Investigating the effect of motility on bacterial predation by a heterotrophic nanoflagellate

MBL - Microbial Diversity Course 2003 Miniproject
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Abstract
Unicellular marine cyanobacteria are the dominant source of fixed carbon in the marine food web with the first step in the flux of this carbon passing primarily through heterotrophic nanoflagellates. Using both motile and non-motile strains of marine *Synechococcus*, the role of bacterial motility on susceptibility to bacterivory was explored. Both direct counts and quantitative-PCR were used to monitor prey cell abundance during time-course experiments with strains fed independently and in combination to the flagellate *Paraphysomonas bandaiensis*. Results illustrate differential predation rates on the strains used with motile cells being cleared at over twice the rate of non-motile cells. One experiment indicates that this difference is intensified when strains are placed in competition. For the environmental isolates used in this comparison, other phenotypic differences, such as size, may explain the differences in selective predation observed, and illustrate the need for more closely controlled comparisons.

Introduction

Oceanic primary productivity is dominated by small unicellular cyanobacteria of the genus *Synechococcus* and *Prochlorococcus* (Waterbury et al. 1986, Chisholm et al. 1988, Li et al. 1992, Landry et al. 1996). While knowledge of the physical and environmental factors that effect the growth of these phytoplankton has been investigated for some time and is reasonably well understood, comparatively little research has focused on the impact of predatory grazers on these globally important primary producers. These cells represent the major input of fixed carbon into the base of the marine food web, thus identifying the factors that affect their consumption by predators is crucial to understanding of the flow fixed carbon into the marine food web.

Due to their small size, unicellular cyanobacteria are unlikely to be consumed directly by mesozooplankton but are certainly ingested by heterotrophic nanoflagellate predators. Heterotrophic nanoflagellates are small 1-10μm eukaryotic predators of both photosynthetic and heterotrophic bacteria. Recent work has begun to outline both physical factors such as UV irradiation (Ochs & Eddy 1998), and biological characteristics such as cell size, morphology, motility, and cell surface hydrophobicity (Monger et al. 1999, Matz at el. 2002) that influence relative susceptibility of cells to predation. Specifically, Matz et al. (2002) report the finding of a significant positive relationship between bacterial swimming speed and increased contact rates between bacteria and flagellate predators. This was offset though, by a corresponding negative relationship between flagellate capture efficiency and bacterial swimming speed resulting in no significant difference in ingestion rates for motile cells compared to non-motile cells.

Reported here are experiments designed to investigate the relationship between motility and predation. In a simplified model system, different strains of marine *Synechococcus* have been added to cultures of the commonly isolated heterotrophic nanoflagellate *Paraphysomonas bandaiensis*. Predation upon *Synechococcus* by *P. bandaiensis* was quantitatively monitored, over relatively short time spans time (<24hrs), by two independent methods. Initial experiments were conducted with a model motile strain (strain WH8102) and a non-motile mutant of WH8102 (strain S1A1[Brahamsha 1996]). Subsequent experiments involved a motile environmental isolate (WH8102) and a non-motile environmental isolate (WH7803).

Methods

Strains and Growth Conditions:

Synechococcus strains WH8102, WH7803, and S1A1-B(Brahamsha 1996), were maintained at 23°C on a 14hr/10hr light dark cycle in SN(Waterbury & Willey 1988) media prepared from local seawater. *Synechococcus* strainWH8102 is a motile strain, which exhibits a unique non-flagellar swimming motility. *Synechococcus* strain S1A1-B is a non-motile mutant strain derived from WH8012. *Synechococcus* strainWH7803 is a naturally non-motile strain. Kanamycin was added at 20μg/ml to the media of mutant strain S1A1-B for maintenance of mutational insertion. *P. bandaiesis* was maintained in 30ml cultures in the same incubator along with *Synechococcus* strains in 0.45μm filtered local seawater + 0.01% yeast extract and a single grain of rice.

