlier method incorporated the use of inert fluorescently labeled microspheres (FLM) as

surrogate prey particles (McMannus and Fuhrman 1986). The discovery that bacterivores discriminate against inert particles led to the use of fluorescently labeled bacteria (FLB) (Sherr et al. 1987), which has been the most widely used method over the past 3 decades. Proof that bacterivores selectively graze manipulated prey, including FLB, (Boenigk et al. 2001a; Gonzalez et al. 1993; Landry et al. 1991), has led to the development of several indirect methods. Estimates from changes in number and volume of food vacuoles using a post-ingestion in-situ fluorescent hybridization process have been used (Eisenmann et al. 1998), as well as monitoring the ingestion of radioactively labeled prey (Zubkov and Sleigh 1996). More recently, bacteria genetically modified to express fluorescent proteins (FP) have been used to represent a naturally grown tracer (Fu et al. 2003; Parry et al. 2001). Rates vary with species and prey concentration, but a general comparison of ciliate ingestion rates attained using FLM or FLB, with rates measured using either an indirect method or FP reveals that newer methods are yielding higher estimates for bacterivory (Figure 1.1). As future methods improve upon existing ones, it will be necessary to re-evaluate protozoan ingestion rates.

Objectives

The purpose of this dissertation was to evaluate the prey density-dependant ingestion rates of marine bacterivorous ciliates grazing both in suspension and in a sedimentary environment. The ciliates *E. vannus*, *E. plicatum*, and *Cyclidium* sp. were used in a series of experiments in which the specific research objectives were to:

(1) Determine the ciliate's initial ingestion rates as a function of suspended bacterial concentration.

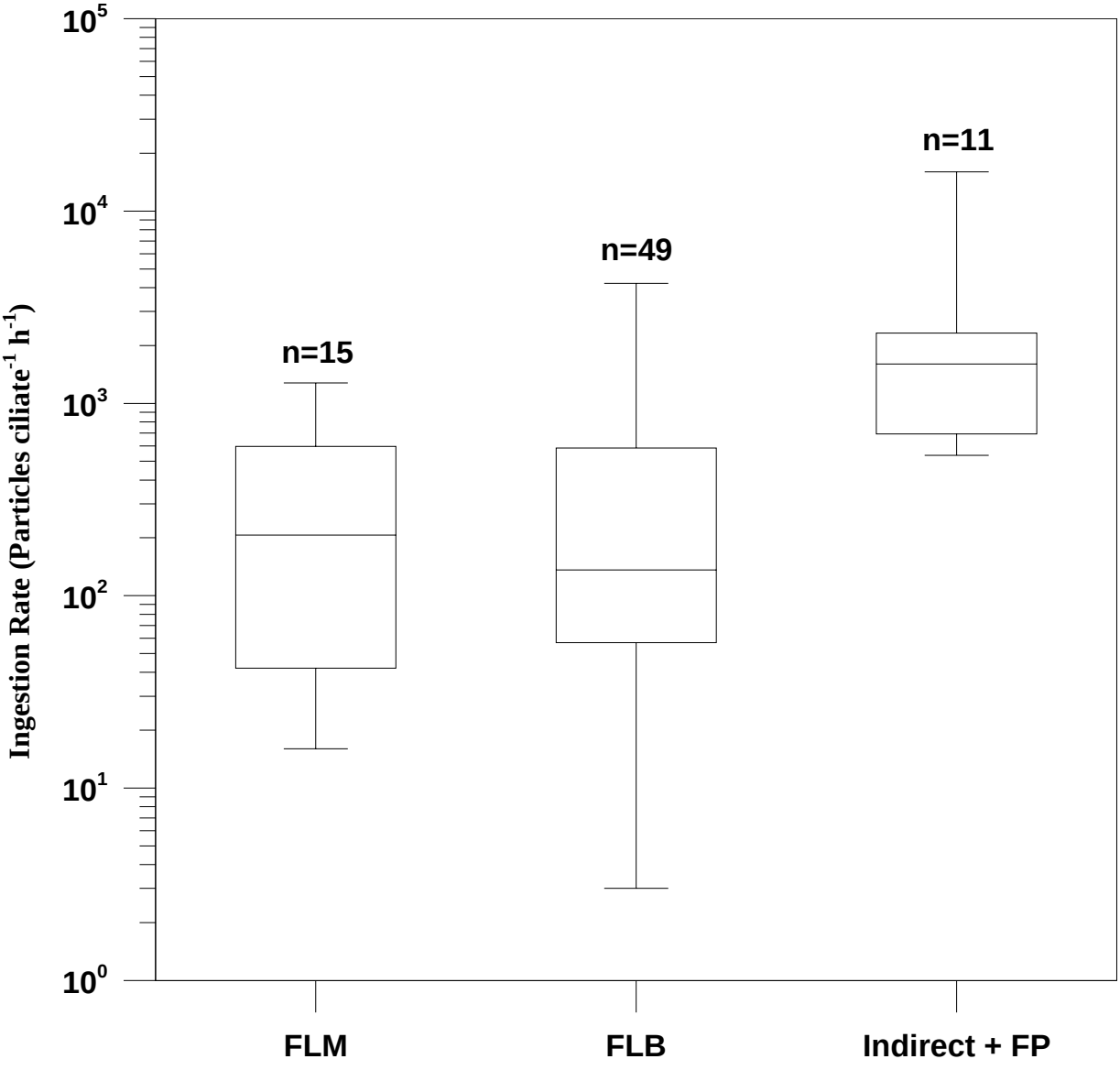
(2) Determine the type of functional response model that describes the ciliate's density-dependant ingestion rates when grazing suspended prey.

(3) Determine the ciliate's steady-state ingestion rates and food vacuole formation rates at different concentrations of suspended bacteria.

(4) Use ingestion rate and food vacuole formation data to determine if the digestion rate of ciliates changes as a function of prey concentration.

(5) Determine if ciliate ingestion rates vary as a function of prey concentration in a sedimentary environment.

Figure 1.1. Ciliate ingestion rates estimated with different methods: fluorescently labeled microspheres (FLM); fluorescently labeled bacteria (FLB); fluorescent protein-expressing bacteria (FP) or an indirect method (changes in food vacuoles, radiolabeled tracers, etc.).



CHAPTER II

The Initial Ingestion Rates and Functional Response of Marine Bacterivorous Ciliates

Abstract

The purpose of this study was to measure the initial ingestion rates of three species of bacterivorous ciliates over a wide range of prey concentrations. The ingestion rates of *Euplotes vannus*, *Euplotes plicatum*, and *Cyclidium* were measured at prey concentrations ranging from 10^4 -10^{10} bacteria ml^{-1} using a single species of bacteria engineered to express green fluorescent protein (GFP). Ingestion rates increased with prey density and ranged from 10^3-10^5 bacteria $ciliate^{-1}$ h^{-1}. Maximum ingestion rates were attained for all predators at a prey concentration of 10^9 bacteria ml^{-1}. Constancy in the number of bacteria consumed per ciliate was observed within 30 s of prey introduction in all experiments, suggesting that short sampling intervals may be required for accurate measurements of protozoan ingestion rates when directly observing prey uptake. A test comparing the rates ascertained using GFP-*Vibrio* as the tracer versus the fluorescently labeled bacteria (FLB) method showed both negative and positive selection for FLB, suggesting that discrimination for or against manipulated prey particles may be species-specific. Ingestion rate data for the ciliates in the current study, as well as from data sets collected from previous studies were best fit by either the Type III or the Type II-with threshold functional response models. It is concluded that the Type III and Type II-with threshold models are a more ecologically realistic for modeling the predator-prey relationship between protozoa and bacteria than the traditional Type II equation.

Introduction

Protozoa are major consumers of bacteria (Sherr and Sherr 2002). As a result, there has been an increase in research investigating the significance of bacterivory in the trophic transfer of energy and the global cycling of nutrients (Caron et al. 1985; Gude 1985; Sherr et al. 1997). Bacterivory can also have a strong influence on prokaryotic diversity, altering the genotypic, phenotypic, and metabolic composition of bacterial communities (Hahn and Hofle 2001; Jurgens and Matz 2002; Jurgens et al. 1999; Kinner et al. 2002; Matz and Jurgens 2003). It is necessary to understand the capability of protozoa in order to determine the overall role that bacterivory plays in the microbial food web.

A traditional way to gauge predation is to measure the consumer's functional response, or how consumption rate changes with respect to prey density (Chow-Fraser and Sprules 1992; Fenchel 1980b; Fenchel 1980c; Hadas et al. 1998; Jurgens and Simek 2000; Moigis 2006). Ingestion rates are usually compared to basic types of prey density-dependant consumption responses, originally described by Solomon (1949). Investigators traditionally described results by fitting their data to either the Type-I (linear), Type II (hyperbolic), or Type III (sigmoid) model originally described by Holling (1966, and references therein). An alternate form of the Type II model is a Type II-with threshold model. This introduces a parameter for a minimum prey density threshold, and is mathematically similar to a Type III equation.

Identifying the type of functional response a predator has provides an assessment of behavior, and has important implications for how the predator-prey relationship impacts ecosystem functioning (i.e. ecosystem stability, prey population control, and biomass turnover). There are two main components that characterize how feeding rates respond to

prey concentration. One is the time it takes to successfully search for and come in contact with prey, which I will call 'search time'. The second component, which I will call 'handling time', is the time it takes to capture, select for (if a selection process is present), and ingest prey. If a predator's search time is constant and handling time is negligible, then ingestion rate increases linearly with increasing prey density until a threshold is reached, producing a Type I response. A Type II response is also generated by a constant search time, but handling time is dependant on prey density causing the grazing rate to increase in a decelerating fashion up to an asymptote, or maximum grazing rate. When both search time and handling time is prey density-dependant we see a sigmoid curve, or a Type III response. For this response, when prey is scarce, search times are long resulting in low ingestion rates. Consumption rate increases with increasing prey concentration initially at an exponential rate, but then the rate of increase is slowed by a limiting factor (i.e. satiation, digestion, clogging of feeding apparatus, etc.).

The most widely used method for measuring ingestion rates of protozoa is to observe the uptake of monodispersed fluorescently labeled bacteria (FLB), a procedure originally described be Sherr et al. (1987). This method involves much manipulation of the prey for staining procedures (hot/cold incubations, freezing/thawing, re-suspension, etc.). Some work has shown that bacterivorous protists may discriminate against surrogate or manipulated prey particles in some instances (Gonzalez et al. 1993; Landry et al. 1991), which may lead to an underestimation of grazer's abilities. A solution to this issue is to use natural prey that have been minimally manipulated, but can still be observed for calculating uptake rates. More recently, bacteria that have been genetically transformed

for the in vivo expression of fluorescent proteins have been used as a natural tracer to measure grazing rates (Fu et al. 2003; Parry et al. 2001; Steinberg and Levin 2007).

The purpose of this study was to investigate the initial ingestion rates of different species of bacterivorous ciliates over a wide range of prey concentrations, and to describe the functional response of each grazer. Uptake was monitored using bacteria that express green fluorescent protein (GFP) in short term grazing incubations over 5-20s sample intervals. A test was also conducted to compare ingestion rates using GFP-bacteria with rates attained using a traditional FLB method.

Methods

Culturing and Preparation of Bacteria

Vibrio sp-nap, originally isolated from Piles Creek, a tributary of the Arthur Kill- NY/NJ Harbor area (Tso and Taghon 1999), was used as prey. Bacteria were maintained in liquid culture (0.2 μm filtered, aged seawater at a salinity of 23, 2.5 g L^{-1} soy peptone, 0.5 g L- yeast extract) on a rotary shaker kept at 75rpm and 20°C. Subcultures of *Vibrio* sp.-nap were obtained that had been transformed to express green fluorescent protein (GFP) and will be referred to as GFP-*Vibrio*. Briefly, a QLAprep DNA miniprep (Quigen) was performed to extract the plasmid DNA from a strain of *Escherichia coli* containing the GFP gene (Gruber et al., in press). All GFP-*Vibrio* were cultured as above with the addition of kanamycin (Sigma®) at a final concentration of 0.1 g L^{-1}.

Fluorescently labeled bacteria (FLB) were prepared after Sherr et al. (Sherr et al. 1987), as modified by Tso and Taghon (Tso and Taghon 1999). GFP-*Vibrio* were harvested between 3-4 days of growth by centrifugation at 7500 × g and washing two

times with phosphate buffer. Cells were re-suspended in 10 ml of buffer, vortexed for five minutes after the addition of 2 mg of 5-(4,6-dichlorotriazin-2-yl) aminofluorescein (DTAF, Sigma®), and incubated for 6 h at 4°C. Following incubation, the bacteria were centrifuged and washed four times to remove any remaining DTAF, and re-suspended in filtered seawater. The suspension was filtered through a 5 μm polycarbonate filter to remove large filaments and aggregates. Cell concentration was determined by epifluorescence microscopy using an Olympus BH-2 microscope (BP490 excitation filter/17AFC + 170515 barrier filter). The FLB stock was diluted to a desired concentration and stored at -20°C until needed (all FLB stocks were used within 6 days of preparation).

Culturing and Preparation of Ciliates

Two species of hypotrich ciliates, *Euplotes vannus* (length = 100 ± 11 μm, width = 57 ± 9 μm) and *Euplotes plicatum* (length = 51 ± 4 μm, width = 32 ± 4 μm), were acquired from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, UK). A species of *Cyclidium* (scuticociliate; length = 26 ± 3 μm, width = 10 ± 2 μm), was isolated from Piles Creek. All protozoa were cultured in an adapted Artificial Seawater for Protozoa medium (CCAP recipe). Ciliate cultures were maintained on an uncharacterized assemblage of bacteria grown on either rice grains or soy extract added as organic substrate. All ciliate cultures were either started or refreshed (sieved, filtered, given fresh media and organic substrate) no more than four days prior to use for grazing experiments. In preparation of an experiment, ciliate cultures were first passed through a mesh sieve (21, 80, and 105 μm mesh for *Cyclidium* sp., *E. plicatum,* and *E. vannus*

respectively) to remove large particles and detritus. Ciliates were then gravity filtered onto a 10 μm polycarbonate filter, rinsed three times with filtered seawater to remove as much extant bacteria as possible, and then re-suspended to desired concentration.

Ingestion Rate Experiments

Vibrio sp-nap and GFP-*Vibrio* cultures were individually harvested by centrifuging (7500 × g) and washing two times, followed by re-suspension with filtered seawater. Bacteria were then filtered through a 5 μm polycarbonate filter to remove large filaments and aggregated bacteria. A diluted sample of each was fixed (1% glutaraldehyde), and the *Vibrio* sp-nap stained with SYBR® Gold (150 μl ml^{-1} of a 20× stain stock in DI; Molecular Probes, Invitrogen Detection Technologies) for 30 minutes. The cell concentration of each suspension was determined by epifluorescence microscopy, and then adjusted to two times the final concentration desired for each trial. An inoculum for the ingestion rate experiments was made by combining the GFP-*Vibrio* and the *Vibrio* sp-nap harvests so that the percentage of GFP-*Vibrio* was adequate for monitoring its uptake (Table 2.1). The percentage of GFP-*Vibrio* used in each experiment was dependant on both the ciliate species and the final desired bacterial concentration. A test for discrimination for or against GFP-*Vibrio* was conducted for each ciliate. Uptake was monitored over time in a trial with a 100% GFP-*Vibrio* inoculum, as well as in a trial using 1:1 GFP-*Vibrio* + *Vibrio* sp-nap inoculum.

Ciliates were added to small test tubes and allowed to acclimate for 3-4 h prior to prey introduction. Samples were run in triplicate for six time durations ranging from 5-80 s (dependant both on the grazer and the bacterial concentration). Bacteria were added

in equal volume to the ciliates in the test tube, diluting both the grazers and the prey to the desired concentrations. At the end of each incubation time the sample was fixed with 1% ice-cold glutaraldehyde.

Immediately after all three replicates for any given incubation time were fixed, samples were gravity filtered onto a 5 μm (*Cyclidium* sp.) or 10 μm (*E. vannus* and *E. plicatum*) polycarbonate filter, over an 8 μm cellulosic backing filter. As filters ran dry the samples were washed one to two times (gently by allowing the pipettes to expel down the side of the filter tower) simultaneously with 1.5-3 ml phosphate buffer solution and 0.15-0.3 ml of sodium borohydride solution (SBH: 1 mg ml^{-1} $NaBH_4$ in cold buffer) to block autofluorescence generated by the glutaraldehyde. A final rinse with 2 ml of buffer was applied to remove any excess SBH. The filters were air dried, and placed on a glass slide in between two generous drops of immersion oil. A cover glass was allowed to settle over the filter without pressure. Filters were scanned using epifluorescence microscopy with either a 10 or 40× objective to locate ciliates. The number of prey consumed per predator was determined by switching to the 100× objective. Only ciliates containing one or more bacteria were included, and between 10 and 15 ciliates per filter were analyzed. Ingestion rates were calculated by linear regression of all samples through those time points that did not reduce the coefficient of determination (R^2) by more than 10% of the R^2 from regression through the initial time point.

For the FLB comparison experiment, a *Vibrio* sp-nap culture was harvested as in the above ingestion experiments, and adjusted to a desired cell concentration. A stock of FLB was thawed, allowed to acclimate to room temperature, and thoroughly homogenized (vortex 5 min.). An inoculum was prepared by adding FLB stock to the

Vibrio sp-nap harvest in the same proportions as in the GFP ingestion rate experiments run at a prey concentration of 10^7 cells ml^{-1} (see Table 2.1). All other experimental and sample processing procedures were performed as described above for the ingestion rate experiments.

Model Comparisons

The following three non-linear types of functional response equations were fitted to the ingestion rate data obtained for *E. vannus*, *E. plicatum*, and *Cyclidium* sp.:

Type II, hyperbolic: $$I = \frac{I'C}{C'+C} \quad (1)$$

Type II, with threshold: $$I = \frac{I'(C - C_t)}{C'+(C - C_t)} \quad (2)$$

Type III, sigmoid: $$I = \frac{I'\left(\frac{C}{C'}\right)^n}{1+\left(\frac{C}{C'}\right)^n} \quad (3)$$

where, I is the density dependant grazing rate, I' is the maximum grazing rate estimated by the model, C represents prey density, C_t is the threshold value in equation 2, and n is the exponential fitting parameter which distinguishes the Type III response from the Type II ($n > 1$ designates a Type III response; $n \leq 1$ reduces to a Type II equation). The C' variable is a fitting parameter in the Type III response, but is equivalent to the prey concentration at which I is at half its maximum in a Type II response (equivalent to the half saturation constant in Michaelis-Mentin kinetics).

A large body of literature was assimilated which investigated ciliate ingestion as a function of food concentration. All data were values that were either directly reported by

the authors, or were extrapolated from results presented as a figure. Each data set was individually fit with equations 1, 2, and 3 and analyzed as described above for the data from the current study. Model fit was determined by the lowest residual sum of squares (Samuelsson et al.) for all data sets. All statistical analysis was done in Statistix 9© (Analytical Software, Tallahassee, FL).

