Marine Microbial Food Webs, 1992, **6** (2) : 79-90

Estimation of Virus Production in the Sea: II. Field Results [1]

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Abstract

Virus production was estimated in samples from diverse marine environments by incorporation of ^{32}P-orthophosphate ($^{32}P_i$) into viral DNA. Rates of virus production in the field were found to vary over three orders of magnitude (from $< 1 \times 10^8$ to 2.3×10^{11} viruses l^{-1} d^{-1}). Due to the possibility of intracellular isotope dilution and filtration losses, these rates should be considered minimum estimates. Virus production displayed a strong onshore-offshore gradient which covaried with bacteria abundance and chlorophyll *a*. Minimum estimates of mortality in two coastal samples (12 and 25 % of bacteria production) suggest that viruses were a significant source of bacteria mortality in these environments. The apparent dependence of virus production on host density suggests that significant phage attack is spatially and temporally episodic in the open ocean (*e.g.* during phytoplankton blooms), but more persistent in the highly productive coastal waters.

Key words: Bacteriophage, Virus, Production, Seawater.

Résumé

Mesure de la production de virus en milieu marin - II. Résultats *in situ*

La production de virus en milieu marin a été mesurée à l'aide d'une méthode d'incorporation du ^{32}P-orthophosphate ($^{32}P_i$) dans l'ADN viral. Les taux de production de virus *in situ* ont varié selon trois ordres de

[1] This work was supported by grants from the US National Science Foundation (SGER; OCE-9204039) to F.A. J.W. was supported by the Swedish Natural Science Research Council and W.P.C. was supported by the Natural Science and Engineering Research Council of Canada.

grandeur (de < 1 x 10^8 à 2,3 x 10^{11} virus l^{-1} j^{-1}), dans les divers environnements marins étudiés. En raison d'une dilution intracellulaire possible de l'isotope et des pertes par filtration, ces valeurs doivent être considérées comme des estimations minimales. La production virale présente un gradient large-côte accentué, qui est à relier à l'abondance bactérienne et à la chlorophylle *a*. Des estimations de mortalité minimales (12 et 25 % de la production bactérienne) suggèrent le rôle important des virus dans la mortalité bactérienne, pour au moins deux environnements côtiers. La dépendance spatio-temporelle, apparente, de la production virale vis-à-vis de la densité en hôtes suggère l'existence d'attaques virales significatives épisodiques, en pleine mer (c'est-à-dire lors des poussées phytoplanctoniques), mais qui persistent dans les eaux côtières hautement productives.

Introduction

Recent studies have implicated viruses as abundant and dynamic members of marine microbial communities (*e.g.*, Bergh, 1989; Børsheim *et al.*, 1990; Bratbak *et al.*, 1990; Heldal and Bratbak, 1991) which may be a significant source of bacterial mortality (Proctor and Fuhrman, 1990, 1991). If so, then phage production may secondarily influence material cycling and food web dynamics since bacteria play a critical role in the ocean's food web structure and biogeochemistry. To assess the importance of viruses in these processes, there is clearly a need for quantitative study of their ecology in the sea.

Techniques have been developed to estimate viral mortality of bacteria (by frequency of infected cells; Proctor and Fuhrman, 1990) and to measure virus decay rates (by the disappearance of viruses after the removal or poisoning of hosts; Heldal and Bratbak, 1991) in natural assemblages. However, due to lack of a method, little is known about the magnitude of the actual gross production of viruses in natural assemblages or how it may be regulated. Using a recently developed radiotracer method for measuring virus production in seawater (Steward *et al.*, 1992), we have estimated virus production rates in a variety of marine environments to obtain information on its variability and possible correlation with other biological variables.

Materials and Methods

Collection of Samples

Seawater samples at offshore and nearshore stations were collected with 5 l Niskin bottles mounted on a rosette sampler during cruises of the R/V *Robert Gordon Sproul* in the Southern California Bight from 17 to 25 September (Stations 301 and 305) and 9 to 10 December 1991 (Stations 2, 5 and 8). Surface water samples from the San Diego area (Los Peñasquitos Lagoon, Mission Bay, and Scripps pier) were collected by hand in sterile, polycarbonate flasks on 21 November and 3 and 5 December 1991, respectively. Sampling locations are presented in Figure 1.

