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# Strong, weak, and missing links in a microbial community of the N.W. Mediterranean Sea

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## Abstract

Planktonic microbial communities often appear stable over periods of days and thus tight links are assumed to exist between different functional groups (i.e. producers and consumers). We examined these links by characterizing short-term temporal correspondences in the concentrations and activities of microbial groups sampled from 1 m depth, at a coastal site of the N.W. Mediterranean Sea, in September 2001 every 3 h for 3 days. We estimated the abundance and activity rates of the autotrophic prokaryote *Synechococcus*, heterotrophic bacteria, viruses, heterotrophic nanoflagellates, as well as dissolved organic carbon concentrations. We found that *Synechococcus*, heterotrophic bacteria, and viruses displayed distinct patterns. *Synechococcus* abundance was greatest at midnight and lowest at 21:00 and showed the common pattern of an early evening maximum in dividing cells. In contrast, viral concentrations were minimal at midnight and maximal at 18:00. Viral infection of heterotrophic bacteria was rare (0.5–2.5%) and appeared to peak at 03:00. Heterotrophic bacteria, as % eubacteria-positive cells, peaked at midday, appearing loosely related to relative changes in dissolved organic carbon concentration. Bacterial production as assessed by leucine incorporation showed no consistent temporal pattern but could be related to shifts in the grazing rates of heterotrophic nanoflagellates and viral infection rates. Estimates of virus-induced mortality of heterotrophic bacteria, based on infection frequencies, were only about 10% of cell production. Overall, the dynamics of viruses appeared more closely related to *Synechococcus* than to heterotrophic bacteria. Thus, we found weak links between dissolved organic carbon concentration, or grazing, and bacterial activity, a possibly strong link between *Synechococcus* and viruses, and a missing link between light and viruses. © 2002 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

## 1. Introduction

Planktonic microbial communities, despite high physiological rates, appear stable on a time scale of days, showing little change in either concentrations or composition. For example, in the open ocean, populations of the photosynthetic prokaryotes *Synechococcus* and *Prochlorococcus* divide about once every 24 h but concentrations change little from day to day [1,2]. Similarly, the community composition of heterotrophic bacteria, as determined using oligonucleotide probes, has been shown to be nearly invariant over a period of days [3]. Thus, production and

mortality generally appear balanced, despite the fact that these terms probably vary over a 24 h period with a factor as obvious as light. For example, light is likely to influence the cell production of heterotrophic bacteria by governing cycles of resources such as the photosynthetic production of dissolved organic carbon (DOC). Conversely, light could be expected to influence bacterial mortality directly through DNA damage [4,5], or indirectly as it is the dominant mechanism of viral destruction or inactivation [6–10]. With regard to bacterial grazers, laboratory studies examining the effects of UV A and UV B radiation on heterotrophic nanoflagellates (HFLAG) have led to the suggestion that grazing in marine surface waters may be inhibited during the day [11,12] while other studies have concluded that light may facilitate digestion, allowing higher grazing of chlorophyll-containing prey such as *Synechococcus* or *Prochlorococcus* [13].

Data on the short-term variability of population con-

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centrations and physiological rates exist, but at least with regard to bulk rates of bacterial production and loss from grazers no consistent patterns emerge. For example, among studies conducted over the past decade, bacterial production reportedly peaks in the early evening [14], or late night, or early morning [15–17], or during midday [18–20], or varies little [21,22] or irregularly [23–26]. As to the variability of fluorescence in situ hybridization (FISH) detection rates (eubacteria and/or archaeobacteria), very little data are available. However, it has been suggested that a great deal of seasonal variability may exist which reflects seasonal changes in cell-specific activity rates [27].

Diel changes in grazing rates on heterotrophic bacteria have been reported and rates found to be significantly higher at night or during the day [28–30]. Parameters directly influenced by light, such as indicators of DNA damage or repair patterns appear more coherent [4,5]. Interestingly, the small number of studies concerning viral abundance suggests that although sunlight is thought to be a major loss mechanism for viruses [7–9,19], in natural systems viral concentrations vary little or irregularly [31–34].

Few or no common patterns may exist because of differences between systems in characteristics as water transparency and the relative importance of allochthonous versus autochthonous carbon. However, even in a given type of system, most studies have examined very few parameters (typically heterotrophic bacterial production and loss to grazers) and have either been limited to a single day/night cycle or based on two to four samplings per 24 h. Thus, in reality, the paradigm of microbial communities as a set of tightly linked populations may be based as much on intuition as evidence.

To examine the nature of the links within planktonic microbial communities, we considered the community of an oligotrophic marine system in which at least one component is characterized by rapid and cyclical growth – the autotrophic prokaryote *Synechococcus*. In the Bay of Villefranche it displays the well-known cycle of early evening cell division but near-constant cell concentration [35,36].

We examined short-term temporal correspondences of abundance and activities of distinct microbial groups in the Bay of Villefranche in September 2001 by sampling every 3 h for 3 days at 1 m depth. We estimated abundance and activity rates of *Synechococcus*, heterotrophic bacteria, viruses and HFLAG. For *Synechococcus*, the frequency of dividing cells was estimated. The metabolic activity of heterotrophic bacteria was quantified in two ways: by the rate of leucine incorporation and by the proportion of cells detectable with FISH using eubacterial or archaeobacterial oligonucleotide probes. Leucine incorporation has been used as a common method of estimating cell production rates [37]. FISH detection is thought to be limited largely by cellular ribosomal RNA content, in

turn usually linked to cellular activity [38]. Viral activity was assessed as the frequency of visibly infected heterotrophic bacteria. Grazing activity of HFLAG on heterotrophic bacteria was estimated as clearance rates measured by the uptake of fluorescently labeled bacteria. We also monitored DOC concentrations, a possible motor driving changes in activities of heterotrophic bacteria.

