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Diel periodicity in *Synechococcus* populations and grazing by heterotrophic nanoflagellates: Analysis of food vacuole contents

Abstract—The relationship between heterotrophic nanoflagellates (HFLAG) and the autotrophic prokaryote *Synechococcus* was examined in the bay of Villefranche (NW Mediterranean) in June 1998. We determined *Synechococcus* concentrations, % of dividing *Synechococcus* cells, concentrations of HFLAG, and numbers of *Synechococcus* cells inside HFLAG food vacuoles in samples taken every 2–3 h over 62 h ($n = 28$). *Synechococcus* cells in division ranged from 10 to 30%, with peak values in late afternoon–early evening hours. Numbers of *Synechococcus* in HFLAG food vacuoles (0.06–0.34 *Synechococcus* HFLAG^{-1}) were not related to concentrations of total *Synechococcus,* or nondividing *Synechococcus* but were negatively related to % of dividing *Synechococcus.* The data suggest there may be a relationship between vulnerability to predation and average *Synechococcus* cell size. HFLAG community grazing pressure, estimated from food vacuole content and HFLAG abundances, ranged from about 0.2% *Synechococcus* stock removed h^{-1} at midnight to 1% *Synechococcus* stock removed h^{-1} at noon.

In a large number of marine systems, the autotrophic prokaryotes, *Synechococcus* and *Prochlorococcus,* are responsible for a majority of the primary production (Wiesse 1993). Often, they show distinct cycles of phased cell division with nighttime maxima in reproducing cells (e.g., Prézelin et al. 1987; Vaulot et al. 1995). Despite strong diel rhythms, the abundances and distributions of picoplankton populations are relatively stable (e.g., Landry et al. 1996). Thus, diel variability in grazing losses has been suggested (Prézelin et al. 1986; Liu et al. 1995, 1997). For an organism that reproduces synchronously, the importance of periodicity of grazer-induced mortality is clear. However, rhythms in grazing activity may affect organisms other than the prey and predator, in trophic levels both below and above the grazer. Periodicity in grazing may be reflected in variability of fluxes of grazer excreta to heterotrophic bacteria and phytoplankton as well as yield periodicity in the formation of biomass for exploitation by higher trophic levels. While the question of periodicity in grazing rates relative to the diel cycle of picocyanobacteria has been identified as deserving attention (Wiesse 1993), it has been largely ignored, due perhaps to methodological problems.

Methods to estimate grazing rates of planktonic protists can be divided into three major classes (Landry 1994): (1) community manipulation approaches such as size fractionation, dilution, or estimating disappearance rates of prey analogs, (2) use of tracer techniques to measure direct uptake rates—for example, fluorescently labeled prey, and (3) inferences from natural populations such as digestive enzyme activity of protists or their food vacuole content. Techniques such as size fractionation or dilution require relatively long incubation times (≥12 h), making them inappropriate for short time-scale studies. In addition, community approaches do not actually identify the predators. Tracer techniques usable with short incubation times, such as estimating direct uptake of fluorescently labeled prey, have problems of potential grazer discrimination; protistan grazers can display considerable variability in prey selection depending on quantities of prey available or physiological state (e.g., Jürgens and Demott 1995; Christaki et al. 1998). We recently examined the feasibility of using food vacuole contents, a method appropriate for short time and space scales, to estimate rates of *Synechococcus* ingestion by HFLAG. HFLAG are generally assumed to be the major consumers of autotrophic picoplankton (Wiesse 1993). We found that estimates of ingestion based on food vacuole content and digestion rate agreed well with independent methods; digestion rates of *Synechococcus* were found to be relatively constant, unaffected by the physiological state of the flagellate (Dolan and Šimek 1998).

Here, we report on periodicity of grazing losses of *Synechococcus,* relative to its diel cycle, using food vacuole content of HFLAG in the Bay of Villefranche (NW Mediterranean). In the bay, *Synechococcus* is an abundant and important component of phytoplankton, estimated to be responsible for about 50% of the primary production during the summer months (Hagström et al. 1988). Previous studies, conducted during the early summer, reported distinct periodicity in *Synechococcus* cell division, with cells dividing in the early evening (Vaulot et al. 1996; Jacquet et al. 1998). We estimated population abundances, % of *Synechococcus* cells in division, and numbers of *Synechococcus* in HFLAG food vacuoles in Bay populations every 2–3 h over a 62-h period.