Feeding experiments:

P. bandaiensis cultures were prepared ~5days before use by inoculating 30ml media with 1ml of a stationary phase culture resulting in working cultures of ~1x10^6 cells · ml^{-1}. Cells numbers were determined for *P. bandaiensis* and *Synechococcus* cells by counts using a hemacytometer and immobilizing onto 0.45μm filters respectively. For feeding experiments, prey cells were added to achieve a final concentration of 100 *Synechococcus/P. bandaiensis* (for competition experiment 50 WH8102/*P. bandaiensis* and 50 WH7803/*P. bandaiensis*). Aliquots were removed at specific time points and split into two for quantification by the two methods described below. 1.0-1.5ml used for direct counts and 1.5 ml used for DNA isolation for subsequent Quantitative-PCR.

Sample processing (cell # quantification):

Direct cells counts: Prior to fixation with 0.5% (final concentration) glutaraldehyde, *P. bandaiensis* cultures were anesthetized with 1.5% (w/v final concentration) NiCl$_2$ for 30seconds to prevent ejection of food vacuole contents (J. Waterbury, F. Valois personal communication). When enumeration of *P. bandaiensis* was required, samples were stained with 5x10^{-4}% proflavine. After fixing and staining, cells were immobilized on black 0.45μm nucleopore filters and visualized by epifluorescence using both a rhodamine filter and an acridine orange filter to enumerate cells. All samples counted within 8hrs of fixation.

Quantitative-PCR: Cells pelleted by centrifugation at ~14K RPM for 10minutes then resuspended with 300μl bead solution provided in MoBio UltraClean Soil DNA kit (MoBio, Solana Beach CA), mixed with beads in vial and frozen until ready to process all samples. DNA extraction procedure performed as per manufacturers instructions for maximum yields with the addition of 2 freeze-thaw cycles at the beginning of the protocol. Quantitative-PCR (Q-PCR) was performed using a Bio-Rad iCycler iQ real time detection system(Bio-Rad, Hercules CA). 25μl reactions were prepared in 96 well plates using Bio-Rad iQ SYBR green supermix, oligos at 1μM, and 1-5μl template DNA. Oligos selected to amplify a small fragment 80-160 bp in length. For all experiments a hot start 2-step amplification reaction was run with 20 sec denaturation at 95°C, 60 sec annealing and extension at 60°C cycled 50 times. All samples run in duplicate including standard curve, which ranged from 5x10^7 targets/reaction to 1x10^7. Oligos used: swmA specific forward (A1) 5'-CTTGGCGTCAAGGAACCAAT-3', swmA specific reverse (A2) 5'- CATTCCTTCCGTGGCAACTC-3', motility mutant specific reverse primer (V1) 5'- TCATACACGGTGCCTGACTG, cyano specific 16S forward (CYA 359F) 5'- GGGGAATYTTCCGCAATGGG -3' (Nubel et. al 1997), universal bacterial 16S reverse (S*Univ 519R) 5'- GWATTACCGCGGCKGCTG -3' (Amann et al. 1995). Controls were performed to confirm that the primer set A1&A2 specifically amplified DNA from motile strain WH8102 and would not amplify WH7803 DNA as well as to confirm that primer set CYA 359F & S*Univ 519R would amplify both WH7803 and WH8102 while not amplifying *P. bandaiensis* culture community DNA.