We hypothesized that, against a background of *Synechococcus* cycles, we would find cycles of production and mortality in heterotrophic bacteria. We expected grazing rates of HFLAG on heterotrophic bacteria to show a diurnal cycle as dividing *Synechococcus* cells became too large to ingest [36]. We expected viral concentrations and infection rates to show a diurnal cycle due to sunlight-induced viral destruction.

We anticipated a tight link between bacterial production and mortality. In the Bay of Villefranche, from summer through autumn, production of heterotrophic bacteria appears to be phosphorus-limited [39]. Short-term variability in bacterial cell production has been linked to nM changes in phosphate concentrations [19]. As grazers of picoplankton are efficient recyclers of phosphorus [40], and exploitation should release organic phosphorus, we expected variability in production or nutritional status of heterotrophic bacteria to follow shifts in total bacterial mortality from grazing or viral lysis.

To our knowledge, no previous study has examined temporal variability of all the components of the microbial loop (autotrophic and heterotrophic bacteria, viruses, HFLAG and DOC) nor employed a sampling regime as comprehensive as at 3 h intervals over 3 days in a natural system.

## 2. Materials and methods

### 2.1. Study site and sampling

The study site was located in Villefranche Bay, Northwestern Mediterranean Sea (43°41'N, 7°19'E). From a point 50 m off the pier of the Station Zoologique, seawater was collected from a depth of 1 m (site depth = 4 m) by filling a 10 l carboy. Water samples were taken every 3 h from 12:00 a.m. September 12, 2001 to 12:00 a.m. September 15, yielding a total of 25 samples. Water temperature varied between 24 and 22°C, and the photoperiod was approximately 12 h.

### 2.2. Bacterial abundance and production

Subsamples were fixed with formaldehyde (2% final concentration); 10 ml aliquots were stained with 4,6-diamino-2-phenylindole (DAPI; final concentration 0.2% wt/v), drawn down on 0.2 µm filters and examined by epifluorescence microscopy (Olympus BX-60). Between 400 and 600 bacteria were counted; thus the 95% confidence limit

for a given estimate is about  $\pm 5\%$ . Heterotrophic bacterial production was estimated from the rate of protein synthesis as determined by the incorporation of  $^3\text{H}$ -leucine into trichloroacetic acid (TCA)-insoluble macromolecular material [37]. For each sampling time, three 10 ml replicates were spiked with 22 nM of leucine and three others were spiked with 42 nM. Each replicate received 2 nM of  $^3\text{H}$ -leucine (specific activity: 51 Ci mmol $^{-1}$ ) and 20 or 40 nM of unlabeled leucine. For each concentration, one of the replicates to which formalin had been added (2% final concentration) served as a control. Samples were incubated in the dark at in situ temperatures for 2 h. We confirmed that leucine incorporation was linear during this period. The live incubations were terminated with formalin and all samples were filtered onto 0.2  $\mu\text{m}$ , 25 mm diameter nitrocellulose filters. Samples were then extracted with 5% TCA for 10 min followed by five 3 ml rinses with 5% TCA. The filters were placed in scintillation vials and 20 ml of Filter Count<sup>®</sup> scintillation cocktail (Packard) was added. Radioactivity was counted with a scintillation counter with counting efficiency corrected for quenching. Results were expressed as pM leucine incorporated l $^{-1}$  h $^{-1}$ . Leucine incorporation rates were translated into incorporation per cell using total heterotrophic bacteria cell concentrations and into bacterial cells produced per hour using the conversion factors of 1 pM leucine = 1.5 ng carbon and 20 fg carbon per bacterial cell. Statistical relationships of leucine incorporation were examined using both bulk and per-cell uptake rates. Relationships were very similar and here only the rates per cell are reported.

### 2.3. Fluorescent in situ hybridization

We employed FISH as a metric of nutritional status as FISH detection rates depend on RNA quantity, which reflects nutritional state and history [41,42]. The proportion of cells with a quantity of rRNA sufficient to allow detection with FISH probes as either eubacteria or archaeobacteria was determined for 15 of the 25 samples. We employed the standard protocol of in situ hybridization with fluorescent oligonucleotide probes on membrane filters [43,44]. For details of the hybridization procedure and error estimates see [45]. Briefly, bacterial cells from 10–20 ml subsamples were concentrated on white 0.2  $\mu\text{m}$  filters (47 mm diameter; Poretics), then fixed on membrane filters by overlaying with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) and stored at  $-20^\circ\text{C}$  [43]. Two oligonucleotide probes (MWG Biotech, Germany) were used: EUB338 for Bacteria [47], and ARCH915 for Archaea [48]. The probes were fluorescently labeled with the indocarbocyanine dye Cy3 (BDS, Pittsburgh, PA, USA). After hybridization, filter sections were stained with DAPI and the percentage of hybridized bacterial cells counted by epifluorescence microscopy. A minimum of 400 cells were examined, thus a counting error of about  $\pm 5\%$  could be expected.

### 2.4. *Synechococcus* abundance and frequency of dividing cells

Subsamples of formaldehyde-fixed water (10 ml) were filtered onto 0.2  $\mu\text{m}$  black polycarbonate filters to estimate the concentration and the frequency of dividing cells (FDC) of *Synechococcus*. 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 1000 $\times$  magnification using the autofluorescence of phycobiliproteins for cell detection. A minimum of 400 single, non-dividing *Synechococcus* cells were counted and cells with a well-developed septum were recorded separately. Counting error could be expected to be about  $\pm 5\%$ .