Results

Ingestion Rate Experiments

Bacteria were clearly visible within the ciliate's food vacuoles, and uptake of bacteria by all three ciliates began immediately after introduction of prey (Figure 2.1). A pattern consisting of a linear increase in the number of prey found in food vacuoles followed by a plateau was seen in all experiments (Figure 2.2). The plateau occurred ≤ 30 s after the inoculation in all experiments. This coincided with signs of digestion, as the degradation of previously ingested bacteria was readily observed along with newly taken in, intact cells. The time it took for the number of prey per predator to level off decreased with increasing prey concentration. Signs of digestion occurred after approximately 15 s or more of uptake, regardless of the grazer or the prey concentration. The tests for discrimination for or against bacteria containing GFP showed that there was no significant difference in the number of bacteria ingested over time whether observing uptake of GFP-*Vibrio* only, or calculating uptake from a 1:1 GFP-*Vibrio* + *Vibrio* sp.-nap inoculum ($\alpha = 0.05$; p-values were 0.83, 0.76, and 0.96 for *E. vannus*, *E. plicatum*, and *Cyclidium* sp. respectively).

The highest prey concentration at which ingestion rates were measured was 10^{10} bacteria ml^{-1}, however, maximum ingestion rates were attained by all grazers at 10^9 bacteria ml^{-1}. The minimum prey concentration that ingestion was observed for *E. vannus* was 10^5 bacteria ml^{-1}, at a rate of 4.5×10^2 bacteria $ciliate^{-1}$ h^{-1} (Table 2.2). *E. vannus* had the highest maximum ingestion rate attaining 1.7×10^5 bacteria $ciliate^{-1}$ h^{-1} at a prey concentration of 10^9 bacteria ml^{-1}. The lowest prey concentration where bacterial uptake was observed was 5×10^4 bacteria ml^{-1} for *E. plicatum*, at a rate of 1.6×10^2 bacteria $ciliate^{-1}$ h^{-1} (Table 2.2). The maximum rate attained for *E. plicatum* was 8.7×10^4 bacteria $ciliate^{-1}$ h^{-1}. Consumption of prey was not observed for *Cyclidium* sp. below 10^6 bacteria ml^{-1}, where bacteria were ingested at 8.1×10^2 cells $ciliate^{-1}$ h^{-1}. *Cyclidium* sp. also had the lowest maximum ingestion rate of 6.8×10^3 bacteria $ciliate^{-1}$ h^{-1}.

The dynamics for grazing experiments using FLB as the tracer were similar to those using GFP-*Vibrio.* Predators immediately began taking up FLB, and the increase in the number of FLB per ciliate leveled off within the same time frames. When comparing the rates ascertained from the two different methods, the results varied between ciliate species. The grazing rate for *Cyclidium* sp. measured using FLB (1.1×10^3 bacteria $ciliate^{-1}$ h^{-1}) was 34% lower than that observed using GFP-*Vibrio* (1.7×10^3 bacteria $ciliate^{-1}$ h^{-1}; Figure 2.3a). The grazing rate measured using FLB for *E. plicatum* (4.8×10^3 bacteria $ciliate^{-1}$ h^{-1}) was 14% lower than the rate determined using GFP-*Vibrio* (5.6×10^3 bacteria $ciliate^{-1}$ h^{-1}; figure 2.3b). In contrast, *E. vannus* comparison obtained an ingestion rate with the FLB (6.8×10^3 bacteria $ciliate^{-1}$ h^{-1}) that was 28% higher than that obtained using GFP-*Vibrio* (5.4×10^3 bacteria $ciliate^{-1}$ h^{-1}) (figure 2.3c).

Model Comparisons

The ingestion rate data obtained for all three ciliates in the current study were best fit by a Type III response model as determined by the RSS (Table 2.3). Ingestion rates for both species of *Euplotes* were next fit best by a Type II-with threshold model, and had the largest RSS for a traditional Type II. The threshold value for the Type II w/threshold for *Cyclidium* sp. was low enough that it was reduced to a traditional Type II model.

Similar results occurred when I modeled the data assimilated from previous investigations on ciliate ingestion rates as a function of prey concentration. Out of 23 individual data sets for various ciliate species, three had the lowest RSS when described by a Type II model, five were best fit by a Type II-with threshold model, and 15 were described best by a Type III functional response (Table 2.3).

Discussion

Ingestion Rates

Traditionally, protozoan ingestion rates have been measured over sampling intervals ranging from 5-10 min for a total of 20 min to hours (Eisenmann et al. 1998; Posch et al. 2001; Sherr et al. 1987; Tso and Taghon 1999; Wieltschnig et al. 2003). Work preliminary to the present study showed that the bacteria in food vacuoles of ciliates became too dense to enumerate in a short amount of time (< 60 s). The current study revealed that this is due to high rates of initial ingestion combined with the initiation of digestion on the same time scale. When studying microbial ecology, attention must be paid to scaling of the variables being measured (Petersen and Hastings 2001). The present results suggest that using the right time scale is critical when directly observing

protozoan ingestion. Initial ingestion rates for all ciliates were obtained within 30 s after prey was introduced, after which the number of prey observed per predator was generally constant (Figure 2.2). Beyond this point it was not possible to determine whether counts were leveling off as a result of digestion alone or due to a combination of digestion and a decrease in ingestion rates. This suggests that short sampling intervals (on the order of seconds) may be required for accurate ingestion rate estimates when directly observing the uptake of prey.

To evaluate how ciliate ingestion rates measured here compare to results from previous investigations, data was tabulated from studies that directly report the ingestion rate (in units of particles ciliate^{-1} h^{-1}), the food particle concentration, and the method used to measure the ingestion rate for various species of ciliate (Table 2.2). The highest rates achieved in this study were substantially higher than any previously reported. It is difficult to determine whether this is due to the range of prey used in the current study, or if the method of measurement provided higher estimates. A graphical comparison reveals that the majority of ingestion rates reported to date have been investigated in a narrow range of prey concentrations (10^6 to 10^7 bacteria ml^{-1}) (Figure 2.4). It can be argued that this is within the range of natural bacterial concentrations typically reported for the ocean, thus anything outside of this range is of no importance. However, bacterial concentrations can reach much higher concentrations in times or areas of high productivity (Santos et al. 2007). Furthermore, bacteria are typically found at concentrations of 10^9 ml^{-1} in benthic environments when scaled per milliliter of pore water (Schmidt et al. 1998). Observations at these particle concentrations are limited, but

estimates from the present study show that ciliates are capable of grazing bacteria at considerable rates in instances where prey density is high.

A few of the higher ingestion rates previously reported were measured using surrogate prey particles (Hadas et al. 1998; Parry et al. 2001; Sanders et al. 1989; Simek et al. 2000; Simek et al. 1996). However, active selection against surrogate tracer food particles has lessened the confidence in grazing rate estimates where these methods are employed (Boenigk et al. 2001a; Landry et al. 1991; McMannus 1991; Parry et al. 2001). The FLB comparison study performed here revealed higher initial ingestion rates when using GFP-*Vibrio* for *Cyclidium* sp. and *E. plicatum*, while a higher rate was obtained for *E. vannus* when ingesting FLB as the tracer. One possible explanation is that mechanisms for active prey selection are species-specific. Boenigk et al. (2002) did show in a test for selection between inert beads and live bacteria that two of the nanoflagellate predators studied showed both active and post-ingestion selection mechanisms, while a third showed no signs of selection. There is evidence that protozoa employ selection using both physical attributes, such as the size and morphology (Jurgens and Matz 2002; Jurgens et al. 1999; Posch et al. 2001), as well as chemical characteristics of the prey cells (Fenchel and Blackburn 1999; Hamels et al. 2004; Tso and Taghon 1999). There are possible physical and chemical changes to the bacteria during FLB preparation that have not been measured yet. These changes may favor selection of FLB by some species (such as *E. vannus*), while causing other species (such as *Cyclidium* and *E. plicatum*) to select against the particles. This, combined with differences in methodology among studies, is a possible explanation for the inconsistencies observed in ingestion rates measured using manipulated prey particles.

Functional Responses

The various behaviors and modes of feeding for ciliates are well studied, and are not restricted to that of a suspension feeder (Patterson et al. 1989; Verni and Gualtieri 1997). However, conventional logic often automatically associates ciliate ingestion with a hyperbolic functional responses (i.e. Type II) (Fenchel 1980c; Jurgens and Simek 2000; Kiorboe et al. 2003; Steinberg and Levin 2007). The ingestion rate data for *E. vannus*, *E. plicatum*, and *Cyclidium* were best fit by a Type III response (Table 2.3). Mathematically, type I and II responses provide the means for removal of prey until they are nearly depleted. This is best realized by analyzing the clearance rate associated with each type of functional response. Clearance rate is a measure of how much prey is 'swept clear' of a given area or volume, and is attained by dividing the ingestion rate of the grazer by the concentration of prey. A Type I response predicts a constant, maximal clearance rate until prey populations reach a concentration of zero, while a Type II predicts a continual increase in clearance as prey density decreases. Both a Type I and II functional response result in maximal clearance of prey at low prey concentrations (Figure 2.5). This is an unrealistic prediction, as the existence of these relationships in a natural system would result in an unstable oscillation of predator and prey, and ultimately to extinction (Morin 1999). Furthermore, this relationship is not feasible for the grazer from an energetic standpoint. An increase in clearance rate is a result of increased activity, while optimal foraging theories dictate that a consumer decrease its activity at time of low food availability to conserve energy (Pyke 1984, and references therein). There has been evidence prior to the present study reporting that clearance rates decline at lower particle concentration (Sanders 1988).

The Type III and a Type II-with threshold functional responses are mathematically equivalent with respect to clearance, and are more ecologically realistic (Figure 2.5). Clearance begins at a threshold prey density > 0, is maximal once resource concentration is no longer limiting, and declines once a mechanical (i.e. clogging of feeding apparatus) or a metabolic (i.e. food vacuole formation, digestion, etc.) process begins to limit ingestion. With these grazing responses, prey populations are allowed a period of recovery due to the depressed consumption rates associated with a reduction in prey, and a more stable coexistence of predator and prey is possible. This is also much more energetically realistic for the predator, where activity is maximal when resources can be optimally utilized.

With the above argument in mind, it can be assumed that a Type I or II response is rare in nature. When the models used to gauge the ciliates in the present study were applied to a collection of data previously reported in the literature, 20 out of 23 of the functional responses were best described by either a Type III, or a Type II-with threshold equation (Table 2.3). If an investigator uses data encompassing too small a prey concentration range to determine the functional responses then a traditional Type II equation would seemingly fit well. Trying to fit a threshold parameter would result in a value near to zero, or possibly a negative number, and this would reduce the Type II-with threshold to a traditional Type II. This is an important realization for the inclusion of bacterivory in larger scale ecological models. Microbial organisms respond rapidly to changes in resources and environmental conditions. Whether or not the functional response component of a model is stable or drives the prey to extinction could have a

significant impact on how we asses bacterivory for the control of bacterial populations and how grazing contributes to nutrient cycling.

Conclusion

The current study presented the highest initial ingestion rates, and encompassed the widest prey range reported in the literature to my knowledge. Results showed that high ingestion rates are attained within seconds of coming in contact with prey, and that digestion begins to act on the same time scale. This suggests that both ingestion and metabolic rates may need to be measured over short sampling intervals to be accurate. Future work is necessary to measure how long protozoa can maintain the ingestion rates reported here. The ingestion rate data of the three species of ciliate studied here, as well as for those reported in many previous studies, gave overwhelming evidence that protozoan grazing can be best described by either a Type III or a Type II-with threshold model. Results here support the use of these prey density-dependant equations over a traditional Type II equation when modeling microbial food web interactions. The probable cause for the frequent use of the less stable Type II model is that data are too few, and measured over too narrow a prey range to measure the predator's full response to changes in prey concentration. Type II predator-prey interactions are less stable, and are probably rare in nature.

Table 2.1. Percentage of GFP-Vibrio used as a tracer for monitoring ciliate ingestion of prey for all experimental prey concentrations; GFP-*Vibrio* presented here as a % of total *Vibrio* for each experiment.

Total Prey (bacteria ml^{-1})	% GFP		
	Cyclidium	*E. plicatum*	*E.vannus*
5.0×10^4	n.a.	100	n.a.
1.0×10^5	n.a.	n.a.	100
5.0×10^5	n.a.	100	n.a.
1.0×10^6	75	n.a.	60
1.3×10^6	n.a.	100	n.a.
2.8×10^6	n.a	100	n.a
5.5×10^6	n.a	100	n.a
1.0×10^7	50	50	15
5.0×10^7	n.a.	15	n.a.
1.0×10^8	20	5	3
1.0×10^9	15	2	1
1.0×10^{10}	15	1	1

Table 2.2. Ingestion rates (particle ciliate^{-1} h^{-1}) and clearance rates (nl ciliate^{-1} h^{-1}) reported for this study and in the literature for bacterivorous ciliates. Also reported are prey particle concentrations, and methods used to monitor ingestion.

Reference	**Taxon**	**Method**	**Particle Concentration (10^6 ml^{-1})**	**Ingestion Rate (particles h^{-1})**
This Study	*Euplotes vannus*	GFP	0.1	472
		GFP	1.0	1,150
		GFP	10.0	5,360
		GFP	100.0	51,800
		GFP	1000.0	167,000
		GFP	10000.0	145,000
	Euplotes plicatum	GFP	0.05	157
		GFP	0.5	450
		GFP	1.3	1.040
		GFP	2.8	1,460
		GFP	5.5	2,590
		GFP	10.0	5,580
		GFP	50.5	26,800
		GFP	100.0	38,200
		GFP	1000.0	87,100
		GFP	10000.0	82,400
	Cyclidium	GFP	1.0	806
		GFP	10.0	1,680
		GFP	100.0	4,640
		GFP	1000.0	6,830
		GFP	10000.0	5,900
Albright et al. (1987)	*Uronema sp.*	FLB	6.8	146
	Euplotes sp.	FLB	10.1	290
	Perotrich sp.	FLB	6.8	4,000
	Scuticociliate sp.	FLB	10.1	1,800
Sherr et al. (1987)	*Scuticociliates*	FLB	n.a.	n.a.
	Oligotrich ciliates	FLB	n.a.	n.a.
Kemp (1988)	*Natural Assemblage*	FLB	3290	525
Sanders et al. (1989)	*Halteria grandinella*	FLM	5.28[##]	1,276
	Strombidium	FLM	5.28[##]	331
	Scuticociliates	FLM	5.28[##]	500
	Epistylis plicatilis	FLM	5.28[##]	1,200
	Vorticella microstoma	FLM	5.28[##]	235
	Vorticella natans	FLM	5.28[##]	1,078
	Stokesia sp.	FLM	5.28[##]	598
	Hateria grandinella	FLM	0.9	n.a.
	Strombidium	FLM	0.9	n.a.
	Vorticella microstoma	FLM	0.9	n.a.
	Hateria grandinella	FLB	0.9	n.a.

	Strombidium	FLB	0.9	n.a.
	Vorticella microstoma	FLB	0.9	n.a.
Simek et al. (1996)	*Pelagohalteria viridis*	FLB	2.2	799
	Halteria grandinella	FLB	3.4	1,580
	Strobilidium hexakinetum	FLB	3.4	380
	Oligotrich sp.	FLB	3.4	440
	Pelagostrombidium fallax	FLB	2.2	136
	Borticella aquadulcis	FLB	3.4	4,200
	Cyrtolophosis mucicola	FLB	3.4	173
	Cinetochilum margaritaceum	FLB	3.4	57
	Cyclidium sp.	FLB	3.4	470
	Urotrich sp	FLB	2.2	10
	Urotrich sp. (small)	FLB	3.4	61
	Balanion planctonicum	FLB	2.2	8
	Coleps sp.	FLB	3.4	63
	Coleps spetai	FLB	2.2	62
Jurgens & Simek (2000)	*Halteria cf. grandinella*	FLB	0.06	n.a.
Simek et al. (2000)	*Halteria spp.*	FLB	4.28	1,782
		FLB	10.5	3,220
Zubkov & Sleigh (1996)	*Euplotes mutabilis*	Radioactive Tracer	30.0	16,000
			30.0	2,320
			6.0	1,600
Eisenmann et al. (1998)	*Tetrahymena sp.*	FISH / vacuole formation	200	4,575
Hadas et al. (1998)	*Cyclidium sp.*	FLB	7.28	35
	Stylonchia sp.	FLB	17.0	1,210
	Colpoda sp.	FLB	28.3	930
		FLB	2.0	1,054
Wilks & Sleigh (1998)	*Euplotes mutabilis*	FLM	0.0001	n.a.
		FLM	0.001	16
		FLM	0.01	n.a.
		FLM	0.05	50
		FLM	1.0	190
Tso & Taghon (1999)	*Cyclidium sp.*	FLB	40.0	68
	Euplotes sp.	FLB	5.0	244
Kisand & Zingel (2000)	*Scuticociliate sp.*	FLB	1.09	44
	Uronema sp.	FLB	1.09	46
	Cyclidium sp.	FLB	1.09	123
	Urotricha furcata	FLB	1.09	25
	Strobilidium	FLB	1.09	62
Ayo et al. (2001)	*n.a.*	FLB	2.90	59
	n.a.	FLB	2.90	179
	n.a.	FLB	2.90	24
	n.a.	FLB	2.90	88

Parry et al. (2001)	*Tetrahymena pyriformis*	FLB*	5.0	1,048
		FLB**	5.0	1,440
		GFP*	5.0	964
		GFP**	5.0	537
		GFP[#]	5.0	693
		FLM	5.0	207
Posch et al. (2001)	*Cyclidium glaucoma*	FLB	8.0	586
Shimeta et al. (2001)	*Cyclidium sp.*	FLB	2.2	n.a.
	Euplotes minuta	FLB	2.2	n.a.
Artolozaga et al. (2002)	*Uronema marinum*	FLB	0.5	33
		FLB	50.0	382
Wieltschnig et al. (2003)	*Natural Assemblage*	FLB	3450.0	98
Cleven (2004)	*Rimostrombidium spp.*	FLB	n.a.	103
	Halteria sp.	FLB	n.a.	194
	Scuticociliates	FLB	n.a.	12
	Epistylis spp.	FLB	n.a.	143
	Balanion/Urotrich sp.	FLB	n.a.	5
	Askenasia spp.	FLB	n.a.	3
	Vorticella	FLB	n.a.	93
	Limnostrombidium	FLB	n.a.	12
Ichinotsuka et al. (2006)	*Strobilidium spp.*	FLM	17.0	23
	Strombidium spp.	FLM	17.0	18
	Cyttarocylis	FLM	17.0	42
	Tintinnopsis	FLM	17.0	47

* = determined from direct counts; ** = determined from change in total population; [#] = determined from change in total fluorescence; [##] = average prey concentration from a wide reported range.