Results

Initial experiments were carried out feeding *Synechococcus* strains independently to the *P. bandaiensis* predator. A logarithmic loss of *Synechococcus* cells is observed for the two strains as detected by both techniques (Fig. 1). Moreover, there was a close correspondence in the rate of grazing predation as monitored by the two methods employed. No clear difference in mortality rate between motile strain WH8102 and the non-motile mutant S1A1-B was observed. Unfortunately, the S1A1-B culture was not growing well and there were not sufficient cells to add a quantity to give equal starting concentrations for the two treatments. The poor condition of the culture also made direct counts difficult due to the variable and inconsistent autofluorescence from the S1A1-B cells. Additionally, for the last time point at nine hours post-addition, no S1A1-B cells could be observed in the directly counted treatment and this data point has been omitted from the linear regression plot.
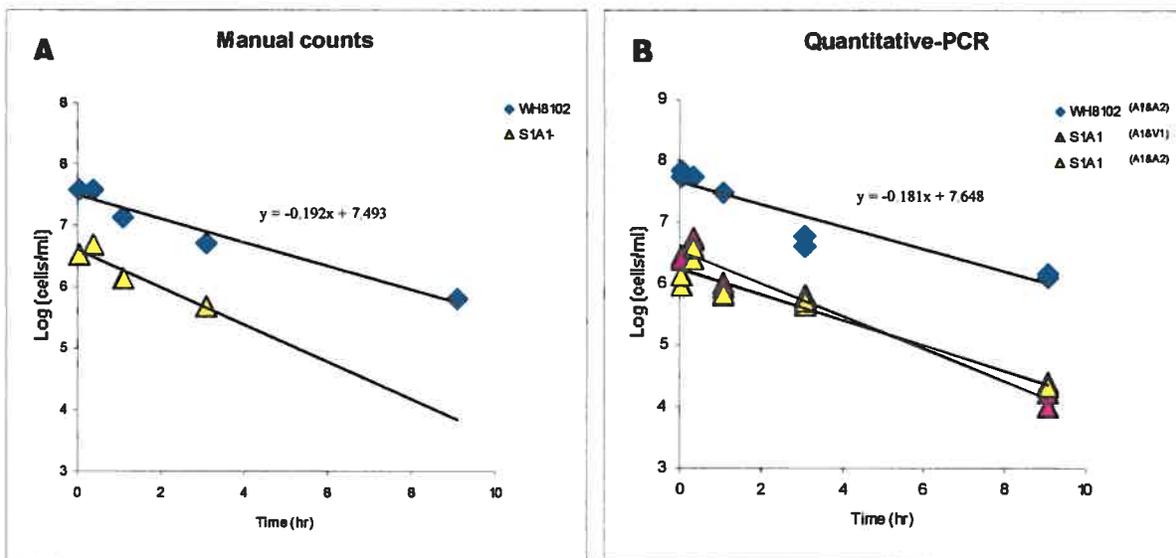

Fig. 1. Time course changes in prey abundance as monitored by direct counts (A) and Q-PCR (B). Strain S1A1 was quantified by Q-PCR using two independent genetic loci denoted by primer pairs listed in parentheses.

Following this first experiment, the non-motile mutant culture S1A1-B died. Another non-motile strain was employed in the following experiment, this time the naturally non-motile environmental isolate WH7803. Once more, the test *Synechococcus* strains were fed independently to the *P. bandaiensis* predator. A close correspondence was again observed between the two methods of quantification (Fig. 2). A distinct difference is observed in the rate at which these two strains are being cleared from the culture. WH8102 is being cleared at a rate of approximately 34% of the Synechococcus stock $\cdot$ hr^{-1} while WH7803 is being cleared at a rate less than half of that (~15% Syn stock $\cdot$ hr^{-1}). Furthermore, the predation rate of *P. bandaiensis* on WH8102 was essentially unchanged from the first to the second experiment (see slope of lines Figures 1A, 1B, 2A, and 2B).