We estimated HFLAG grazing rates based on food vacuole content and a previously determined digestion rate constant (Dolan and Šimek 1998). Grazing losses due to HFLAG predation appear highest during hours when *Synechococcus* cells in division are least frequent. A possible explanation for the phenomenon observed, a ''size-refuge'' of dividing *Synechococcus,* is proposed.

The study site was Villefranche Bay, NW Mediterranean Sea (43°41′N, 7°19′E). From a point 75 m off the pier of the Station Zoologique, seawater was pumped from a depth of 2 m (site depth = 8 m) using a continuous flow pumping system. An industrial peristaltic pump (Delasco DSC 12, PVM Pompes) was connected to 100 m of aged polyvinyl chloride (PVC) tubing (20-mm ID) run along the seabed (depth = 1.5 to >8 m) to the sampling site. Water was drawn at a rate of 4 liters min^{-1}, yielding a transit time (bay to pump) of about 8 min. Water was collected from a 4-liter Nalgene bottle placed in-line before the pump; thus, water sampled had not passed through the pump. Water samples were taken every 2–3 h from 1200 h on 22 June 1998 to 0000 h on 25 June 1998. A total of 28 samples were taken.

Table 1. Summary statistics of measured and estimated parameters. *Synechococcus* (Syn) and other concentrations (Conc) are in 10^3 ml^{-1}, HFLAG food vacuole content is in units of *Synechococcus* per flagellate, HFLAG clearance rates are nl flagellate^{-1} h^{-1}, and HFLAG community grazing is in units of % *Synechococcus* standing stock (nondividing cells) removed hourly.

Parameter	Range	Average ± SD	Median	*n*
Total Syn Conc	16.7–37.4	26.6 ± 4.91	26.3	28
Nondividing Syn Conc	12.5–33.3	22.2 ± 4.68	21.8	28
% dividing Syn	9.7–29.6	16.8 ± 5.60	14.9	28
HFLAG Conc	0.6–1.9	1.1 ± 0.28	1.1	28
HFLAG fd vac: overall avg	0.03–0.34	0.16 ± 0.075	0.17	28
HFLAG fd vac: Syn FDC ≤ 15%	0.06–0.34	0.19 ± 0.065	0.19	14
HFLAG fd vac: Syn FDC ≥ 15%	0.03–0.32	0.13 ± 0.07	0.12	14
HFLAG clr rate: overall avg	0.6–10.6	4.2 ± 2.02	3.8	28
HFLAG clr: Syn FDC ≤ 15%	1.6–7.0	4.6 ± 1.56	4.8	14
HFLAG clr: Syn FDC ≥ 15%	0.6–10.6	3.6 ± 2.39	3.1	14
HFLAG clr: DAY	1.5–10.6	4.8 ± 2.02	4.8	19
HFLAG clr: NIGHT	0.6–4.3	2.7 ± 1.07	2.8	9
HFLAG clr: non-div Syn	0.8–11.8	4.9 ± 2.25	4.7	28
HFLAG community grazing	0.08–1.4	0.6 ± 0.37	0.5	28
AFLAG Conc	0.5–2.47	1.4 ± 0.59	1.3	28

One hundred-milliliter samples were preserved using calcium $CaCO_3$-buffered formalin (2% final conc); other fixatives were tested and found inferior (*see below*). Samples were kept refrigerated and in darkness; all slides were prepared within 2 weeks of sampling. Subsamples (10 and 20 ml) were filtered onto 0.2- or 0.8-μm–pore black polycarbonate filters for counts and examinations of *Synechococcus* and nanoflagellates, respectively. Slides were examined using a Zeiss Axiophot epifluorescence microscope equipped with blue and green filter sets. Using the green filter set, orange-red *Synechococcus* cells were counted under ×1,000 magnification via autofluorescence of phycobiliproteins for cell detection. A minimum of 400 single, nondividing *Synechococcus* cells were counted, and cells with a well-developed septum were recorded separately. Subsamples for flagellate counts were stained with DAPI; slides were prepared and kept frozen until examination within 6 weeks of sampling. A minimum of 100 flagellates, representing a sum of similarly abundant HFLAG and autotrophic nanoflagellates (AFLAG) were enumerated.

