AN ABSTRACT OF THE THESIS OF

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Abstract approved:_____

Barry Sherr

By identifying the relationships between abundance and metabolic activity in a bacterial community, we advance our knowledge of the marine carbon cycle. In the present study, our goal was to examine the role of protist grazing on the metabolic activity of γ-*Proteobacteria* in the upwelling system off the Oregon coast. Metabolically active γ-*Proteobacteria* were identified and quantified by a technique which combines microautoradiography and fluorescence in situ hybridization. The experiment consisted of three treatments: a control, a cubitainer filtered to enhance the effects of grazing, and a cubitainer filtered to reduce the grazing pressure on heterotrophic cells. In the three treatments, changes in the γ-*Proteobacteria* population were independent of the entire bacterial community. We found different γ-*Proteobacteria* population patterns in each experimental treatment at the beginning and over the course of a 2 day incubation. In the reduced grazing environment, the increase in γ-*Proteobacteria* abundance was 11 times greater than the increase in the enhanced grazing environment. This suggests that γ-*Proteobacteria* are vulnerable to grazing pressures. Additionally, our data suggest that metabolically active γ-*Proteobacteria* were targeted by grazing protists. Understanding the marine microbial communities through which carbon flows depends upon further defining the relationships between grazers and specific phylogenetic bacterial groups.

Key Words: metabolic activity, microautoradiography, γ-*Proteobacteria*

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The Influence of Grazing on Active γ-*Proteobacteria* Cells

By

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I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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INTRODUCTION

The microbial food web plays an integral role in the flow of carbon in the ocean, an important consideration in light of increasing levels of atmospheric carbon dioxide. Bacteria, which are a component of this food web, are responsible for decreasing the amount of organic carbon transported to the depths of the ocean, where the carbon can be sequestered indefinitely (Legendre & Févre 1995). Heterotrophic bacteria consume organic carbon and, as a result of respiration, convert approximately 85% of the carbon to carbon dioxide (Williams 2000). Consequently, carbon recycling in the ocean is responsible for 1) limiting the downward flux of carbon, and 2) reducing the flux of carbon dioxide from the atmosphere to the ocean due to equilibrium constraints. As the details of this process are not yet fully known and because the microbial component is known to be important, it is necessary to understand what regulates metabolic activity of marine bacterial assemblages.

Prior to the 1990s, microbial food web processes were treated as a black box, suggesting individual characteristics of a community were irrelevant in determining the larger food webs and processes. However, a significant shift in research has occurred towards dissecting the distinctive elements of bacterial populations (Suzuki 1999, del Giorgio et al. 1996). Categorizing bacteria into distinct subgroups provides a more versatile and complete approach to understanding the microbial community. The rich diversity of prokaryotes in the ocean can be differentiated by characteristic rRNA sequences (Giovannoni & Rappé 2000).

The near constant number of bacteria present in a local area suggests that regulating factors influence the microbial population (Andersen & Fenchel 1985). Two

mechanisms are generally used to explain population control: 1) top-down control and 2) bottom-up control. Top-down control corresponds to grazing and viral lysis. Fuhrman and Noble (1995) suggest that bacterial populations are influenced by both viruses and protists in similar proportions in coastal ecosystems. Bottom-up control limits bacterial numbers by the availability of resources. Whether top-down or bottom-up control exerts more control on bacterial abundance and activity is unclear and probably varies in space and time. Gasol et al. (2002) reported that bacteria in nutrient rich ecosystems were predominantly maintained by bottom-up regulation. Conversely, Sanders et al. (1992) noted in eutrophic conditions, bacterial abundance was more directly linked to grazing pressures. This project focuses on the shelf region of the ocean, which represents a nutrient rich environment.

Predatory grazing of protists on bacteria alters the representatives present in a community. In experimentally manipulated conditions, Suzuki (1999) showed that protists have the ability to preferentially graze on specific phylogenetic groups of bacteria. The 0.8 micron filtered sample, which limited the number of protists, showed a significant difference in the patterns of bacterial diversity in comparison to the unfiltered sample. These changes occurred between the 24 and 48 hour incubation times. Bacterial populations are capable of rapid shifts in population due to the large diversity of species, and short generation times (Kirchman 2000).

Feeding of protists on diverse types of prey can be selective (Jurgens & Matz 2002), thus contributing to the naturally occurring diversity of a bacterial assemblage. However, different bacterial populations subjected to the same protist did not result in similar community changes (Vázquez-Domínguez et al. 2005). In addition, changes in

the metabolic activity of bacteria were not correlated with the presence of protists. However, Vázquez-Domínguez et al. (2005) conducted the experiment under conditions of nutrient abundance.