### 2.5. Heterotrophic nanoflagellate abundance and grazing rate

Grazing on bacterioplankton was estimated using fluorescently labeled bacterioplankton (FLB, [49]), concentrated from Rimrov reservoir water according to [50]. Bacterioplankton were starved for 3 weeks prior to staining to reduce cell volumes to 0.05–0.09  $\mu\text{m}^3$ . FLB uptake experiments were run for each sampling. 100 ml samples were dispensed into 250 ml flasks and FLB added to yield a final concentration of  $2 \times 10^5$  ml $^{-1}$  (about 20% of bacterial natural abundance) and incubated at in situ temperature for 30 min. 25 ml subsamples for protozoan enumeration and tracer ingestion determinations were taken and fixed by adding 0.5% of alkaline Lugol's solution, immediately followed by 2% borate-buffered formalin (final concentrations) and several drops of 3% sodium thiosulfate to clear the Lugol's color [49]. 20 ml subsamples were stained with DAPI, filtered through 1  $\mu\text{m}$  black filters (Poretics), and inspected by epifluorescence microscopy. Non-pigmented, HFLAG and plastidic flagellates were differentiated. 50–100 HFLAG were inspected for FLB ingestion in each sample. An average clearance rate was calculated for each sample by dividing the average number of FLB ingested by the concentration of FLB. Given a Poisson distribution of FLB inside the population, we estimate the 95% confidence limit of individual heterotrophic nanoflagellate ingestion rate estimates to be  $\pm 20\%$ . To estimate total grazing, we multiplied the uptake rate of HFLAG by their in situ abundance.

### 2.6. Dissolved organic carbon measurements

For the DOC measurements, 10 ml aliquots of filtered (GF/F, Whatman) sample were collected in pre-combusted glass ampoules. The sample was then acidified with 85%  $\text{H}_3\text{PO}_4$  to a pH  $< 1$  and the ampoule flame-sealed. The samples were stored at  $4^\circ\text{C}$  in the dark until analysis. DOC concentrations were measured with a Shimadzu TOC-5000 total organic carbon analyzer (see [26], for ex-

ample) and certified reference materials (D.A. Hansell, University of Miami) were used to calculate the machine blank and to assess the performance of the machine on the measurement days.

### 2.7. Viral concentrations

Virus-like particles were counted by epifluorescence microscopy using the fluorochrome Yo-Pro [51] and a modification of the Hennes and Suttle method [52] that produces reliable counts of free viruses in aquatic ecosystems [53]. The filters were transferred to glass slides, covered with single drops of a solution of 50% glycerol, 50% PBS (0.05 M Na<sub>2</sub>HPO<sub>4</sub>, 0.85% NaCl, pH 7.5), and 0.1% *p*-phenylenediamine (made fresh daily from a frozen 10% aqueous stock solution; Sigma) on 25 mm square coverslips. This mountant minimizes fading [7]. All working solutions (i.e. stain, double-distilled water, mountant, fixatives) were filter-sterilized immediately before use, using Anotop 10 units (Whatman) equipped with 0.02 µm inorganic membranes and sterile syringes. In addition, a blank was routinely examined to control for contamination of the equipment and reagents. The virus-like particles were counted using an Olympus HB2 microscope equipped with a 100/1.25 Neofluar objective lens and a wide blue filter set. The size, the distinctive shape and very much brighter fluorescence of bacteria clearly distinguished these particles from viruses. Triplicate counts of subsamples yielded standard deviations of < 5%.

### 2.8. Estimating frequencies of virus-infected bacteria and subsequent mortality

In formalin-fixed samples, the bacteria contained within 8 ml subsamples were harvested by ultracentrifugation onto grids (400 mesh NI electron microscope grids with carbon-coated Formvar film) using a Centrikon TST 41.14 swing-out rotor at 70 000×*g* for 20 min at 4°C [54,55]. Each grid was then stained for 30 s with uranyl acetate (2% wt/wt) and examined in a JEOL 1200EX transmission electron microscope (TEM) operated at 80 kV at a magnification of ×40 000. Because of the high acceleration voltage, we were able to identify bacterial cells containing mature phages. A cell was considered infected when the phages inside could be clearly recognized by their shape and size. At least 300 bacterial cells were inspected per sample to determine an infection rate or frequency of visibly infected cells. Due to equipment problems, only samples from the first 15 time points were processed.

To estimate the impact of viruses on bacterial mortality, the frequency of visibly infected cells (FVIC, as a percentage) was related to the frequency of infected cells (FIC) and virus-induced bacterial mortality (VIM, as a percentage per generation), first using the relationship relating

FVIC to FIC presented by Weinbauer et al. [56]:

$$\text{FIC} = (9.524 \times \text{FVIC}) - 3.256$$

Then the relationship relating FIC to VIM formulated by Binder [57]:

$$\text{VIM} = (\text{FIC} + 0.6 \text{FIC}^2) / (1 - 1.2 \text{FIC})$$

The relationship is based in part upon the determination of the fraction of the latent period that elapses before the appearance of intracellular virus particles [58]. The VIM figure was translated into an hourly cell loss rate by multiplying VIM by hourly cell production based on leucine incorporation.

### 2.9. Data analysis

Data were standardized, relative to 24 h averages, in the following manner. For each variable, a 24 h average was calculated for each of the three sampled 24 h periods (e.g. 12:00 Sept. 12 to 12:00 Sept. 13). Each data point was then expressed as a percentage of the corresponding 24 h average value. The variables, as percentages, were normalized using square root arcsine transformation. The standardized, normalized variables were examined using a one-way analysis of variance (ANOVA) to test for effects of time of day as a 'treatment' and correlation analysis to examine correspondence between variables. We also employed non-parametric Spearman rank correlation analysis to test for simple correspondence between variables using untransformed data.

