

# Production and decay of viruses in aquatic environments

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**ABSTRACT:** The quantitative significance of aquatic viruses in coastal and in lake water was investigated. The number of viruses in marine surface waters was found to change on a diurnal basis along with changes in number of bacteria and bacterial activity. By inhibiting the production of viruses, we were able to measure viral decay rates up to  $1.1 \text{ h}^{-1}$  in marine systems, and up to  $0.6 \text{ h}^{-1}$  in a freshwater lake, for the majority of the viral population. A minor fraction (4 to 40 %) of the viral population was found to have decay rates lower than  $0.05 \text{ h}^{-1}$ . The fraction of bacteria containing mature virus particles ranged from 2 to 16 %, and the number of viruses released from these bacteria was on average about 50 (range 10 to 300). From these results we estimate that phages may lyse 2 to 24 % of the bacterial population per hour. Phages may thus be a major cause of bacterial mortality in aquatic ecosystems and may have a significant impact on the carbon and nutrient flow in aquatic food webs.

## INTRODUCTION

The number of viruses in marine waters has been shown to be  $10^4 \text{ ml}^{-1}$  or higher (Torrella & Morita 1979). It was later demonstrated that the number of viruses may vary both with location and time from less than  $10^4$  to more than  $10^7 \text{ ml}^{-1}$  (Sieburth et al. 1988, Bergh et al. 1989, Proctor & Fuhrman 1990, Bratbak et al. 1990), and that up to 7% of marine bacteria may contain mature phage particles (Proctor & Fuhrman 1990). The quantitative importance of viruses in aquatic ecosystems is however still poorly understood.

Currently there is no method available for measuring rates of viral production in aquatic environments. The method we introduce in this paper is in principle similar to methods that have been used for measuring bacterial growth and protozoan grazing: by arresting production (or consumption) we can measure consumption (or production) and thereby estimate the production (or consumption). Several different treatments, including specific eucaryotic and procaryotic inhibitors, filtration and dilution have been used for arresting bacterial production and protozoan grazing (Wright & Coffin 1984, Ducklow & Hill 1985, Sanders & Porter 1986, Sherr et al. 1986). In this study we used cyanide to inhibit the production of viruses and, by

observing how fast the viral particles decay and disappear, we could estimate the production rate.

The reason for using cyanide as an inhibitor in this investigation was that its mode of action is well known and that it is a very efficient inhibitor of aerobic respiratory bacteria. Cyanide has in addition been widely used as an inhibitor in viral research and it has been shown not to inactivate free phage particles (Doermann 1952). Cyanide inhibits the production of phages while cell lysis and release of mature phage particles may continue (Doermann 1952, Symonds 1957). The full effect of cyanide may therefore not be observed before bacteria containing mature phage particles at the start of the experiments have lysed. Any change in viral concentration seen initially in the decay experiments will therefore depend on the balance between viral production and decay in the water at the time of sampling.

The rate of viral-induced mortality may be estimated from the rate of viral production when the burst size, i.e. number of viruses released from each individual host organism, is known. The burst size of bacteria may be estimated by inducing lysis from without and counting the phages released.

In this paper we present the results from a diurnal study where we made observations of short-time variations in viral numbers, enabling us to make minimum estimates of viral production and decay. Furthermore,

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we measured the rate of viral decay in several different environments and have estimated the rate of viral-induced bacterial mortality. Results, and their implications, are discussed in relation to current methods for measuring bacterial production and protozoan grazing, and in relation to our current view on the structure and function of microbial food webs.

## MATERIALS AND METHODS

**Diurnal study.** A diurnal study of bacteria and viruses was performed in water from the bay off the Department of Marine Biology (University of Bergen) adjacent to Raunefjorden, Western Norway (Bratbak et al. 1990), on May 5 to 6, 1990. Polyethylene tanks of 60 l capacity were used as enclosures (sea bags) to ensure that any change would be due to biological activity and not to patchiness of drifting surface water. Two enclosures, one wrapped in several layers of black plastic foil to exclude light, were filled with surface water at night, 11 h before start of the experiment and moored in the sea to a floating frame. Both enclosures were sampled every second hour for 24 h.