Separate slides were prepared for HFLAG food vacuole examinations and processed within 2 weeks of sampling. For each time-course sample, a minimum of 100 HFLAG cells were examined. DAPI-stained cells were located under blue-light excitation, HFLAG distinguished from AFLAG based on the absence of chloroplasts, and food vacuole content examined using the green filter set to detect *Synechococcus* inside HFLAG.

HFLAG food vacuole content was examined in formalin-fixed samples following tests of alternative fixatives: hexamine-buffered foramalin, cold gluteraldehyde, and Lugol's formalin decolorization technique (Sherr and Sherr 1993). In subsamples treated with different fixatives, 60–100 HFLAG were examined. The Lugol's decolorization technique did not yield reproducible results, as the Lugol's solution had a strong bleaching effect on *Synechococcus* autofluorescence. The highest cell content values were obtained with $CaCO_3$-buffered formalin (0.26 ± 0.54 *Synechococcus* flagellate^{-1}), followed by hexamine-buffered formalin (0.20 ± 0.48 *Synechococcus* flagellate^{-1}) and gluteraldehyde (0.16 ± 0.42 *Synechococcus* flagellate^{-1}).

Correlation analysis was used to examine the relationship between measured variables: population abundances, % of dividing *Synechococcus,* and HFLAG food vacuole contents. HFLAG food vacuole contents and calculated clearance rates, relative to the frequency of dividing *Synechococcus,* were examined using *t*-tests comparing the two sets of values found to occur above and below the median of % of dividing *Synechococcus.* This analysis is similar to, and gave the same results as, post hoc analysis of variance (ANOVA) tests. For each time point, ingestion rate estimates for HFLAG were made, assuming that ingestion and digestion were in steady state, by multiplying *Synechococcus* HFLAG^{-1} by a digestion rate constant of 1.1% min^{-1}. The digestion rate was determined in a previous study (Dolan and Šimek 1998), conducted with a mixture of flagellates and *Synechococcus* from the bay at a temperature close to those measured in this study (22°C). HFLAG clearance rates were calculated by dividing *Synechococcus* concentration, either total cells or nondividing cells, by estimated ingestion rates. Temporal changes in the community grazing of HFLAG on *Synechococcus* were examined by estimating aggregate quantities of *Synechococcus* removed by the HFLAG community relative to the stock available.

Throughout the study period, water temperature varied from about 22 to 24° C, with lowest temperatures in the early daylight hours (data not shown). Weather conditions were relatively constant and sunny, except for the last day, which was partly cloudy. Population abundances were in the range of the low 10^4 and 10^3 cells ml^{-1} for *Synechococcus* and flagellates, respectively; of the three populations examined, AFLAG were the most variable, followed by HFLAG, with *Synechococcus* displaying the least variability (Table 1). There were no clear diel patterns in population abundances, although AFLAG concentrations appeared somewhat higher in samples taken during daylight hours (Fig. 1). In contrast,

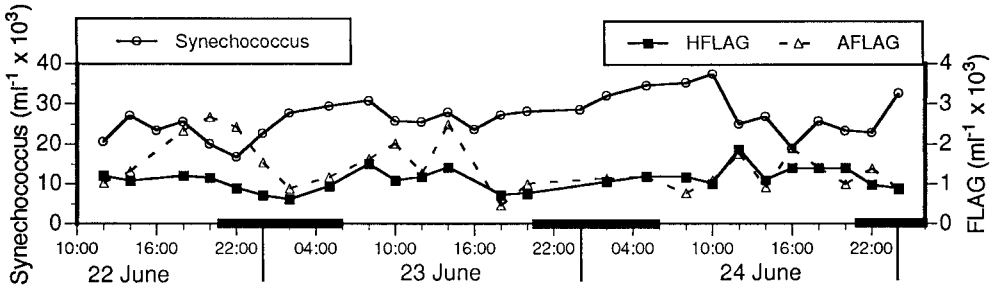

Fig. 1. Temporal changes in concentrations of HFLAG, AFLAG, and *Synechococcus* in the Bay of Villefranche. Dark bars denote nighttime hours.

there was a clear diel cycle in the frequencies of dividing *Synechococcus* cells (Fig. 2). Peaks of about 30% of the total *Synechococcus* population were found in samples taken at dusk with % in division decreasing to about 10% during daylight hours.