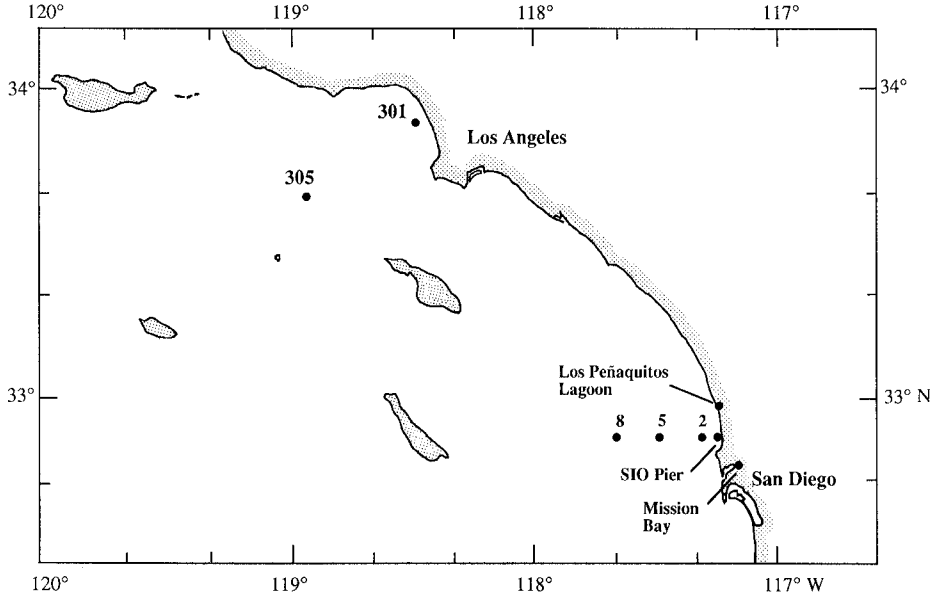

Fig. 1. Sampling sites in the Southern California Bight. Station names and coordinates are : "305" (33° 45' N, 118° 55' W), "301" (33° 54' N, 118° 26' W), "8" (32° 52' N, 117° 40' W), "5" (32° 52' N, 117° 28' W), "2" (32° 52' N, 117° 17' W), Scripps pier (32° 53' N, 117° 15' W), Mission Bay (32° 47' N, 117° 13' W) and Los Peñasquitos Lagoon (32° 55' N, 117° 15' W).

Measurements

Virus production was estimated by incorporation of $^{32}P_i$ into viral DNA (Steward *et al.*, 1992) using the chemical hydrolysis protocol for distinguishing viral DNA. Briefly, unfiltered or 0.8 µm filtered triplicate seawater samples were incubated in the dark at *in situ* temperature (± 2 °C) with $^{32}P_i$ (10 µCi ml^{-1} final). At specific time points, subsamples were withdrawn and syringe filtered (0.2 µm Acrodisc® HT) then treated with nucleases to degrade dissolved nucleic acids. Samples were syringe filtered a second time, split into 900 µl aliquots and the viruses precipitated with $(NH_4)_2SO_4$ at 0 °C. Pellets were washed once with 50 % saturated ammonium sulfate then resuspended in phosphate buffer. Duplicate aliquots were treated with NaOH (0.5 N at 60 °C) to degrade RNA or trichloroacetic acid (TCA; 5 % at 100 °C) to degrade RNA and DNA. After cooling on ice, the NaOH-treated aliquot was acidified with TCA. Both replicates were incubated on ice then centrifuged (16 000 x g) to pellet the precipitated macromolecules. Pellets were washed once with cold 5 % TCA then assayed for radioactivity by scintillation counting. The difference in activity between the replicates was taken as the activity in viral DNA.

