Marine Microbial Food Webs, 1992, **6** (2) : 57-78.

Estimation of Virus Production in the Sea: I. Method Development [1]

Grieg F. STEWARD [2], Johan WIKNER [2,3], David C. SMITH [2], William P. COCHLAN [2,4] and Farooq AZAM [2]

[2] *Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, La Jolla, California 92093-0202, USA.*

Present addresses:
[3] *Umeå Marine Scientific Center, Box 3124, S-903 04 Umeå, Sweden.*
[4] *Hancock Institute for Marine Studies, University of Southern California, Los Angeles, California 90089-0373, USA.*

Abstract

A method for estimating virus production in seawater was developed and tested in the field. The rates of virus production were determined on the basis of the rate of ^{32}P-orthophosphate ($^{32}P_i$) or ^{3}H-thymidine (^{3}H-TdR) incorporation specifically into the nucleic acid of the viruses released during the incubation period. The ^{3}H-TdR-incorporation method was designed to specifically detect the production of DNA-containing bacterial viruses. The $^{32}P_i$-incorporation method has the *potential* to measure the production of DNA and RNA viruses of bacteria, algae and protozoa. The $^{32}P_i$-incorporation protocols developed in this study, however, measure only the production of DNA viruses. The method provides sensitive detection of virus production with high precision and low blanks. Conversion factors relating label incorporated to viruses produced were derived empirically from culture and field measurements. Due to variable and potentially biased losses of viruses, the accuracy of the method could not be determined in the present study. Virus production measured by this method must be considered minimum estimates at present. This method should be of value in studies of the ecology of viruses in the sea and the roles of viruses in the ocean's biogeochemical dynamics.

Key words: Bacteriophage, Virus, Production, Method, Seawater.

[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.

0297-8148/92/02/57/22/$4.20/ © Institut océanographique

Résumé

Mesure de la production de virus en milieu marin - I. Mise au point d'une méthode

Une méthode de mesure de la production virale dans l'eau de mer a été mise au point et testée en conditions naturelles. Les taux de production virale ont été calculés d'après les taux d'incorporation spécifique du ^{32}P-orthophosphate ($^{32}P_i$) ou de la ^{3}H-thymidine (^{3}H-TdR) dans les acides nucléiques des virus libérés au cours de la période d'incubation. La méthode d'incorporation de la ^{3}H-TdR a été conçue pour détecter spécifiquement les bactériophages à ADN. La méthode d'incorporation du $^{32}P_i$ permet, elle, en principe de mesurer la production de virus, à ADN ou à ARN, des bactéries, des algues et des protistes. Cependant, le protocole d'incorporation du $^{32}P_i$ développé ici ne permet de dénombrer que les virus à ADN. La méthode présente une grande sensibilité, avec une précision élevée et un faible bruit de fond. Les facteurs de conversion reliant la quantité d'isotope incorporé à la production de virus ont été déduits expérimentalement de mesures à partir de cultures ou en mer. La validité de la méthode n'a pu être déterminée dans cette étude, du fait des pertes en virus, variables et potentiellement biaisées. La production de virus mesurée doit être considérée comme une estimation minimale. Cette méthode devrait être utile pour les études abordant l'écologie des virus en mer, ainsi que le rôle des virus dans la dynamique biogéochimique des océans.

Introduction

Recent studies have demonstrated high virus abundance in seawater (Bergh *et al.*, 1989; Børsheim *et al.*, 1990; Bratbak *et al.*, 1990; Heldal and Bratbak, 1991; Paul *et al.*, 1991; Wommack *et al.*, 1992; Cochlan *et al.*, in press). Together with the demonstration of a high incidence of virus-infected bacteria in the sea (Proctor and Fuhrman, 1990, 1991), these data suggest that viral attack may account for a large fraction of bacteria mortality in aquatic environments. Since bacteria play major roles in the ocean's food web structure and biogeochemistry, bacterial viruses (also known as bacteriophage or phage) may secondarily influence material cycling and food web dynamics. To assess the importance of viruses in these processes, there is a need for quantitative studies of population dynamics of viruses in the sea. This requires the development of methods for measuring virus production in aquatic samples as has been done for ecological studies of phytoplankton and bacteria production.

Information on the ecology of marine viruses has been limited due to methodological constraints. Plaque assay has been used to study the ecology of bacterial viruses in natural marine ecosystems (Ahrens, 1971; Moebus, 1991). However, plaque assay requires the isolation of a host for each virus to be assayed. This constrains such studies to a limited number of viruses making it difficult to infer their impact on the entire bacterial community. The dynamics of an entire viral assemblage can be monitored by transmission electron microscopy (TEM) (Bratbak *et al.*, 1990; Heldal and Bratbak, 1991), but TEM, like plaque assay, measures only net changes in abundance. Virus production may be underestimated due to the simultaneous decay of viruses.

Virus decay itself has been used as a proxy for virus production (Heldal and Bratbak, 1991). In that study, virus production was inhibited by removing or poisoning the host organisms. With a demonstration of a steady state for virus numbers in a

control sample, the disappearance of viruses in poisoned or host-free samples was assumed equal to the original rate of production. However, the direct measurement of gross production of viruses in mixed assemblages has not been possible for lack of a technique.

We have developed a radiotracer method for measuring virus production in seawater samples. This paper describes the method and its calibration. The following paper (Steward *et al.*, 1992) reports field measurements in Southern California coastal waters and discusses their ecological implications.

Approach

We adopted an approach similar in principle to the radiotracer method for measuring bacteria production (*e.g.* Fuhrman and Azam, 1980). Tracer uptake by the host secondarily labels the viruses, and tracer incorporation specifically into the nucleic acid of released viruses can be calibrated to yield estimates of virus production. This seemingly straight-forward approach, however, involves several technical challenges including the choice of tracer, isolation of labeled phage nucleic acid from the much larger pools of labeled contaminants and the development of a suitable calibration system.