Samples for counting of viruses and bacteria (100 ml) were preserved with 1 % glutaraldehyde (final concentration). Bacteria were counted in the epifluorescence microscope after staining 5 ml subsamples with DAPI (Porter & Feig 1980). Viruses were harvested onto electron microscope grids in duplicate by centrifugation and counted in the transmission electron microscope as previously described (Børsheim et al. 1990, Bratbak et al. 1990). Thymidine (TdR) incorporation (Fuhrman & Azam 1982) was measured in duplicate samples (10 ml) incubated for 30 min in situ with 15.5 nmol  $^3\text{H}$ -labeled TdR  $\text{l}^{-1}$ . Glutaraldehyde (1 % final concentration) was added to blanks and at the end of incubations. Radioactivity in cold TCA-precipitated material collected on 0.2  $\mu\text{m}$  pore size cellulose nitrate filters (Sartorius) was

measured. Data on global radiation was obtained from measurements at Geophysical Institute, Department of Meteorology, University of Bergen.

**Viral decay experiments and induction of lysis in bacteria.** Water samples for these experiments were collected with a Ruttner water sampler between 08:00 and 10:00 h at the following locations (Table 1): in Raunefjorden, Western Norway (Bratbak et al. 1990); at the Dept of Marine Biology harbour, which lies in a bay adjacent to Raunefjorden, 20 km southwest of Bergen (Bratbak et al. 1990); in Bergen harbour area; and in Lake Kalandsvannet which is a 3.4  $\text{km}^2$  oligotrophic freshwater lake with a mean depth of 37 m, 20 km south of Bergen. The experiments were in most cases started immediately after sampling and always within 1 h after sampling.

All incubations for decay experiments were made in 1 or 5 l bottles and at in situ temperature. The viral decay, i.e. decrease in viral concentration over time, was recorded after inhibiting production of new viruses by adding KCN to a final concentration of 2 mM (Doermann 1952, Symonds 1957). The pH of the KCN stock solutions were adjusted to 7.0 for use in freshwater, and to 8.5 for use in seawater. Samples (100 ml) for counting of viruses were withdrawn at intervals down to 30 min. The samples were preserved and the viruses counted as described for the diurnal study. To investigate possible effects of cyanide on the viral decay measurements we performed control experiments where the viral production was arrested by removing possible host organisms by centrifugation at 16 000  $\times g$  for 20 min. The experimental protocol was otherwise unchanged. The viral decay rate was calculated from the log-linear part of the decay curves using linear regression.

Bacteria containing mature phage particles were lysed from without using streptomycin (Symonds 1957, Symonds 1968). This made it possible to count both the frequency of bacteria containing mature phage particles and the number of phages released from the bac-

Table 1 Numbers of bacteria and viruses, virus decay rates and percent bacteria with mature phage particles

Location (Norway)	Date (1990)	Depth (m)	Temperature ( $^{\circ}\text{C}$ )	Bacteria ( $10^6 \text{ ml}^{-1}$ )	Viruses ( $10^7 \text{ ml}^{-1}$ )	Viral decay rate (SE, n) <sup>a</sup> ( $\text{h}^{-1}$ )	Bacteria with mature phage particles (%)
Dept of Manne							
Biology harbour	6 Jun	Surface	12	4.4	4.4	0.30 (0.01, 4)	ND
Bergen Harbour	8 Jun	1.5	13	2.9	5.9	1.1 (0.2, 3)	ND
Raunefjorden	19 Jun	Surface	14	1.4	3.5	0.49 (0.02, 6)	13
Raunefjorden	19 Jun	5	14	1.5	2.8	0.55 (0.03, 5)	14
Raunefjorden	19 Jun	25	8	0.7	1.1	0.26 (0.002, 3)	8
Lake Kalandsvannet	17 Aug	0.5	17	3.0	20.2	0.35 (0.05, 3)	16
Lake Kalandsvannet	28 Aug	1	16	4.1	15.4	0.64 ( - , 2)	4
Lake Kalandsvannet	28 Aug	25	5	0.8	1.9	0.33 (0.08, 4)	2

<sup>a</sup> SE: standard error; n: number of data points used in calculation; ND: not determined

teria. Water samples (100 ml) were incubated immediately after sampling with 0.3 % w/v streptomycin (Sigma No. S-6501) for 1 h and then harvested onto grids in duplicate and prepared for electron microscopy as described for total counting of viruses (Børsheim et al. 1990, Bratbak et al. 1990).