Table 2.3. Best-fit values for Type II (eq. 1), Type II w/ threshold (eq. 2), and Type III (eq. 3) functional response models for ingestion rate, I (bacteria ciliate^{-1} h^{-1}) as function of bacterial concentration, C (cells mL^{-1}) of bacterivorous ciliates from this study and others (see footnotes for references); C_t = threshold value; C' and n are fitting parameters; RSS = residual sum of squares.

species	Model	I'	C'	C_t	n	RSS
E. vannus[1]	2	3.19×10^{5}	2.48×10^{8}	----	----	2.21×10^{9}
	thresh	3.18×10^{5}	2.42×10^{8}	3.40×10^{6}	----	2.15×10^{9}
	3	2.91×10^{5}	1.53×10^{8}	----	2.77	7.64×10^{7}
E. plicatum[1]	2	1.16×10^{5}	1.22×10^{8}	----	----	9.92×10^{7}
	thresh	1.16×10^{5}	1.20×10^{8}	5.30×10^{5}	----	9.79×10^{7}
	3	1.14×10^{5}	1.13×10^{8}	----	1.13	8.32×10^{7}
Cyclidium sp.[1]	2	7.30×10^{3}	1.03×10^{7}	----	----	7.32×10^{5}
	thresh	Reduces to type 2		----	----	----
	3	7.24×10^{3}	1.03×10^{7}	----	1.09	7.15×10^{5}
Tintinnopsis fimbriata[2]	2	1.01×10^{3}	2.85×10^{5}	----	----	7.75×10^{4}
	thresh	Reduces type 2		----	----	----
	3	Reduces type 2		----	----	----
					----	----
Tintinnopsis parvula[2]	2	5.78×10^{1}	9.16×10^{4}	----	----	2.81×10^{2}
	thresh	Reduces type 2		----	----	----
	3	5.54×10^{1}	8.28×10^{4}	----	1.08	2.79×10^{2}
Tintinnopsis tubulosa[2]	2	1.38×10^{2}	1.40×10^{5}	----	----	1.17×10^{3}
	thresh	1.37×10^{2}	1.31×10^{5}	1.83×10^{3}	----	1.12×10^{3}
	3	1.26×10^{2}	1.07×10^{5}	----	1.19	1.04×10^{3}
Eutintinnus pectinis[3]	2	1.70×10^{2}	3.20×10^{1}	----	----	1.27×10^{4}
	thresh	1.58×10^{2}	1.65×10^{1}	1.08×10^{1}	----	1.09×10^{4}
	3	1.48×10^{2}	2.83×10^{1}	----	2.12	9.72×10^{3}
Helicostomella subulata[4]	2	1.45×10^{2}	2.14×10^{1}	----	----	2.16×10^{3}
	thresh	1.38×10^{2}	1.51×10^{1}	4.1	----	1.83×10^{3}
	3	1.32×10^{2}	1.87×10^{1}	----	1.4	1.96×10^{3}
Glaucoma scintillans[5]	2	9.94×10^{2}	6.08×10^{7}	----	----	2.74×10^{4}
	thresh	9.48×10^{2}	5.24×10^{7}	1.83×10^{6}		2.70×10^{4}
	3	8.62×10^{2}	4.51×10^{7}	----	1.18	2.65×10^{4}
Cyclidium sp.[6]	2	2.92×10^{4}	1.17×10^{10}	----	----	7.36×10^{5}
	thresh	2.38×10^{4}	7.75×10^{9}	2.71×10^{8}	----	2.38×10^{5}

[1] This study

[2] (Spittler 1973), Data from Figure 2

[3] (Heinbokel 1978), Data from Figure 7

[4] (Heinbokel 1978), Data from Figure 8, experiment 9

[5] (Fenchel 1980c), Data from Figure 2 (upper)

[6] (Fenchel 1980c), Data from Figure 5

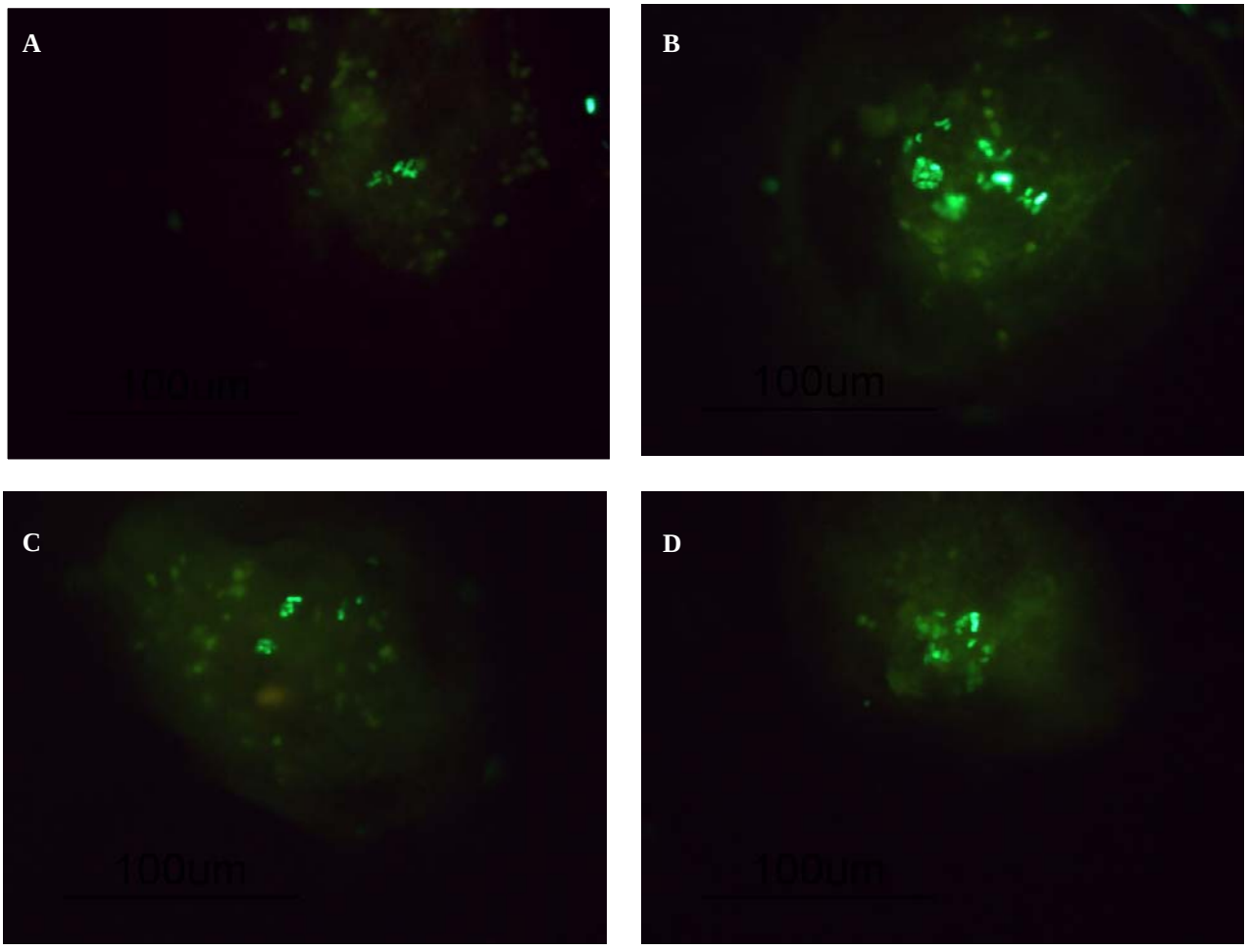
	3	2.08×10^{4}	6.24×10^{9}	----	1.2	$5.13E+0^{5}$
Tintinnopsis acuminate[7]	2	1.63×10^{2}	5.69×10^{1}	----	----	1.77×10^{2}
		2.91×10^{2}	9.40×10^{1}	----	----	1.08×10^{3}
	thresh	Reduces to type 2		----	----	----
	3	Reduces to type 2		----	----	----
Tintinnopsis vasculum[8]	2	7.53×10^{2}	6.51×10^{1}	----	----	1.08×10^{4}
		8.80×10^{2}	5.18×10^{1}	----	----	3.68×10^{4}
	thresh	7.12×10^{2}	4.46×10^{1}	1.15×10^{1}	----	6.91×10^{3}
		8.20×10^{2}	2.94×10^{1}	1.46×10^{1}	----	2.41×10^{4}
	3	6.59×10^{2}	5.34×10^{1}	----	1.58	4.99×10^{3}
		7.64×10^{2}	4.51×10^{1}	----	1.89	1.81×10^{4}
Lohmanniella spiralis[9]	2	2.29×10^{3}	1.46×10^{5}	----	----	1.36×10^{5}
	thresh	2.21×10^{3}	1.30×10^{5}	3.46×10^{3}	----	1.33×10^{5}
	3	2.04×10^{3}	1.14×10^{5}	----	1.15	1.32×10^{5}
Lohmanniella spiralis[10]	2	2.18×10^{2}	9.75×10^{3}	----	----	9.45×10^{2}
	thresh	1.88×10^{2}	4.25×10^{3}	1.82×10^{3}	----	1.63×10^{2}
	3	1.68×10^{2}	5.57×10^{3}	----	2.02	2.15×10^{2}
Strobilidium spiralis[11]	2	1.94×10^{-1}	1.09×10^{2}	----	----	1.94×1^{-3}
	thresh	1.82×10^{-1}	8.07×10^{1}	9.43	----	1.64×1^{-3}
	3	1.57×10^{-1}	7.05×10^{1}	----	1.59	1.28×10^{-3}
Tintinnopsis dadayi[11]	2	1.60×10^{-1}	1.41×10^{2}	----	----	1.76×10^{-3}
	thresh	1.46×10^{-1}	9.66×10^{1}	1.24×10^{1}	----	1.52×10^{-3}
	3	1.26×10^{-1}	8.61×10^{1}	----	1.52	1.44×10^{-3}
Strombidium sp.[12]	2	4.46×10^{-2}	1.47	----	----	2.32×10^{-5}
	thresh	3.80×10^{-2}	9.17×10^{-1}	1.07×10^{-1}	----	1.99×10^{-5}
	3	3.50×10^{-2}	8.85×10^{-1}	----	1.28	2.15×10-5
Strombidium sp.[13]	2	5.19×10^{-2}	1.78	----	----	4.42×10^{-5}
	thresh	4.25×10^{-2}	1.02	1.15×10^{-1}	----	3.46×10^{-5}
	3	3.30×10^{-2}	6.13×10^{-1}	----	1.70	3.39×10^{-5}
Strombidium sp.[14]	2	4.77×10^{-3}	1.78	----	----	2.59×10^{-7}
	thresh	3.92×10^{-3}	1.02	1.51×10^{-1}	----	1.20×10^{-7}
	3	2.97×10^{-3}	6.13×10^{-1}	----	1.70	1.01×10^{-7}
Halteria cf. *grandinella*[15]	2	8.03	1.32×10^{6}	----	----	5.49×10^{-1}

[7] (Verity 1985), Data from Figure 3a; at 25°C (top value), at 25°C (bottom value)
[8] (Verity 1985), Data from Figure 3b; at 10°C (top value), 15°C (bottom value)
[9] (Jonsson 1986), Data from Figure 2
[10] (Jonsson 1986), Data from Figure 3
[11] (Verity 1991), Data from Figure 1a
[12] (Verity 2000), Data from Figure 4a
[13] (Verity 2000), Data from Figure 4b
[14] (Verity 2000), Data from Figure 5
[15] (Jürgens and Simek 2000), Data from Figure 3a, 1.8 μm-beads

	thresh	7.54	1.03×10^{6}	6.56×10^{4}	----	3.18×10^{-1}
	3	6.26	7.65×10^{5}	----	1.58	6.79×10^{-2}
Halteria cf. *grandinella*[16]	2	5.61	5.30×10^{5}	----	----	4.35×10^{-2}
	thresh	Reduces to type 2		----	----	
	3	5.29	4.66×10^{5}	----	1.05	4.18×10^{-2}
Cyclidium sp.[17]	2	1.22×10^{4}	1.83×10^{8}	----	----	4.97×10^{6}
	thresh	1.21×10^{4}	1.73×10^{8}	3.29E+06	----	4.81×10^{6}
	3	8.53×10^{4}	9.55×10^{7}	----	2.65	6.98×10^{5}
Cyclidium sp.[18]	2	6.55×10^{2}	1.98×10^{7}	----	----	8.70×10^{3}
	thresh	6.48×10^{2}	1.86×10^{7}	$3.63E+0^{5}$	----	8.29×10^{3}
	3	5.92×10^{3}	1.50×10^{7}	----	1.35	7.33×103
Metopus es[19]	2	4.15×10^{3}	2.62×10^{7}	----	----	3.25×106
	thresh	Reduces type 2		----	----	----
	3	Reduces type 2		----	----	----
Plagiopyla nasuta[20]	2	4.25×10^{3}	3.70×10^{-7}	----	----	5.04×106
	thresh	4.02×10^{3}	2.67×10^{7}	2.20×10^{6}	----	4.71×106
	3	3.67×10^{3}	2.38×10^{7}	----	1.40	4.47×106
Plagiopyla nasuta[21]	2	3.53×10^{2}	1.26×10^{6}	----	----	3.17×105
	thresh	3.49×10^{2}	1.14×10^{6}	6.88E+04	----	3.15×105
	3	3.04×10^{2}	8.28×10^{5}	----	2.95	2.89×105

[16] (Jürgens and Simek 2000), Data from Figure 3a, 2.76 μm-beads
[17] (Sanders 1988), Data from Table 2, 0.6 μm-beads
[18] (Sanders 1988), Data from Table 2, 0.93 μm-beads
[19] (Massana et al. 1994), Data from Figure 5
[20] (Massana et al. 1994), Data from Figure 4a
[21] (Massana et al. 1994), Data from Figure 4b

Figure 2.1. Images showing the amount of GFP-*Vibrio* at 10 and 20 s after introduction of bacteria for *E. plicatum* (A and B), and *Cyclidium* sp. (C and D).



10 s

20 s

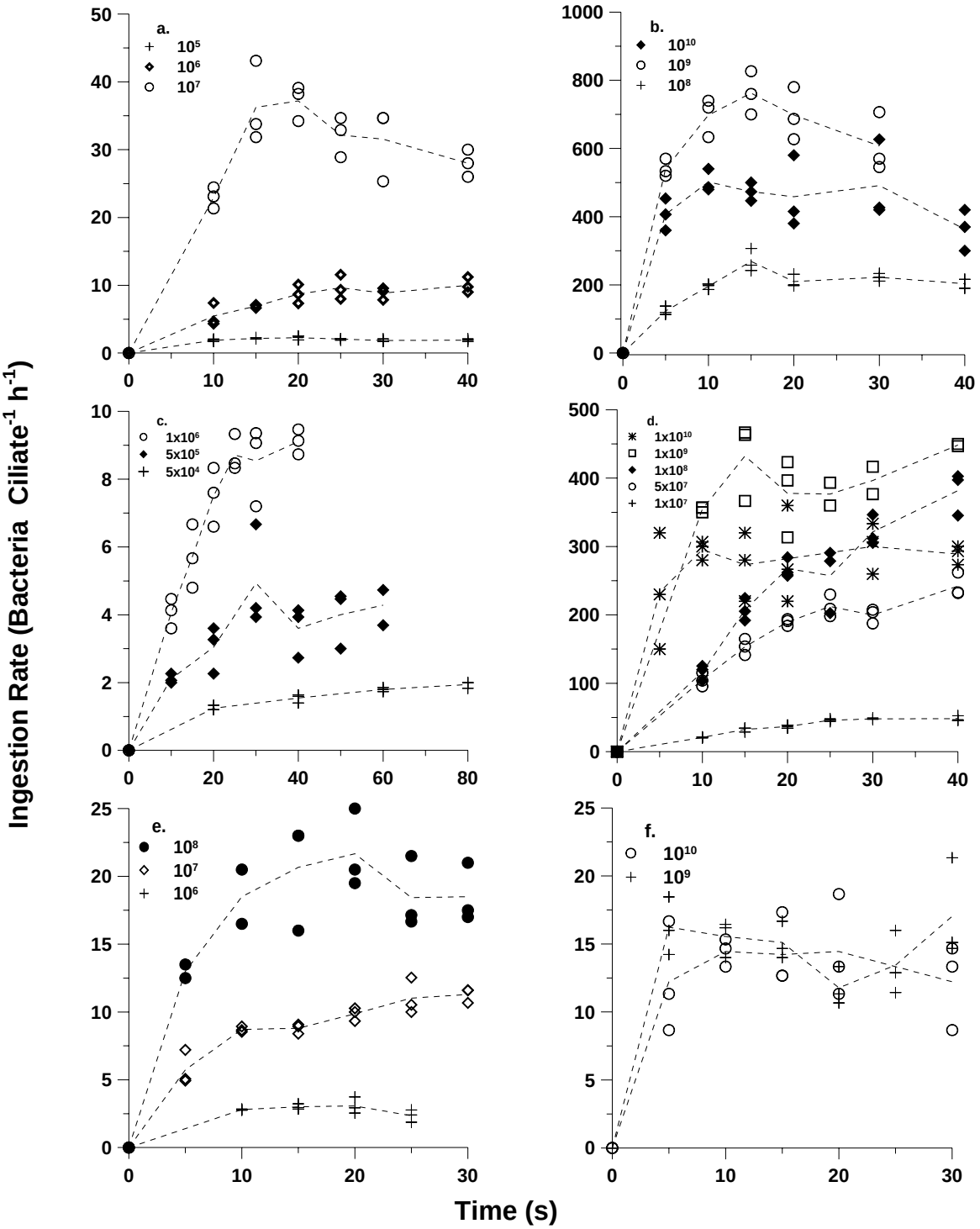


Figure 2.2. Initial ingestion data for *E. vannus* (a-b), *E. plicatum* (c-d), and *Cyclidium* sp. (e-f) at various concentrations of bacteria; (dashed lines = average trend for n=3 replicates.

Figure 2.3. Ingestion of GFP-*Vibrio* (solid line) and FLB (dashed line) for *Cyclidium* sp. (a), *E. plicatum* (b), and *E. vannus* (c).