• Choice of tracer

The choice of tracer depends on the goal of the study. ^{3}H-TdR and $^{32}P_i$ were considered. ^{3}H-TdR should be mainly taken up by bacteria (Fuhrman and Azam, 1982) and hence it can be used to specifically measure bacteriophage production, but its use will not include RNA phages. $^{32}P_i$ will be incorporated into both DNA and RNA but it will label phytoplankton and protozoa in addition to bacteria (Faust and Correll, 1976; Harrison *et al.*, 1977). We first developed a protocol based on $^{32}P_i$ incorporation into both DNA and RNA phages. Size-fractionation will allow rough separation of bacteria from larger organisms, although loss of particle-attached bacteria must be considered (Steward *et al.*, 1992). We have subsequently used ^{3}H-TdR to specifically measure DNA phage production.

• Isolation and quantification of viral nucleic acids

Discrimination of viral nucleic acids from various other labeled pools is a challenging task. The labeled contaminants include intact and lysed bacteria, subcellular organelles, and released macromolecular pools including dissolved nucleic acids. The contaminants can contain several orders of magnitude more label than the viruses and must be removed with extreme efficiency. Intact hosts and larger cell debris might be removed by filtration (0.2 μm; low-protein-binding filters to maximize virus passage). The filtration recovery should be determined to correct for loss of viruses. Dissolved labeled nucleic acids could be removed by nuclease digestion (viral nucleic acids would be protected from digestion because they are enclosed within the capsid proteins). Viruses must then be separated from the nucleotides produced by the

nuclease digestion, from unincorporated label, as well as from dissolved DNA which may have escaped digestion. We considered and attempted several methods (not presented) and decided on $(NH_4)_2SO_4$ precipitation of viruses (and, unavoidably, proteins as well) under conditions which do not precipitate nucleic acids (Ziai *et al.*, 1988).

Further separation of viral nucleic acid from remaining contaminants (mainly proteins) was necessary and employed two different approaches. 1) Chemical hydrolysis using hot NaOH to degrade RNA or hot TCA to degrade both DNA and RNA (Kennell, 1967 and references therein). 2) Specific degradation of nucleic acids by enzymes. Samples were treated to release nucleic acids from the viral capsids and the released nucleic acids degraded with nucleases. Loss of label from the macromolecular (cold TCA precipitable) fraction should be a measure of label in viral nucleic acid.

• Conversion factor

To derive virus production rates from tracer incorporation experiments requires a factor relating label incorporated to viruses produced. This may be calculated *a priori* based on the tracer specific activity and a virus's molar content of that molecule. In a field sample, however, the molar content may not be known. A more direct approach is to empirically relate incorporation of label in viral DNA to increases in virus numbers as determined by TEM. However, this requires a situation where virus production greatly exceeds virus decay so that the net production, as observed by TEM, will be close to the gross production. This situation may occur naturally or may be created by experimental manipulation (see methods).

In developing the virus production method, we started with a conservative multi-step approach to minimize error from contaminants. With subsequent use of the method, we have found some steps which are unecessary or which may be necessary only in some situations. These are mentioned or discussed as appropriate in the text.

Materials and Methods

Solutions and Media

Solutions were prepared with purified water (Milli-Q®, Millipore). Phosphate buffer pH 3.5 (PB) containing 250 mM $NaH_2PO_4 \cdot H_2O$ and 10 mM $MgCl_2 \cdot 6H_2O$ was adjusted to pH 3.5 with HCl then filtered through a 0.2 µm pore size membrane filters (HAWP, Millipore). Saturated $(NH_4)_2SO_4$ (approximately 4 M) was prepared by adding 767 g $(NH_4)_2SO_4$ to 1 liter PB and stirring overnight at room temperature (20-24 °C). Excess $(NH_4)_2SO_4$ and particulates were removed by filtration (as above). Fifty-percent saturated $(NH_4)_2SO_4$ was prepared by diluting the saturated solution 1:1 with PB. Stock solutions of DNase (Sigma, 1 U μl^{-1}), RNase (Sigma, 1 U μl^{-1}) and micrococcal nuclease (MNase, Worthington, 5 U μl^{-1}) were prepared in water and

stored at -20 °C. A stock solution containing Tris (hydroxymethyl) aminomethane (Tris; 100 mM), ethylene glycol-bis(ß-aminoethyl ether) N, N, N', N'-tetraacetic acid (EGTA; 10 mM), and ethylenediamine tetraacetic acid (EDTA; 10 mM) was adjusted to pH 8 (10X TEGED), autoclaved, stored at room temperature and diluted to the working solution of 1X TEGED as needed. A stock carrier solution was prepared according to Hollibaugh (1988) which contained sheared calf thymus DNA, bovine serum albumin (BSA), and *Torula* yeast RNA (all at 1 mg ml^{-1}), 1 mM TdR and 0.1 mM NaN_3. A second, similar carrier solution was prepared, but without BSA.

Collection of Field Samples

Surface water samples from Mission Bay, San Diego (32° 47' N, 117° 13' W) and the Scripps Institution of Oceanography pier in La Jolla (32° 53' N, 117° 15' W) were collected by hand in sterile, polycarbonate flasks. Samples were transported to the laboratory immediately after collection in a covered cooler to shield samples from sunlight. For samples transported from Mission Bay, the cooler was filled with water from the collection site to serve as a thermal buffer.

Virus Production

• Radiotracer incorporation

$^{32}P_i$ (8500-9120 Ci $mmol^{-1}$) and ^{3}H-TdR (methyl-^{3}H; 81 Ci $mmol^{-1}$) were obtained from New England Nuclear. Seawater samples were incubated with carrier-free $^{32}P_i$ (10 µCi ml^{-1} final concentration) or ^{3}H-TdR (20 nM final concentration) in 50 ml-capacity, sterile, polypropylene tubes in the dark at *in situ* temperature (± 2 °C) and sampled periodically.

• Determination of label specifically incorporated in viral DNA

A schematic diagram of the method showing the chemical and enzymatic hydrolysis procedures is shown in Figure 1.