Samples of seawater for phosphate analysis were filtered through rinsed (Milli-Q-purified H_2O) GF/F filters and stored frozen until assayed. The concentration of

molybdate-reactive phosphorus was determined according to Parsons *et al.* (1984). Phosphate concentrations were used to calculate the effective specific activity of $^{32}P_i$ in each sample (activity vol^{-1} divided by $[PO_4^{3-}]$ vol^{-1}). Rates of $^{32}P_i$ incorporation were then converted to rates of phosphate assimilation based on the effective specific activity of $^{32}P_i$ in the sample.

Virus production rates were calculated from the incorporation of phosphate into viral DNA using a field conversion factor (5×10^{19} viruses mole-P_i^{-1}) derived from the factor presented in Steward *et al.*, (this issue). Since the samples in this study were processed by a protocol which results in 40 % lower recovery of the incorporated ^{32}P than that used to derive the conversion factor (Steward *et al.*, this issue), a corrected factor of 1.25×10^{20} viruses mole-P_i^{-1} has been used here.

Counts of bacteria and viruses, measurement of chlorophyll *a* (chl *a*), and assay of bacterial secondary production were as indicated in Steward *et al.* (this issue).

Statistics

Rates of label incorporation were determined by least-squares linear regression. The 95 % confidence intervals for the slope of the regression line were taken as the rate error and used to calculate significance of x-intercepts. Spearman's nonparametric rank correlation method was used to determine the coefficients and significance of correlations (data were not normally distributed).

Results and Discussion

Incorporation of $^{32}P_i$ into DNA viruses was generally well fitted by least-square linear regression after an initial lag of 5-6 h (Fig. 2). The lag in appearance of labeled viruses is expected to reflect the turnover time of the nucleotide pools used for viral DNA synthesis (which can include host DNA), but may also reflect that portion of the latent period between cessation of viral DNA synthesis and release of intact viruses. Since filtration efficiency was not measured at all stations, the rates are not corrected for filtration loss and may, therefore, be substantially underestimated (Steward *et al.*, 1992).

Rates of virus production were highest (up to 2.3×10^{11} viruses l^{-1} d^{-1}) in the waters of Los Peñasquitos Lagoon and Mission Bay (Table I). Rates were also quite high at the nearshore stations, but declined dramatically offshore where they were not significantly different from zero in 6 of 8 samples. Shortest turnover times of the virus pool (0.57 and 0.98 d) were at the nearshore stations. Our rates overlap, but are generally lower than, the rates implied by the virus decay experiments of Heldal and Bratbak (1991). Based on their observation that > 60 % of the virus community degraded at the measured rates, we convert their data to minimum production estimates ranging from 0.4 to 14×10^{11} l^{-1} d^{-1}. The corresponding maximum turnover times range from 0.06 to 0.23 d.