## 3. Results

### 3.1. Temporal patterns

Temporal changes in concentrations are shown in Fig. 1. Over the 3 day period, bulk concentrations of all the organisms considered varied by about ± 50%. In contrast, DOC concentrations varied within a relatively narrow range of 92–115 µM. It should be noted that 'dissolved' carbon included any organic matter that passed through the (GF/F) filters. Regular oscillations were apparent only with regard to the concentrations of *Synechococcus* and viruses; *Synechococcus* was maximal from 0:00 to 3:00 and viruses from 15:00 to 18:00. Thus, the cycle of *Synechococcus* was as expected. However, if light is a significant loss factor for viruses, viral abundance varied in an unexpected manner of lower concentrations at night.

Most rates (Fig. 2) varied more than concentrations. Leucine incorporation rates varied between about 50 and 250 fM leucine cell<sup>-1</sup> h<sup>-1</sup> × 10<sup>-6</sup>. The portion of DAPI-counted cells detectable with the eubacteria probe varied between 45 and 65%; archaeobacteria were found at the limit of detection (< 2%? DAPI cells). Interestingly, the shifts in proportions of bacterial cells detectable with the

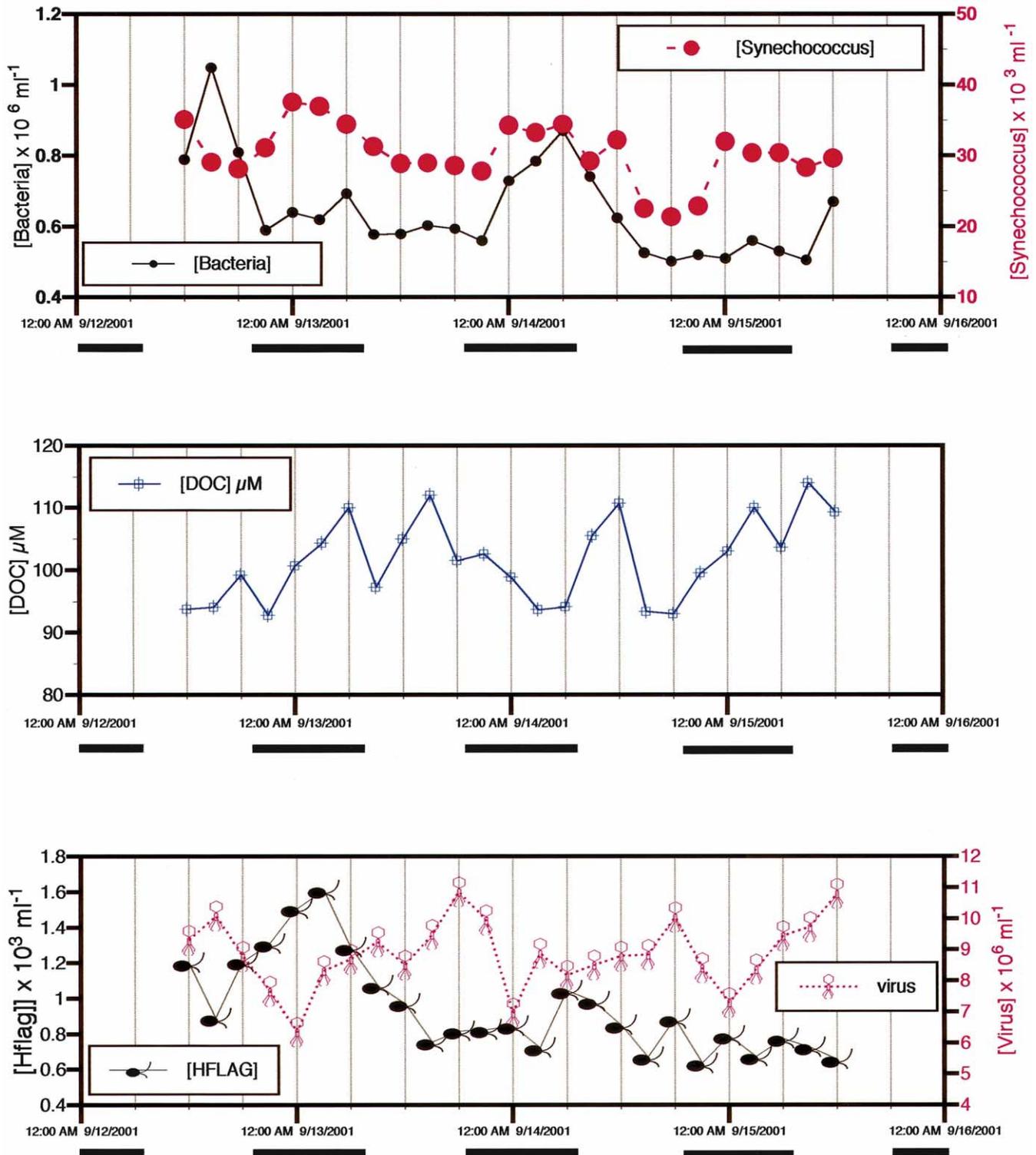
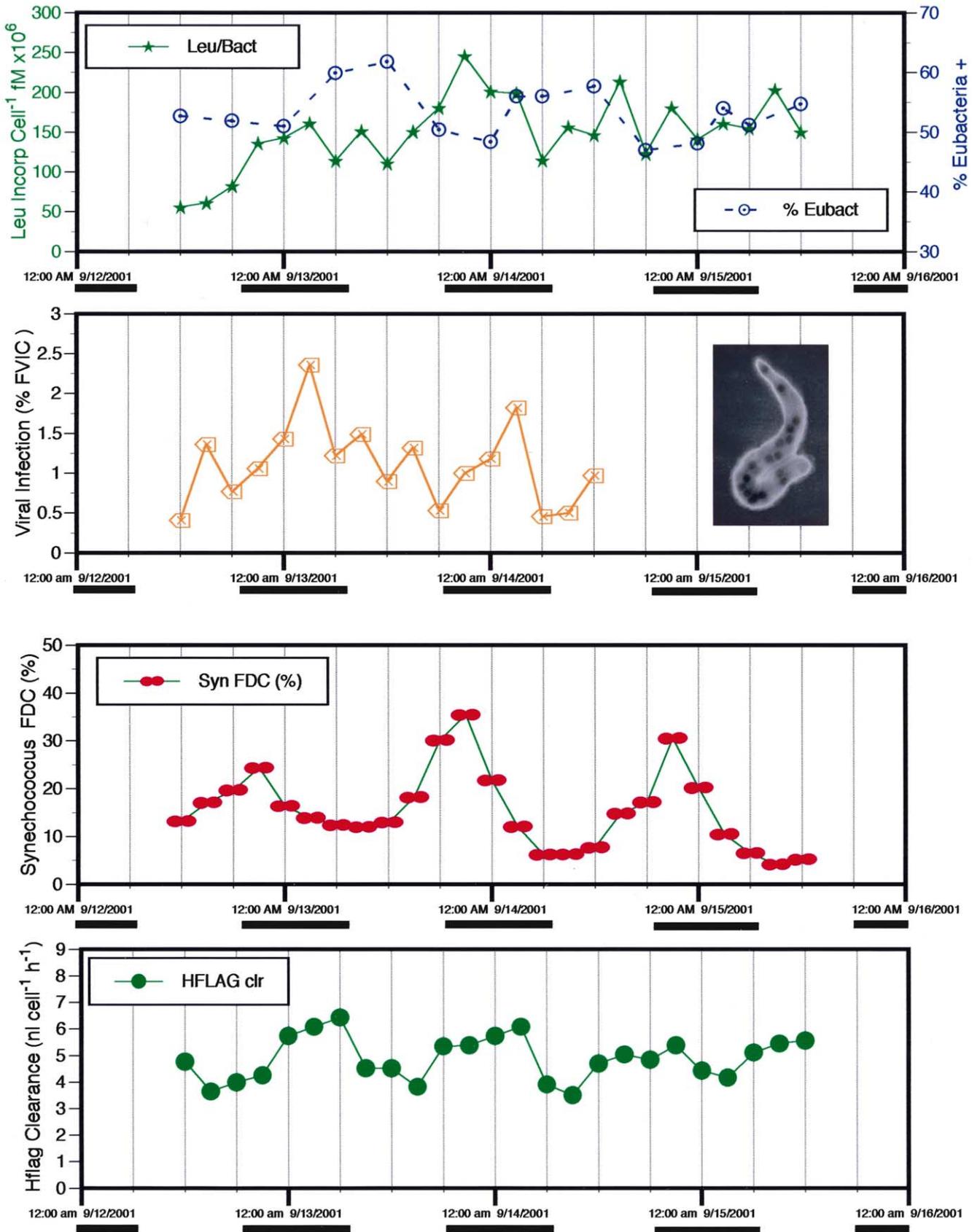