A — Fed Individually - Manual counts

$y = -0.174x + 8.045$

B — Fed Individually - Q-PCR

$y = -0.172x + 8.714$

C — Competition Q-PCR
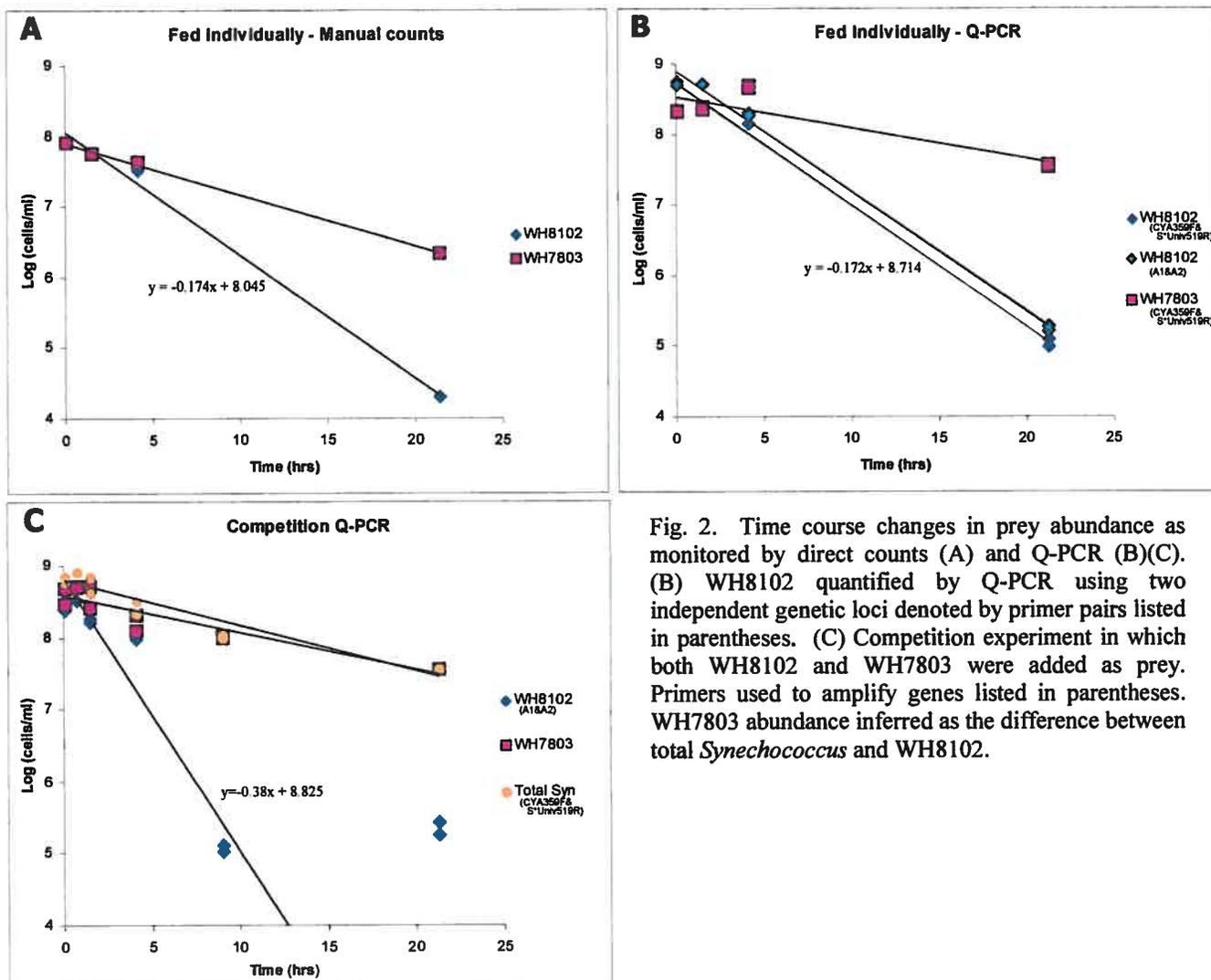
$y = -0.38x + 8.825$

Fig. 2. Time course changes in prey abundance as monitored by direct counts (A) and Q-PCR (B)(C). (B) WH8102 quantified by Q-PCR using two independent genetic loci denoted by primer pairs listed in parentheses. (C) Competition experiment in which both WH8102 and WH7803 were added as prey. Primers used to amplify genes listed in parentheses. WH7803 abundance inferred as the difference between total *Synechococcus* and WH8102.

Observations of directly counted cells at the final time point illustrate another difference between the predation of *P. bandaiensis* on WH8102 as compared to WH7803. After incubating WH8102 21 hours with *P. bandaiensis*, very few *Synechococcus* cells remain and those seen are unicellular and well dispersed. In contrast the WH7803 fed culture contains primarily clumps of cells ranging from just a few to greater than fifty cells per aggregate. Although isolated single cells are still observed the majority of the prey cells remaining are found associated into multicellular clusters (Fig 3).

Preliminary experiments were all designed around the goal of feeding strains to a predator in combination to observe what happens to predation rate in a competitive situation. Figure 2C shows the change over time in both total *Synechococcus* and strain WH8102 along with the inferred concentration of WH7803. WH7803 concentration is assumed to be the difference between the total *Synechococcus* concentration and WH8102 concentration.