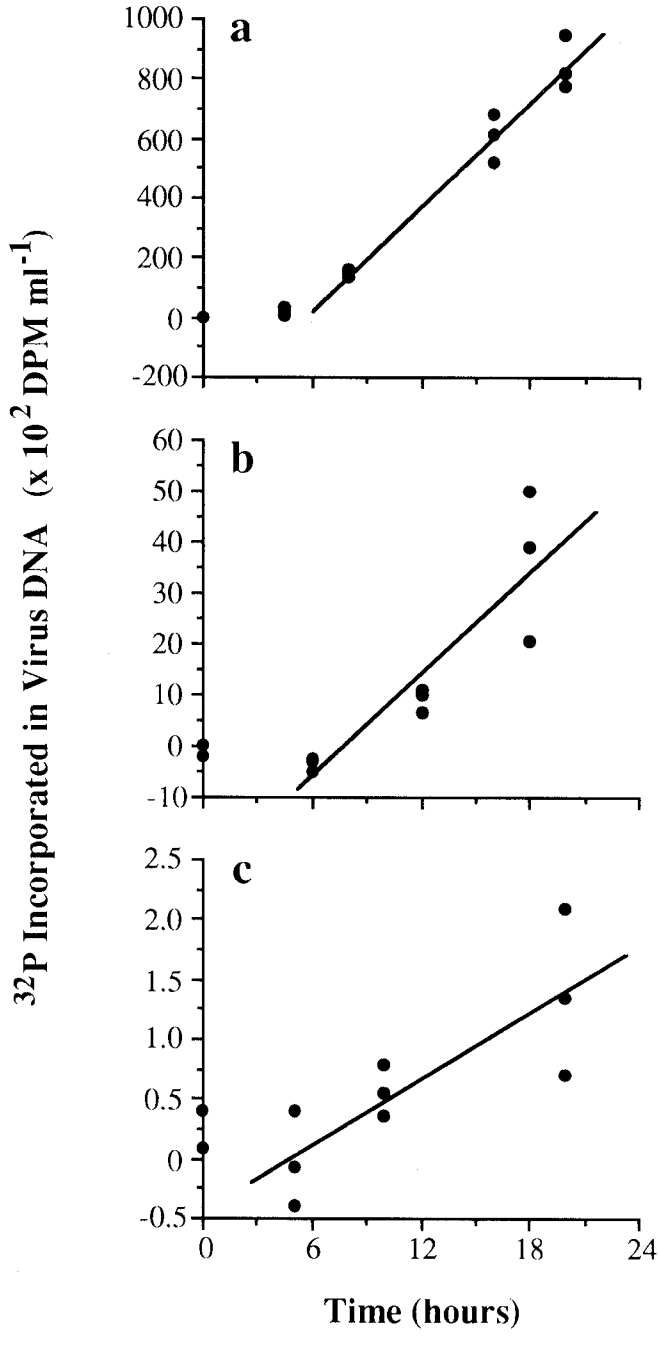

Fig. 2. Time courses of $^{32}P_i$ incorporation into virus DNA in samples from a) Peñasquitos Lagoon, b) coastal Station 301 and c) offshore Station 5 in the Southern California Bight. Rates of increase were determined by least-squares linear regression (excluding points before 5 hours due to the apparent lag phase).

TABLE I. – Summary of virus and bacteria production and abundance. Bacteria production was measured at the mid-point of the incubation for the virus production assays. Viral mortality of bacteria was calculated as the ratio of virus production to bacteria production divided by the burst size. The range of mortality is based on a range of burst sizes (10 to 300) observed for marine bacteria (Heldal and Bratbak, 1991).

Location and Sampling depth		*Virus Production* (± 95% C.I.) (x 10^9 virus l^{-1} d^{-1})	*Viruses* (x 10^9 l^{-1})	*Virus Turnover Time* (d)	*Bacteria Production* (x 10^9 l^{-1} d^{-1})	*Bacteria* (x 10^9 cells l^{-1})	*Estimated Mortality* (%)
Coastal Stations							
Peñasquitos Lagoon		230 (± 40)	620	2.7	3.1	14.1	25 - 740
Mission Bay		35 (± 12)	137	3.9	0.94	3.9	12 - 372
Scripps Pier		-0.52 (± 0.63)†	11.7	—	0.65	0.69	—
Nearshore Stations (0.9 to 3 km from shore)							
Station 301	10m	24 (± 11)	13.7	0.57	n.d.	2.1	—
Station 2	2m	12 (± 9.6)	11.7	0.98	3.0	0.48	1.3 - 40
Offshore Stations (9 to 46 km from shore)							
Station 5	2m	0.61 (± 0.38)	18.3	30	0.19	0.34	1.1 - 32
Station 8	2m	2.8 (± 4.7)†	15.3	—	0.22	0.42	—
Station 305	5m	1.4 (± 1.3)	12.4	8.9	0.48	0.87	0.97 - 29
	10m	0.63 (± 1.9)†	12.0	—	1.7	0.71	—
	30m	-1.1 (± 2.1)*†	10.7	—	0.078	0.81*	—
	50m	0.01 (± 0.22)*†	5.7	—	0.021	0.46*	—
	700m	-1.47 (± 2.6)†	1.1	—	0.0063	0.07	—
	900m	1.1 (± 5.6)†	2.5	—	0.0041	0.11	—