Distinct physical traits enable bacteria to evade predation, a significant factor in molding the community's composition. Jurgens & Matz (2002) reported that size is an integral component for the selection of prey: small cells have an advantage due to reduced encounter rates, while the large cell ingestion rate is limited by the protist clearance regulations. Consequently, bacterial populations have bimodal size distributions during high predation. In addition, Beardsley et al. (2003) showed that the large γ-*Proteobacteria* had disproportional losses in a population study, evidence of size selective mortality. Beardsley's research suggests γ-*Proteobacteria* typically grow rapidly in the absence of predators, yet are selectively fed on when predator populations increase.

In pelagic ecosystems, the number of bacterial cells is not equivalent to the number of active bacterial cells (Cottrell & Kirchman 2000). Metabolically active cells, in the process of growing and dividing, are larger than their dormant counterparts (del Giorgio et al. 1996). Studies indicate active bacteria are targeted by heterotrophic nanoflagellates (Sherr et al. 1994). Protists can discriminate between bacteria due to biochemical surface structures, which Jurgens & Matz (2002) suggest could be indicative of the physiological state of the prey.

The present study examined the role of protist grazing on the metabolic activity of γ-*Proteobacteria* in the upwelling system off the Oregon coast. The cellular uptake of radioactive leucine, an amino acid labeled with tritium, was used to identify

metabolically active cells. Active γ-*Proteobacteria* cells were identified and quantified with a technique that combines microautoradiography and fluorescence in situ hybridization (MICROFISH also referred to as STAR-FISH or MAR-FISH). In FISH, fluorescently labeled oligonucleotide ribosomal RNA probes were used to define different phylogenetic groups. Microautoradiography is a visual technique used to detect the emission of radioactive energy from individual bacterial cells.

MATERIALS AND METHODS

Sample collection and experimental setup

These experiments were conducted in September 2004. Water was collected using General Oceanics 5 L Niskin bottles mounted on a rosette equipped with a SeaBird SBE 911+ CTD and a SeaTech fluorometer. Water was collected from a depth of 10 m at a site on the Oregon shelf, 5 km from shore, bottom depth 54 m (HH1, 44ºN 124.2ºW). Three experimental setups were established in 9.5 L low-density polyethylene cubitainers. Cubitainer 1 was a whole seawater control (referred to as 'control' in this paper). Cubitainer 2 was a 1:1 mix of whole seawater with seawater gravity filtered through a 5 μm Nitex mesh screen. Cubitainer 3 was a 1:1 mix of seawater vacuum-filtered twice through 0.8 μm polycarbonate filters. Cubitainer 2 was filtered to enhance the effects of grazing by reducing the number of predators on the nanoflagellates. Cubitainer 3 was filtered to reduce the grazing pressure on heterotrophic cells. The experiment commenced immediately following water collection and lasted for 4 days. The cubitainers were incubated in a darkened on-deck incubator flushed continuously with surface seawater. During the experiment, water temperature in the on-deck incubator averaged 17ºC. Samples were collected daily from the cubitainers, starting with time zero and ending 4 days later; only the first three sampling points were analyzed for this project to study time periods of interest noted from an additional project. At each sampling time, water was collected for cell counts, and microautoradiography combined with fluorescence in situ hybridization (MICROFISH). MICROFISH was used to identify and quantify metabolically active γ-*Proteobacteria* cells.

Methods used to count the heterotrophic nanoflagellates

Samples for enumeration of flagellates were preserved by a three-step process as previously described (Sherr and Sherr 1993); cells were first preserved with 0.05% (final concentration) alkaline Lugol's solution, followed by 0.1% (final concentration) sodium thiosulfate, and finally 2% (final concentration) of borate-buffered formalin. Samples were incubated at 4 ºC for 24 hours, stained with DAPI (25 μg ml^{-1} final concentration) for 10 min and then filtered onto black 0.8 μm polycarbonate filters (Sherr et al. 1993). These filters were viewed with an Olympus BX-61 epifluorescence microscope with the appropriate filter sets for DAPI-stained cells and chlorophyll autofluorescence.

Processing of samples for microautoradiography and fluorescence in situ hybridization (MICROFISH).

We have modified existing protocols combining microautoradiography and in situ fluorescent hybridization (Longnecker et al. 2006). Whole water samples were incubated immediately after collection with 40 nM of ^{3}H-leucine (specific activity 170 Ci $mmol^{-1}$, Perkin Elmer Life Science Products) in the dark for one hour at the situ water temperature. Samples were fixed with 0.2% w/v paraformaldehyde (final concentration), allowed to sit for at least 10 min in the dark, and then quick-frozen and stored in liquid nitrogen. Samples were transferred to a -80ºC freezer after the return to shore until sample processing.