CHEMICAL HYDROLYSIS. Samples (3 ml) were filtered (0.2 µm, Acrodisc® HT Tuffryn syringe filters, Gelman) to remove labeled, unlysed bacteria. In order to degrade dissolved nucleic acids, samples were incubated for one hour at room temperature (21-24 °C) with a mixture of DNase (1 U $µl^{-1}$), RNase (1 U $µl^{-1}$), and micrococcal nuclease (5 U $µl^{-1}$). The efficacy of nucleases in seawater was tested with purified phage lambda DNA as a substrate (not shown). (In initial field measurements, samples were then filtered a second time; this step has been eliminated as unnecessary.) Samples which were not immediately processed further were fixed with formalin (2 % final, v:v) and kept on ice.

Samples were split into three 900 µl aliquots and $(NH_4)_2SO_4$ precipitated to concentrate viruses as well as to remove unincorporated label and any residual, dissolved nucleic acids not degraded by the nucleases. BSA (50 µg ml^{-1} final) was

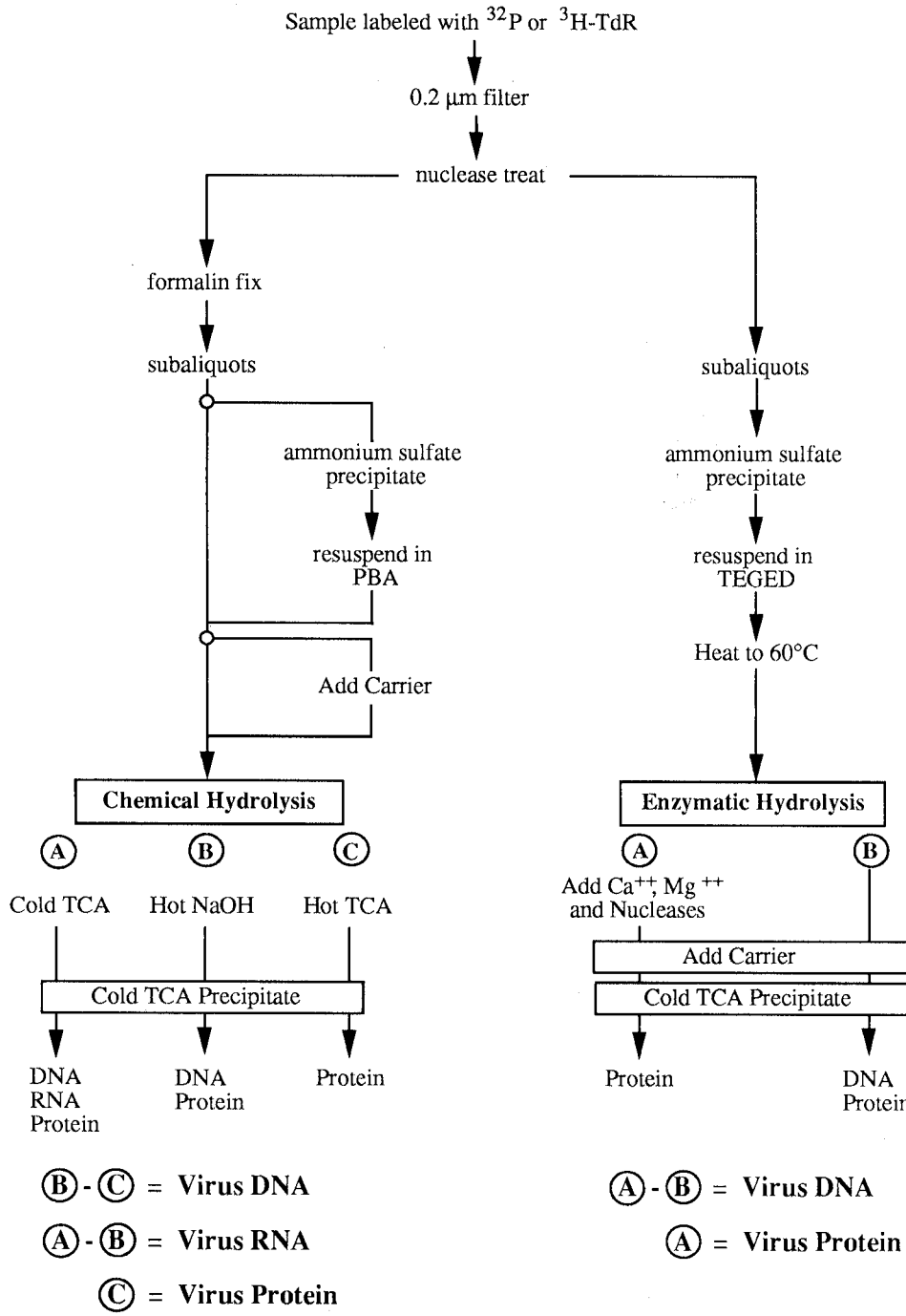

Fig. 1. Flow diagram of the virus production assay for ^{32}P and ^{3}H-TdR. Branch points (indicated by the open circles) indicate tested variations of the chemical hydrolysis protocol.

added as a coprecipitant prior to the addition of an equal volume of saturated $(NH_4)_2SO_4$. Viruses (and other proteins) were precipitated by incubation on ice for at least 20 min. Using a microcentrifuge kept in a 5 °C refrigerator, the precipitates were pelleted by centrifugation for 20 min at 16 000 x g then the supernatant was aspirated. (In initial field measurements, the pellet was washed by adding 50 % saturated $(NH_4)_2SO_4$, then repeating the ice-incubation, centrifugation (but only 10 min each) and aspiration steps; this wash has now been eliminated as unnecessary.)

The pellets from each set of three aliquots were resuspended in PB then treated by addition of either an equal volume of 10 % TCA (2 aliquots) or 1 N NaOH (1 aliquot). One of the aliquots with TCA was incubated at 0 °C (no hydrolysis), and the other at 100 °C (DNA and RNA hydrolyzed) for one hour. The aliquot with NaOH was incubated at 60 °C (RNA hydrolyzed) for one hour then cooled and acidified by addition of 200 µl of 100 % TCA. All aliquots were then incubated on ice at least 10 min and the precipitated macromolecules pelleted by centrifugation at 16 000 x g and 5 °C for 10 min. Supernatants were aspirated then the pellets washed by adding 5 % TCA, vortexing briefly, and repeating the ice incubation, centrifugation, and aspiration steps.