† Rates not statistically different from zero ($p > 0.05$)

* Data is for 0.8 μm prefiltered samples

— Calculation not applied to rates not significantly different from zero or where data not available

n.d. No data

There was good correlation of virus production with bacteria numbers and chl *a* suggesting a dependence of virus production on the trophic state of the environment (Fig. 3). Virus production was not, however, correlated with the bacterial specific growth rate. These correlations may reflect the host density dependence of virus production. Such a relationship would be expected whether the production is by lytic growth or induction of lysogens. The converted values from the data of Heldal and Bratbak (1991; see above), with one outlier excluded (June 6 sample), also reveal a strong correlation between virus production and bacteria abundance (Fig. 3b), although this relationship was not noted in that report. Least-squares linear regression of both data sets results in slopes that differ by a factor of ≈ 25, but have positive x-intercepts (0.47 and 0.72 x 10^9 bacteria l^{-1}). Regression of our data for chl *a* results in an x-intercept of 0.27 μg l^{-1}.

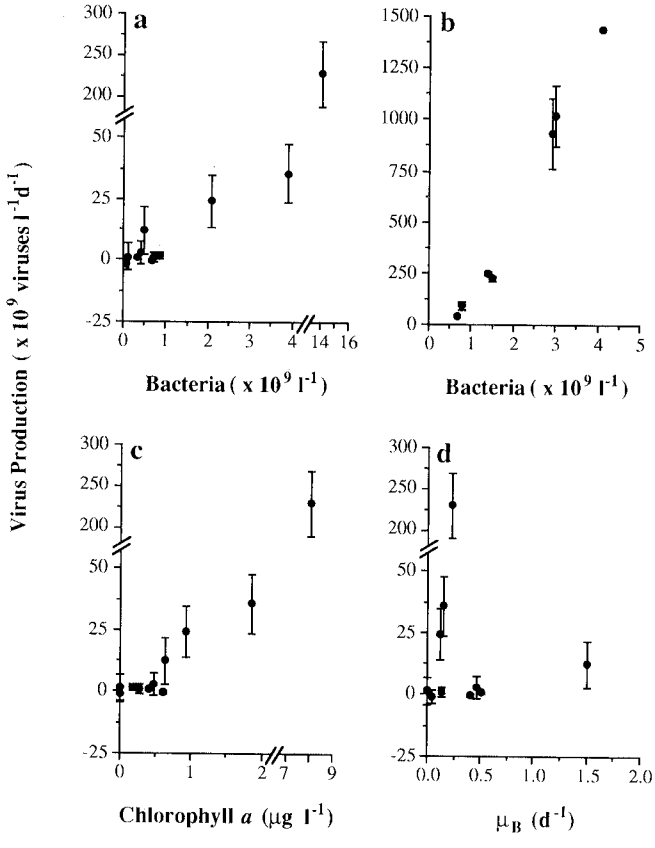

Fig. 3. Virus production *vs.* a) bacteria ($r = 0.74$, $p < 0.05$, $n = 11$), b) bacteria ($r = 0.96$, $p < 0.01$, $n = 7$), c) chl *a* ($r = 0.78$, $p < 0.05$, $n = 11$), and d) bacterial specific growth rate ($r = 0.14$, $p > 0.5$, $n = 11$). Data in panel b is from Heldal and Bratbak (1991) with virus production calculated from decay rates. Error bars are 95 % confidence intervals (a,c,d) or standard deviation (b).