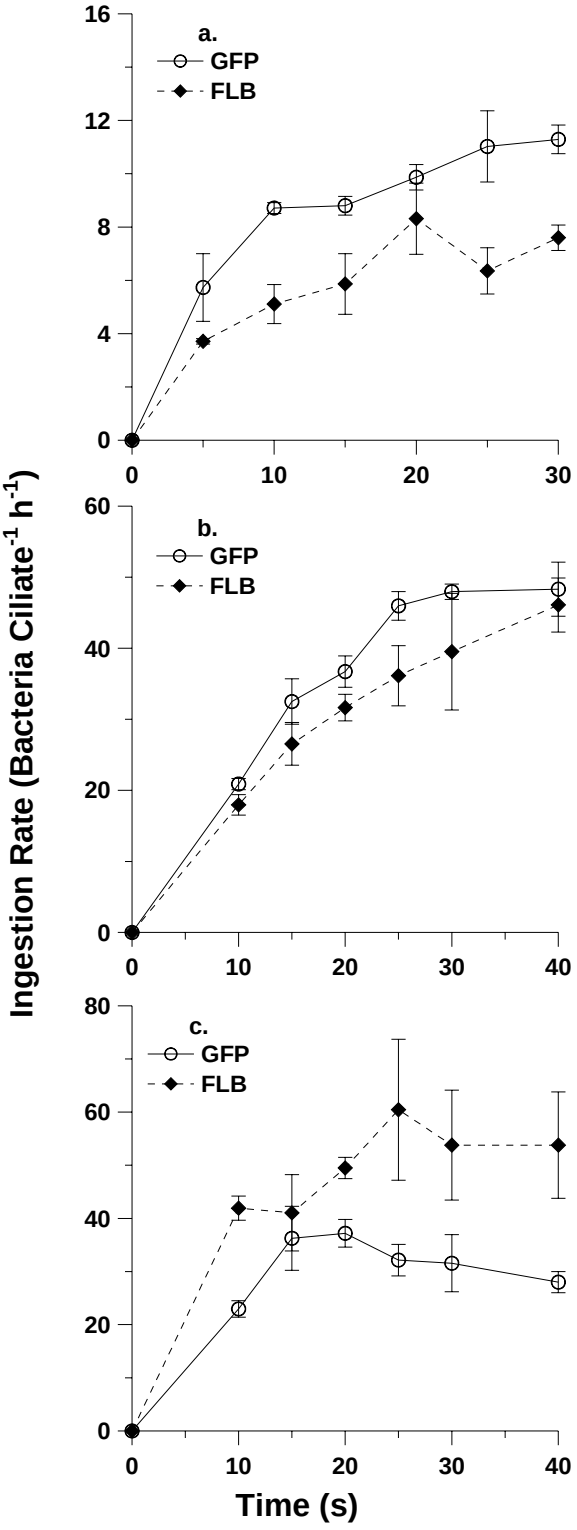


Figure 2.4. Ciliate ingestion rates measured as a function of prey concentration from the present study (open circles) and previous studies (closed diamonds).

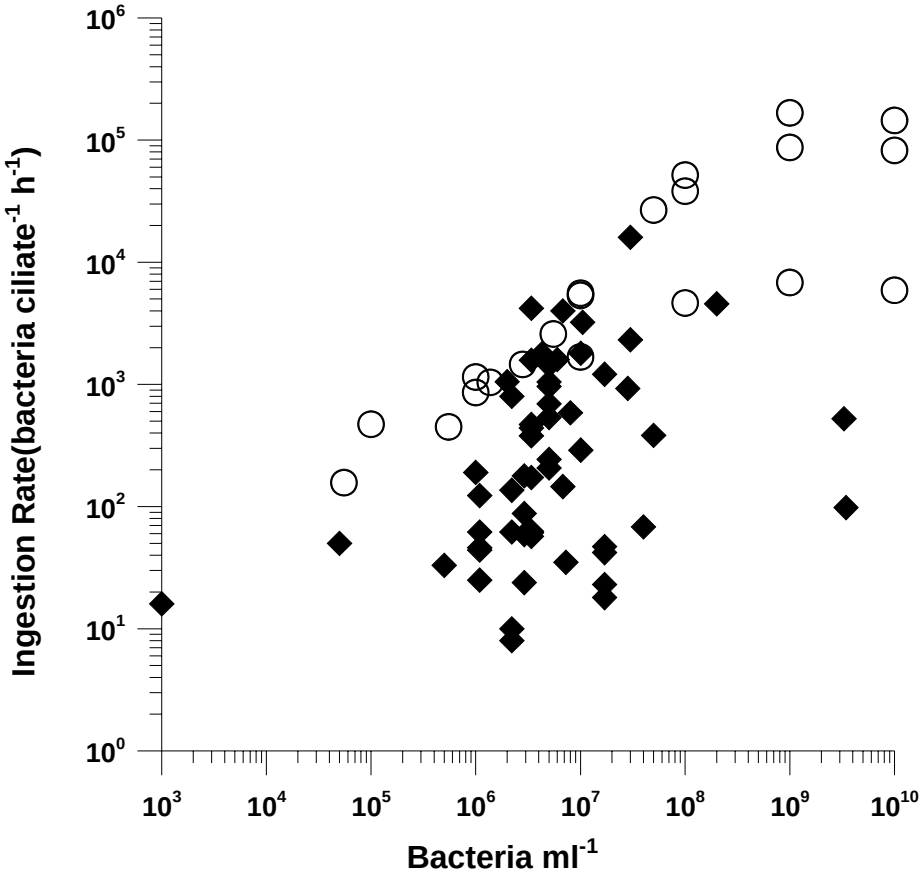
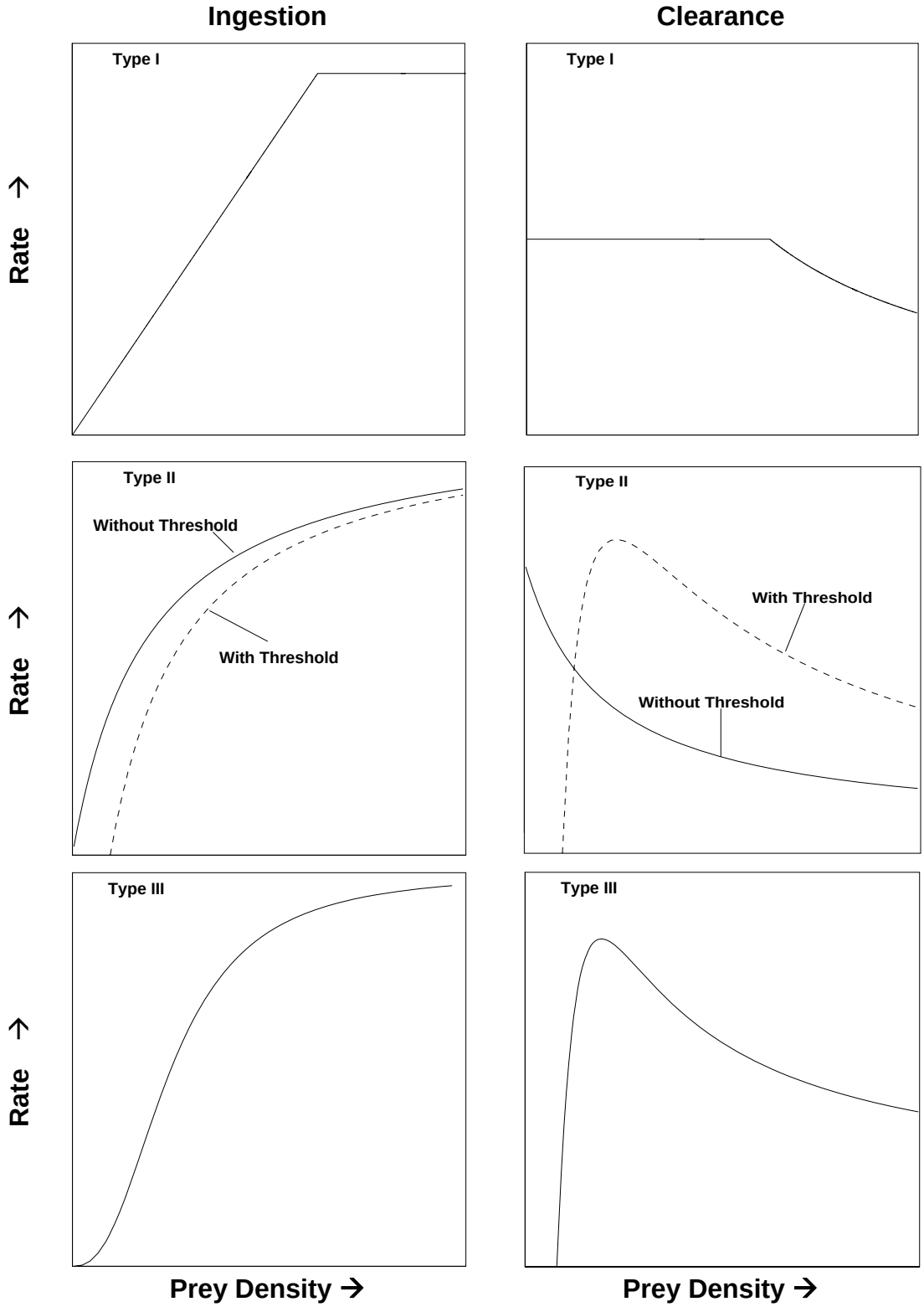


Figure 2.5. Theoretical representation of ingestion and clearance rate for a Type I, II with (dashed line) and without (solid line) a threshold value, and III feeding response to prey density.



CHAPTER III

Estimating Ciliate Digestion Using Direct Measurements of Ingestion Rates and Food Vacuole Formation

Abstract:

In the current study measurements of ingestion and food vacuole formation were used to investigate digestion rates for the marine bacterivorous ciliates *E. vannus*, *E. plicatum*, and *Cyclidium* sp. Bacteria expressing green fluorescent protein were used as a natural prey tracer to calculate ingestion rates, vacuole formation rates, and to estimate the maximum number food vacuoles for each grazer at a low (10^6-10^7 bacteria ml^{-1}) and a high (10^9 bacteria ml^{-1}) prey concentration. The resultant data were used to estimate the time it takes to reach maximum food vacuole capacity, and subsequently the digestion rate of the predators. Food vacuole content and ingestion rates were higher at high prey concentrations. Digestion rates had a negative correlation with food vacuole content and thus with prey concentration. This refutes the idea that digestion occurs as a rate constant and is independent of food vacuole content. The estimates for ingestion rates and digestion efficiency predict that ciliates are capable of processing nearly 100% of their consumed prey per hour, and consequently exerting a substantial amount of top-down control on bacteria at typical water column predator and prey concentrations. However, ciliates will need to either exhibit elevated digestion rates or exist at higher abundances to exert the same top-down pressure in environments containing higher prey concentrations.

Introduction

The qualitative importance of protozoan bacterivory in the marine food web is well known (Sherr and Sherr 1994; Sherr and Sherr 2002). Protozoa directly impact bacteria by removing them from the environment, and so there has been a focus on quantifying protozoan ingestion rates (Ayo et al. 2001; Huws et al. 2005; Sherr and Sherr 1994). It is necessary to be able to estimate how much bacterial biomass is assimilated into grazer biomass (becoming a potential "trophic link" to higher levels in the food chain) and how much is excreted as regenerated nutrients (becoming a "trophic sink" to fuel further production) (Caron 1991; Zubkov and Sleigh 1996), before we can quantify the ecological and biogeochemical role of protozoan bacterivory. Therefore, it is also necessary to characterize rates at which protozoa process and metabolize prey.

There are many examples of the use of food vacuole content and digestion rates for estimating ingestion rates in protozoa (Dolan and Coats 1991; Konigs and Cleven 2007; Li et al. 2001). This method assumes that the change in food vacuole content (F) is dependant on the balance between the grazer's ingestion rate (I) and digestion rate (D):

$$dF/dt = I - D \qquad (1)$$

Digestion rate is assumed to equal the food vacuole content times a digestion rate constant (k) (i.e. $D = k \times F$), thus when food vacuole content reaches steady state, $dF/dt = 0$, and:

$$I = kF \qquad (2)$$

Estimating ingestion rates using this method requires that k is constant and independent of F . However, it has been shown that food content processing is in fact a function of prey concentration (Sherr et al. 1988; Zubkov and Sleigh 1996).

Furthermore, there is evidence that the initial number of ingested prey impacts estimates of k, and that the total number of ingested prey is also a function of prey concentration (Fenchel 1980d; Fok et al. 1988; Harding 1937). It follows then, that it is unlikely that k is independent of F. A better understanding is needed of the relationship between protozoan ingestion and digestion, as well as knowledge of any interspecific differences in that relationship.

Quantifying processes for microbial organisms is inherently difficult due to the scale of the system. Protozoa are adapted to optimize food capture and processing in an environment with patchy and rapidly changing resources. Ciliates are capable of achieving high initial rates of ingestion ($>10^5$ bacteria ciliate^{-1} h^{-1}) within seconds of encountering high prey concentrations (see Chapter 2), and these rates vary as a function of prey concentration. Maintaining ingestion rates of this magnitude would require that digestion occur at similar rates and that it also vary as a function of prey concentration. The purpose of this study was to determine how the food vacuole formation and ingestion rates of three species of bacterivorous ciliates change over time as a function of prey concentration, and to use the results to estimate digestion rates.

Methods

Bacteria

Vibrio sp.-nap was used as prey and was maintained in liquid culture (0.2 µm filtered, aged seawater at a salinity of 23, with 2.5 g L^{-1} soy peptone, and 0.5 g L^{-1} yeast extract) on a rotary shaker kept at 75rpm and 20°C. Subcultures of *Vibrio* sp.-nap that had been transformed to express green fluorescent protein (GFP) were obtained (Gruber in press),

and will be referred to as GFP-*Vibrio*. *Vibrio* sp.-nap and GFP-*Vibrio* cultures were maintained and prepared for experiments as previously described (Chapter 2).

Ciliates

Two species of hypotrich ciliates, *Euplotes vannus* (length = 100 ± 11 μm, width = 57 ± 8 μm) and *Euplotes plicatum* (length = 51 ± 4 μm, width = 32 ± 4 μm), were acquired from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, UK). A species of *Cyclidium* (scuticociliate; length = 26 ± 3 μm, width = 10 ± 2 μm), was isolated from Piles Creek, NJ. All protozoa were cultured in an Artificial Seawater for Protozoa medium (ASWP) adapted from the CCAP. Ciliate cultures were maintained and prepared for experiments as previously described (Chapter 2).

Ingestion Rate Experiments

Ingestion rates were measured using a modification of previously described methods (Chapter 2). Protozoa were kept at low densities ($<10^3$ cells ml^{-1}) to limit the reduction of the overall prey throughout the course of an experiment. GFP-*Vibrio* were added after ciliates had been feeding on the non-fluorescent *Vibrio* sp.-nap for varying durations (10, 60, and 120 min. for *Cyclidium*; 10, 60, and 3,600 min. for *E. plicatum* and *E. vannus*). The pulse of labeled prey permitted the measurement of ingestion rate constancy as a function of feeding duration. Two prey concentration were used, 10^7 and 10^9 for *Cyclidium*, and 10^6 and 10^9 for *E. plicatum* and *E. vannus*. The proportions of added GFP-*Vibrio* were adjusted so that overall prey concentration was not altered and so the observation of prey within food vacuoles was optimized (Table 3.1**)**. All other

procedures for the ingestion trials, sample processing, and ingestion rate calculation were previously described (Chapter 2).

Vacuole Formation and Digestion rates

Ciliates were harvested using the same procedure as in the ingestion experiments. Ciliates were added to small test tubes and allowed to acclimate for approximately 3 hours prior to prey introduction. GFP-*Vibrio* cultures were harvested and were added to the ciliates to achieve the same prey concentrations as in the ingestion rate experiments. Three replicates were preserved with 1% (by volume) ice-cold glutaraldehyde between 15 and 30 min after prey introduction. Fixed samples were immediately gravity filtered onto a 5 μm (low prey concentrations) or 10 μm (high prey concentrations) polycarbonate filter. Filters were washed one to two times simultaneously with 2-4 ml of phosphate buffer and 0.2-0.4 ml sodium borohydride (1 mg ml^{-1}) to block autofluorescence. Filters were removed, air dried, and placed on a slide between two drops of immersion oil. A cover glass was allowed to settle over the filter without applying pressure. Filters were analyzed immediately using epifluorescence microscopy, and the number of food vacuoles per ciliate was recorded for 15-20 ciliates per filter until a total of 50 ciliates were observed. Only ciliates containing one or more discernable, intact food vacuole were analyzed. This sampling procedure was repeated at 30-60 min intervals until a noticeable plateau in the number of vacuoles per ciliate was observed. The mean number of vacuoles per ciliate was calculated for each time point. The maximum number of vacuoles per ciliate (V_{max}) at each prey concentration included all samples after food vacuole formation reached a plateau, whose combined mean was not

significantly greater than that of the samples from the previous time point (ANOVA, significance level of 0.05). Rates of food vacuole formation were determined by linear regression through all samples preceding the time points used to estimate V_{max}.

Food vacuole content at equilibrium was taken as the combined units of prey within all vacuoles when the ingestion rate and the number of vacuoles were constant. This is equal to the product of the time it takes to reach V_{max} (T_{max}) times the ciliates steady-state ingestion rate (ie. $F = I \times T_{max}$). I estimated T_{max} for each prey concentration by dividing the observed V_{max} by the rate of vacuole formation. From equation 2 we derive $k = I/F$, and with the appropriate substitutions for F, digestion rates were directly calculated by:

$$k = \frac{1}{T_{max}} \qquad (3)$$

Results

Ingestion Rate Experiments

The uptake of GFP-*Vibrio* was clearly visible. A linear increase of prey within food vacuoles was observed over a course of 15-30 s for all ciliates (Figure 3.1). An observed abatement in prey increase was always accompanied by distinct signs of the cellular breakdown of ingested bacterial cells. Average ingestion rates of all three grazers remained relatively stable from the first through the final time trial for each particular prey density (Figure 3.2). Ingestion rates after 10 min averaged 0.95 and 1.36×10^3 bacteria individual^{-1} h^{-1} for *Cyclidium* at low and high prey concentrations respectively. The lowest prey concentration used for *Cyclidium* sp. grazing experiments was 10^7 cells ml^{-1}. This was following several attempts where ingestion of bacteria at a density of 10^6

ml^{-1} was not observed. Experimental protocols did not allow for the percentage of GFP-*Vibrio* required to observe uptake by *Cyclidium* sp. at 10^6 bacterial ml^{-1} (Chapter 2). Rates for *E. plicatum* for the low and high prey concentration trials were calculated to be 0.92 and 16.77×10^3 bacteria h^{-1} respectively, and were 1.11 and 13.18×10^3 bacteria h^{-1} respectively for *E. vannus*.

Vacuole Formation and Digestion Rates

Food vacuoles were clearly defined and the change in the number of vacuoles per ciliate was discernable over time (Figure 3.3). One to two newly formed vacuoles were usually present, and were distinguishable from older ones due to a brighter green fluorescence of the GFP and clear outlines of the most recently ingested GFP-*Vibrio* cells. As vacuoles matured, GFP was denatured by the digestion process, and a dull fluorescence produced mainly by autofluorescence induced by the glutaraldehyde became noticeable. The number of vacuoles per individual increased linearly with time for all grazers (Figure 3.4) until a maximum number of vacuoles was attained. Vacuole formation rates as well as the maximum number of vacuoles increased with increasing prey concentration for *E. vannus* and *E. plicatum* (Table 3.2). The maximum number of vacuoles at high prey density was considerably higher than at low prey density for *Cyclidium* sp., but rates of vacuole formation for the two prey concentrations were similar.