Samples were filtered onto 0.2 μm polycarbonate filters placed on top of a 0.45 μm backing filter. The volume filtered ranged from 8 to 10 ml. The filters were cut using a razor blade into eight sections and mounted onto glass slides. A Cy3-labeled oligonucleotide probe, GAM42a, was used to specifically target a region of rRNA found

in γ-*Proteobacteria*. During the hybridization step, the samples were incubated overnight at 42°C. The washes were done at 48°C. The hybridization buffer contained 0.9 M NaCl, 20 mM Tris (pH = 7.4), 0.01% SDS, and 30% formamide. Following the initial hybridization, slides were washed twice with 20 mM Tris (pH = 7.4), 0.01% SDS, 5 mM EDTA and 0.102 M NaCl concentrations as in Manz et al. (1992). Washes were for 10 min each, followed by 10 min in 5 μg ml^{-1} DAPI at 4°C, and 2 min in 4°C Milli-Q. The filters were allowed to dry before the next processing step. In the darkroom with a red light at least 1 m away, new slides were dipped into an EM-1 emulsion (Amersham Pharmacia Biotech) and the filter pieces placed face-down onto the emulsion. The slides were incubated at 4°C for 12-14 hours. Following the incubation, the slides were developed for 4 min in Kodak D-19 developer, stopped in Milli-Q water for 10 sec, placed in Kodak fixer for 5 min, and washed in Milli-Q for 5 min. After the slides were dry, the filter was removed from the slide, Citifluor was placed onto the area where the filter had been located, and a coverslip was attached to the glass slide using nail polish.

Slides were viewed on an Olympus BX-61 epifluorescence microscope using the filter sets appropriate for DAPI and Cy3-labeled cells. Sufficient images were captured with a SensicamQE CCD camera (Cooke Corporation, Auburn Hills, MI) to count at least 500 DAPI-stained cells. 10 fields per sample were captured except in cubitainer 2, time 1 where 16 fields were taken. Image Pro-Plus was used to filter the images and then threshold each image to define the location of the cells or silver grains. The masks generated by Image Pro Plus were imported into Matlab to determine where the silver grains overlapped with the DAPI-stained and/or probe-stained cells. Cells which were touching silver grains were identified as cells which had assimilated ^{3}H-leucine and were

therefore biosynthetically active. Cells which were stained with both DAPI and the Cy3-labeled probes were identified as probe-positive cells.

Calculations were made to determine the absolute and relative abundances of the different probe-stained populations, and the absolute and relative abundances of probe-stained populations assimilating leucine. The proportion of each probe-stained population as a percent of the total bacterial assemblage was calculated by dividing the number of probe-positive cells by the number of DAPI-stained cells for each sample (percent abundance). The percent of each probe-positive group assimilating leucine was calculated by dividing the number of cells which were DAPI-stained, probe-positive, and assimilating leucine by the number of DAPI-stained and probe-positive cells (percent assimilating). This later calculation indicates what percent of γ-*Proteobacteria* was biosynthetically active. Standard deviations were calculated using a formula for the propagation of error (Bevington & Robinson 2003) as follows:

$$\frac{\Delta z}{z} = \sqrt{\frac{(\Delta x)^2}{x} + \frac{(\Delta y)^2}{y}}$$

Where z is the % abundance or % assimilating and is equal to x divided by y, as described above. Δx and Δy are the standard deviations associated with x and y, respectively. Δz is the standard deviation calculated for z. Standard deviations were then converted to standard errors for each calculation.

RESULTS

The abundance of heterotrophic nanoflagellates in the unfiltered seawater did not increase on the second day, but rather decreased by three percent from day 1. In cubitainers 2 and 3, the heterotrophic nanoflagellate numbers increased similarly, increasing approximately by a factor of 2.3 from day 1 to 2 (Figure 1). By day 2, the number of nanoflagellates in the enhanced grazing cubitainer was 37% greater than the control population. By 48 hrs, the nanoflagellate count in the reduced grazing population was 16% smaller than the control population.