The significant (based on 95 % confidence intervals) positive x-intercepts suggest the presence of a threshold host-density for virus production. A threshold host-density of *ca.* 10^4 c.f.u. ml^{-1} has been reported previously for production of lytic phages in culture (Wiggins and Alexander, 1985). More recently, however, lytic growth has been shown to be possible at specific host densities at least as low as 10 ml^{-1} (Kokjohn *et al.*, 1991). In addition, phage production resulting from the induction of lysogens should be possible, though not necessarily sustainable, at any non-zero host density. Therefore, explanations other than host-density thresholds should be considered in interpreting the positive x-intercepts. It is possible that the relationship between host density and phage production is obscured when bacteria abundance is very low, because virus production is below the detection limit of the assay.

Since our data reveal community-level relationships of complex assemblages, their interpretation is more difficult than data from experiments with specific phage-host systems. Nonetheless, since the vast majority of the marine environment is oligotrophic, with low bacteria and phytoplankton densities, the correlations we observed strongly suggest that virus production in the open ocean is generally low.

$^{32}P_i$ added to seawater is taken up by both bacteria and phytoplankton so that virus production from both of these sources should be detected by this method. Samples were size-fractionated to assess the relative importance of the two host types. Virus production was compared in parallel samples either unfiltered or prefiltered (0.8 μm) to remove most phytoplankton (Fig. 4). Prefiltration of samples consistently resulted in lower rates (28 ± 23 % of unfiltered samples; mean ± s.d., n = 4). However, in addition to removing phytoplankton, the prefiltration resulted in a proportional loss of bacteria. At these sites phytoplankton or particle-attached bacteria could have been a significant source of virus production. We hypothesize that colonized particles, because of the high local bacteria density, may be sources of significant virus production relative to the free-living bacteria. Our production data and the previous demonstration of significant numbers of infected bacteria associated with particulate material (Proctor and Fuhrman, 1991) are consistent with this hypothesis.

Phage production rates may be used to calculate phage-induced bacteria mortality provided the burst size is known. Given the errors inherent in measuring both virus and bacteria production and the potential for high variability in burst size, the accuracy of mortality estimates derived in this way is uncertain. With this caveat in mind, we present mortality estimates (as a percent of bacteria production) for samples with significant virus production using the range of burst sizes (10-300) found by Heldal and Bratbak (1991) for marine bacteria (Table I). The viral mortality of bacteria at the nearshore and offshore stations ranged from as low as 0.97 % to as high as 40 % of bacteria production, depending on the assumed burst size. Using the mean burst size of

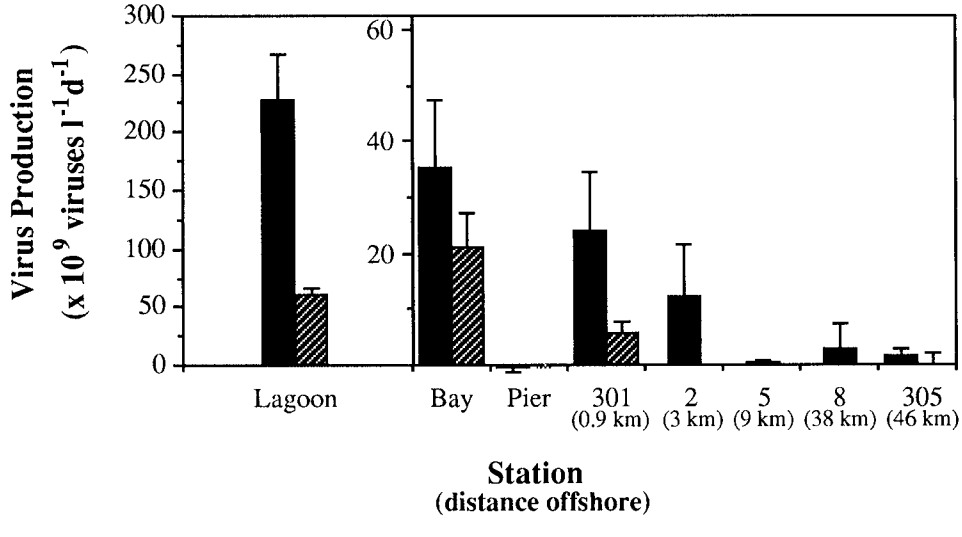

Fig. 4. Virus production rates in unfiltered (solid columns) and 0.8 μm prefiltered (striped columns) samples. Errors bars are 95 % confidence intervals. Note that there are no prefiltered samples for stations 2, 5 and 8.