Samples were radioassayed by liquid scintillation spectrometry. To minimize self-absorption, ^{3}H-TdR-labeled pellets were solubilized with 10 µl of 1 N NaOH prior to adding scintillation cocktail (Ecoscint, National Diagnostics). To avoid chemiluminescence, these samples were then reacidified with 10 µl of 1 N HCl. Samples were counted in a Beckman model LS6000TA scintillation counter (with half-life correction for $^{32}P_i$-labeled samples). Activity in DNA, RNA and protein fractions was calculated according to Figure 1.

In an abbreviated version of the protocol, the $(NH_4)_2SO_4$ precipitation step was eliminated. Samples were filtered and nuclease treated as described. After nuclease treatment, three 900 µl aliquots were transferred to microcentrifuge tubes. NaOH (0.5 M final; from 10 M stock) or TCA (5 % final; from 100 % stock) was added directly to the aliquots. More concentrated stocks of NaOH and TCA were used to minimize the final volume. This was necessary to allow room for the subsequent addition of TCA to reacidify the NaOH-treated samples. Incubations for acid/base hydrolysis, reacidification of NaOH-treated samples, and subsequent TCA precipitation were as described above.

ENZYMATIC HYDROLYSIS. Samples were processed through the $(NH_4)_2SO_4$ precipitation step (above) except that they were not fixed with formalin (since they were to be treated with nuclease) and were processed immediately. The $(NH_4)_2SO_4$ precipitated pellets were resuspended in 500 µl of 1X TEGED to inhibit DNAse and micrococcal nuclease, and to destabilize viruses, by chelating Mg^{++} and Ca^{++} (Cunningham *et al.*, 1956; Adams, 1959; Junowicz and Spencer, 1973). To facilitate the release of nucleic acids from the viruses, samples were heated at 60 °C for 20-25 min (similar to Richardson *et al.*, 1988). Samples were cooled to room temperature and to one of each duplicate aliquots was added 1/10 volume of a solution containing 100 mM each of $CaCl_2$ and $MgCl_2$ (to obtain a 10-fold molar excess relative to the chelators) and 15 µl of nuclease mix. The other replicate served as a control. Aliquots

were incubated at room temperature for one hour. After addition of 50 µl of carrier solution (without BSA), an equal volume of 10 % TCA was added and the aliquots precipitated (TCA), washed, and prepared for scintillation counting as described for the chemical hydrolysis method. The radioactivity in viral DNA was calculated as the difference between DPM in the control and the enzymatically digested aliquots.

Effect of Carrier

In experiments to test the effect of carrier in the chemical hydrolysis procedure, 50 µl of carrier solution (without BSA) were added to the samples just prior to chemical hydrolysis. In the standard procedure, carrier solution without BSA was used because the samples already contained BSA from the $(NH_4)_2SO_4$ precipitation step. In the abbreviated version where samples are not first $(NH_4)_2SO_4$ precipitated, the carrier solution with BSA is used.

Filtration Recovery

Seawater samples collected from Scripps pier and Mission Bay were sampled for virus counts before and after filtration through a syringe filter as described for virus production. To minimize volume-related errors, only 3.3 ml of seawater was passed through a given filter (as for virus production). The mean virus counts of filtered and unfiltered, triplicate samples were compared to determine filtration efficiency.

Test of $^{32}P_i$ Incorporation Method in Culture

In $^{32}P_i$-incorporation experiments with two previously isolated phage-host systems (PHS), label in phage DNA was assayed by a procedure involving electrophoresis (details presented in Wikner *et al.*, submitted). Briefly, samples were processed as for the virus production assay (above) through the ammonium sulfate precipitation and wash steps. Pellets were then resuspended in electrophoresis buffer, combined with loading buffer, heated to 65 °C for 10 min, then the phage DNA separated from labeled contaminants by agarose gel (0.5 %) electrophoresis. Position of the phage DNA in the gels was determined using purified DNA from each isolate as markers which were visualized by staining with ethidium bromide. Following autoradiography (to demonstrate separation of the phage genome from labeled contaminants) phage DNA bands were excised from the gel and assayed by scintillation counting. Parallel samples were assayed by the chemical hydrolysis method. A comparison of the two methods is presented in this paper.

Conversion Factors

• Unmanipulated Sample

Using a sample from Scripps pier taken on 4 August 1992, field conversion factors were determined by relating change in virus abundance (determined by TEM) to

changes in $^{32}P_i$ or ^{3}H-TdR incorporated (determined by the chemical hydrolysis protocol without $(NH_4)_2SO_4$ precipitation or carrier addition). Virus and bacteria numbers (in parallel, unlabeled samples) and activity incorporated into viral DNA were determined at 0, 15.5 and 20.5 hours. Bacteria production was determined at 15.5 hours. The activity in viral DNA was corrected for precipitation loss by dividing by the mean precipitation efficiency factor (0.76) for three marine bacteriophage isolates (Wikner *et al.*, submitted). In a plot of moles P_i or TdR incorporated ml^{-1} *vs.* viruses ml^{-1}, the conversion factor is taken as the inverse of the slope of the regression line.

• Manipulated Sample

To minimize the underestimation inherent in a field calibration based on net changes in virus abundance, a second ^{3}H-TdR field calibration was performed with a natural bacteria population diluted into water with a reduced virus abundance. A reduction in the size of the initial pool of viruses should allow more sensitive detection of newly produced viruses by TEM. Seawater from Mission Bay was centrifuged at 2 500 x g for 15 minutes and the supernatant (low-speed) reserved. A portion of the supernatant was transferred to ultracentrifuge tubes and centrifuged at 55 000 rpm in a Beckman 70Ti rotor (207 000 x g average) for 2 hours. Reserved low-speed supernatant (2.7 ml) was diluted into the high-speed supernatant (130 ml) and split into unlabeled (for virus and bacteria counts) and labeled (with ^{3}H-TdR for virus production assay) aliquots in sterile 50 ml polypropylene tubes. Subsamples were taken at 0, 12, 18 and 36 hours to determine virus and bacteria counts and incorporation of ^{3}H-TdR in viral DNA by the chemical hydrolysis protocol (without $(NH_4)_2SO_4$ precipitation or carrier addition).