Fig. 1. Temporal changes in concentrations of organisms (HFLAG = heterotrophic nanoflagellates) and dissolved organic carbon (DOC) in the Bay of Villefranche, September 12–15, based on samples from 1 m depth.

eubacteria probe appeared unrelated to the considerable variations in leucine incorporation rates. Regular oscillations were not evident, but within a given 24 h period a maximum value was recorded from the midday samples. Our estimates of the clearance rates of HFLAG, overall, fell largely between 4 and 6  $\text{nl cell}^{-1} \text{h}^{-1}$ .

Rates of viral infection ranged from roughly 0.5 to 2.5% of the stock of heterotrophic bacteria. Based on data from two of the three cycles, peak infection rates occurred at 03:00. Recalling that virus abundance peaked at about 18:00 (Fig. 1), the time between virus contact and the occurrence of TEM-detectable infection would then



appear to be either 9 h or a multiple of (9+24) h. However, the patterns must be interpreted with caution as the number of infected cells detected was insufficient to reliably distinguish minima and maxima of infection.

For *Synechococcus*, the expected pattern of cell division in the early evening was evident from the FDC data. Rough calculations of *Synechococcus* cell production from the FDC data correspond well with the night-time increases in cell concentrations. The 35% of cells in division out of a total of 30 000 cells ml<sup>-1</sup> does about yield the increase of 10'000 cells ml<sup>-1</sup> detected from 18:00 to 24:00 (shown in Fig. 1).

The results of the ANOVA analysis as a test for an effect of 'hour of day' on the relative magnitude of the parameters are given in Table 1. These, in general, provided statistical confirmation of patterns evident from casual inspection of Figs. 1 and 2. There was a significant effect of 'time of day' for estimates of: (a) concentrations of *Synechococcus*, (b) concentrations of viruses, (c) *Synechococcus* FDC, (d) viral infection rates, and (e) the proportion of DAPI counts detectable with the eubacteria probe.

### 3.2. Relationships between variables

Few strong relationships between microbial parameters were apparent, whether as temporal correspondences (values transformed into percentage of the 24 h average) or as absolute changes in magnitude. The Spearman rank correlation of simple correspondences of absolute magnitudes (Table 2) showed the strongest relationships to be a positive relation between specific leucine incorporation rates and HFLAG concentrations and a negative relation of the concentrations of viruses and *Synechococcus*. Concentrations of bacteria and *Synechococcus* were positively related while proportions of bacteria detectable with the eubacteria probe were negatively related to *Synechococcus* FDC.