50 reported by Heldal and Bratbak (1991), the mortality estimates for those stations ranged from 6 to 8 %. This is in the range of mortality implied by %-infected bacteria (4-18 %) in a Sargasso Sea sample (Proctor and Fuhrman, 1990) using the low and high conversion factors (Proctor and Fuhrman, 1991). Using the mean burst size, estimates of phage-induced mortality in the lagoon and bay samples were 146 % and 75 % of bacteria production, respectively. The *minimum* estimates for these sites (25 % and 12 %), respectively, strongly suggest that viruses were an important source of mortality in those samples.

Although the above data were not corrected for filtration losses, from data collected so far it appears that such losses are greatest in waters of highest productivity (Paul *et al.*, 1991; Steward *et al.*, 1992). Paul *et al.* (1991) speculate that this is a result of higher particle loads to which viruses may adsorb. Occlusion of pores by particulates could also contribute to the increased filtration losses observed in more productive waters. This implies that our highest rates may be more seriously underestimated than the lower rates. For instance, recoveries in highly productive waters could be 10 % or less while recovery in oligotrophic waters may approach 100 % (Paul *et al.*, 1991; Steward *et al.*, 1992). Given the apparent relationship between filtration recovery and trophic state of the environment, this would not change the general patterns of phage production we observed, but would imply that the trends are even more dramatic and that viral mortality of bacteria is higher than estimated, especially at the coastal stations.

We did not determine production of RNA viruses due to a large background in the RNA fraction which results when using the current protocol (Steward *et al.*, 1992). However, marine RNA viruses are probably rare; in 2 separate surveys, 1 of 32 and none of 75 marine phages examined had an RNA genome (Hidaka, 1975; Frank and Moebus, 1987, respectively). Even the viruses of eukaryotic algae, in contrast to those of land plants, seem to be predominantly DNA-containing (*e.g.*, Van Etten *et al.*, 1991), although the nucleic acid type of many isolates has not yet been determined. If the results of these surveys are generally indicative of their abundance, then RNA viruses would be expected to contribute little to total virus production in the sea. Nevertheless, direct testing of this hypothesis would be desirable.

Bacterial specific growth rate (μ_B) can affect virus production estimates by influencing the specific activity of intracellular nucleotide pools used for viral DNA synthesis. The relationship between μ_B and relative specific activity of bacteriophage has recently been modeled (Wikner *et al.*, submitted). In this model, two sources of nucleotides for viral DNA synthesis are considered: one in which viral DNA is synthesized from the host's free nucleotide pools and a second in which the host genome is degraded and used as a pool of nucleotides. The specific activity of the nucleotide pools at any given time depends both on the specific growth rate of the host and the sizes of the pools. According to the model, in cases where viruses rely only on the host's small pool of free nucleotides, replicating viruses quickly approach the specific activity of the medium. If viruses rely on nucleotides from the degraded host genome they have a specific activity close to that of the host DNA which lags behind that of the free nucleotide pool.

Applying this model as a correction to field data is not yet possible. To do so requires 1) knowledge of the source of nucleotides used by the actively replicating viruses and 2) measurement of the specific growth rate of those particular bacteria in the assemblage which are serving as hosts for phage production. Although experiments with marine phage isolates in culture (Wikner *et al.*, submitted) indicated that the host genome was a significant source of nucleotides for all three phages tested, experiments with many more isolates are required to test the generality of this conclusion. In addition, autoradiographic analysis of ^{3}H-TdR uptake by marine bacteria (Fuhrman and Azam, 1982) suggests that there are differences in μ_B within assemblages so that the mean specific growth rate measured for the assemblage may not reflect the growth rate of phage-producing bacteria.