The formation of food vacuoles at 10^6 bacteria ml^{-1} was only discernable in *E. vannus*. Only one to three vacuoles were visible through all time points. No distinctly formed vacuoles were visible at any time at 10^6 bacteria ml^{-1} for either *Cyclidium* or *E. plicatum* suggesting that digestion was occurring faster than a food vacuole could be filled to its

entirety. As a result, rates of formation for low prey concentrations were measured for these two species at 10^7 bacteria ml^{-1}. The maximum vacuole formation rates reached were 42.6, 28.8, and 13.2 vacuoles min^{-1} for *Cyclidium*, *E. plicatum*, and *E. vannus* respectively. There was a positive relationship between T_{max} and prey concentration for all three ciliates **(**Table 3.2). Thus, digestion rates displayed an opposite trend, and showed an inverse relationship with both prey concentration and ingestion rate. A k value > 1 h^{-1} was calculated for *E. vannus* at a prey concentration of 10^6 bacteria ml^{-1}. All other k values were ≤ 0.83 h^{-1} (Table 3.2).

Discussion

Ingestion Rates and Food Vacuole Dynamics

I adapted the procedure from Chapter 2 to compare the initial prey consumption dynamics for *E. vannus*, *E. plicatum*, and *Cyclidium* reported there, to those measured here after the ciliates had been feeding for varying periods of time. After 10 min of grazing at a high prey concentration (10^9 bacteria ml^{-1}) the rates of uptake were 5× lower for *Cyclidium* sp. and *E. plicatum*, and 13× lower for *E. vannus*, than their respective initial ingestion capabilities (Figure 3.2; initial ingestion rates at time = 0 taken from Chapter 2). Rates remained relatively stable through the final time point. In contrast, ingestion rates over time at low prey concentrations showed little variation from their respective initial rates reported in Chapter 2. Food vacuole observations showed that a maximum number of food vacuoles (V_{max}), was reached under all conditions. Steady state food vacuole content was attained or both the low and high prey trials of all three

grazers, as ingestion rates remained stable well beyond the time that was required to attain the maximum number of food vacuoles (T_{max}; see Figure 3.2).

Early investigations argued that protozoan ingestion capabilities were regulated primarily by mechanical variables, such as the functional capabilities of the oral cilia (Fenchel 1980c; Fenchel 1980d). A previous study revealed that at high prey densities the ciliates are capable of ingesting bacteria at high rates initially (Chapter 2), but within minutes their uptake decreased until finally reaching a steady state rate in the present study. This implies that a processing step (i.e., food vacuole formation or digestion) is limiting the grazer's ingestion capabilities at high prey concentrations, rather than a consumption mechanism. Being that ingestion rates are maintained after V_{max} is reached, it is likely that digestion is the limiting variable at high prey concentrations. At low prey concentrations ingestion rate did not change over time. Additionally, full food vacuoles were not observed at 10^6 bacteria ml^{-1} for two out of the three grazers. This suggests that at lower prey concentrations, search time (i.e., the time it takes to successfully search for and come in contact with prey) is what limits ingestion rather than food processing.

Digestion and Prey Concentration

Digestion rates are usually measured directly by monitoring the disappearance of the prey from within food vacuoles (Boenigk et al. 2001b; Capriulo and Degnan 1991; Jezbera et al. 2005; Sherr et al. 1988). Digestion rate dynamics of protozoa have been reported to be described by both a linear (Li et al. 2001; Sherr et al. 1988) and an exponential decay model (Dolan and Simek 1997; Dolan and Simek 1998; Jezbera et al. 2005). However, the protocols of many studies observe digestion after separating the predator from their

prey (Dolan and Simek 1998; Jezbera et al. 2005; Li et al. 2001), and thus do not represent natural conditions. If digestion rate is in fact dependant on food content and no new prey are being ingested then the disappearance of prey would occur exponentially. In other instances continual feeding is simulated by washing the system free of tracer, and placing the grazers in a system with non-tracer prey. In either case it is not likely that digestion rates measured are representative of steady state values after lengthy manipulation, given the rapidity with which protists respond to changes in environmental conditions and prey concentrations. Here I report that ingestion rates do not drop off even after there is no further increase in the number of food vacuoles per cell. This leads to the conclusion that digestion is occurring in a linear fashion at a rate that is equal to ingestion.

The most direct way to determine food vacuole content would be to calculate it based on the total food vacuole volume and prey volume. Vacuole volume not only varies with changes in prey concentration (Eisenmann et al. 1998; Fok et al. 1988) and environmental conditions such as pH (Mills 1931) and temperature (Li et al. 2001; Sherr et al. 1988), but can also change depending on which stage of processing the vacuoles are in (Ramoino et al. 1996; Zubkov and Sleigh 1996). Prey also undergo changes in volume as they progress through stages of digestion. Consequently, estimating average prey and vacuole volume for the determination of vacuole content is problematic. In the present study, digestion rate was calculated as the reciprocal of the time it takes to reach steady state food vacuole content. If ingestion rates are constant, then food vacuole content is at steady state when the maximum number of vacuoles is reached. I was able to directly calculate food vacuole formation rates as well as the maximum number of vacuoles, and

thus obtain an estimate for T_{max}. The resulting k values fall within the range of values reported elsewhere (Capriulo and Degnan 1991; Dolan and Coats 1991; Dolan and Simek 1997; Jezbera et al. 2005; Sherr et al. 1988). However, while ciliate digestion rates are often reported as being invariable with changes in the amount of consumed prey (Dolan and Simek 1997), results from this study reveal that k has negative relationship with F (Table 3.2). This must be considered when inferring ingestion rates from measured digestion.

That k varies with prey concentration also has important implications for how ciliates turn over bacterial biomass and control prey populations in environments of various prey densities. By multiplying k by 100 an estimate of digestion efficiency is obtained in the form as a percentage of ingested prey that is digested per hour. The k value of *E. vannus* predicts that > 100% of its consumed prey is digested per hour at 10^6 bacteria ml^{-1}, and this is supported by the low accumulation of food vacuoles (Table 3.2). This may actually be an underestimation of k for *E. vannus* at this prey density, as the maximum number of food vacuoles was already attained by the first sampling point. A higher food vacuole formation rate results in smaller T_{max}, and thus a higher k. Digestion rates for *E. plicatum* and *Cyclidiuim* sp. were only obtained at 10^7 bacteria ml^{-1}. However, the lack of food vacuole formation at 10^6 bacteria ml^{-1}, despite an appreciable ingestion rate, leads to the assumption that these two species can also digest 100% of their consumed prey per hour at this concentration. As ingestion rates increase the predator's digestion capabilities cannot balance the uptake. A possible explanation for this is that the energy required to search for and subsequently handle food particles at high prey density limits the amount of energy that can be devoted to metabolic processes. Optimal foraging

theory dictates that a consumer exhibit higher levels of activity as food becomes more available. As a result the cell stores an increasing amount of prey (i.e. an increase in F), but a smaller percentage of that food content is actually being processed per hour and ingestion decreases to compensate.

The present data shows that while digestion efficiency goes down as prey concentration goes up, ingestion rates continue to rise. This is evidence that protozoa are adapted to maximize utilization of resources at each particular prey density. As a result, ciliates have the potential to play a significant role in the turnover of bacterial biomass. Water column bacterial abundances average approximately 10^6 bacteria ml^{-1} (Ducklow 1999). This value can increase at times or locations of higher productivity (Santos et al. 2007), and can be as high as 10^9 bacteria ml^{-1} at sediment interfaces (Schmidt et al. 1998). For a prey concentration of 10^6 cells ml^{-1} to persist, bacteria must maintain a growth rate which accounts for population losses. The three ciliate species in the present study maintain a measured steady ingestion rate in the range of 10^3 bacteria h^{-1} at a prey concentration of 10^6 bacteria ml^{-1}. Ciliate abundance in the open ocean is typically reported to be within the range of 10-100 ml^{-1} (Ichinotsuka et al. 2006), but they can attain numbers on the order of 1000 ml^{-1} in productive and benthic environments (Kemp 1988; Wieltschnig et al. 2003). Assuming a ciliate abundance of 100 cells ml^{-1}, and that 100% of the predator population is actively feeding, loss to grazing would represent 10% of the total hourly bacterial turnover. If we do the same calculations for bacterial densities above 10^6 ml^{-1}, the percentage of population turnover due to grazing is approximately an order of magnitude less for every order of magnitude increase in prey abundance. This may imply that at typical water column prey concentrations, ciliates can

exert a substantial top-down control on bacteria. In comparison, for them to have the same impact in areas of high productivity or in the sediments they will need to either have higher abundances, or display higher ingestions rates.

Conclusion

In the current study it was shown that at high prey densities ($> 10^7$ bacteria ml^{-1}) , ciliates rapidly (< 10 min) adapt their ingestion to a steady-state rate that is lower than their initial uptake capability. At prey concentrations $< 10^7$ bacteria ml^{-1}, there is little or no difference between the uptake capabilities and their steady-state ingestion rates. Contrary to some reports, digestion rates proved to be directly related to food vacuole content and consequently a function of prey concentration, showing a negative relationship with both prey abundance and ingestion rate.

Calculated digestion rates estimated that the ciliates are capable of digesting 100% of their food vacuole content h^{-1} at prey densities of approximately $\leq 10^6$ bacteria ml^{-1}. From this we estimated that at typical ocean bacterial densities of 10^6 bacteria ml^{-1} ciliates are capable of contributing significantly to both bacterial biomass turnover and prey population control. In higher prey environments however, digestion becomes limiting and ciliates would either have to be present in higher abundances or exhibit higher rates of ingestion to be considered a top-down control on bacteria.

Here data was obtained in systems containing one predator and one prey at a time. In reality there is competition between multiple predators for prey. Also, it is known that various species and categories of prey are available to bacterivores, and they are likely to feed on some or all of them to various degrees. Furthermore, our assessment on the

ability of ciliate bacterivores to both turn over bacterial biomass and control bacterial populations was based on the assumption that 100% of the grazer population is actively feeding. We monitored active grazers in one stage of the current experiments. The results showed that 95% of the population is feeding actively for both species of *Euplotes* after 10 minutes of grazing at prey densities $\geq 10^6$ bacteria ml^{-1}, but only 67% of *Cyclidium* appeared to feed actively under the same conditions. It can not be concluded whether any of the assumptions made here will be observed in nature when ciliates compete against other bacterivores for food. Rather, results from this experiment are a measure of capability. The next logical step towards understanding the microbial food web is to apply some of the results laboratory studies are generating to the designing of natural in situ experiments.

Table 3.1. Percentage of GFP-*Vibrio* contributing to the total prey population in ingestion experiments for all ciliate species.

Species	Prey Density (bacteria ml^{-1})	GFP
E. vannus	10^6	60%
	10^9	10%
E. plicatum	10^6	70%
	10^9	20%
Cyclidium	10^7	60%
	10^9	30%

Table 3.2. Values for vacuole formation rate (± SE); maximum number of vacuoles, V_{max} (± SD); the time required to attain maximum vacuoles, T_{max} (h); and digestion rate, k (h^{-1}) as a function of prey density for all three species of ciliate.

Species	bacteria ml^{-1}	Vacuole Formation Rate (h^{-1})	R^2	V_{max}	T_{Vmax}	k
E. vannus	10^6	2.3 ± 0.2	0.81	1.4 ± 0.6	0.6	1.67
	10^9	13.2 ± 0.4	0.91	38.0 ± 10.7	2.9	0.34
E. plicatum	10^7	16.9 ± 0.5	0.86	26.9 ± 8.1	1.6	0.63
	10^9	28.8 ± 1.0	0.90	59.1 ± 16.6	2.1	0.48
Cyclidium	10^7	42.6 ± 0.8	0.93	51.1 ± 12.0	1.2	0.83
	10^9	40.2 ± 0.8	0.94	73.8 ± 17.0	1.8	0.56

Figure 3.1**.** Prey uptake data for *E. vannus* (a-b), *E. plicatum* (c-d), and *Cyclidium* (e-f) after various durations of grazing.

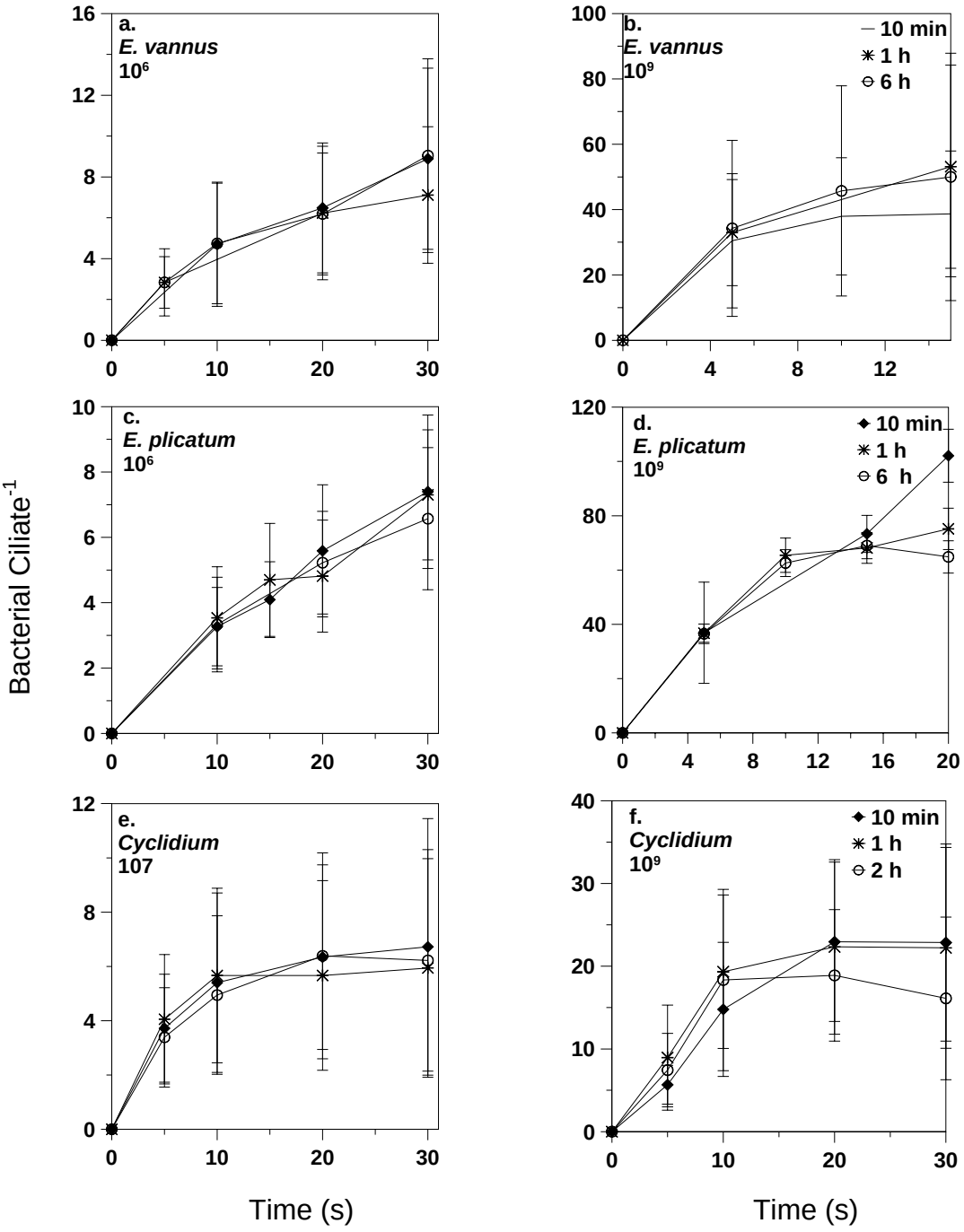


Figure 3.2**.** Ingestion rates for *E. vannus*, *E. plicatum*, and *Cyclidium* sp. as a function of grazing duration (open symbols). The point in time were the maximum number of food vacuoles is reached (T_{max}) is indicated for each prey density; Initial ingestion rates (closed symbols) taken from Chapter 2.