[*Synechococcus* strain WH7803] = [Total *Synechococcus*] − [*Synechococcus* strain WH8102]
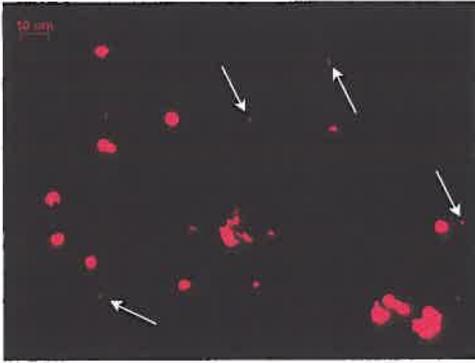
Fig. 3. Epifluorescence micrograph of remaining *Synechococcus* sp. strain WH7803 cells 21 hours post feeding to *P. bandaiensis*. Majority of cells present in large multicellular cluster with individual cells indicated by arrows.

Within the first 10 hours the concentration of WH8102 dropped dramatically, much more rapidly than observed when fed in isolation to *P. bandaiensis*. Interestingly, at the final time point WH8102 abundance had not dropped any further and may even indicate that these cells are increasing in number after having reached a minimum. As a means of comparison, a linear regression line was fit to all points except this last time point. Calculating a predation rate based on this line yields a rate of approximately 50% of the WH8102 stock hr $^{-1}$. While the predation rate of *P. bandaiensis* on WH8102 appears to have increased when in competition no obvious change in predation rate on WH7803 is seen when in competition.

Discussion

These results show the promise of Q-PCR as a tool for quantifying different populations of cells in applications where microscopic differentiation of cells is not possible. In both experiments the rate of predation observed by direct counts very closely matched that determined by Q-PCR. In the first experiment, the absolute value of cell concentrations correlated very well between these two methods of quantification. This close correspondence did not hold up in the second experiment with Q-PCR giving values up to an order of magnitude greater than direct counts. This disparity was likely due to difficulties in obtaining accurate DNA concentrations of nucleic acid isolated using the MoBio kit for use in generating a standard curve. In spite of this possible source of error, Q-PCR remains a robust means for measuring relative levels of specific populations.

Experiments in which different strains of *Synechococcus* were fed independently to *P. bandaiensis* show a distinct difference in the rate at which this flagellate ingests particular cells. Additionally, the one competition experiment conducted suggests that this discrepancy between predation rates is magnified when strains are fed in combination. While several recent reports have documented flagellates grazing at different rates on *Prochlorococcus* as compared to *Synechococcus* (Guillou at al. 2001, Christaki et al. 2002, Worden & Binder 2003) the results presented here show that grazing predation may have a significant influence on community structure at an even finer level. These results suggest that motile cells may be at a disadvantage to non-motile cells in regard to predation susceptibility.

Matz et al. (2002) investigated the effect of motility on predation and showed an increased encounter frequency for motile cells but corresponding decrease in capture efficiency by the flagellate, the net result being no change in net ingestion of motile cells compared to non-motile ones. The authors suggest that the decreased capture efficiency may be due to "run and reversal" motion typical of many marine bacteria with reversals

acting as an active predator avoidance strategy. If this is the case, motile marine *Synechococcus* spp. offer an interesting comparison as these cells swim in seemingly random loopy paths never reversing directions or tumbling to reorient. Thus the above results are consistent with the possibility that motile *Synechococcus* have an increased encounter frequency with predators due to motility but gain no offsetting reduction in escape frequency due to their swimming behavior.

While the aim of this study was to investigate the role of motility in susceptibility of bacterial populations to predation, various other differences between these two strains may be dominating the basis of differential predation observed. While both strains are marine *Synechococcus,* reports have shown this group to be highly divergent (Toledo & Palenik 1998, Toledo et al. 1999) and there are other phenotypic characteristics differing between these two strains such as pigment content. While motility is another clear difference, it seems likely that there may be other differences which the flagellates can discern that we cannot. These strains do not differ markedly in size but even small differences may be sufficient to create a selective difference such as that observed. *Synechococcus* sp. strain WH7803 is a notoriously "clumpy" strain (E. Webb, personal communication) which may be an important factor for its apparent resistance to grazing. Observations at the end of the feeding experiment show a conspicuous difference between the two strains under investigation. While WH8102 had largely vanished, WH7803 had declined to 2-3% of the original starting concentration. It is not clear if clustering occurred in response to grazing pressure or if this fraction of WH7803 cells were clustered into multicellular groups prior to incubation. In either case, this observation lends some credibility to the hypothesis that size selective feeding resulted in the observed differences in survival of these two strains.