The majority (>90%) of the HFLAG cells were 3–5 μm in maximum dimension (fixed cells). There were no obvious morphotypes always containing or never containing *Synechococcus,* with the exception of a heterotrophic dinoflagellate (15-μm length) never found to contain *Synechococcus.* AFLAG with ingested *Synechococcus* (mixotrophs) were relatively rare (0–5% of AFLAG cells). Numbers of *Synechococcus* found inside HFLAG cells ranged from 0.03 to 0.34 *Synechococcus* HFLAG^{-1} (Fig. 2). The median value of frequency of dividing *Synechococcus* was about 15% (Table 1). Average cell contents of HFLAG (Table 1) were significantly greater in samples in which *Synechococcus* in division was ≤15% compared to samples in which *Synechococcus* in division was >15% (*t*-test, $P = 0.036$). Quantities of *Synechococcus* in HFLAG were lower in nighttime samples (0.11 ± 0.52 *Synechococcus* HFLAG^{-1} $n = 9$) compared to daylight samples (0.19 ± 0.72 *Synechococcus* HFLAG^{-1} $n = 19$) but were not significantly different (*t*-test, $P = 0.08$).

Correlation analysis of measured variables revealed few significant relationships. Population abundances were not related to one another, with the exception of *Synechococcus* and AFLAG concentrations (Table 2). HFLAG food vacuole contents were not related to either total *Synechococcus* numbers or concentrations of nondividing *Synechococcus.* However, HFLAG food vacuole contents were negatively related to frequencies of dividing *Synechococcus* (Table 2; Fig. 3).

Calculation of ingestion rates based on food vacuole contents yielded estimates ranging from 0.02 to 0.2 *Synechococcus* HFLAG^{-1} h^{-1}. Clearance rates (Table 1) calculated on the abundance of total *Synechococcus* cells, i.e., the sum of dividing and nondividing cells, were significantly ($P = 0.047$) higher in samples containing <15% dividing *Synechococcus* compared to samples with higher division frequencies and were higher in day compared to night samples ($P = 0.031$). However, differences in grazing rates were not apparent when only the nondividing population was considered as available prey. Clearance rates, calculated using the abundance of nondividing *Synechococcus* cells in the samples, showed no significant differences between night and day samples ($P = 0.130$) nor between samples with more or less than 15% dividing *Synechococcus* ($P = 0.578$). Thus, the apparent differences in clearance rates on the total *Synechococcus* population were linked to the abundance of dividing cells, as there were no significant changes in filtration rates calculated using the concentrations of nondividing cells.

Microbial abundances and the pattern of *Synechococcus* cell division conformed to expectations. The early summer concentrations of *Synechococcus* and HFLAG (Table 1) are typical for surface waters of oligotrophic marine systems (e.g. Davis et al. 1985) and similar to previous reports on Villefranche Bay populations concerning June and July abundances, whether based on microscopic enumerations (Ferrier-Pagés and Rassoulzadegan 1994) or for *Synechococcus* using flow cytometry (Vaulot et al. 1996; Jacquet et al. 1998). The pattern of *Synechococcus* cell division with a distinct dusk–early evening peak in % cells in division (Fig. 2), while perhaps not common to all *Synechococcus* populations (e.g., Carpenter and Campbell 1988), has been de-