## RESULTS

### Counting accuracy and precision

The accuracy of our virus counting method was tested using the coli phage T<sub>4</sub> as a standard. The number of plaque-forming units in a fresh lysate was found to be 85 to 90 % of the number of countable phage particles (data not shown). This discrepancy may be due to the presence of inactive phages or to the presence of clumps of active phages which will give rise to only one plaque (Luria et al. 1951). The conclusion is nevertheless that our counting method gives us close to 100 % recovery of virus particles.

When counting viruses in natural waters (Fig. 1) the standard deviation of parallel preparations was 1 to 29 % (mean: 16%), and the counting error was 4 to 13 %. The precision of our virus counting method may also be judged from the standard error of the decay rates (Table 1). On this basis we conclude that our virus data are sufficiently precise and accurate.

### Diurnal study

Results from the diurnal study are shown in Fig. 1. The pattern of change in bacterial concentration was similar in the 2 enclosures (Fig. 1B, C). The concentration decreased from the start of the experiment (09:00 h), reached a minimum in the afternoon at 15:00 h and increased throughout the evening and night.

The viral concentration in the light-exposed enclosure (Fig. 1B) peaked at noon and during the daylight period (06:00 to 21:00 h) was significantly correlated with the global radiation (Fig. 1A) ( $r = 0.725$ ,  $n = 0.8$ ,  $p < 0.05$ ). The viral concentration in the dark enclosure (Fig. 1C) showed a marked diurnal variation with a maximum value in the afternoon (15:00 to 17:00 h). The number of viruses in this enclosure was correlated with thymidine incorporation (Fig. 1D) ( $r = 0.584$ ,  $n = 12$ ,  $p < 0.05$ ) and with thymidine incorporation per bacterium ( $r = 0.799$ ,  $n = 12$ ,  $p < 0.01$ ). We found no significant correlation between number of bacteria and number of viruses.

From the changes in viral concentration observed in the diurnal study (Fig. 1B, C) we may calculate viral production rates up to  $0.36 \text{ h}^{-1}$ , and viral decay rates up to  $0.32 \text{ h}^{-1}$ . These rates are minimum estimates since they are based on net changes in viral concentration.