Results using isogenic populations of a wild-type strain and specific mutants derived from this strain were unfortunately inconclusive. Such an experiment would eliminate the uncertainty of potential differences between strains. The results from this miniproject indicate that the methods employed here should be able to detect differential grazing by predators and may be successfully employed, with appropriate cultures, to tease apart finer details of flagellate grazing preferences.

Cited References
Amann RI, Ludwig W, Schleifer KH (1995) Phylogenetic identification and *in situ* detection of microbial cells without cultivation. Microbial Reviews 59:143-169

Brahamsha B (1996) An abundant cell-surface polypeptide is required for swimming by the nonflagellated marine cyanobacterium Synechococcus. Proceedings of the National Academy of Sciences of the United States of America 93:6504-6509

Chisholm SW, Olsen RJ, Zettler, ER, Waterbury JB, Goericke R, Welschmeyer N (1988) A novel free living prochlorophyte occurs at high cell concentration in the oceanic euphotic zone. Nature 334:340-343

Christaki U, Courties C, Karayanni H, Giannakourou A, Maravelias C, Kormas K, Lebaron P (2002) Dynamic characteristics of *Prochlorococcus* and *Synechococcus* consumption by bacterivorous nanflagellates. Microbial Ecology 43:341-352

Guillou L, Jacquet S, Chretiennot-Dinet MJ, Vaulot D (2001) Grazing impact of two small heterotrophic flagellates on *Prochlorococcus* and *Synechococcus*. Aquatic Microbial Ecology 26:201-207

Landry M, Kirshtein J, Constantinou J (1996) Abundances and distributions of picoplankton populations in the central equatorial Pacific from 12N to 12S, 140N. Deep-Sea Research Part II Oceanographic Research Papers 43:871-890

Li WKM, Dickie PM, Irwin BD, Wood AM (1992) Biomass of bacteria, cyanobacteria, prochlorophytes and photosynthetic eukaryotes in the Sargasso Sea. Deep-Sea Research 39:501-519

Matz C, Boenigk J, Arndt H, Jurgens K (2002) Role of bacterial phenotypic traits in selective feeding of the heterotrophic nanoflagellate *Spumella* sp. Aquatic Microbial Ecology 27:137-148

Monger BC, Landry MR, Brown SL (1999) Feeding selection of heterotrophic marine nanoflagellates based on surface hydrophobicity of their picoplankton prey. Limnology and Oceanography 44:1917-1927

Nubel U, Garcia-Pichel F, Muyzer G (1997) PCR primer to amplify 16S rRNA genes from cyanobacteria. Applied and Environmental Microbiology 63:3327-3332

Ochs CA & Eddy LP (1998) Effects of UV-A (320-399nm) on grazing pressure of a marine heterotrophic nanoflagellate on strains of unicellular cyanobacteria *Synechococcus* spp. Applied and Enviromental Microbiology 64:287-293

Toledo G, Palenik B (1997) *Synechococcus* diversity in the California current as seen by RNA polymerase (rpoC1) gene sequences of isolated strains. Applied and Environmental Microbiology 63:4298-4303

Toledo G, Palenik B, Brahamsha B (1999) Swimming marine *Synechococcus* strains with widely different photosynthetic pigment ratios form a monophyletic group. Applied and Enviromental Microbiology 65:5247-5251

Waterbury JB, Watson SW, Valois FW, Franks DG (1986) Biological and ecological characterization of the marine unicellular cyanobacterium *Synechococcus*. Canadian Bulletin of Fisheries and Aquatic Sciences:71-120

Waterbury JB, Willey JM (1988) Isolation and growth of marine planktonic cyanobacteria. Methods in Enzymology 167:100-105

Worden AZ & Binder BJ (2003) Application of dilution experiments for measuring growth and mortality rates among *Prochlorococcus* and *Synechococcus* populations in oliogtrophic evvironments. Aquatic Microbial Ecology 30:159-174