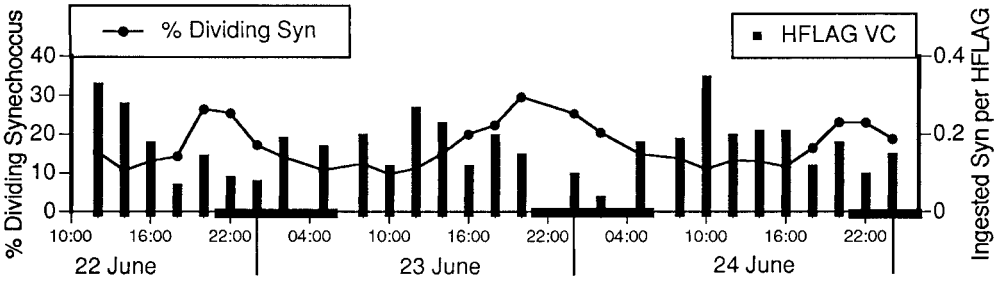

Fig. 2. Temporal changes in the % of dividing *Synechococcus* cells (% dividing Syn) and average numbers of *Synechococcus* found inside HFLAG food vacuoles (VC) in the Bay of Villefranche. Dark bars denote nighttime hours.

Table 2. Results of correlation analysis of measured variables.

Variable 1	Variable 2	Correlation coef	*P* value
% div Syn	HFLAG FV	−0.485	0.008
Tot Syn	HFLAG FV	+0.195	0.323
Nondiv Syn	HFLAG FV	+0.333	0.084
Tot Syn	HFLAG Conc	−0.810	0.703
Tot Syn	AFLAG Conc	−0.504	0.009
HFLAG Conc	AFLAG Conc	+0.359	0.074

scribed for Villefranche populations. Vaulot et al. (1996) found a similar pattern based on 10 time-course samples over a 30-h period in late June 1993, as did Jacquet et al. (1998) using samples obtained at 30-min intervals over 7 d in early July 1996.

Similarly, the overall grazing rates we estimated conformed with data from the few previous field studies of marine HFLAG predation on cyanobacteria. Feeding rates calculated from HFLAG food vacuole content and an experimentally determined digestion rate (Dolan and Šimek 1998), translated into terms of clearance rates (~1–11 nl HFLAG^{-1} h^{-1}, Table 1), agree well with rates derived using different methods and in different systems. For example, Caron et al. (1991), using size fractionation and metabolic inhibitors, estimated average community clearance rates of heterotrophic nanoplankton feeding on cyanobacteria to range from 5 to 17 nl h^{-1} cell^{-1} in Vineyard Sound, Massachusetts. Based on uptake of fluorescently labeled *Synechococcus* in estuarine mesocosms, Christoffersen (1994) reported clearance rates ranging from 0.5 to 27 nl heterotrophic nanoflagellate^{-1} h^{-1}. Rates of 2.5–9 nl flagellate^{-1} h^{-1} were calculated from dilution experiments by Landry et al. (1984) for HFLAG feeding on *Synechococcus* in Kaneohe Bay, Hawaii. It should be noted that these rates, lower than those often reported in laboratory studies, are averages for heterogeneous communities, likely containing forms that do not feed on cyanobacteria as well as perhaps specialized feeders.

While our estimates of grazing rate appear robust, they are subject to two sources of error: estimates of food vacuole content and error associated with estimating digestion rates. We evaluated different fixatives in an attempt to minimize fixation artifacts (*see Methods*), but we can not reject the possibility of having underestimated numbers of *Synechococcus* inside HFLAG. The number of HFLAG examined in a single sample (100) was dictated by the need to rapidly process samples using a time-consuming technique. We chose to process a large number of samples, allowing a longer temporal coverage, at the price of individual sample precision. It should be noted that no previous studies of protistan predators and their prey have been conducted with a similar intensity of sampling over a comparable period of time.

Grazing rates were calculated assuming a constant digestion rate of 1% cell contents h^{-1}. The rate was determined in an earlier study using a mixed assemblage of HFLAG from the Bay of Villefranche digesting *Synechococcus* at a temperature close to that encountered in our field study.