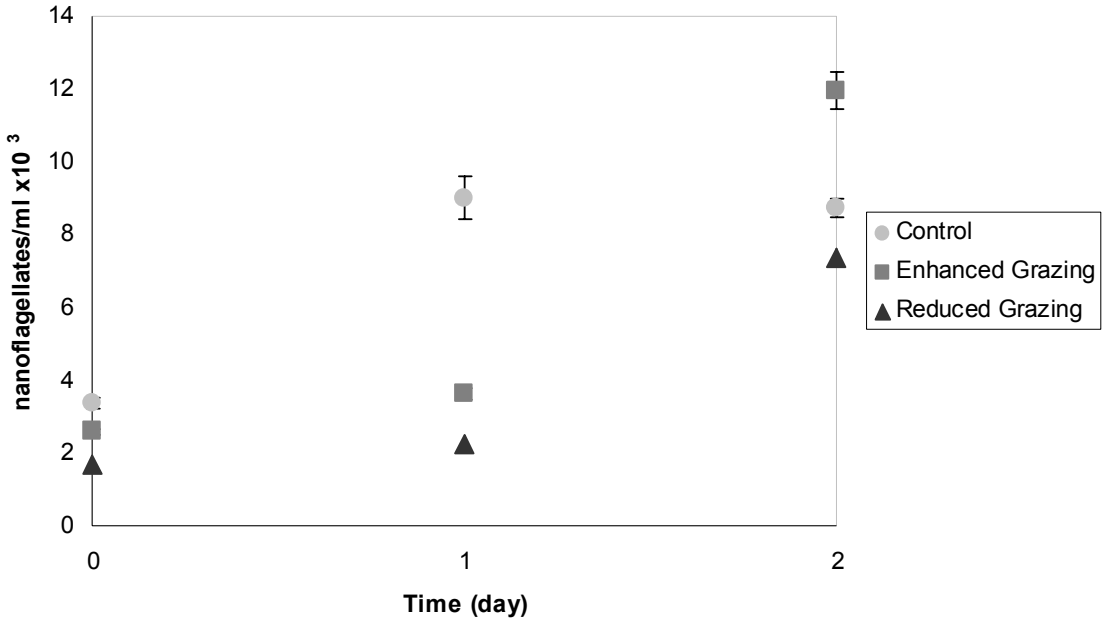

Figure1. Heterotrophic nanoflagellate population as a function of day, with standard errors.

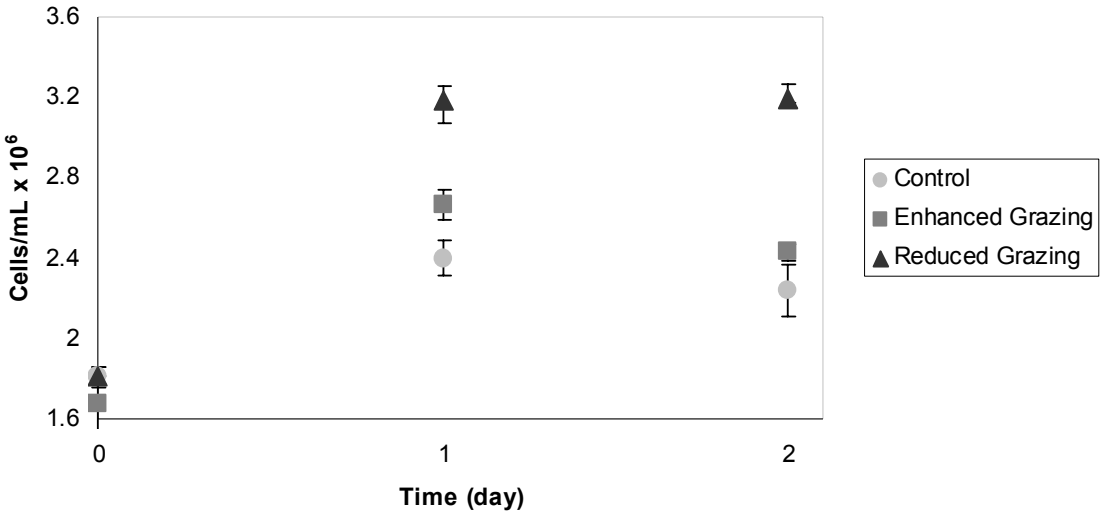

Figure 2. Abundance of heterotrophic bacterial cells from DAPI cell count, with standard errors

Over a period of two days, the heterotrophic bacterial assemblage in the control and in the enhanced grazing cubitainer reached a maximum at day 1 and decreased slightly by day 2 (Figure 2). In contrast, the population of heterotrophic bacterial cells in the reduced grazing cubitainer had an initial increase following time 0 and remained relatively constant from day 1 to day 2. The abundance of heterotrophic cells in both the enhanced and reduced grazing cubitainers was greater than the control population after the onset of the experiment.