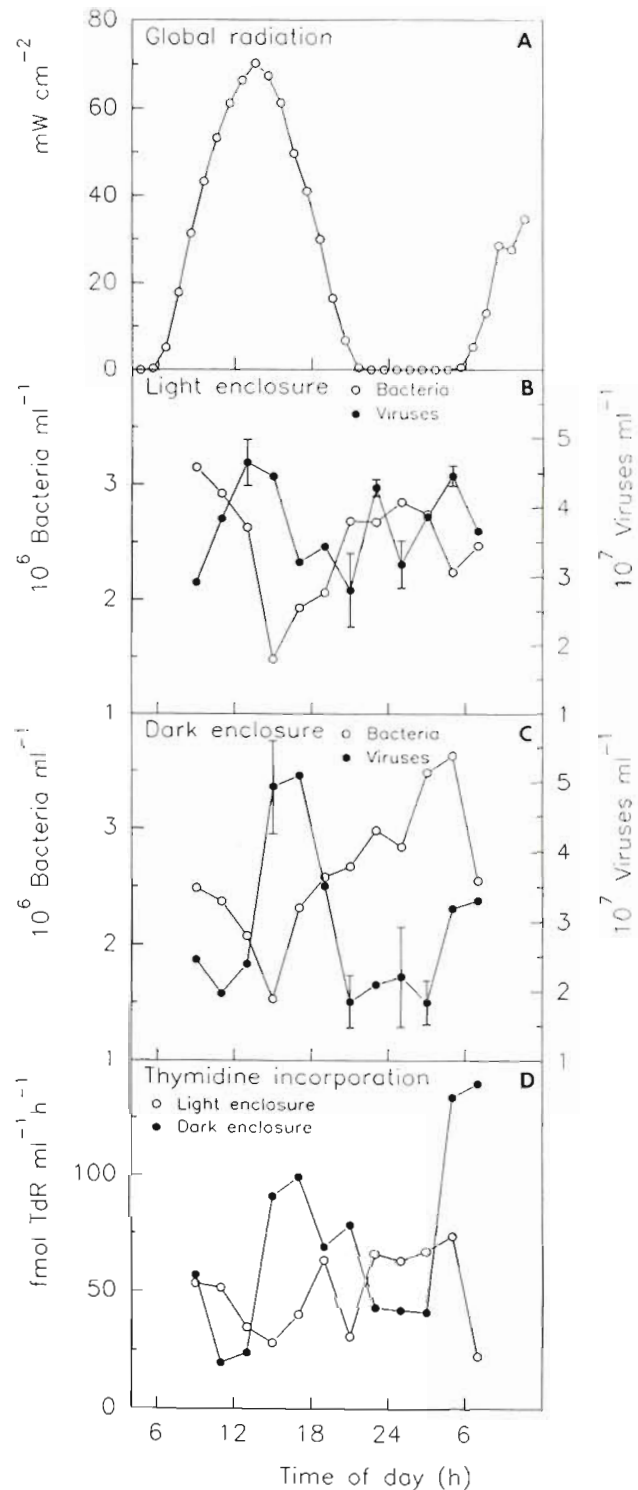


Fig. 1. Diurnal changes in (A) global radiation, (B, C) number of bacteria and viruses, and (D) thymidine (TdR) incorporation in one light-exposed and one dark 60 l seawater enclosure, Raunefjorden, western Norway, May 5 to 6, 1990

### Viral decay experiments and induction of lysis in bacteria

Cyanide has no significant effect on the viral decay rate compared to the decay rate observed when removing possible host organisms by centrifugation (Fig. 2A).

In the decay experiments shown in Fig. 2A, B we

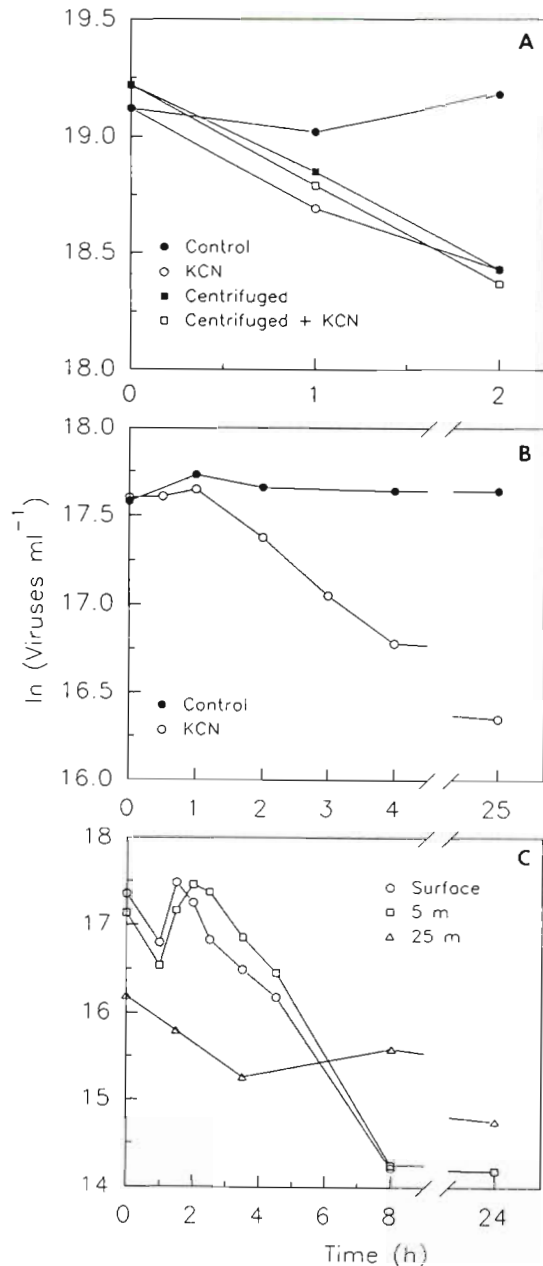


Fig. 2. Changes in viral counts as a function of time after arresting the production of viral particles. (A) Comparative study of viral decay after cyanide addition and after removing possible host organisms by centrifugation. Sample from Lake Kalandsvannet, Norway, August 17, 1990. (B, C) Viral production inhibited with cyanide. Samples from (B) Dept of Marine Biology harbour, June 6, and (C) Raunefjorden, June 19, 1990

observed no significant changes in viral concentration in the control bottles receiving no treatment. This indicates that production and decay of viruses in these cases were in balance during the incubation.