Despite these uncertainties, we examined the potential effect of varying specific growth rates on our phage production estimates. We assumed 1) that, as a limiting case, all nucleotides were derived from the host genome and 2) that specific growth rate was uniform for the assemblage. The potential variation in specific activity due to differences in μ_B were then calculated using the equation ($SA_{rel} = 1 - \exp(-(at+b)\mu_B)$, where SA_{rel} is the specific activity relative to that expected with no isotope dilution, $a = 0.466$, $b = 0.6167$ and μ_B is in units of h^{-1} (Wikner *et al.*, submitted). The variable t is the time after addition of label and, for our analysis, was chosen to correspond to the approximate mid-point of the incubations (*i.e.*, 10 h). The virus production data were then reanalyzed after correction for the predicted isotope dilution at each station. Only the six stations which had rates of label incorporation significantly greater than zero were included in the correction and reanalysis.

The correction did not affect our general conclusions regarding onshore-offshore trends nor correlation of virus production with other biological variables. On the contrary, after the correction, correlation of virus production with bacteria abundance was more significant and the results were better fitted by a least-squares linear regression (Fig. 5). The most notable effect of the correction was to increase virus production rates by approximately an order of magnitude on average. The highest rate of measured production then becomes 2.5×10^{12} (lagoon) and the fastest turnover time becomes 1 h (Sta 301). These rates are closer to the very high rates implied by the decay experiments of Heldal and Bratbak (1991), but lead to seemingly unrealistic estimates of virus-induced bacteria mortality (*e.g.*, minimum of 272 % and 130 % for Peñasquitos Lagoon and Mission Bay, respectively). The order-of-magnitude increase in production rates implied by the model-based correction for specific growth rate should be viewed with reservation given the current uncertainty in the underlying assumptions. Despite the uncertainty regarding the magnitude of phage production (due both to questions regarding filtration efficiency and intracellular isotope dilution) our conclusions regarding the patterns of production and its correlation with other variables remain unchanged.

In conclusion, we have found that virus production in the ocean varies dramatically, but systematically, exhibiting strong correlations with bacteria abundance and chl *a*. The linear correlation and positive x-intercept of virus production *vs.* bacteria concentration raise the intriguing possibility that there is a threshold host density for

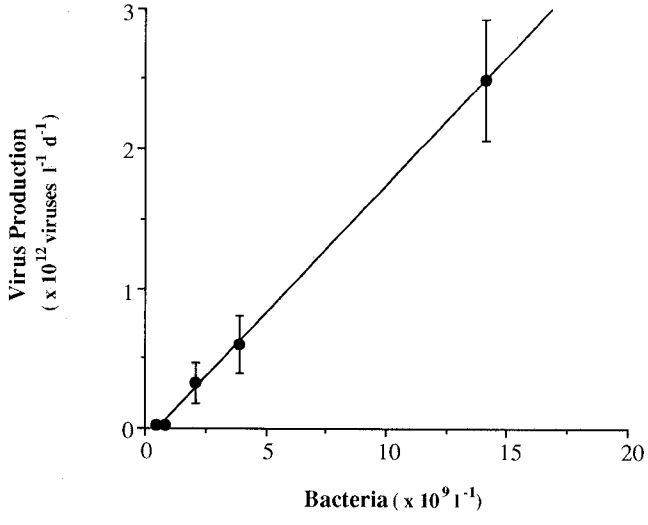

Fig. 5. Least-squares linear regression of virus production *vs.* bacteria abundance after correcting virus production for predicted isotope dilution (r^2 = 0.999, x intercept = 0.5 x 10^9 bacteria l^{-1}).

virus production in the sea. The apparent dependence on bacteria density further suggests that there may be micro-environments of high local bacteria density (such as colonized particles) which might support virus production even in oligotrophic waters. Given the importance of ocean margin productivity as a significant source of organic carbon for the deep sea (*e.g.*, Walsh, 1991), these results have implications for cross-shelf exchange and the spatiotemporal patterns of carbon fluxes in the ocean. Bacteriophage dynamics should, therefore, be considered in ocean-basin level studies of biogeochemical dynamics.

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