Other Measurements

The concentration of molybdate-reactive phosphorus was determined according to Parsons *et al.* (1984). Chlorophyll *a* (Chl *a*) was measured according to Holm-Hansen and Riemann (1978) in samples filtered on Whatman GF/F glass fiber filters. Bacteria production was measured by ^{3}H-TdR incorporation (Fuhrman and Azam, 1982) or by tritiated leucine (^{3}H-Leu) incorporation (Kirchman *et al.*, 1985; Simon and Azam, 1989) using a micro-assay method (Smith and Azam, 1992).

Samples for bacteria counts were fixed with borate-buffered formalin (2 % final). Samples were stained with diamidinophenylindole (DAPI, 1 μg ml^{-1} final) then treated by addition of Tween 80 (polyoxyethylene (20) sorbitan monoleate) and brief sonication to disperse particle-attached bacteria (Yoon and Rosson, 1990). After allowing at least 10 min for staining, samples were filtered onto black Nuclepore membranes (0.2 μm) and enumerated by epifluorescence microscopy (Porter and Feig, 1980). Viruses in glutaraldehyde-fixed seawater samples were counted by TEM as described in detail elsewhere (Cochlan *et al.*, in press as modified from Nomizu and Mizuike, 1986). Briefly, viruses were sedimented directly onto Formvar-coated, 200 mesh copper grids (Ted Pella) which were rendered hydrophilic by high-voltage

glow discharge (Hayat and Miller, 1990). Grids were stained with uranyl acetate (0.5 % w/v) and rinsed thrice in purified H_2O (Milli-Q, Millipore). Air-dried grids were examined by TEM at 40,000 to 82,000 X magnification and viruses identified on the basis of morphology and staining characteristics.

Results

Comparison of Protocols

Figures 2 and 3 show comparisons between enzymatic and chemical hydrolysis protocols for processing field samples. In Figure 2 the chemical hydrolysis protocol was tested with and without the ammonium sulfate precipitation step as well as with and without the addition of carrier.

• Effect of Carrier

Seawater from Scripps pier was incubated with $^{32}P_i$ or ^{3}H-TdR and assayed for label incorporation after 0 and 17 h (Fig. 2a-d). The effect of carrier in the chemical hydrolysis method was found to depend on whether the $(NH_4)_2SO_4$ precipitation step was included. For both $^{32}P_i$ and ^{3}H-TdR, chemical hydrolysis had the lowest and most consistent blanks when the $(NH_4)_2SO_4$ step and addition of carrier were included (Fig. 2a,b). When assaying tracer incorporation at T_{17h}, carrier addition yielded higher and more precise values if the $(NH_4)_2SO_4$ step was included. Without the $(NH_4)_2SO_4$ step, carrier addition had little effect on assay of $^{32}P_i$ incorporation (Fig. 2c), but it decreased the yield and increased the variability when assaying ^{3}H-TdR incorporation (Fig. 2d).

• Comparison of Enzymatic and Chemical Hydrolyses

In the same labeling experiment as above, additional samples taken at T_{17h} were processed by enzymatic hydrolysis for comparison with chemical hydrolysis. Since the enzymatic hydrolysis protocol includes $(NH_4)_2SO_4$ precipitation and carrier addition, the most valid comparison of its incorporation yield is with the chemical hydrolysis protocol which also includes these steps (Protocol B, Fig. 2). In this case the two methods agree very well (less than 17 % and 11 % discrepancy between the methods for $^{32}P_i$ and ^{3}H-TdR, respectively).

In a second experiment, the time courses of $^{32}P_i$ and ^{3}H-TDR incorporation into viral DNA were measured in parallel by enzymatic hydrolysis and by the chemical hydrolysis protocol (without $(NH_4)_2SO_4$ precipitation or carrier addition). In the case of ^{3}H-TdR, linear regression of DPM in viral DNA determined by the two methods results in a slope not significantly different from 1 (Fig. 3a). In the case of $^{32}P_i$ (Fig. 3b), chemical hydrolysis consistently yielded 31 % higher incorporation than the enzymatic hydrolysis (slope = 1.31). An additional sample was processed by the chemical hydrolysis protocol as it was originally developed and used in the field (Steward *et al.*, 1992). This includes a second filtration after the nuclease digest, a wash of the