The decay observed in the KCN bottles during the first 2 to 8 h of the incubations can be described as exponential (Fig. 2). Viral decay rates, which may be calculated from the log-linear part of the decay curves, ranged from 0.3 to 1.1 h<sup>-1</sup> (Table 1). These rates are in agreement with the minimum rates estimated from the diurnal study. The rate of viral decay decreased to less than 0.05 h<sup>-1</sup> after 4 to 8 h when 4 to 40 % of the initial viral population remained (Fig. 2B, C). The majority of the viral population (> 60 %) thus has a relatively short turnover time, while a minor fraction (< 40 %) is relatively refractile. This latter fraction was usually dominated by larger viruses with head diameter > 60 nm.

Both in Lake Kalandsvannet and in Raunefjorden, the rate of viral decay was found to be higher above the thermocline than below the thermocline (Table 1). This may indicate that the viral decay is directly or indirectly related to physical, chemical or biological factors in the environment.

A high viral decay rate implies a high rate of production of new viruses and thus that host organisms containing mature viral particles should be frequent. Lysing infected bacteria from without using streptomycin we found that 2 to 16 % of the bacteria contained mature phage particles (Table 1). These phage particles (Fig. 3) appeared similar to the free viral particles counted in the diurnal study and in the decay experiments, suggesting that the free viral population was dominated by phages. The number of phages released from lysed bacteria was on the average about 50 (range 10 to 300).

## DISCUSSION

### Diurnal study

It is reasonable to believe that most phages in aquatic environments are temperate rather than virulent (Freifelder 1987, Bratbak et al. 1990). The rate of phage production will thus depend on the rate of induction in lysogenic bacteria rather than on the rate of infection. If phage production in many lysogenic bacteria is induced by the same environmental factors we may expect the phage production in aquatic environments to be synchronous, and thus that the number of free phages and the number of bacteria containing mature phage particles fluctuate.

The results from the diurnal study (Fig. 1) show that the viral population density in marine waters may change significantly on a time scale of hours. The viral



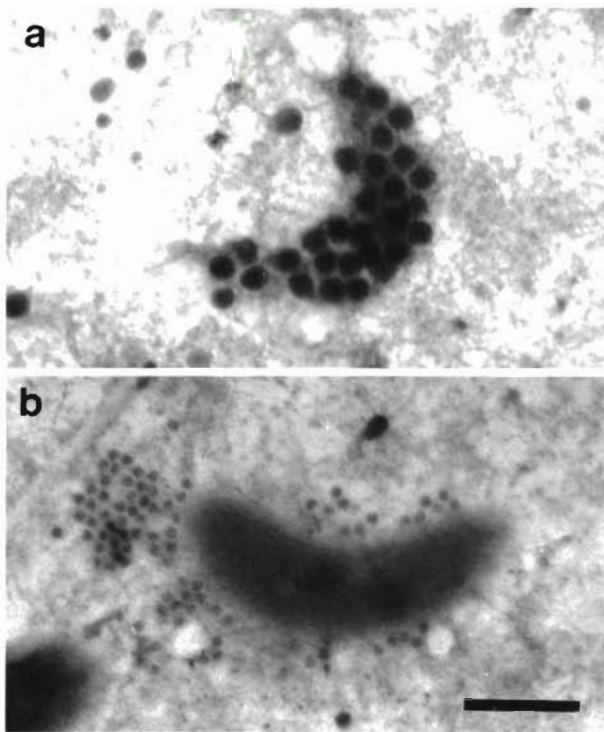


Fig. 3. Artificially lysed bacteria. (a) Bacterium with phage particles, head diameter ca 100 nm. (b) Bacterium surrounded by phage particles, head diameter ca 30 nm. Scale bar = 0.5  $\mu\text{m}$

population is thus a dynamic member of the microbial community in aquatic ecosystems and may change on the same time scale as other microbial parameters (e.g. primary production, bacterial numbers, thymidine incorporation and dissolved DNA concentration) in some environments (Paul et al. 1988, Jeffrey et al. 1990). Light and nutrient availability are among the environmental factors which may induce lysogenic bacteria to produce and liberate viruses. A simple interpretation of the correlations between viral numbers and light and between viral numbers and thymidine incorporation in the diurnal study (Fig. 1) is that the viral production is governed directly or indirectly by light during daytime, while in the dark it is governed by the bacterial activity. However, the enclosure experiment was not run with replicates and it is therefore not possible to reject the hypothesis that the results obtained are differences between 2 similar bags. The results may thus also be interpreted in terms of diurnal rhythms rather in terms of light as a factor per se.