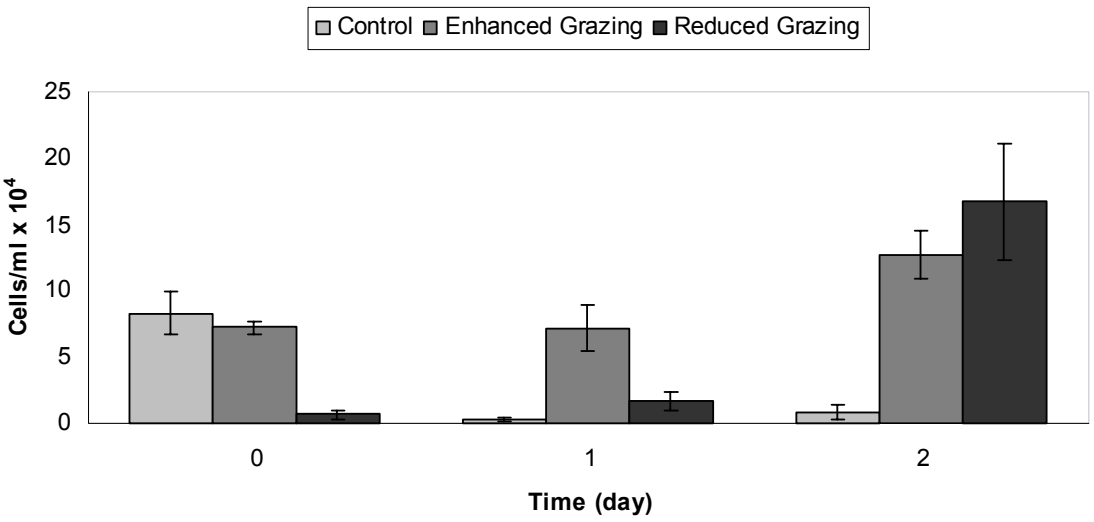

Figure 3. Absolute abundance of γ-*Proteobacteria* with standard errors

The pattern of γ-*Proteobacteria* abundance between the three treatment groups was different (Figure 3). In the enhanced grazing cubitainer, the abundance of γ-*Proteobacteria* remained relatively constant until day 2, when the population increased 78%. In contrast, the γ-*Proteobacteria* population in the reduced grazing cubitainer was constantly increasing. The abundance of γ-*Proteobacteria* at day 2 was almost 9x greater than at day 1. The maximum abundance of γ-*Proteobacteria* cells occurred in the reduced grazing cubitainer at day 2, with 170,000 cells per milliliter.

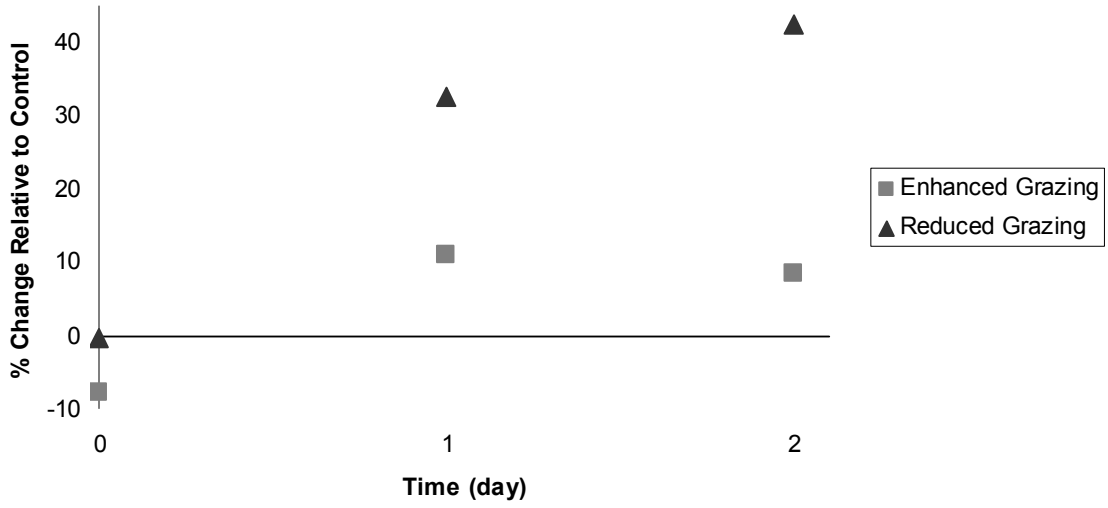

Figure 4. Abundance of heterotrophic bacterial cells normalized to control population