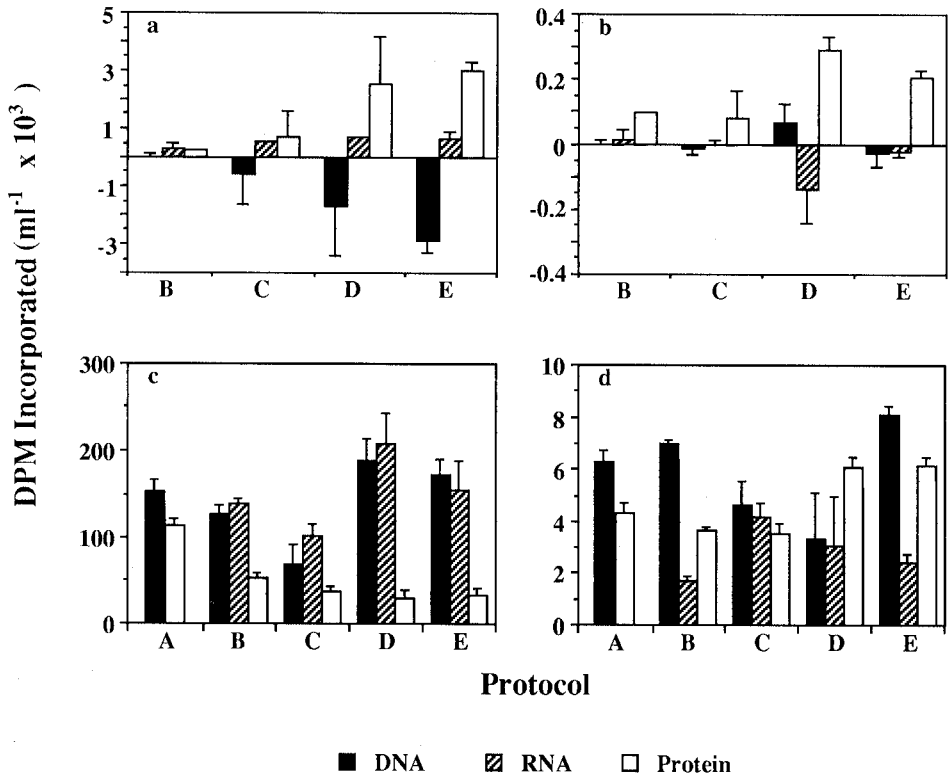

Fig. 2. Comparison of label incorporation into macromolecules as determined by the enzymatic (A) and chemical (B-E) hydrolysis protocols. Chemical hydrolysis was tested with and without the $(NH_4)_2SO_4$ precipitation step as well as with and without the addition of carrier (branch points in Fig. 1) as follows: B, with carrier, with $(NH_4)_2SO_4$; C, no carrier, with $(NH_4)_2SO_4$; D, with carrier, no $(NH_4)_2SO_4$; E, no carrier, no $(NH_4)_2SO_4$. Data are for labeling with ^{32}P (a,c) or ^3H-TdR (b,d) after 0 (a,b) and 17 (c,d) hours of labeling.

$(NH_4)_2SO_4$ precipitated protein pellet with 50 % saturated $(NH_4)_2SO_4$, and no addition of carrier. Processing by this method results in activity which is 40 % of the activity obtained by the abbreviated chemical hydrolysis protocol.

Test of $^{32}P_i$-Incorporation Method in Culture

Comparison of chemical hydrolysis and electrophoretic analysis of samples from $^{32}P_i$-incorporation experiments with cultures showed excellent agreement between the

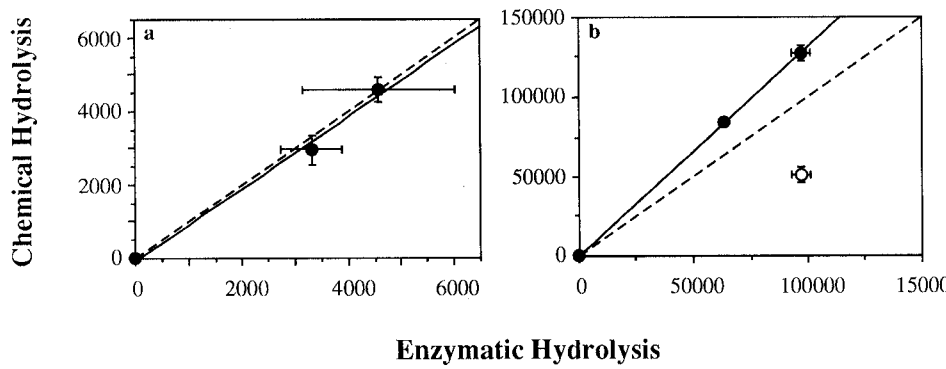

Fig. 3. Comparison of the DPM incorporated into virus DNA as assayed by the chemical (without the ammonium sulfate step) and enzymatic hydrolysis protocols in field samples with a) ^{3}H-TdR or b) ^{32}P-orthophosphate. An additional point in panel b (open circle), shows activity assayed by the chemical hydrolysis method as it was originally developed (including a second filtration after the nuclease treatment, ammonium sulfate precipitation, a wash of the ammonium sulfate precipitate and no addition of carrier). The dotted lines represent a slope of 1.

methods for PHS Ø21/50 (Fig. 4). In an experiment with a second isolate (Ø21/43) a high $^{32}P_i$ background was found in the DNA fraction when determined by chemical hydrolysis.

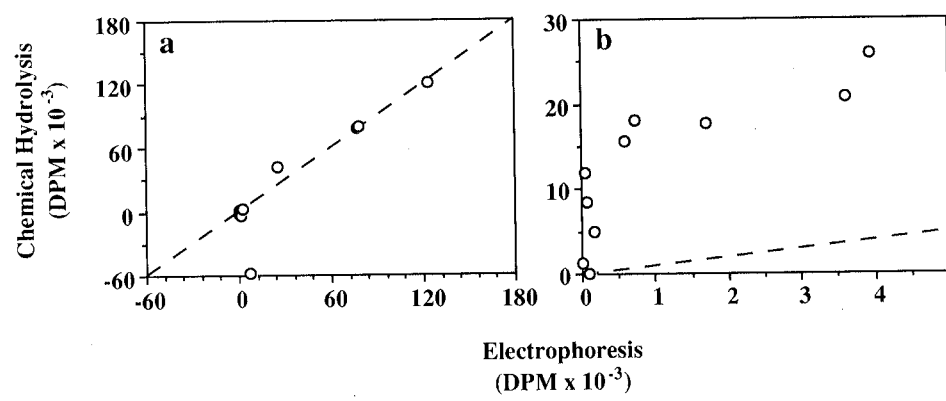

Fig. 4. Incorporation of ^{32}P in virus DNA as determined by acid/base hydrolysis *vs.* electrophoretic separation during time course labeling experiments in cultures of a) PHS Ø21/50 and b) PHS Ø21/43. The dotted line represents a slope of 1.

Filtration Recovery

Bacteria in the field samples were efficiently removed by filtration as assayed by colony forming units (CFUs). In experiments with a cultured host bacterium and with a natural bacteria assemblage, CFUs were reduced to below detection in both cases (*i.e.* < 0.05 % and < 0.025 % of the initial value, respectively). The filter was found not to be toxic to bacteria as determined by diluting seawater into filtered seawater and comparing observed and expected CFUs (not shown). Recoveries for natural viral assemblages (determined by TEM) ranged from 113 % to 9.5 % for three experiments with water from Scripps pier. Recovery of viruses in an experiment with water from Mission Bay was 6.9 % (Table I).