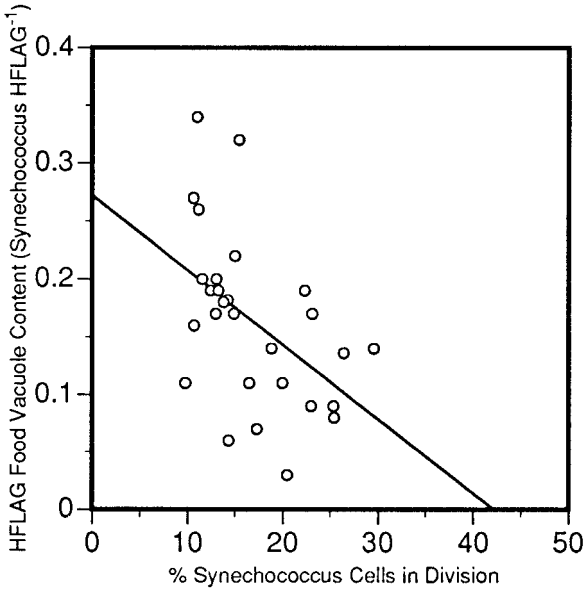

Fig. 3. Scatterplot of HFLAG food vacuole content and % *Synechococcus* cells in division; the two variables were negatively related ($P = 0.008$).

Hence, it would appear a reasonable rate to assume. We can not reject the possibility that digestion rates vary over short time scales. However, laboratory studies have shown that digestion of *Synechococcus* in at least one HFLAG, *Bodo saltans,* is the same in cells of different growth phases (Dolan and Šimek 1998). Recently, preliminary evidence was presented suggesting that digestion efficiency may be higher in daylight hours in heterotrophic dinoflagellates and ciliates (Strom 1998). If digestion rates in HFLAG are higher in daylight hours, the day/night differences in grazing rates we estimated would increase, as food vacuole content was higher in daylight hours.

Our finding that HFLAG grazing rates on nondividing *Synechococcus* did not change with time was difficult to predict. Existing data on patterns of HFLAG feeding on bacteria are ambiguous, and there is very little information on diel variability of HFLAG feeding on autotrophic picoplankton. Diel studies of predation on marine heterotrophic bacteria have shown that, while grazing pressure changes over a 36-h period, it may peak during the day or at night (Wikner et al. 1990). In a study that followed Villefranche Bay populations isolated in a carboy for 24 h, bacterivory was estimated four times; it was lowest in the morning and it peaked in the afternoon (Wikner et al. 1986).

With regard to predation on autotrophic prokaryotes, recent laboratory studies examining the effects of ultraviolet (UV)-A and UV-B radiation have led to the suggestion that HFLAG grazing in marine surface waters may be inhibited during the day (Ochs 1997; Ochs and Eddy 1998). However, data on natural populations are, to our knowledge, limited to a study in estuarine mesocosms that indicated higher grazing rates during the day. Christoffersen (1994) estimated HFLAG grazing in using uptake of fluorescently labeled *Synechococcus;* rates were estimated at 2–6-h intervals over 24 periods, and daytime clearance rates were about double

the nighttime rates (Christoffersen 1994). Our clearance rate estimates, when based on the abundance of both dividing and nondividing *Synechococcus,* were also about twice as high for day compared to night values (Table 1). However, we believe the difference is because, in Villefranche Bay, dividing cells are more abundant at night, and HFLAG appear not to graze, or to graze at low rates, dividing *Synechococcus* cells.

Food vacuole contents varied inversely with % dividing Synechococcus (Fig. 3). Clearance rates calculated on total *Synechococcus* were lower in samples with <15% dividing *Synechococcus,* regardless of time. In contrast, clearance calculated on abundance of nondividing *Synechococcus* was not significantly different in day and samples or with regard to more or less than the median value of dividing *Synechococcus.* Hence, finding significant differences depended on including quantities of dividing *Synechococcus.*

The absolute quantities of *Synechococcus* found in HFLAG food vacuoles likely reflect to some extent the availability of other prey items such as picoeukaryotes and heterotrophic bacteria, which were not visible in food vacuoles. However, the pattern of *Synechococcus* in food vacuoles varying inversely with % dividing *Synechococcus* (Fig. 2) was clear and may reflect cyclical changes in the vulnerability of *Synechococcus* to predation related to the average cell size of *Synechococcus.* We have no data to directly examine this hypothesis, but the data of Jacquet et al. (1998) on cyclical changes in relative size of Villefranche populations lend support to the idea. From flow cytometry analysis, clear cycles in right-angle light-scatter measurements (a relative measure of size) were apparent, with minima of average cell size at dawn and maxima in the early evening of July 1996. Our data show maximum food vacuole content in the morning and minima at night.