An overall negative correlation between number of bacteria and number of viruses could be expected if the bacterial population was controlled by viral-induced lysis. This correlation may be obscured, however, if the phage production in subpopulations of bacteria with widely different burst sizes is controlled by different environmental parameters, or if the viral decay rate is

high compared to the sampling interval or to the bacterial growth rate. Protozoan bacterivory, which is a ubiquitous phenomenon in marine ecosystems, may also obscure the correlation between bacterial and viral numbers.

The bacteria in the 2 enclosures may be estimated from the measured thymidine incorporation to grow at an average rate of 0.6  $\text{d}^{-1}$  (range: 0.2 to 1.4  $\text{d}^{-1}$ ), assuming a conversion factor of  $1 \times 10^{18}$  cells per mol TdR incorporated. These growth rate estimates are reasonable compared to literature data on bacterial growth in aquatic ecosystems (Ducklow & Hill 1985, Moriarty 1986). However, molecular fractionation and isotope dilution analysis was not performed, and we did not determine an empiric conversion factor relating thymidine incorporation to cell production for the environment we were investigating. The overall range in published empiric conversion factors is more than 10-fold, whereas conversion factors determined using high (> 10 nM) thymidine concentrations, and conversion factors determined for particular environments during a particular season, are often more congruent (Riemann et al. 1987, Bell 1990, Riemann & Bell 1990). There is addition a wide discrepancy (>> 10-fold) between theoretical and empirical conversion factors (Bell 1990). Considering the uncertainty in the conversion factor, our bacterial growth rate estimates cannot be regarded as more accurate than to within a factor of 3 to 5.

#### Viral decay experiments and induction of lysis in bacteria

Using the approach we applied to measuring viral production, it must be ascertained that the processes measured are not affected by the treatment of the samples. This is a serious problem when dealing with biological processes such as bacterial growth or protozoan grazing (Taylor & Pace 1987, Tremaine & Mills 1987a, b). Using cyanide we reduced the possibility that biological processes may affect the measured decay. If the viral decay to some extent, directly or indirectly, depends on energy-requiring biological processes, the rate of decay would be systematically underestimated. Nevertheless, considering the fact that cyanide has been widely used as an inhibitor in viral research and the results from the control experiment showing that cyanide has no significant effect on the viral decay rate, we conclude that cyanide is an adequate metabolic inhibitor for our viral decay experiments.

A steady decrease in viral concentration was in some decay experiments not observed until 1 to 2 h after addition of cyanide (Fig. 2b, c). This may be explained

as a consequence of the fact that cyanide inhibits the production of phages while cell lysis and release of mature phage particles may continue (Doermann 1952, Symonds 1957).

The fate of the viral particles is unknown. One possibility is that their nucleic acid is ejected or otherwise released. Any empty capsids will not be recognized by the methods we have used. Release of DNA and RNA from cells during lysis and from free phage particles may contribute to the concentration of nucleic acids in aquatic habitats (Karl & Bailiff 1989). Paul et al. (1988) observed a diel periodicity in microbial activity and in dissolved DNA in a subtropical estuary. The dissolved DNA production lagged behind the maximum in bacterial activity by ca 4 h. These observations may be explained by a diel periodicity in viral production and by release of DNA from decaying viral particles.

Streptomycin is an inhibitor of protein synthesis and will also stop phage maturation (Symonds 1957). High concentrations of streptomycin will in addition induce bacterial lysis (Symonds 1968). We have no reason to believe that all bacteria are affected to the same extent by the streptomycin treatment. The fraction of bacteria containing mature phage particles detected by this method must therefore be regarded as a minimum estimate. The number of mature phage particles released from the lysed bacteria must also be regarded as a minimum estimate of burst size because the streptomycin terminates phage maturation and lyses the cells before the latent period is over.

#### Rate of bacterial growth and mortality

Protozoan grazing is generally assumed to be the most important cause of bacterial mortality in aquatic ecosystems. The rate of grazing may however not always be sufficient to explain the mortality of bacteria and does not always balance the bacterial production (Servais et al. 1985, McManus & Fuhrman 1988, Pace 1988, Servais et al. 1989, Sherr et al. 1989). This discrepancy may be due to viral-induced bacterial mortality.