TABLE I. – Recovery of marine phages after filtration through a 0.22 µm Acrodisc®. Recovery of field assemblages was determined using TEM to count viruses before and after filtration. Recoveries are presented as the fraction of virus abundance in filtered relative to unfiltered samples.

Station	*Date*	*n*	*mean*	*s.e.*
Scripps pier	31 Mar 92	3	0.19	0.05
Scripps pier	1 Apr 92	3	0.095	0.04
Scripps pier	4 Aug 92	3	1.13	0.57
Mission Bay	1 Apr 92	3	0.068	0.03

Conversion Factors

• Unmanipulated Field Sample

Conversion factors for $^{32}P_i$ and ^{3}H-TdR incorporation were calculated from parallel experiments with water collected from the pier at Scripps Institution of Oceanography. The activity (2.22×10^{10} DPM l^{-1}) added to the $^{32}P_i$-labeled samples was divided by the measured phosphate concentration of the sample (5.5×10^{-8} M ± 6 %) to yield an effective specific activity of 4.0×10^{17} DPM mole-P_i^{-1}. The concentration of TdR in the seawater is assumed to be negligible so that the effective specific activity of ^{3}H-TdR was calculated from the stock (1.8×10^{17} DPM mole-TdR^{-1}). Values obtained for DPM ml^{-1} incorporated into viral DNA were corrected for 24 % loss during $(NH_4)_2SO_4$ precipitation (Wikner *et al.*, submitted). Filtration recovery in this sample was not distinguishable from 100 % (Table I), hence, no correction for loss was made. Dividing the corrected DPM ml^{-1} by the appropriate effective specific activity results in moles P_i or TdR incorporated ml^{-1}. In a plot of moles P_i or TdR incorporated ml^{-1} *vs.* viruses ml^{-1} (Fig. 5) the conversion factors are computed as the inverse of the slope of the regression lines. The resulting factors are 5.0×10^{19} viruses mole-P_i^{-1} and 6.17×10^{20} viruses mole-TdR $^{-1}$.

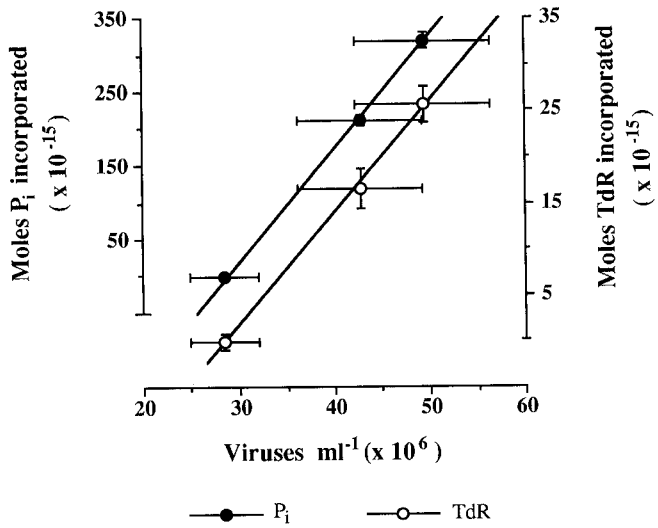

Fig. 5. Moles P_i (closed circles) and TdR (open circles) incorporated *vs.* virus abundance in a parallel labeling experiment of Scripps pier water. Virus production was measured by the short method using ^{32}P and ^{3}H-TdR. Virus abundance was determined by TEM of parallel unlabeled samples.

• Manipulated Field Sample

Changes in bacteria and virus numbers and moles TdR incorporated into bacterial or viral DNA are presented in Figure 6. Incorporation into viral DNA was corrected for precipitation loss as above, but it was not necessary to correct it for filtration loss since samples for both ^{3}H-TdR incorporation and TEM counts were 0.2 μm-filtered. One exception was the T_{0h} TEM sample which was not filtered so that the initial virus count is expected to be too high relative to the subsequent time points. In addition, after 18 h viruses showed an apparent decrease. Only periods where virus numbers are increasing can be used for determining a conversion factor. For these reasons, only the T_{12h} and T_{18h} points were used to calculate the virus conversion factor for this experiment. The net increase in viruses was divided by the net increase in moles TdR incorporated between 12 and 18 hours to obtain a conversion factor of 2×10^{21} viruses mole-TdR $^{-1}$ which is *ca.* 3 times higher than that obtained with the unmanipulated sample above. A conversion factor for bacterial incorporation of ^{3}H-TdR during the same time period (12 to 18 h) was calculated for comparison with the virus factor. Dividing moles TdR incorporated by change in bacteria numbers results in a factor of 1.1×10^{19} bacteria mole-TdR^{-1}.

Discussion

Our results demonstrate the feasibility of using ^{3}H-TdR as well as $^{32}P_i$ incorporation for measuring phage production in seawater samples. The choice of method (^{3}H-TdR or $^{32}P_i$) and of the protocol for specifically quantifying label

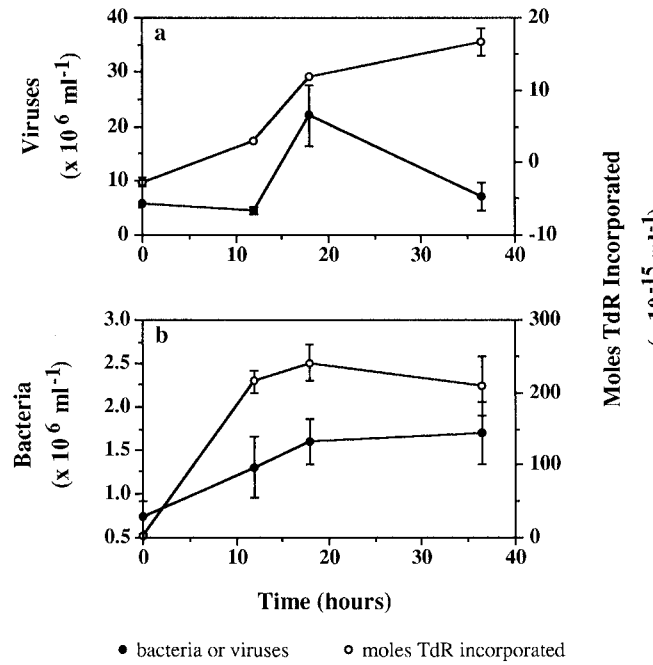

Fig. 6. Change in a) virus and b) bacteria abundance (closed circles) and incorporation of ^{3}H-TdR into virus and bacteria DNA (open circles) during incubation of a seawater culture prepared with water from Mission Bay.

incorporation into viral DNA (*i.e.*, with or without $(NH_4)_2SO_4$ precipitation; by chemical or enzymatic hydrolysis) depends on the goal of the study and requirements for precision and detection limit. These issues and the question of calibration are discussed below.