Correspondences of temporal shifts, examined using correlation analysis of time-averaged parameters, revealed a larger number of significant relationships (Table 3). The proportion of bacteria detected with the eubacteria probe was again negatively related to *Synechococcus* FDC but was also positively related to DOC concentrations. In contrast, leucine incorporation rates, while not related to DOC, showed a negative relationship with bacterial concentrations and a positive relationship with HFLAG clearance rates. Interestingly, viral infection rates were positively related to leucine incorporation rates and con-

centrations of *Synechococcus*, but not proportions of eubacteria probe-positive cells.

### 3.3. Production and mortality

For heterotrophic bacteria, production and loss rates were of the same order of magnitude of 10<sup>3</sup> cells ml<sup>-1</sup> h<sup>-1</sup>, with estimates of bacterial cell production, overall, greater than estimates of cell loss. Flagellate- rather than virus-induced mortality dominated bacterial cell loss, based on our estimates of HFLAG clearance rates and abundance and mortality from viral lysis (Fig. 3). Mortality from viruses, based on infection frequencies, appeared to reach peak values between 24:00 and 06:00. Our highest estimate of viral mortality was about 25% of cell production. However, it should be recalled that all of our estimates involve unverified conversion factors and/or assumptions.

For *Synechococcus*, FDC data and oscillations of cell concentrations yielded production and mortality estimates of about 33% of the stock per day. Based on our estimates of HFLAG concentrations and clearance rates, flagellates likely consume only about 15% of the stock per day. The remaining mortality may be due to viral lysis because not only is a source of *Synechococcus* mortality missing, but so too is a source of virus production.

Consideration of the magnitude of the oscillations in viral concentrations and the quantities of virus-infected heterotrophic bacteria leads to the conclusion that there is likely to be a source of virus other than heterotrophic bacteria. The oscillations suggest a viral production rate of at least 3 × 10<sup>6</sup> viruses per day (Fig. 1). The peak FVIC data translate, using common factors (e.g. [56]), into an absolute infection rate of about 15%. We estimated a heterotrophic bacterial cell production rate of about 1.2 × 10<sup>5</sup> cells per day. To obtain a viral production rate of 3 × 10<sup>6</sup> per day from 15% of 1.2 × 10<sup>5</sup> bacteria per day would require burst sizes of over 150 particles cell<sup>-1</sup> compared to common figures of < 50 phages cell<sup>-1</sup> [56]. There then appears to be an excess production of viruses of about 2 × 10<sup>6</sup> ml<sup>-1</sup> per day. We can provide an estimate of *Synechococcus* mortality not due to HFLAG grazing as total *Synechococcus* production minus HFLAG grazing, calculated as total community HFLAG clearance. The resulting estimate is 15 000 *Synechococcus* ml<sup>-1</sup> per day. A mortality rate of 15 000 *Synechococcus* ml<sup>-1</sup> per day to viral lysis could produce 2 × 10<sup>6</sup> viruses per day given a burst size of 133 viruses per *Synechococcus*. Furthermore, the negative correlation between viral and *Synechococcus* concentra-

Fig. 2. Temporal changes in activities of microorganisms in the Bay of Villefranche, September 12–15, based on samples from 1 m depth. For heterotrophic bacteria, the top panel shows <sup>3</sup>H-leucine incorporation rate per cell and the percentage of DAPI-stained bacteria detected by FISH with the eubacteria probe EUB338. Below is shown the frequency of cells found to be visibly infected with viruses by examining whole cells using TEM; the inset photo shows a *Vibrio*-like bacterium containing about 20 phage particles. *Synechococcus* (Syn) growth variability was estimated as the frequency of dividing cells (FDC) and the grazing activity of HFLAG as clearance rates (HFLAG Clr).

Table 1  
Results of ANOVA analysis used to test for differences with the hour of day

Variable	Hour DF	Residual DF	F-value	P-value
[Bact]	7	17	0.73	0.650
<b>[Syn]</b>	<b>7</b>	<b>17</b>	<b>10.06</b>	< <b>0.001</b>
[DOC]	7	17	0.49	0.826
<b>[Virus]</b>	<b>7</b>	<b>17</b>	<b>4.42</b>	<b>0.006</b>
[HFLAG]	7	17	1.12	0.397
<b>Syn FDC</b>	<b>7</b>	<b>17</b>	<b>13.17</b>	< <b>0.0001</b>
Leu cell <sup>-1</sup>	7	17	1.89	0.135
<b>% Eubact+</b>	<b>4</b>	<b>10</b>	<b>6.13</b>	<b>0.009</b>
<b>FVIC</b>	<b>7</b>	<b>9</b>	<b>4.17</b>	<b>0.025</b>
HFLAG Clr	7	17	0.77	0.619

All estimates were percentages of the appropriate integrated 24 h average value, transformed for normalization. Concentrations of bacteria, *Synechococcus*, dissolved organic carbon, viruses, HFLAG: [Bact], [Syn], [DOC], [Virus], [HFLAG]; rates of leucine incorporation per bacterial cell: Leu cell<sup>-1</sup>; percent of DAPI bacterial counts as positive with the eubacteria probe: % Eubact+; frequency of visibly infected bacterial cells: FVIC; clearance rates of heterotrophic nanoflagellates on bacteria: HFLAG Clr. Parameters in bold showed a significant variation with the time of day.

tions (Tables 2 and 3) suggests that *Synechococcus* cells might act as a sink for viral particles.