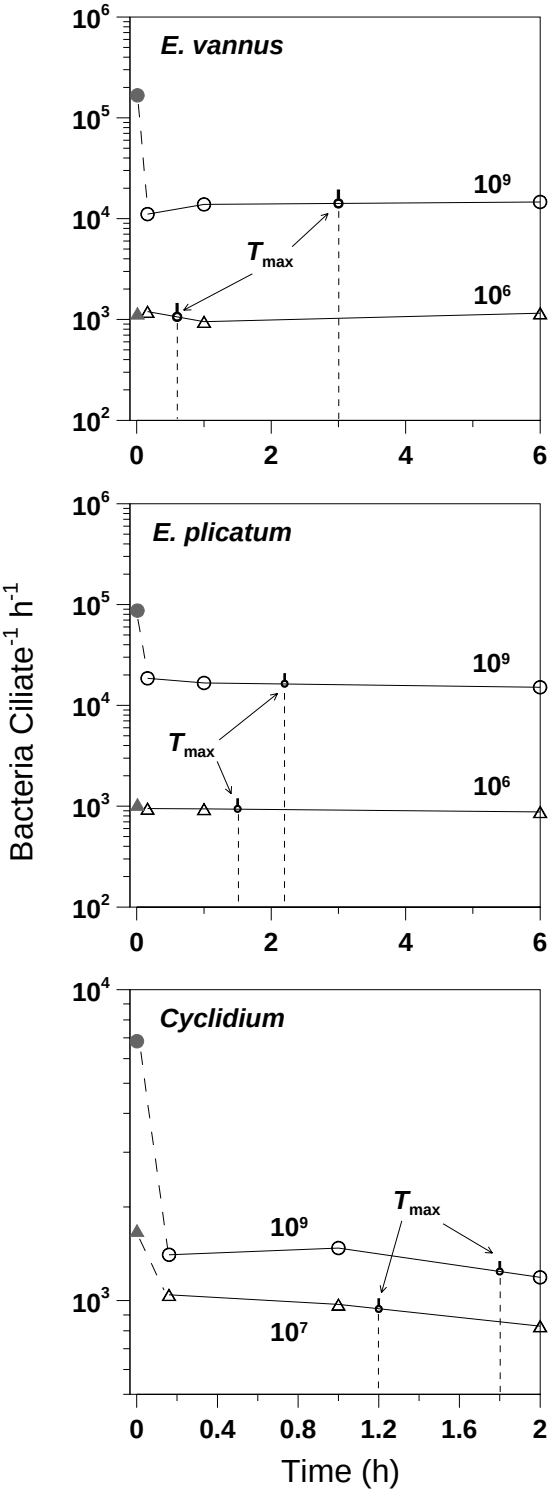
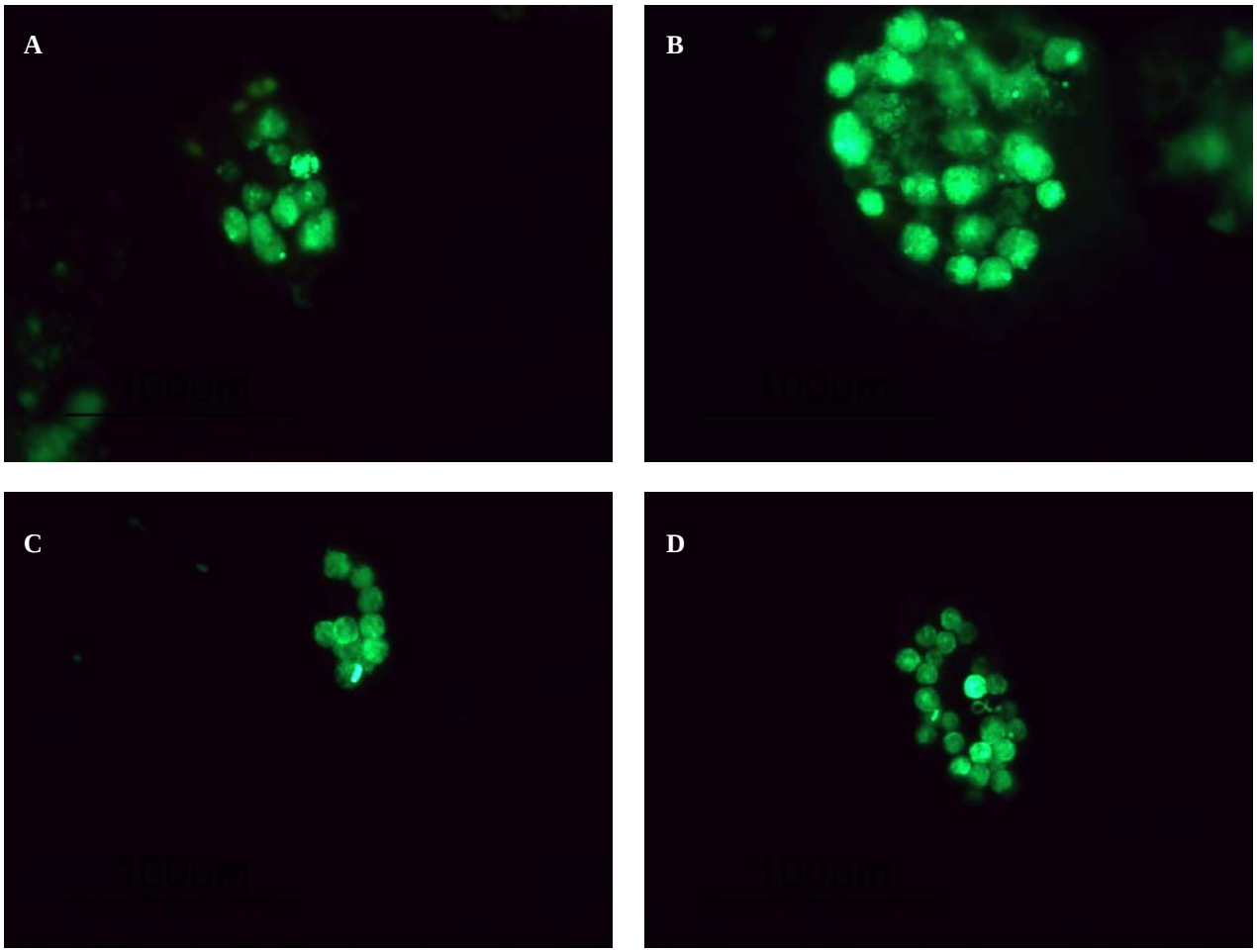
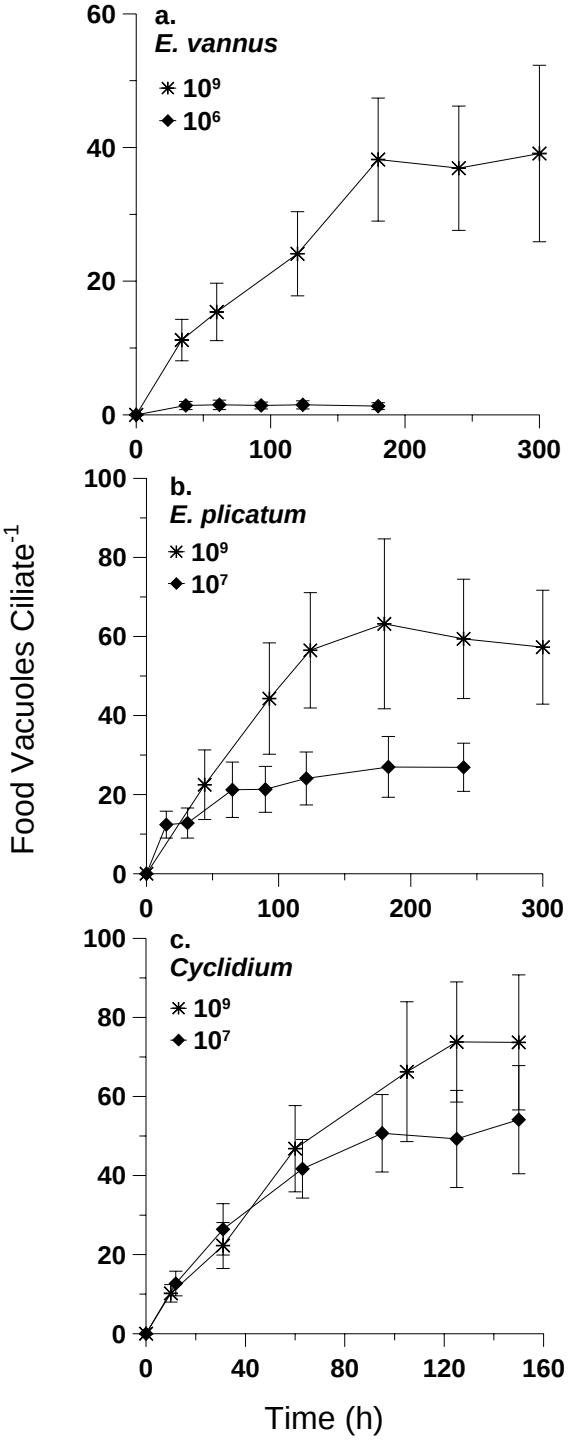


Figure 3.3. Images showing the number of food vacuoles accumulated after 10 and 60 min of grazing for *E. plicatum* (A and B), and *Cyclidium* sp. (C and D).



10 min 60 min

Figure 3.4. Number of food vacuoles as a function of time for *E. vannus* (a), *E. plicatum* (b), and *Cyclidium* sp. (c), at two different prey concentrations.



CHAPTER IV

Benthic Bacterivory of Marine Ciliates as a Function of Prey Concentration

Abstract

This purpose of the current study was to investigate how ingestion rates of the benthic bacterivorous ciliates *E. vannus*, *E. plicatum*, and *Cyclidium* sp., vary with prey concentration in a sedimentary environment. Using bacteria that were transformed to express green fluorescent protein as a natural tracer, prey were cultured in sediment microcosms to concentrations ranging from 10^8-10^{10} bacteria per milliliter of pore water (ml^{-1} PW). Ingestion rates increased with prey density for all three ciliates, ranging from 10^2-10^5 bacteria $ciliate^{-1}$ h^{-1}. A decline in ingestion rates at a prey density of 10^8 bacteria ml^{-1} PW suggests a possible prey concentration threshold below which the ciliate's ability to graze sediment bacteria diminishes. Ingestion rates at a typical sediment prey concentration (10^9 ml^{-1} PW) were approximately 2× higher for both species *Euplotes*, and 27× higher for *Cyclidium* sp. than rates reported for these species when grazing on suspended prey at similar concentrations. I propose that the ingestion rates of ciliates are higher in sediment than in suspension because the growth of bacteria on particle surfaces presents localized areas of high prey density allowing ciliates to ingest at greater rates. Additionally, the reduced amount of effort spent on search time and capture of prey when grazing sediment surfaces as opposed to filtering prey from suspension, allows energy to be diverted to increase the rates metabolic processes (i.e. digestion rate).

Introduction

The microbial loop and its role in the cycling of energy, carbon, and nutrients in the water column has been recognized for many decades (Azam et al. 1983). The importance of bacterivory in the microbial food web is well established. Protozoan grazing is not only a top-down control over bacterial populations (Sherr and Sherr 1994; Sherr and Sherr 2002), but also has a stimulatory effect on bacterial activity (Barsdate 1974; Caron 1991; Johannes 1965), and can alter the diversity of a bacterial community (Jurgens and Matz 2002; Jurgens et al. 1999; Ronn et al. 2002). There are a limited number of quantitative studies on the effects of protozoan grazing in a benthic environment. The quantity of organic matter as well as microbial abundances in a benthic environment can be much higher than in the water column. Bacterivory has been shown to accelerate the rates of decay of organic matter in detrital systems (Fenchel 1970; Fenchel and Jorgensen 1977; Patterson et al. 1989), and it is likely that protozoan grazing has a large impact on fate of organic matter in sedimentary environments.

Measuring a consumer's functional response, or how consumption rate changes with prey concentration, provides an assessment of both feeding behavior and the impact that consumer might have on an ecosystem (i.e. ecosystem stability, prey population control, and biomass turnover) (Chow-Fraser and Sprules 1992; Holling 1966; Jurgens and Simek 2000; Moigis 2006; Solomon 1949). That ingestion rates of protozoa grazing in suspension increase with prey concentration is well documented (see Chapters 2 and 3). We know much less about this dynamic in the benthic environment where protozoan grazing includes feeding on bacteria that are free, as well as, those attached to particles. Observations of ciliate feeding patterns show that ciliates do not necessarily forage

evenly over surfaces, but may continually feed in distinct patches (Fenchel and Blackburn 1999; Huws et al. 2005; Lawrence and Snyder 1998). This occurs even when adjacent areas contain much higher prey densities. This suggests that when protozoa feed on surfaces, higher prey concentrations are not necessarily associated with higher ingestion rates.

Early assumptions were that protozoan ingestion rates in a benthic habitat would be higher than in the water column due to the high density of bacteria that colonize particle surfaces (Fenchel 1975; Fenchel 1980b). Estimates for protozoan ingestion in sediments however, have either been similar or low compared to measurements in suspension (Fischer et al. 2006; Konigs and Cleven 2007; Starink et al. 1994b). However, much of the research measuring benthic protozoan bacterivory has utilized monodispersed fluorescently labeled bacteria (FLB) (Parry et al. 2001; Posch et al. 2001; Shimeta et al. 2001; Wieltschnig et al. 2003) which does not represent the consumption of attached prey. This combined with the possible selection against artificial food particles (Boenigk et al. 2001a; Gonzalez et al. 1993; Landry et al. 1991) could result in underestimated feeding rates of benthic protozoa.

The direct effects of prey density on rates of protozoan bacterivory in a sedimentary system have not been quantified. Comparisons of previous research on the subject are of limited utility due to differences in methodology and inconsistencies in the units used to report variables such as predator and prey concentration. Devising methods for accurate determinations are inherently difficult due to the scale and complexity of sedimentary environments. Studies in Chapters 2 and 3 (this dissertation) showed that the use of bacteria transformed for expression of green fluorescent protein (GFP) as a natural prey

tracer permitted good estimates of grazing ability in suspension. The purpose of this study was to investigate the effect of prey concentration on protozoan bacterivory in a sedimentary environment. Bacteria expressing GFP were cultured in benthic microcosms and ingestion rates of three species of marine ciliates were measured as a function of sediment prey concentration.

Methods

Bacteria

A subculture of *Vibrio* sp.-nap that had been transformed for the expression of green fluorescent protein (GFP) (see Chapter 2), hereafter referred to as GFP-*Vibrio,* was used as prey in all grazing trials. The original strain of *Vibrio* sp.-nap was isolated from Piles Creek, a tributary of the Arthur Kill- NY/NJ Harbor area (Tso and Taghon 1999), GFP-*Vibrio* cultures were maintained on Zobell's media as previously described (Chapter 2).

Ciliates

Two species of hypotrich ciliates, *Euplotes vannus* (length = 100 ± 11µm, width = 57 ± 8 µm) and *Euplotes plicatum* (length = 51 ± 4 µm, width = 32 ± 4 µm), were acquired from the Culture Collection of Algae and Protozoa (CCAP, Dunstaffnage Marine Laboratory, UK). A species of scuticociliate, *Cyclidium* sp. (length = 26 ± 3 µm, width = 10 ± 2 µm), was isolated from Piles Creek. All protozoa were cultured in an Artificial Seawater for Protozoa medium (ASWP) adapted from a CCAP recipe. Ciliate cultures were maintained on an uncharacterized assemblage of bacteria growing on either rice grains or

soy extract added as organic substrate. All ciliate cultures used in grazing experiments were no more than four old.

Sediment Preparation

Sediment was collected from a tidal estuary on the Manasquan River, New Jersey. Mineral particles from the sediment were wet sieved, washed, and sorted to a size class of 177-210 μm. For each treatment, 0.5 g of sediment was added to 10 ml serum bottles (three replicates × four time points). This resulted in an average sediment depth of 1.0 mm (± 0.1 mm). This sediment depth removed the possibility of anoxia and allowed re-suspension of preserved ciliates and free pore bacteria without physically disturbing the sediments. The low prey treatments (treatment 1) received 1 ml of seawater only. To achieve three successively higher concentrations of bacteria, 1 ml of Zobell's media was added at concentrations of 1, 25, or 100% to bottles for treatments 2, 3 and 4 respectively. All bottles were autoclaved, allowed to cool, and received a sterile addition of 0.5 g L^{-1} of kanamycin. A GFP-*Vibrio* culture was prepared by centrifuging (7500 × g, 10 min), washing two times, and diluting to an optical density of 0.05 at a wavelength of 550nm (approximately 10^7 cells ml^{-1}). All serum bottles were inoculated with 10 μl of the GFP-*Vibrio* harvest, covered with a foam stopper, and placed in a humid chamber on a rotary shaker for 48 h.

Following the incubation period, the medium was aspirated from all serum bottles. The sediment was washed 4 times by gently adding 2 ml of sterile seawater down the side of the serum bottle, followed by aspiration. Preliminary experiments showed that more washes did not alter the bacterial population any further.

Ingestion Rate Experiments

Ciliate cultures were harvested and prepared for experiments as previously described (Chapter 2). Experiments began by inoculating all replicates with 0.2 ml of ciliate harvest. This volume resulted in complete saturation of the sediment pore space, but left no overlying fluid. This allows for all calculations to be normalized to units per milliliter of pore water (ml^{-1} PW). All time-zero replicates were sacrificed by gently adding 3.8 ml of ice-cold 1% glutaraldehyde in phosphate buffer. Any suspended ciliates were allowed to settle and a sample was transferred from the overlying fluid to a test tube for the estimation of free bacteria (representing bacteria suspended in the pore water only). The remaining overlying fluid was withdrawn and expelled several times with a pipette, only slightly disturbing the sediment layer to re-suspend the ciliates. An aliquot was transferred from the overlying fluid to individual test tubes for the estimation of ciliate abundance. This sampling process was repeated at 2, 4 and 6h. All samples were covered and stored at 4°C for no more than 24 h before sample processing.

Sample Processing

I modified the procedure from Epstein and Rossel (1995) for enumerating total sediment bacteria in our microcosms. The glutaraldehyde-fixed sediment was sonicated in the serum bottles using a Branson Sonifier 250 (output 25 Watts; 10% duty cycle) for 3× 30 seconds. The bottles were chilled on ice between each sonic treatment. Large particles were allowed to settle, after which a sample was transferred to a micro-centrifuge tube. Samples were centrifuged for 2.5 min at 70 × g to settle any remaining fine sediment

particles, and a sample of the overlying fluid was taken to estimate the total bacterial concentration (attached plus free). Control tests showed that no loss of suspended bacteria occurs from this process (data not shown).

Samples for both the total and free bacteria were filtered onto black, 0.22 μm polycarbonate filters. Filters were placed on slides between drops of immersion oil, covered with a glass slip, and stored at –20°C until the time of enumeration. Filters were scanned using an epifluorescence microscope (BP490 excitation filter/17AFC + 170515 barrier filter) at 1000× magnification. All ciliate samples were enumerated in a Sedgewick Rafter Cell (ProSciTech). Bright field microscopy was used for *E. vannus* and *E. plicatum* at 60× magnification, while C*yclidium* sp. samples were first stained with SYBR Gold® (75μl ml^{-1} of a 20× stain stock in PB; Molecular Probes, Invitrogen Detection Technologies), and then enumerated with an epifluorescent microscope at 100× magnification.

Statistical Analysis

The counts for both the total and suspended bacteria populations were standardizes as numbers per milliliter of PW (ml^{-1}PW). Abundance of bacteria attached to sediment particles was calculated as the difference between the total and suspended counts for each sample. Ingestion rates for the entire predator population (bacteria h^{-1}) were calculated by linear regression through all data points for each time series. Per capita ingestion rates (bacteria $ciliate^{-1}$ h^{-1}) were calculated using the mean number of ciliates across all trials and all time points. All statistical analysis was performed using Statistix 9© (Analytical Software, Tallahassee, FL).

Results

Total initial bacterial abundance increased between treatments 1 and 4 for all experiments (Table 4.1). Prey ranged from 10^8-10^{10} bacteria ml^{-1} PW for all experiments, however, the concentration of bacteria in treatment 2 (1% media) was never substantially higher than in treatment 1 (SW only). The contribution of attached bacteria to the total population was always > 96% relative to free bacteria, regardless of the initial prey concentration or the total amount of bacterial loss (Table 4.2).

The mean abundance of all *E. vannus* treatments for all time points was 6,931 (± 672) cells ml^{-1} PW (Figure 4.1), and the difference between individual means was not significant ($\alpha = 0.05$, $p = 0.83$). Total average prey loss in the *E. vannus* experiments increased with bacterial concentration (Table 4.1), ranging from 10^6-10^9 bacteria ml^{-1} PW. This loss was due to decreases in concentration of both the attached and free portions of the bacterial population (Figure 4.2). The concentration of free bacteria was always ≥ 2 orders of magnitude less than the attached bacteria, leading to the conclusion that any changes in free bacterial concentration were small relative to the total prey loss. Per capita ingestion rates also increased as a function of prey concentration for *E. vannus* (Table 4.1). *E. vannus* had the highest ingestion rate of all three predators in treatment 4 (> 10^5 bacteria $ciliate^{-1}$ h^{-1}), and the lowest ingestion rate for treatment 1 (< 10^2 bacteria $ciliate^{-1}$ h^{-1}). The standard error was high enough for treatment 1, however, to conclude that the calculated ingestion rate for that prey density was not significantly different from zero (t-test, $p = 0.31$).