If the viral population density is to be maintained, we may, from the data given in Table 1, estimate that phages must lyse 2 to 24 % of the bacterial population per hour, assuming a burst size of 50 to 100 new phages per lysed bacterium and (conservatively) that 50 % of the viral population decays at the rates indicated. The bacteria must grow at a rate of 0.4 to 6 d<sup>-1</sup> to balance this viral-induced mortality. These growth rate estimates are relatively high compared to the bacterial growth rates measured in most aquatic ecosystems (Ducklow & Hill 1985, Moriarty 1986) indicating that current estimates of protozoan grazing may be overestimated, or that current estimates of bacterial produc-

tion may be underestimated. The diel periodicity in the viral concentration we observed in the diurnal study suggests a diel periodicity in the rate of production or in the rate of decay of the viral particles. We cannot therefore claim that our viral decay measurements, which were all made on water samples taken in the morning, are representative of the daily average.

The phages released in a single burst may be calculated to contain 1.5 to 3 times as much DNA as a single bacterium, assuming the burst size to be 50 to 100 and the DNA content of individual bacteria and phages to be 2.6 and 0.08 fg respectively (Fuhrman & Azam 1982, Børshheim et al. 1990). Considering the rates of virus production we have estimated, this suggests that the basis for estimating the rate of bacterial growth in aquatic ecosystems from the rate of DNA synthesis (Fuhrman & Azam 1980, Fuhrman & Azam 1982) may be questioned.

<sup>3</sup>H-thymidine incorporation has been widely used for measuring the rate of DNA synthesis in bacteria in aquatic environments (Moriarty 1986, Riemann & Bell 1990). In its current form, this method does not discriminate between production of bacterial DNA, viral DNA and dissolved DNA released during lysis of bacteria. The thymidine method therefore in principle measures total DNA synthesis in bacteria. Rates of thymidine incorporation are converted to cell production using conversion factors. Conversion factors may be derived from simultaneous measurements of thymidine incorporation and observations of cell number increase in cultures of natural bacterial populations where bacterivory has been prevented by filtration and dilution (Riemann & Bell 1990). Cells lost due to viral-induced lysis will not be included in the calibration and the cell number increase is therefore net production. The conversion factor will thus relate the total rate of DNA synthesis in bacteria to the net bacterial cell production. Consequently, viral-induced bacterial mortality will contribute to the variability in the conversion factors (Bell 1990, Riemann & Bell 1990) and to the uncertainty in the production estimates.

The methods used for measuring the protozoan grazing will not detect viral-induced bacterial mortality. Most phages are presumably temperate and present as prophages in bacteria, and their possible impact on the bacterial population is therefore not affected by dilution or filtration (Wright & Coffin 1984, Ducklow & Hill 1985). Prokaryotic inhibitors (Sanders & Porter 1986, Sherr et al. 1986) will also inhibit phage production and thus preclude detection of any viral-induced mortality. Eucaryotic inhibitors (Taylor & Pace 1987) will in principle not affect the viral production, and the viral-induced mortality will be the same in the experimental bottle as in the control bottle, i.e. viral-induced mortality is not detected.



These considerations on production and consumption of bacterial biomass make it important to distinguish between net and total bacterial production. Net production refers to the bacterial biomass that may be harvested as particles by protozoan grazing. Total production includes in addition the biomass of the viral particles and the organic material released from the bacteria as dissolved organic material during lysis. The bacterial production removed by viral-induced lysis may enter the pool of dissolved organic material (DOM) utilized mainly by bacteria and be recycled in a bacteria-phage-DOM loop (Bratbak et al. 1990). One implication of this is that the bacterial production released due to viral-induced lysis is in part self-sustained. Assuming 50 % respiration, the carbon input required for phage production will be half that required for harvestable (net) production.

Viruses uncouple production and consumption of particulate biomass and they increase the production of DOM. They may thus be a key factor for understanding the transformation between the particulate, colloidal and dissolved fractions of organic material in seawater (Sugimura & Suzuki 1988, Isao et al. 1990). The high rate of viral decay suggests that transformation rather than transduction may be the predominating pathway for genetic exchange between bacteria in natural aquatic environments. The ecological significance of algal and protozoan viruses remains to be investigated.

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