#### 4. Discussion

The data gathered over 3 days indicated that many of the microbial parameters estimated varied significantly and regularly with time (Figs. 1 and 2, Table 1). As previously described for the Bay of Villefranche [35,36], we found cyclical changes in *Synechococcus* FDC and concentration, but a stable population over a period of days. We found a distinct rhythm in viral concentrations and infection rates among heterotrophic bacteria but, unexpectedly, viruses showed peak abundance at midday. Our data also suggested that for heterotrophic bacteria, although bacterial concentrations and leucine incorporation varied irregularly, the proportion of cells detectable using the eubacteria probe showed a midday peak.

It is tempting to attribute the shifts in the proportions of probe-positive cells to shifts in DOC concentrations as a correlation was found (Table 3). The magnitude of DOC change (20 µM) is more than an order of magnitude greater than rough estimates of carbon fixation or respiration based on changes in cell concentrations, so the mechanism

driving shifts in DOC is likely to be exterior to the planktonic food web examined. Benthic input cannot be excluded as our sampling site was in relatively shallow waters. However, in a previous study at the same site [35], water movement through the sampling site was postulated to occur with a period of about 17 h, corresponding to the inertial frequency at 43°N and the period of 17 h roughly agrees with DOC shifts. In addition, the shifts in FISH detection rates must be interpreted with caution as the source of variability in these rates is unclear. The FISH probe indicates nutritional status, but it is distinct from estimates of instantaneous activity such as leucine incorporation, which measures amino acid incorporation into macromolecules.

In single cells, FISH probe signal intensity is directly related to cellular rRNA content [59]. The fraction of cells detectable using the eubacteria probe (archaeobacteria were barely detectable) should then equal the fraction of cells that each contain a minimum number of ribosomes equal to about 5 fg of RNA [42]. For a bacterial cell, ribosome number can reflect nutritional condition as well as nutritional history. Presently available data, although quite limited, indicate that a considerable variability exists among bacterial species with regard to ribosome dynamics, in terms of both production and destruction rates, with

Table 2  
Results of Spearman rank non-parametric correlation analysis used to test for simple correspondence among estimated variables

	<i>n</i> = 25 [Syn]	<i>n</i> = 25 Syn FDC	<i>n</i> = 25 [DOC]	<i>n</i> = 25 [Virus]	<i>n</i> = 25 [HFLAG]	<i>n</i> = 25 Leu cell <sup>-1</sup>	<i>n</i> = 15 % Eubact+	<i>n</i> = 17 FVIC	<i>n</i> = 25 HFLAG Clr
[Bact]	<b>0.496*</b>	-0.161	-0.139	-0.085	<b>0.434*</b>	<b>-0.420*</b>	0.359	-0.211	-0.138
[Syn]		-0.278	0.040	<b>-0.526**</b>	0.506*	-0.248	0.225	0.279	0.248
Syn FDC			-0.386	-0.089	0.064	0.071	<b>-0.643*</b>	0.069	0.028
[DOC]				0.054	-0.201	0.155	0.462	-0.089	0.065
[Virus]					-0.355	0.077	-0.017	-0.071	0.001
[HFLAG]						<b>0.554**</b>	0.102	0.071	-0.84
Leu cell <sup>-1</sup>							-0.264	0.314	0.428*
% Eubact+								0.082	-0.039
FVIC									0.414

See Table 1 for an explanation of abbreviations. Asterisks denote significance levels: \**P* = 0.05, \*\**P* = 0.001.

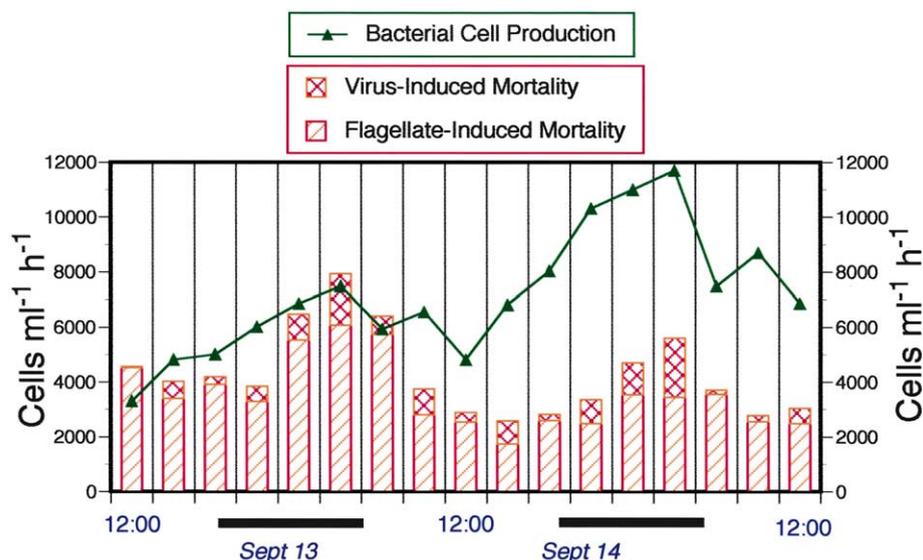


Fig. 3. Temporal changes in estimated values of heterotrophic bacterial cell production based on  $^3\text{H}$ -leucine incorporation rates, and bacterial mortality from flagellate grazing based on FLB uptake rates, and viral lysis based on frequencies of visibly infected bacteria.

changes in nutritional status [42,60]. The possession of a minimum number of ribosomes may be a poor predictor of instantaneous activity. In addition, detectability of ribosomes can vary with other factors, ranging from rRNA architecture to membrane permeability [61]. Thus, we cannot exclude, for example, the possibility of a coincidental correlation of DOC and a midday increase in membrane permeability of bacteria, perhaps UV-mediated [62]. Overall, the eubacteria probe data, while showing that the resolution of genetic probes may vary with the time of day, are difficult to interpret. It should be noted that other studies, in which leucine incorporation and eubacteria probe detection rates were examined in different locations, found no relationship [63].