The mean number of cells for *E. plicatum* was 4,143 (±1,247) ml^{-1} PW (Figure 4.1), and the difference between means for all treatments did not vary significantly for the

duration of the experiment ($\alpha = 0.05$, $p = 0.91$). Total consumption of bacteria increased for *E. plicatum* as prey density increased (Table 4.1). The concentration of free bacteria increased slightly in all *E. plicatum* treatments, while the concentration of attached always decreased (Figure 4.3). Ingestion rates for *E. plicatum* increased with prey concentration (Table 4.1). *E. plicatum* had the highest per capita ingestion rate of all three grazers for treatment 1 and treatment 2. In contrast to the experiments with *E. vannus* and *Cyclidium* sp., the prey concentrations in treatments 1 and 2 for the *E. plicatum* experiment did not drop below 2×10^8 bacteria ml^{-1} PW and were the most similar to each other. As a result, *E. plicatum* showed the least amount of change in rate between treatments 1 and 2.

Cyclidium sp. abundance averaged 8,651 (±1,708) cells ml^{-1} PW (Figure 4.1) across all time points for all treatments. The difference between individual means of all samples was not significant ($\alpha = 0.05$, $p = 0.73$). Total loss of prey increased with increasing prey concentration (Table 4.1). The change in the concentration of free bacteria revealed no pattern, decreasing in treatments 1 and 3, and increasing slightly in treatments 2 and 4 (Figure 4.4). The concentration of attached bacteria decreased for all treatments. The ingestion rates for *Cyclidium* sp. increased with prey concentration, but were the lowest of all three grazers for treatment 4.

Discussion

The three species of ciliates in the present study displayed a functional feeding response to changes in prey density in a sedimentary environment. The lowest ingestion rates were on the order of 10^2 bacteria $ciliate^{-1}$ h^{-1} and increased continuously to rates of 10^4

and 10^5 bacteria ciliate^{-1} h^{-1} at prey concentrations > 10^{10} bacteria ml^{-1} PW. The prey concentrations achieved in treatments 1 and 2 for experiments with *E. vannus* and *Cyclidium* sp. fell below 2×10^8 bacteria ml^{-1} PW, which coincided with a substantial decline in consumption rate (Table 4.1). The same decline in ingestion rate did not occur in experiments containing *E. plicatum* where prey concentrations did not fall below 2×10^8 bacteria ml^{-1} PW. The magnitude of the decrease in consumption rate for *E. vannus* and *Cyclidium* sp. from treatment 2 to treatment 1 suggests a possible threshold where prey concentration begins to limit appreciable uptake of benthic bacteria by ciliates.

Considering that there may be processes that are masked from our measurements (growth of attached or free bacteria, attachment or detachment of existing bacteria), the exact proportion of consumption due to grazing on attached versus grazing on free bacteria can not be directly calculated. For this reason we report consumption rates for total bacteria. It is likely that bacteria from both the attached and the free pools were ingested to some degree, however the total loss of prey was ≥ 1 order of magnitude larger than the measured change in the free bacterial pool. All evidence supports that the majority of bacterial loss was due primarily to the removal and consumption of attached bacteria.

Alternate possibilities for how prey were consumed are that, either both attached and free bacteria were grazed at equal rates, or that the majority of the bacterial loss was a result of consumption of the free bacterial pool. Results from this study suggest that we can discount that both attached and suspended bacteria were grazed at equal rates. The changes in concentration of free bacteria were small relative to the attached pool (Figures 4.2-4.4) regardless of the total loss. Equal rates of grazing would have resulted in a

dramatic reduction in the suspended prey pools. This would have been particularly evident in the high prey density treatments (1 and 2) where total prey loss was always one or two orders of magnitude higher than the total size of the free bacterial population.

The second scenario is that grazing of bacteria suspended in the pore water accounted for the majority of bacterial loss. The suspended pool of bacteria was a small percentage of the total bacteria in all cases (Table 4.2). The change observed in the attached bacterial population was too great to conclude that change in bacterial dynamics occurred solely from the removal of free bacteria. A possible explanation is that detachment of bacteria from the sediment was occurring at a rate exactly equal to consumption. If detachment was the process limiting ingestion capability then the grazers would likely supplement this by feeding on bacteria already in suspension. Free bacterial concentrations were well within the ciliate's ability to graze upon (Figures 4.2-4.4), but this would have resulted in a greater reduction in the suspended population than was measured. If detachment was occurring at rates higher than the ciliate's uptake capability, then the excess of detached bacteria would result in an increase in the free bacterial concentration. This would account for the increase in free bacterial concentration observed in the *E. plicatum* experiment. However, this should have resulted in a continual increase in the free bacterial pool. Furthermore, this was not observed in the experiments for E. *vannus* and *Cyclidium*. It is more likely that the protozoa are actively removing the bacteria from the particles. Bacterial growth could possibly supplement these processes. However, any remaining growth substrate was removed from the system. While regeneration of nutrients such as nitrogen and phosphorus by the grazers might have stimulated growth, bacteria do not use the carbon

directly regenerated by ciliate grazers at any detectable level (Gruber and Taghon, in press).

To best analyze how the benthic bacterivory rates estimated here compare to grazing on bacteria in suspension, I consider the studies from Chapters 2 & 3, which investigated the same three species of ciliates. Compared here are ingestion rates for three different conditions: initial ingestion rates on suspended bacteria (Chapter 2), steady-state ingestion rates on suspended bacteria (Chapter 3), and ingestion rates on sediment bacteria (Table 4.1). The comparison is made with results obtained at a prey density centered around 10^9 ml^{-1}, which is a typical sediment bacterial concentration found when scaled to pore water volume (Schmidt et al. 1998).

Measurement of initial ingestion rates (uptake rate upon introduction of prey) revealed that *E. vannus*, *E. plicatum*, and *Cyclidium* sp. are capable of consuming suspended prey at rates of 1.7×10^5, 8.9×10^4, and 6.8×10^3 bacteria $ciliate^{-1}$ h^{-1} respectively (see Chapter 2). The same three species of ciliates were determined to have steady state ingestion rates of 1.3×10^4, 1.7×10^4, and 1.4×10^3 bacteria h^{-1} respectively, once digestion becomes limiting (see Chapter 3). When grazing free prey under similar conditions, the steady state ingestion rate of each grazer was much lower than the initial rate. Ingestion rates on sediment bacteria for both *Euplotes* species were lower than their initial rates on suspended bacteria, but higher than steady state rates (Figure 4.5). The ingestion rate of *Cyclidium* sp. in sediment was substantially higher than either the initial or steady state rates on suspended bacteria, suggesting a predisposition for grazing on surfaces. This is contrary to reports that *Cyclidium* have lower benthic grazing capabilities and are primarily suspension feeders (Fenchel 1980b; Tso and Taghon 1999).

Additionally, the ingestion rates among species in sediment were relatively similar when compared to either of the rates measured in suspension. Different species of ciliate can differ greatly in their ability to interact and feed both in suspension and on surfaces (Ricci 1989). The sediment possibly acts to equalize the capability of three species which vary substantially in morphology and behavior. Unfortunately we do not have data for all three of these conditions to compare at other prey concentrations.

Ingestion rates at steady state for both *Euplotes* species in sediments were more than twice the rates observed in suspension, and > 27× higher for *Cyclidium* sp. This supports original hypotheses for benthic grazing, which predicted that rates of consumption would be higher than in suspension (Fenchel 1975; Fenchel 1980b). It was assumed that because ingestion rates are related to prey density and that bacteria in sediments are more concentrated compared to water column bacteria, that ciliates could conceivably consume more bacteria by "grazing the lawn". Digestion rates of ciliates decrease with prey concentration, which limits their uptake capabilities when feeding in suspension (see Chapter 3). It follows that for rates of grazing in sediments to surpass those in suspension that digestion rates must also be higher in a sedimentary environment. I propose that grazing in a sedimentary environment requires considerably less effort for search and handling time compared to filtering bacteria from suspension. This would allow grazers to divert energy usually utilized for search and capture of prey to metabolic processes, thus increasing rates of digestion. Additionally, I propose that sediment surfaces act as a microhabitat which makes prey available to grazers in a manner that is analogous to how solid surfaces make dissolved organic matter available to bacteria. It has long been recognized that bacterial activity associated with growth on inert surfaces

is higher than in suspension (Zobell 1943). Adsorption of DOM to inert surfaces increases the local concentration gradient of the substrate, allowing for higher rates of uptake (van Loosdrecht et al. 1990). The growth of bacteria on sediment surfaces effectively causes the same phenomenon for bacterivores. A localized concentration of prey allows for higher ingestion rates. Rather than using cilia as a filtering tool to concentrate suspended prey for uptake, grazers are able to use their cilia as a raking tool to consume an already concentrated prey supply.

In the present study, ingestion rates were much higher in a sedimentary system when compared to the same species of ciliates feeding on suspended prey. In contrast, previous reports of benthic ciliate ingestion rates have been generally low (Table 4.3). One hypothesis is that the hydrodynamics associated with the presence of sediment particles can have a negative effect on grazing (Young et al. 1994). It has been suggested that particle surfaces may act as a spatial refuge, implying that attachment is a form of grazing resistance (Jurgens and Gude 1994). Yet others have suggested that there may be a considerable increase in the effort required to graze in sediments due to navigating pore structure in search of prey (Wang et al. 2005; Young et al. 1994) or for the physical removal of attached bacteria (Artolozaga et al. 2002). It is difficult to compare the rates of consumption reported here for treatments 1 and 2 to values from the literature. There are inconsistencies among studies in the units used to report predator and prey concentrations, as well as, ingestion rates in benthic environments. However, ingestion rate values for typical sediment prey concentrations measured in the present study, fall well above previously reported rates of benthic bacterivory by ciliates regardless of the bacterial concentration or experimental conditions.

A possible explanation for reported rates of benthic bacterivory being historically low lies in previous methodology. The methods typically used to estimate benthic bacterivory rates incorporate the use of FLB (Table 4.3). A large body of research shows that protozoa may select for or against artificial prey (Gonzalez et al. 1993; Landry et al. 1991; Parry et al. 2001) and that FLB in general can be problematic (Boenigk et al. 2001a). The use of direct observation (microscopic videography), a natural tracer (such as fluorescent proteins), or the use of an indirect method (changes in total population, post-ingestion FISH, etc.) typically produce higher estimates for rates of bacterivory than when using a surrogate prey (i.e. microspheres or FLB) (see Chapter 2). Furthermore, most studies utilize FLB that are added as a suspension rather than truly attached to the sediment. Starink et al. (1994b) measured twice the ingestion rate when the procedure stained both free and attached bacteria in the system, as opposed to only adding suspended FLB. Results from the current study showed that most of the prey loss occurred in the attached population. If the ingestion rates of bacterivores are lower when feeding in suspension, then the use of suspended, artificial tracers in the sediments will underestimate benthic consumption rates.

Conclusion

The ingestion rates for three species of bacterivorous ciliates displayed a strong positive relationship with prey concentration in a sedimentary environment. Ingestion rates in sediment were 2-27 times higher than ingestion rates measured for the same species at similar prey concentrations when grazing in suspension. I propose that consumption rates of ciliates are higher in sediment due to the concentration of prey associated with

bacteria growing on particles. The result is that energy is diverted from the search, capture, and concentration of prey to rates of uptake and digestion. The benthic ingestion rates observed at typical sediment bacterial concentrations were also substantially higher than rates previously reported at any prey concentration. This was likely due to the culturing of a natural prey tracer directly in the sediment microcosms, rather than using a surrogate prey particle that was primarily in suspension.

Table 4.1. Summary of initial bacterial abundance, total bacterial loss, and ingestion rates for all treatments (Tr. 1-4) for all ciliate experiments.

Species	Tr.	Abundance (SD) (10^8 ml^{-1} PW)	Loss (SD) (10^7 ml^{-1} PW)	Ingestion (SE) (10^2 ciliate^{-1} h^{-1})
E. vannus	1	1.2 (0.3)	0.6 (2.3)	0.8 (3.4)
	2	1.5 (0.3)	3.0 (1.7)	6.5 (3.0)
	3	24.9 (3.2)	121.0 (16.1)	273.2 (40.5)
	4	115.8 (4.9)	457.1 (43.0)	1,074.1 (165.5)
E. plicatum	1	2.7 (0.7)	4.5 (5.1)	19.5 (12.2)
	2	2.9 (0.7)	6.6 (3.4)	20.7 (12.9)
	3	39.6 (1.6)	126.4 (17.8)	397.8 (91.5)
	4	176.9 (28.6)	192.6 (109.2)	821.6 (461.2)
Cyclidium sp.	1	1.9 (0.3)	3.1 (1.8)	6.1 (3.1)
	2	2.9 (0.4)	9.6 (3.7)	17.0 (4.4)
	3	53.3 (12.4)	211.6 (96.0)	371.7 (171.4)
	4	182.8 (32.1)	357.7 (156.6)	617.6 (332.9)

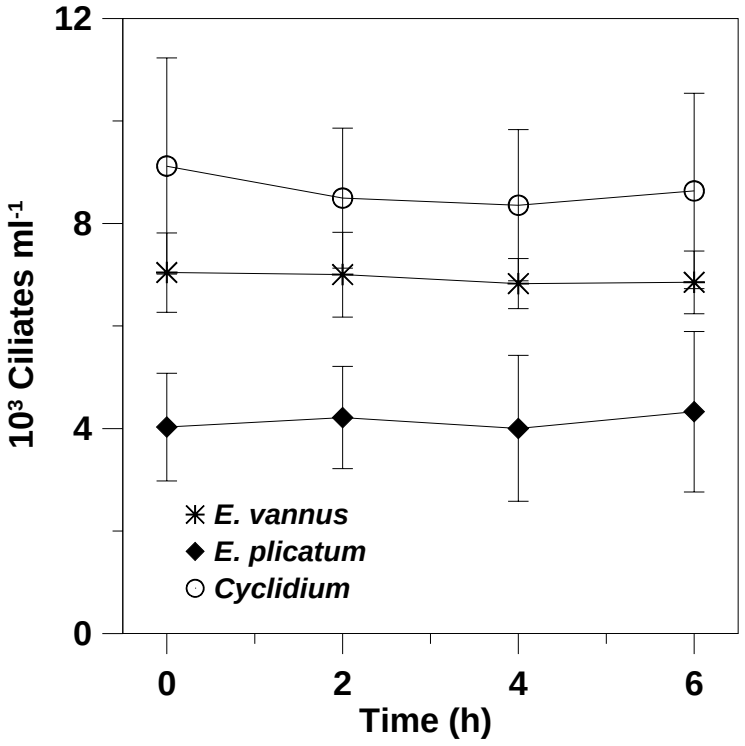
Table 4.2. Initial and final relative percent contributions (±SD) of attached and free bacteria for treatments 1-4 for all three ciliates.

		Treatment 1		Treatment 2		Treatment 3		Treatment 4	
Species	Time	% Attached	% Free	% Attached	% Free	% Attached	% Free	% Attached	% Free
E.vannus	Initial	97.9 (0.01)	2.1 (0.01)	96.9 (0.02)	3.1 (0.02)	99.2 (0.01)	0.8 (0.01)	99.6 (0.0)	0.4 (0.0)
	Final	98.3 (0.0)	1.7 (0.0)	97.8 (0.0)	2.2 (0.0)	99.1 (0.0)	0.9 (0.01)	99.8 (0.0)	0.2 (0.0)
E. plicatum	Initial	98.0 (0.01)	2.0 (0.01)	97.6 (0.01)	2.4 (0.01)	98.3 (0.0)	1.7 (0.0)	99.0 (0.0)	1.0 (0.0)
	Final	96.9 (0.01)	3.1 (0.01)	97.0 (0.0)	3.0 (0.0)	96.7 (0.01)	3.3 (0.01)	97.2 (0.0)	2.8 (0.0)
Cyclidium sp.	Initial	97.4 (0.01)	2.6 (0.01)	98.9 (0.0)	1.1 (0.0)	99.4 (0.0)	0.6 (0.0)	99.6 (0.0)	0.4 (0.0)
	Final	98.1 (0.01)	1.9 (0.01)	97.9 (0.0)	2.1 (0.0)	99.4 (0.01)	0.6 (0.0)	99.5 (0.0)	0.5 (0.0)

Table 4.3. Ciliate ingestion rates (bacteria protist^{-1} h^{-1}) reported in previous investigations for ciliates grazing particle associated bacteria; Rates are the maximum reported for each taxonomic group from each study.