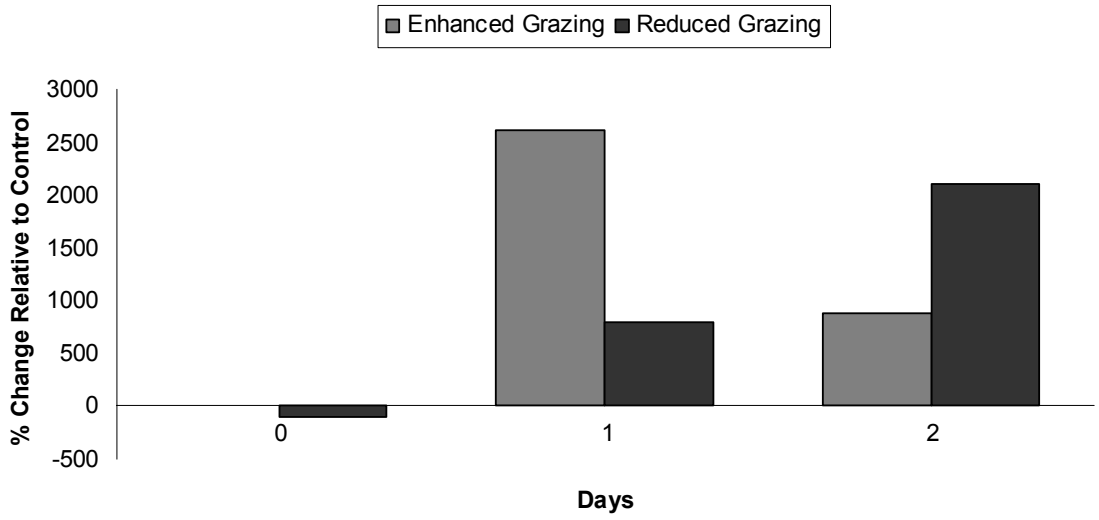

Figure 5. Absolute abundance of γ-*Proteobacteria* normalized with the control population. [Data at time 0 for the enhanced grazing cubitainer is plotted at -13% change relative to control population.]

The enhanced and reduced grazing cubitainers were normalized by calculating the percent change relative to the population in the control. Both the abundance of the γ-*Proteobacteria* and the total heterotrophic bacterial assemblage showed an analogous increase relative to the control population (Figures 4 and 5).

	Time (day)	Abundance (cells per milliliter)	Standard Errors
Control			
	0	19000	4800
	1	48	630
	2	990	700
Enhanced Grazing			
	0	2200	990
	1	3200	1500
	2	9200	2700
Reduced Grazing			
	0	48	420
	1	4400	3000
	2	110000	27000

Table 1. Abundance of active γ-*Proteobacteria*, where active cells are defined as cells which had assimilated leucine

Bacterial cells which assimilated leucine were identified as active cells. The abundance of active γ-*Proteobacteria* cells increased most dramatically over the two day period in the reduced grazing cubitainer (Table 1). At day 2 in the reduced grazing cubitainer, the greatest number of γ-*Proteobacteria* per milliliter actively incorporated radioactive leucine. In the enhanced grazing cubitainer, there was nearly a 2-fold

increase of γ-*Proteobacteria* cells which were actively assimilating leucine from day 1 to day 2.

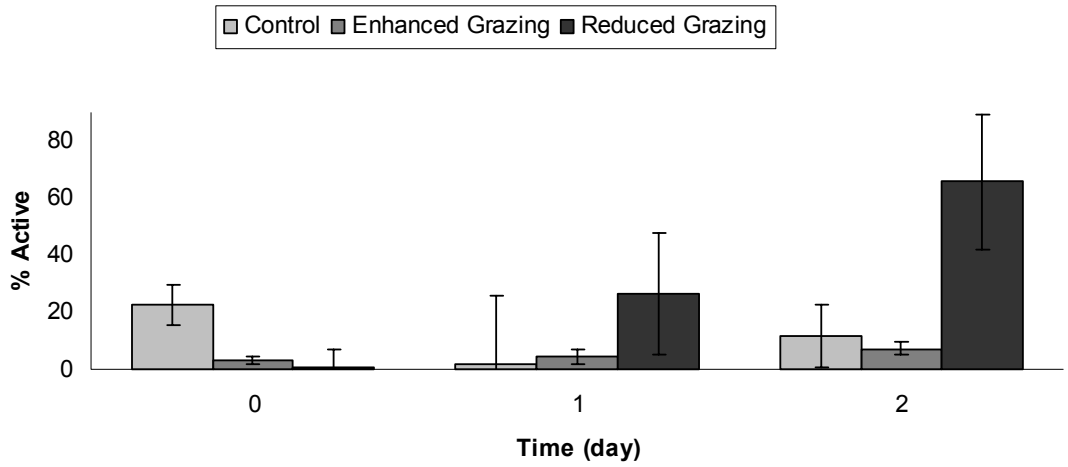

Figure 6. Percent of γ-*Proteobacteria* population which assimilated leucine, with standard error

Relative activity is defined as the percent of the total number of γ-*Proteobacteria* that were identified as active cells. As seen in Figure 6, the percentage of active γ-*Proteobacteria* cells in the reduced grazing cubitainer increased over the 48 hour period. This indicates that not only did the abundance increase (Figure 3), but also, a larger percentage of the increasing population was actively assimilating leucine. On the other hand, in the reduced grazing cubitainer, a larger number of cells were active at day 2, yet relative to the increase in abundance, the percentage of active γ-*Proteobacteria* cells was maintained constant at 10%.