The oscillations in viral numbers did not appear to reflect light-mediated viral destruction, as maximum virus abundance appeared at midday. Again, it is tempting to ascribe a direct relationship between correlated parameters, in this case virus and *Synechococcus* abundance. Our rough calculations of the production of viruses not

due to lysis of heterotrophic bacteria, combined with estimates of the mortality of *Synechococcus* not due to HFLAG grazing, suggest that the magnitude of virus concentration oscillations could be due to phage production through *Synechococcus* mortality. The burst size required would be about 130 phages per *Synechococcus* cell, which is within the range of reported values [64].

In natural systems, no diel patterns of viral abundance have been found [32–34]. On the other hand, very similar patterns to that presented here of circadian variability in viral numbers have been reported from mesocosm experiments among bacteriophages [65], as well as among viruses thought to infect *Emiliana huxleyi* [66]. However, two cautionary notes must be added. Firstly, the oscillations necessitate a synchronized production of viruses with lysis rates increasing as *Synechococcus* cells begin to divide (see Figs. 1 and 2) and we can provide no explanation as to why this should occur. Secondly, the few reports addressing virus-induced mortality of *Synechococcus* provide little evidence that viral lysis is the dominant source

Table 3  
Results of correlation analysis used to test for correspondence between temporal changes in estimated variables

	$n=25$ [Syn]	$n=25$ Syn FDC	$n=25$ [DOC]	$n=25$ [Virus]	$n=25$ [HFLAG]	$n=25$ Leu cell $^{-1}$	$n=15$ % Eubact+	$n=17$ FVIC	$n=25$ HFLAG Clr
[Bact]	0.154	-0.365	-0.177	0.175	-0.282	<b>-0.576**</b>	0.205	-0.203	-0.278
[Syn]		<b>-0.477*</b>	0.362	<b>-0.420*</b>	0.345	0.065	0.210	<b>0.488*</b>	0.268
Syn FDC			-0.313	-0.056	-0.229	0.197	<b>-0.698**</b>	0.024	0.121
[DOC]				0.060	0.110	0.111	<b>0.555*</b>	0.161	0.118
[Virus]					<b>-0.435*</b>	-0.155	0.154	-0.156	-0.042
[HFLAG]						0.023	0.001	-0.078	0.092
Leu cell $^{-1}$							-0.207	<b>0.588*</b>	0.546**
% Eubact+								0.153	-0.041
FVIC									0.498*

All estimates were expressed as percentages of the appropriate 24 h average and then transformed for normalization. For an explanation of abbreviations see Table 1. Asterisks denote significance levels: \* $P=0.05$ , \*\* $P=0.001$ .

of mortality [64]. For example, strain-specific phages were enumerated in the Woods Hole area and the conclusion reached that viruses were a minor source of *Synechococcus* mortality [67]. Hence, our tentative presentation of a strong link between viruses and *Synechococcus* requires confirmation through either virus production studies or a determination of infection rates.

We found some evidence of a link between the activities of grazers and producers in the form of leucine incorporation and HFLAG grazing rates. Clearance rates of flagellates covaried with cell-specific bacterial production (Fig. 2, Tables 2 and 3). Clearance rates of HFLAG vary with bacterial prey size [68–70], which most likely varies with leucine incorporation. However, we estimated clearance rates based on the ingestion of FLB of constant size. The absolute magnitude of HFLAG clearance rates may be higher, if the bacterial grazers discriminated against FLB. Indeed, one possible explanation of the covariance of leucine incorporation and ingestion of FLB is that selectivity of grazers shifted with bacterial activity because prey quality varied with activity. Thus, variability in clearance rates may not represent shifts in rates of water flow but rather shifts in selectivity. Changes in selectivity with shifts in preferred prey abundance (in absolute or relative terms) have been documented [71,72].

Given the uncertainties involved in extrapolating FLB ingestion to the ingestion of natural living bacteria and the assumptions involved in estimating bacterial mortality from FVIC data, we could only hope to establish temporal trends and approximate magnitudes of bacterial mortality. Hence, it was remarkable to find that our estimates of bacterial mortality (total community grazing by HFLAG plus mortality attributable to viral lysis) were comparable in magnitude to bacterial production (Fig. 3). On the other hand, we found little evidence of a tight link, on the scale of hours, between bacterial mortality and production. HFLAG grazing, according to our estimates, dominated bacterial mortality – a common finding [73]. Temporal trends in aggregate grazing impact loosely followed aggregate bacterial production, in a similar manner to the relationship between HFLAG clearance rates and bacterial cell-specific leucine incorporation rates. Viral mortality appeared to be more variable than production or flagellate-induced mortality and was independent of bacterial production. It is worthwhile recalling that viral mortality is calculated first from FVIC data which yield mortality in the form of a percentage per generation and is then multiplied by the cell production rate to obtain a rate in terms of cells per hour. The viral mortality rate then reflects variability in infection rates as well as production rates.

Overall, based on intensive sampling over an extended period, we discovered sparse evidence to support the links commonly presented in schematic diagrams (for example, between DOC and bacteria and HFLAG; viruses and bacteria) as being ‘tight’, at least in terms of displaying similar temporal variations. In contrast, we found intriguing

relationships between the autotrophic prokaryote *Synechococcus* and viruses rather than with viruses and light. We discovered that oligonucleotide probe sensitivity may vary with the time of day. Thus, we found weak links between DOC concentration, or grazing, and bacterial activity, a possibly strong link between *Synechococcus* and viruses, and a missing link between light and viruses.

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