Reference	Method	Taxon	Ingestion Rate
Albright et al (1987)	FLB	scuticociliate	16.4
		Euplotes sp.	12.4
Kemp (1988)	FLB	Ciliate assemblage	525
Starink et al. (1994a)	Stained Sediment	Total protozoa	61
Eisenmann et al. (1998)	FISH / vacuole volume change	*Tetrahymena sp.*	1,641
Lawrence & Snyder (1998)	Microscopic Videography	*Euplotes* sp.	882
Tso & Taghon (1999)	FLB	*Euplotes sp*	176
Artolozaga et al. (2002)	FLB	*U. marinum*	27.2
Wieltschnig et al. (2003)	FLB	Ciliate assemblage	97.6
Fischer et al. (2006)	FLB	Ciliate assemblage	11
Konigs & Cleven (2007)	FISH / Food Vacuole Content	*Pluronema*	86
		Vorticella sp	38
		C. margaritaceum	78
		other / scuticociliates	150

Figure 4.1. Average ciliate abundance of all treatments over time for *E. vannus*, *E. plicatum*, and *Cyclidium* sp. in ingestion rate experiments



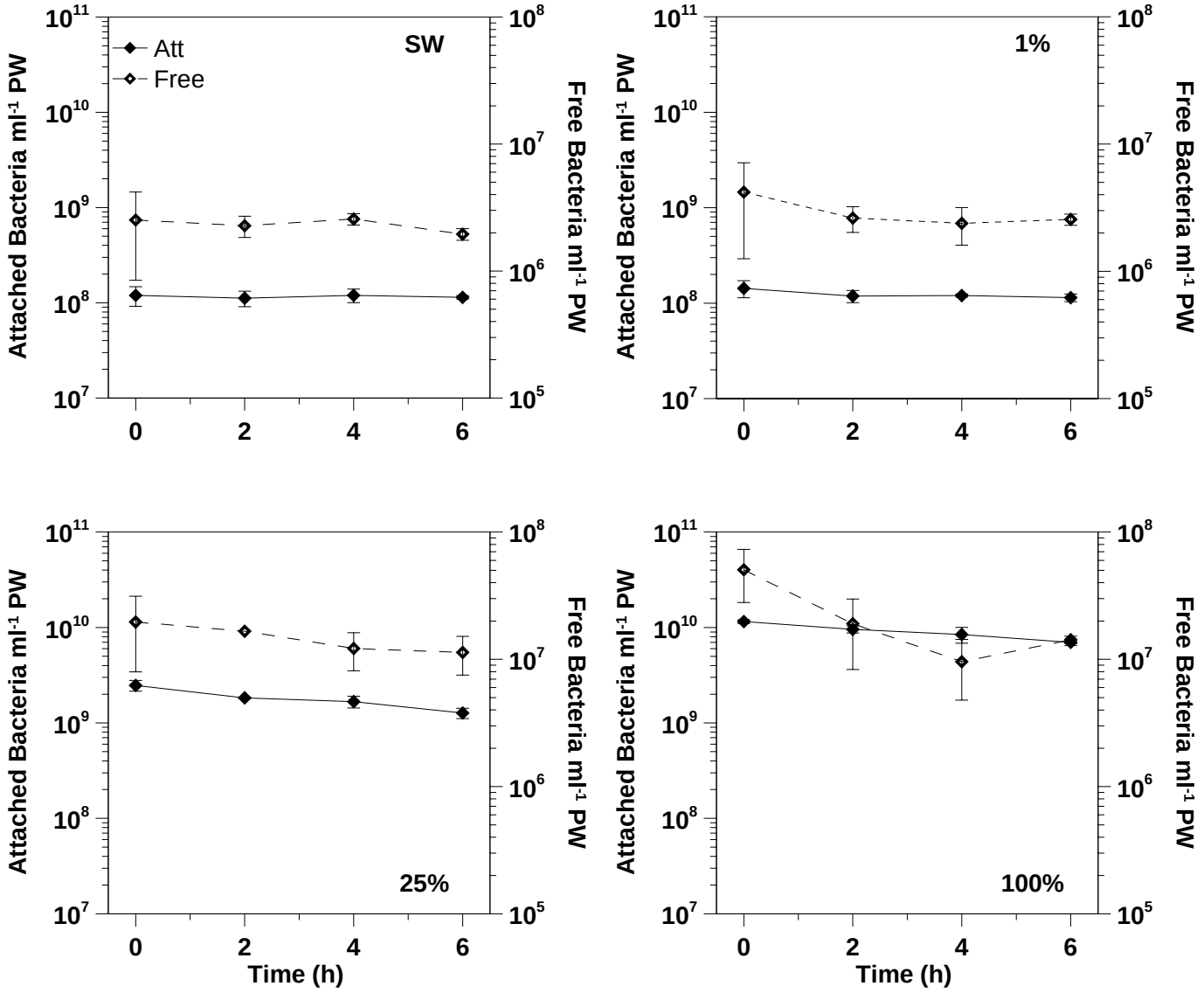


Figure 4.2. Concentration of attached (closed symbols) and free (open symbols) bacteria over time for *E. vannus* ingestion trials in all four treatments (seawater only, 1%, 25%, and 100% media)

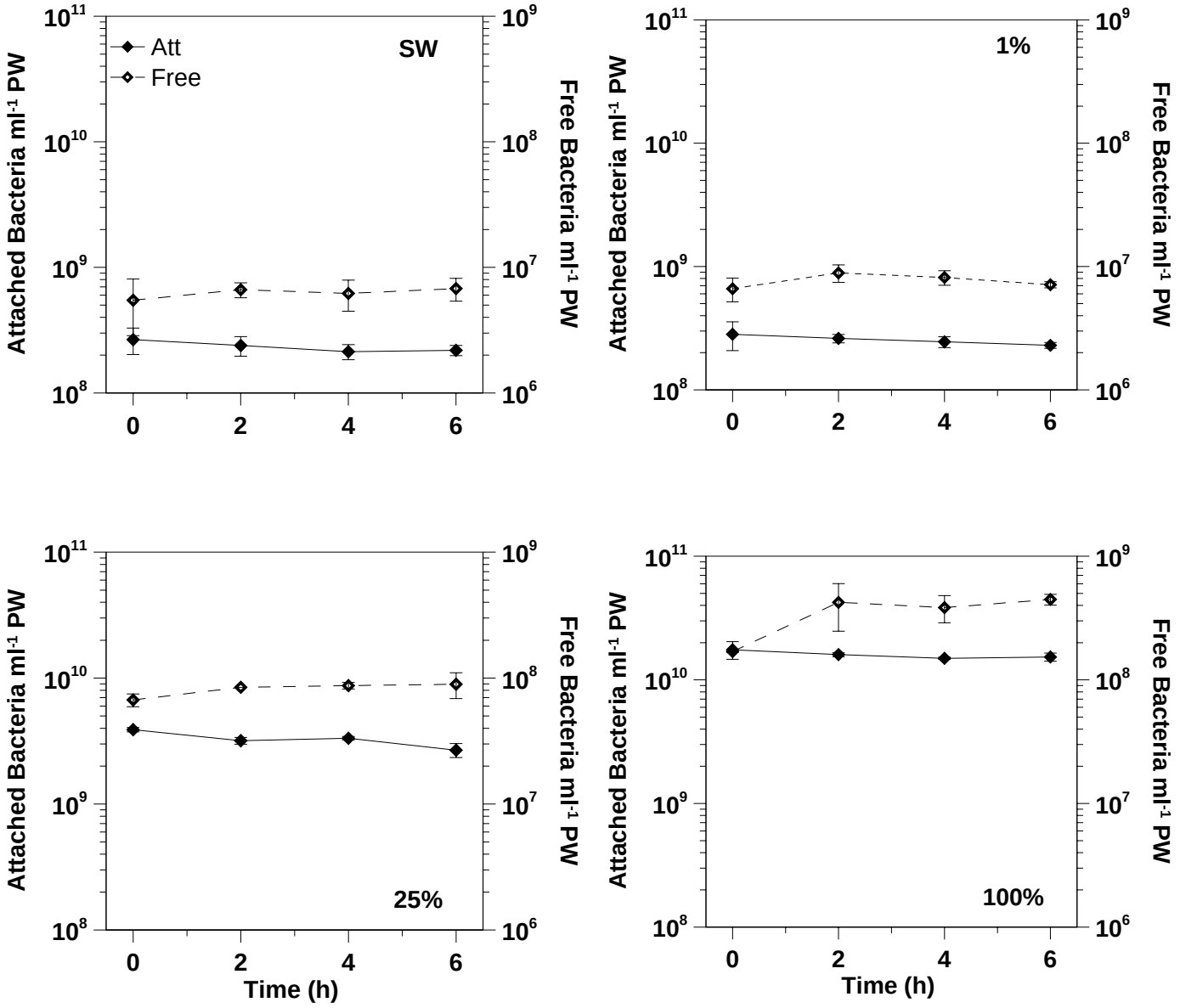


Figure 4.3. Concentrations of attached (closed symbols) and free (open symbols) bacteria over time for *E. plicatum* ingestion trials in all 4 treatments (seawater only, 1%, 25%, and 100% media)

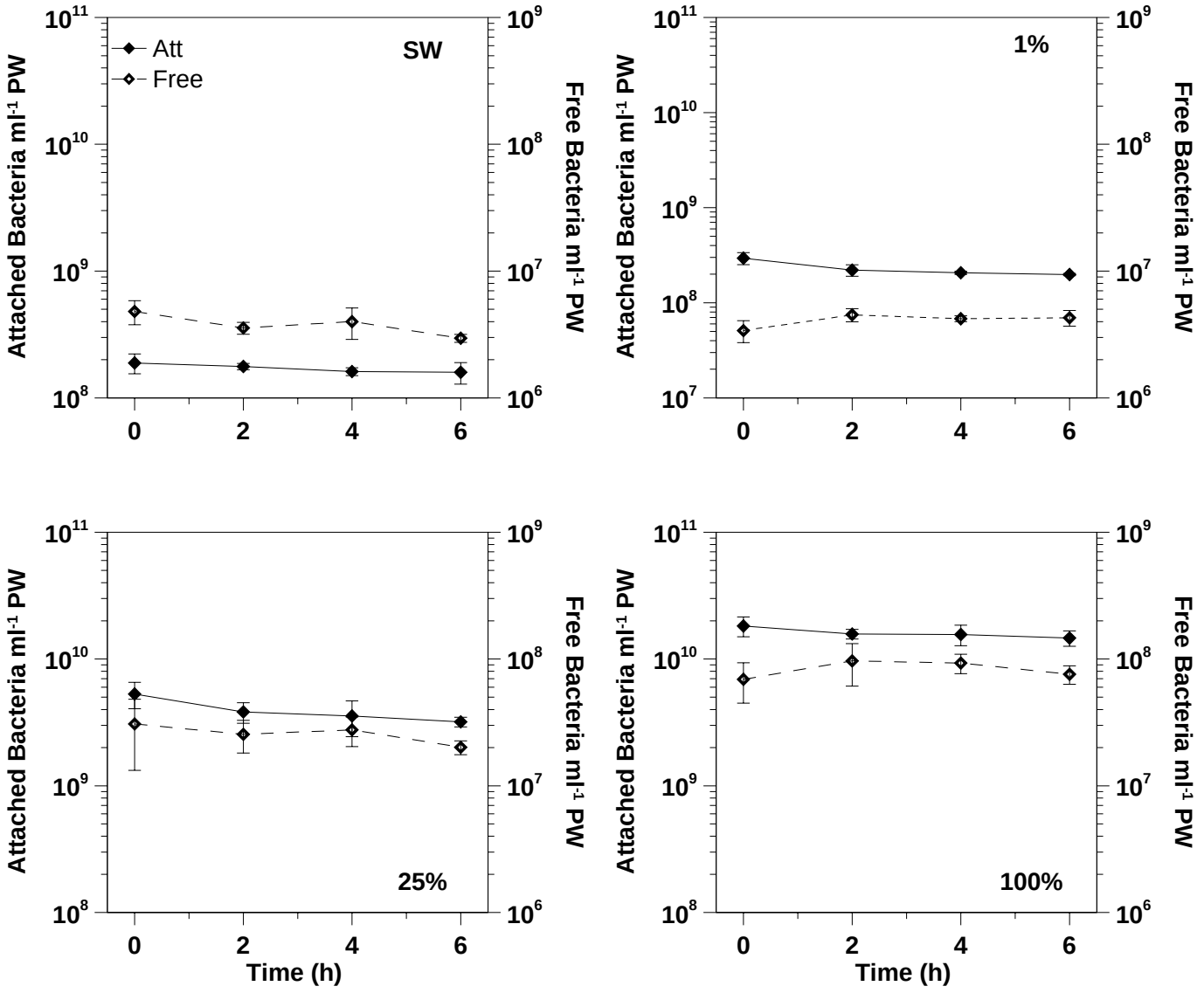
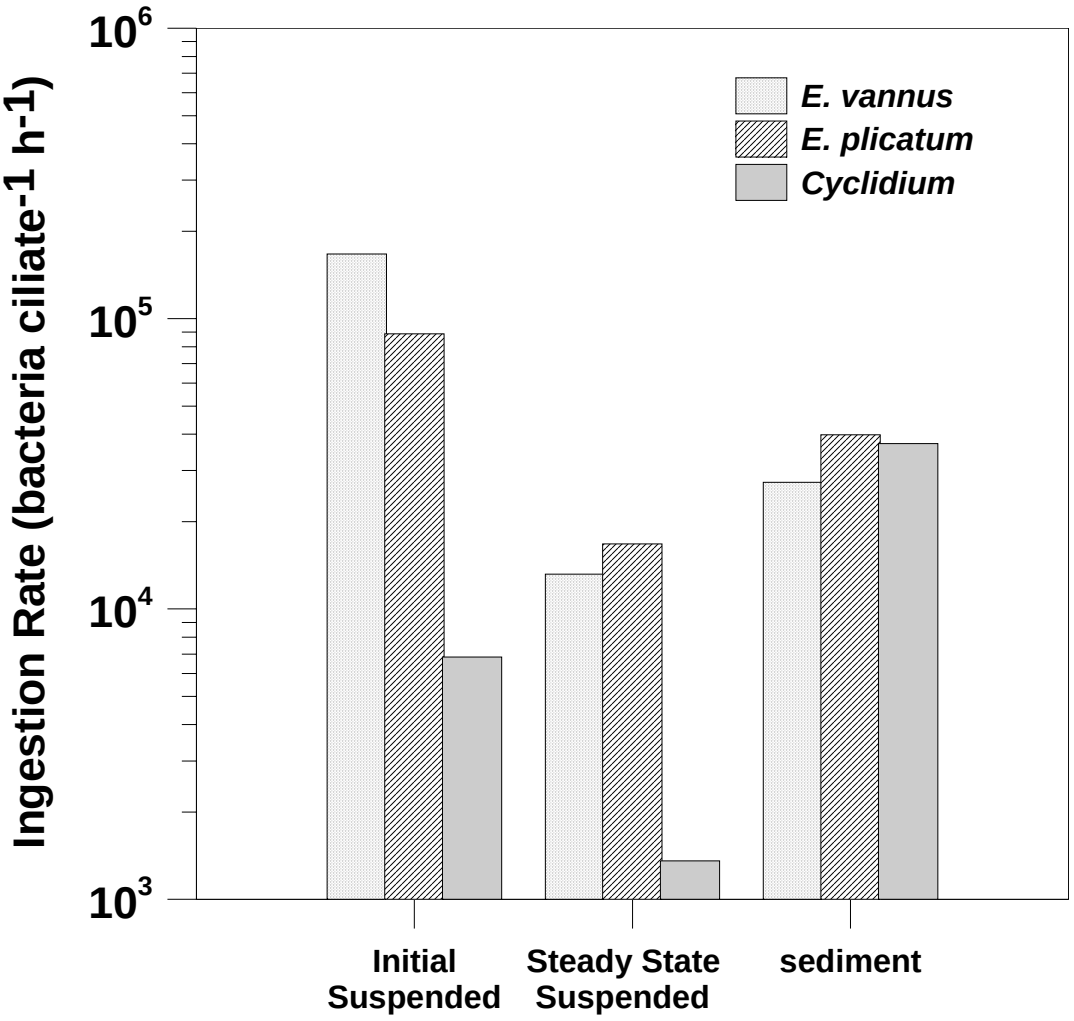


Figure 4.4. Concentrations of attached (closed symbols) and free (open symbols) bacteria over time for *Cyclidium* ingestion trials in all 4 treatments (seawater only, 1%, 25%, and 100% media)

Figure 4.5. A comparison of initial (data from Chapter 2) and steady state (data from Chapter 3) ingestion rates for *E. vannus*, *E. plicatum*, and *Cyclidium* sp. when grazing suspended bacteria, with ingestion rates when grazing in sediment (data from Table 4.1, present study).



CHAPTER V

Conclusion

On Measuring Microbial Dynamics

The scale and complexity of microbial ecosystems require that care be taken not to overlook or misinterpret pertinent mechanisms. Work presented here suggests that the time frame with which microbial interactions are monitored has an impact on observed results. Recently, more attention is being paid to the pitfalls of monitoring microscopic dynamics in general, specifically when using fluorescence microscopy (Pearson 2007). One source of bias when investigating protozoan bacterivory, the type food particle used as a tracer, was also addressed in the present investigations. The development and implementation of the experiments presented in this dissertation revealed multiple sources for potential bias beyond the prey particle used. Primarily, the manipulation of a sample post fixation results in damage to some portion of the grazer population, causing the number of grazers containing ingested prey to be highly variable between replicates. The processing steps that seemed most significant to this study included the transfer of samples, filter rinsing steps, the use of a vacuum for filtration, and the application of pressure to cover slips in preparation of slides. Great care with sample preparation and a trained eye was required to assure that only intact predator cells containing prey within un-ruptured food vacuoles were included in the data.

Another source of underestimation was the autofluorescence generated by the predator's cell due to the fixation process. The pros and cons of various preservatives, and the proper situations to use them have been reviewed elsewhere (Karayanni et al.

2004; Sherr and Sherr 1993). Work preliminary to this dissertation confirmed that the use of glutaraldehyde resulted in the least amount of instantaneous predator cell loss, reduced egestion of consumed prey, and provided the most reliable long term storage. However, over short periods of time autofluorescence developed that was sufficient enough to mask the majority or all of the consumed prey. To combat this, I modified a procedure for the use of a strongly reducing agent, sodium borohydride (SBH) (Baschong et al. 2001; Clancy and Cauller 1998), into a filter rinsing step. This resulted in a marked decrease in autofluorescence that persisted indefinitely, and allowed for uninhibited observation of all ingested prey. This procedure had to be optimized for each predator as the accumulation of autofluorescence and the sensitivity to the SBH solution varied between species.

Very often investigators develop procedures to combat methodological issues similar to those described here, but the details are left out of publications in an attempt to be concise. It is necessary to report these findings either along with the study results, or as a methodological publication. This will decrease the possibility of future investigators reporting misinterpreted results. An increase in procedural detail will also increase the likelihood that another investigator can reproduce results. Choosing the correct method, and optimizing sampling procedures for each individual situation is critical for accurate results gathered by microscopy. Overall, the rate of advancement of microbial ecology will depend on attention to detail, and whether or not results and procedures are reported in a way that promotes improvement by the next researcher.

Prospectus

There are implications for studying microbial interactions beyond characterizing the energy and nutrient cycling within the microbial food web itself. It is becoming an important tool to study the ecology of microbial systems and then relate them to macro ecological systems that are to cumbersome, or that act on too long a time scale to study effectively. The three studies reported in the present dissertation applied basic ecological concepts to microbial microcosms, thus lending support to the use of such proxies. However, as the field of oceanography pays an increasing amount of attention to marine microbial dynamics, research appears to have a stronger laboratory and computer modeling component, while less and less of the empirical data and models are being tested in the field. We need to know more specifics about phagotrophic protists and their interspecific interactions as they are in-situ. This will undoubtedly rely on improvement of laboratory procedures for the application of methods in the field. The incorporation of techniques such as fluorescent proteins and post ingestion FISH are likely to be a powerful tool for facilitating this transition back to the field. There must be a call for the development of protocols to integrate modern molecular procedures used to monitor protozoan bacterivory in the laboratory, to be able to monitor these interactions in nature.

Advancement will also be dependant on improving our capability to monitor microbial interactions in stable, multitrophic laboratory experiments. Certain insights into the effects of diversity and competition will need to be assessed in a controlled system. Ecological studies of protozoan bacterivory often treat bacterial diversity as a black box, or characterize them generally through DNA analysis. The incorporation of fluorescent proteins (FP) has allowed me to directly monitor predator-prey dynamics using a natural

prey with an in-vivo tracer. A suite of fluorescent proteins, including yellow FP, indigo FP, and a green FP that becomes blue once exposed to UV light are available for empirical use. Inclusion of multiple such tracers will permit an increase in the biocomplexity of future systems, making it conceivable to investigate a broader range of ecological interactions at the microbial scale, such as prey selection, apparent competition and ecosystem stability. A much more quantitative evaluation of energy flow is also needed to determine the fate of bacterial biomass that has been consumed by protozoa. Recently, continuous cultivation of multitrophic microbial systems have proven to be stable for nearly 60 days (Posch et al. 2001). A multi-trophic, chemostat style system would permit the elucidation of population dynamics, consumption rates, as well as, biomass and nutrient turnover all from one experimental system. A combination of laboratory and in-situ studies addressing complexity ranging from single species to communities will undoubtedly reveal many and varied insights into the structure and functioning of marine microbial foodwebs.

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