DISCUSSION

Treatment effects on bacterial abundance:

The abundance of γ-*Proteobacteria* in the three treatments was not similar to the response of the total, DAPI-stained heterotrophic bacterial assemblage. Presumably, the protist grazers had a differential effect on the γ-*Proteobacteria* relative to the total bacterial assemblage. It is interesting to note, however, that a similar trend was observed after normalizing each population in treatments 2 and 3 against their respective populations in the control treatment.

Pernthaler et al. (1996) showed that when flagellates are introduced, the community shifts from one composed of large bacteria, to a population of small bacteria. They hypothesized that the small cells are more able to escape predation. Beardsley et al. (2003) also reported a proportionally greater loss of γ-*Proteobacteria* due to size selective mortality. Significant evidence of size selective feeding exists, which results in bimodal distributions of a natural bacterial population, where cells are either large or small (Jurgens & Matz 2002). Our data suggests that increasing the predator population does not result in an immediate decrease in γ-*Proteobacteria* population. Thus, γ-*Proteobacteria* may initially have a different mechanism other than size to avoid the immediate predation of nanoflagellates. Future analysis will look at how the presence of grazers affected the size of γ-*Proteobacteria*. The size of the bacteria can be estimated from the images in Image Pro-Plus for preliminary data.

Gasol et al. (2002) suggested that in a eutrophic environment, the bacteria population is maintained by bottom-up regulation. According to these authors, in eutrophic shelf regions, the water column bacteria should be controlled by the concentration and availability of nutrients in patches where their activities have brought

down the nutrients below a certain threshold. Our experiment was completed in a closed environment, and is not a complete representation of eutrophic conditions. However, the slight decrease in the control population of the heterotrophic bacteria cells at day 2, might suggest the nutrient concentration was decreasing as in naturally occurring micropatches. It is predicted that in a eutrophic environment, the function of predators is merely to regulate the percent abundance of phylogenetically diverse bacteria in the community. In our research, the shifts in the γ-*Proteobacteria* abundance, which were distinct from the changes in the heterotrophic bacteria community, suggest that predators control the abundance of specific phylogenetic groups. Future work will investigate the bacteria communities from the oligotrophic offshore region. In an oligotrophic environment, the bacteria population is thought to be controlled by predators. Additionally, the bacteria are most likely dependent on predation to replenish the nutrient pool by the release of organic carbon (Gasol et al. 2002).

The abundance of the DAPI-stained bacterial cells which also were labeled with the γ-*Proteobacteria* oligonucleotide probe changed during the two day study and the pattern of change was different between the three treatment groups. The control γ-*Proteobacteria* population had an inverse relationship with the nanoflagellate population; as the nanoflagellate population increased at day 1, the γ-*Proteobacteria* population decreased. In the absolute γ-*Proteobacteria* cell count of both the enhanced and reduced grazing cubitainer, the population increased despite an increase in the predator population. However, the abundance of predators was initially reduced following filtration in both filtered samples. During this short period of reduced grazing, the bacteria might have increased in population prior to the regeneration of predators in the

cubitainers. This deviation could also suggest that the larger grazers were the primary grazers of the γ-*Proteobacteria*; in both the enhanced and reduced cubitainers the larger grazers had been screened out.

Ŝimek et al. (2006) indicated that the γ-*Proteobacteria* group has significantly faster growth rates than the bulk heterotrophic bacteria community. In his study, the doubling time of the γ-*Proteobacteria* was 21 hours compared to 23 hours for the heterotrophic bacterial assemblage as a whole (by contrast, the doubling time of heterotrophic nanoflagellates was 10 hours). An increase in the percent abundance of a proteobacterial phylotype in a high predator environment was viewed by Ŝimek as the selection of bacterial groups with intrinsically high growth rates. Ŝimek reported fast turnover in a specific β-*Proteobacteria* 'Rhodoferax' sp. BAL47 cluster, similar to the γ-*Proteobacteria*. Ŝimek et al. (2006) proposed that β-*Proteobacteria* are 'uptake specialists' which were described by Thingstad et al. (2005) as having a high growth potential yet being vulnerable to protist grazing. Specifically, in a grazing enhanced environment, this β-*Proteobacteria* group had the fastest intrinsic growth rate but the lowest realized production. The γ-*Proteobacteria* in our study had the greatest increase in population when faced with reduced levels of predation. The γ-*Proteobacteria* population therefore had similar traits to the "uptake specialist."