A hypothesis of higher predation rates with lower average cell size for *Synechococcus* is consistent with data from studies on size selection of prey HFLAG, as *Synechococcus* cells are near the upper limit of prey size, which is efficiently cleared by most HFLAG. When presented with prey of bacteria of different sizes, HFLAG are known to show exponential increases in clearance rates with the size of bacterial prey (e.g., Gonzalez 1996). Interestingly, the size of the HFLAG appears independent of the size of bacterial prey ingested, with both small and large flagellates preferentially ingesting the same large bacteria (e.g. Gonzalez et al. 1990). However, apparent selection does not extend to very large bacteria (e.g., Chrzanowski and Šimek 1990; Šimek and Chrzanowski 1992). HFLAG clearance rates decrease abruptly when prey volumes exceed 0.6–1.2 μm^3, depending on the species (Jürgens and Gûde 1994). We did not attempt to measure average *Synechococcus* cell volumes in this study, but rough measurements indicate a cell volume for nondividing *Synechococcus* of about 1 μm^3 and for dividing cells, 1.5–2 μm^3. Dividing *Synechococcus* cells may be too large for many HFLAG to ingest, and the HFLAG population in our study, dominated by cells 3–5 μm in diameter, does not appear unusual (Sherr and Sherr 1991).

Studies of HFLAG feeding on heterotrophic bacteria have shown that dividing bacteria (considerably smaller than dividing *Synechococcus*) are removed at higher rates than nondividers (Sherr et al. 1992). These studies have led to the observation that HFLAG were removing the production more than the standing stock, as the larger, producing cells are removed preferentially (Sherr et al. 1992). For *Synechococcus,* dividing cells may enter a size-refuge (Jürgens and Gude 1994) with regard to HFLAG, which may preferentially remove smaller newly produced cells. Thus, HFLAG feeding on *Synechococcus* may be characterized as removing the production rather than the producers.

Although HFLAG are generally assumed to be the primary consumers of picoplankton production (Wiesse 1993), our HFLAG clearance rates multiplied by HFLAG concentrations provide a rough estimate of about 14% the nondividing *Synechococcus* population removed per day (Table 1). Our clearance rates may be underestimates, but they appear to be of the correct order of magnitude. It may be of interest to compare HFLAG consumption rates with *Synechococcus* production rates.

We did not attempt to estimate *Synechococcus* division rates using % dividing cells because this approach involves a number of assumptions and uncertainties (e.g., Vaulot 1992). However, the similarity in peaks of dividing *Synechococcus* we detected corresponded in timing and magnitude with previous reports of peaks in phase G2 *Synechococcus* cells, which gave estimates of about one doubling per day (Vaulot et al. 1996; Jacquet et al. 1998). While peaks in % dividing cells need not correspond with % cells in G2, it would appear reasonable to assume that during our study period, *Synechococcus* was probably dividing about once a day. Thus, given our estimates of HFLAG consumption rates, it appears that most *Synechococcus* production is either consumed by organisms other than HFLAG or exported. We found few ingested *Synechococcus* among AFLAG. A large fraction of *Synechococcus* production could be available for viral lysis (e.g., Suttle et al. 1990) or trophic levels higher than that of HFLAG. In marine systems, other potentially significant consumers of cyanobacteria include mucous net feeders such as appendicularians and ciliate microzooplankton. As neither the *Synechococcus* nor the HFLAG populations appeared unusual in terms of abundances and activities, our results suggest that the role of HFLAG as the dominant consumers of *Synechococcus* populations in marine systems may require revision.

John R. Dolan

Marine Microbial Ecology Group
CNRS ESA 7076
Station Zoologique, B.P. 28
F-06230 Villefranche-Sur-Mer, France

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Karel Šimek

Hydrobiological Institute
Czech Academy of Sciences
Ná sadkách 7
CZ-37005 České Budějovice, Czech Republic

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