In research conducted by Vázquez-Domínguez et al. (2005), the bacteria community shifted from a population dominated by edible species to a population resistant to predation. These changes in the bacterial assemblage are both genotypic and phenotypic in nature. Although there was an increase in absolute abundance of the γ-*Proteobacteria* population in both the enhanced and reduced grazing cubitainers, the

largest increase occurred in the reduced grazing cubitainer. The reduced grazing cubitainer also had the lowest number of heterotrophic nanoflagellates and should consequently have had the lowest grazing rate. The data suggests the γ-*Proteobacteria* population is unable to resist predation.

Gasol et al (2002) suggested that in filtration schemes, it is possible for the targeted microorganisms to squeeze through the filter pores, and thus may exist in the sample. Vázquez-Domínguez et al. (2005) showed that different protists altered parallel bacteria communities uniquely. Our data show that each cubitainer housing a different starting biotic environment resulted in different abundances of γ-*Proteobacteria.* If the filtering scheme merely alters the types of protists present in the sample, the results provide additional support to the study conducted by Vázquez-Domínguez et al.

Sanders et al. (1992) suggested that in most aquatic systems there is a near constant ratio of 1000 bacteria for every heterotrophic nanoflagellate. However, the heterotrophic nanoflagellate pressure on bacteria is not as tight as originally presumed, i.e. the heterotrophic nanoflagellates response to a change in number of bacteria does not occur immediately. In an experiment conducted by Gasol et al. (2002), the heterotrophic bacteria population increased in 24 hours, yet the heterotrophic flagellate population did not respond for three to five days. Our study was conducted for 48 hours, and due to the loose coupling of the populations suggested by Gasol et al., our data might be more indicative of bottle effects than predator and prey relationships. The bottle effect is the tendency of stressed bacteria to adhere to the cubitainer surface to create a biofilm. The development of a biofilm would reduce the abundance of microorganisms in the sample, and provide an inaccurate representation of the population (Costerton *et al.* 1999).

Consequently, population dynamics may not be indicative of in situ growth dynamics in the first 48 hours of incubation.

Treatment Effects on Metabolic Activity:

The epifluorescence microscope-based index of the incorporation of radio-labeled substrates such as leucine into bacterial macromolecules (termed autoradiography) coupled with the FISH technique (termed MICROFISH in this paper) provides a way to identify individual cells of specific phylogenetic affiliation which are metabolically active or inactive in a natural water sample. In terms of activity the index gives a qualitative *yes* or *no* answer but does not quantify the cell's activity level.

At time 0, 22.8 % of the γ-*Proteobacteria* in the control had incorporated tritium labeled leucine. This number is higher than the 6.5% of active bacteria reported by del Giorgio et al. (1996) from in situ samples in the Bay of Blanes on the Mediterranean coast of northern Spain. del Giorgio et al. also stated that in the absence of grazing, over three-fourths of the bacteria were active. Our study corresponds with similar numbers. For example, at day 2 in the reduced grazing cubitainer over 65% of the γ-*Proteobacteria* cells were actively incorporating leucine.

In the study by del Giorgio et al. (1996) there was strong selective grazing on metabolically active cells. There was also a net increase in the number of inactive cells, indicating a mechanism for cells to enter an inactive state. del Giorgio et al. suggest the heterotrophic nanoflagellates were responsible for the overall bacterial losses and the removal of active cells. Additionally, del Giorgio showed that the active cells were 2.2 times bigger in volume than the inactive cells and grazing on active cells was over four

times greater than the grazing on inactive cells. In our research the relative percentage of active γ-*Proteobacteria* cells in an enhanced grazing environment did not change. Yet, in the reduced grazing environment, there was an increase in the abundance of active γ-*Proteobacteria* cells at day 2. This indicates that there was additional grazing pressure on active γ-*Proteobacteria* over the course of two days.

The percentage of γ-*Proteobacteria* incorporating leucine in the enhanced and reduced grazing cubitainers was greater than in the control population. Sherr et al. (1999) showed an increase in metabolic activity in incubated samples, which did not correspond with the in situ metabolic activity. It was suggested the difference was due to an artifact of filtration which may have acted to increase the amount of substrate available for bacteria consumption. With current methods it is difficult to change the predation pressure without also impacting substrate/nutrient recycling. It is not possible to discriminate between the negative impact of grazers and the positive effect of grazer mediated recycling. Thus, the increase in metabolically active γ-*Proteobacteria* cells after filtering might be a result of reduced grazing pressure, increased nutrient supply, or a combination of the two.

Our study adds to the understanding of the intricate relationships that exist between protists and γ-*Proteobacteria.* While our data supports the claim that metabolically active γ-*Proteobacteria* are vulnerable to grazing pressures, any further analysis is limited, due to lack of information regarding the level of activity. Additionally, the suggestion that changes in the γ-*Proteobacteria* population are independent of the changes in the total heterotrophic bacterial community suggests the

need for further investigation to define how phylogenetic composition of such systems change as a result of perturbation.

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