Choice of Isotope

The advantages of $^{32}P_i$ are that it has a high specific activity and that it is incorporated by both phytoplankton and bacteria into both DNA and RNA. However, further investigation is needed to establish that phytoplankton viruses are measured by this method. There are also potential errors due to the possible hydrolysis of polyphosphates (Harold, 1966) or phosphorous-protein bonds (Anthony and Spector, 1972 and references therein) during the chemical hydrolysis protocol. The latter phenomena may explain the high background in the RNA fraction which prevents assay of RNA virus production by the current chemical hydrolysis procedure. However, from the good agreement between the enzymatic and chemical hydrolysis protocols in the field, this does not seem to affect measurements of $^{32}P_i$ incorporation into DNA. It may be possible to measure RNA virus production by modification of the enzymatic hydrolysis protocol. Other disadvantages of $^{32}P_i$ are that it is a high-energy

beta emitter which requires careful shielding and that it requires measurement of the phosphate concentration of each sample assayed. The requirement for phosphate measurement introduces an uncertainty into production estimates since molybdate-reactive phosphate may include an unknown fraction of organically-bound phosphorous (*e.g.*, Chamberlain and Shapiro, 1969; Tarapchak *et al.*, 1982). The advantages of ^{3}H-TdR are that it is more specifically incorporated by heterotrophic bacteria into DNA and that it is a low energy beta emitter which does not require shielding. Possible disadvantages are that it cannot be used to measure RNA virus production and it may not be incorporated by all bacteria (*e.g.*, Pollard and Moriarty, 1984).

Protocol Performance

For accurate measurement of label incorporated into viral DNA, corrections should be made for virus losses (mainly due to filtration and $(NH_4)_2SO_4$ precipitation). Virus loss can be incorporated as part of the empirical calibration factor if it is found to be constant between samples. The filtration recovery for three isolated marine bacteriophages seeded into seawater was consistently high (91 % ± 8 recovery; Wikner *et al.*, submitted), but our results with natural assemblages showed much higher variability. This may be due, in part, to variable particle loads in environmental samples which result in low filtration efficiency by occlusion of filter pores and/or adsorption of viruses (Paul *et al.*, 1991). It may be necessary to check filtration recovery of representative samples for each new environment and, ideally, for each sample. A difficulty in correcting for filtration loss is the potential for biased loss for different types of viruses. The use of an average loss calculated for an assemblage is only valid as a correction if loss is random for all types of viruses. Electron microscopy may be used to assess the degree of bias by determining losses separately for different morphological groups in natural assemblages.

Virus recovery with $(NH_4)_2SO_4$ precipitation for three phage isolates was 76 % (Wikner *et al.*, submitted). This mean recovery was used to correct for precipitation losses in virus production assays. As with filtration, there is the potential for biased losses in the $(NH_4)_2SO_4$-precipitation step. If recoveries for filtration and/or precipitation were found to be severely biased, it would be difficult to properly correct samples for losses. However, uncorrected samples could still be used to obtain minimum estimates (Steward *et al.*, 1992). Further investigation of recovery is needed to adequately assess the accuracy of the method.

One aspect in which our method performs exceptionally well is in yielding low background for both $^{32}P_i$ and the ^{3}H-TdR. Mean background at T_0 was approximately 100 DPM for ^{3}H-TdR. Background for $^{32}P_i$ depended on the treatment. Cold TCA-treatment resulted in background counts of *ca.* 500 DPM and the hot NaOH- or TCA-treated samples had background counts *ca.* 200 DPM. Since counts in DNA are obtained by subtraction of counts obtained by different treatments, the blanks for incorporation were even lower. For ^{3}H-TdR, the DNA blank was as low as 0.3 DPM and for $^{32}P_i$ the DNA blank was as low as 29 DPM when the $(NH_4)_2SO_4$ step was

included (Fig. 2a,b). If the $(NH_4)_2SO_4$ precipitation step is omitted, the DNA blanks are larger, more variable and often negative. However, in this experiment, the absolute value of even the highest blanks was only *ca.* 2 % of the label incorporated at 17 h for both $^{32}P_i$ and ^{3}H-TdR (Fig. 2). Therefore, it may be necessary to include the $(NH_4)_2SO_4$ precipitation only in samples with very low virus production.

The low error between triplicate assays indicates the excellent precision obtainable with the $^{32}P_i$- and ^{3}H-TdR-incorporation methods (Fig. 2). Highest precision is obtained by including the $(NH_4)_2SO_4$ precipitation and using carrier nucleic acids (7 % and 2 % error for $^{32}P_i$ and ^{3}H-TdR, respectively). Excellent precision was also found for the chemical hydrolysis protocol without $(NH_4)_2SO_4$ precipitation when no carrier was added (11 % and 4 % for $^{32}P_i$ and ^{3}H-TdR, respectively). Our replicates were true replicates (separately labeled samples) not replicate assays of the same sample. Thus, both inter- and intra-sample variability were low. If some of the observed error is attributable to inter-sample variability, then the precision of the assay is even higher than indicated by the measurement errors.

The accuracy of the method depends both on the accuracy with which the protocol measures label in viral DNA and the accuracy of the conversion factor (the latter is discussed later). It is critical that the method should measure viral DNA uncontaminated by other labeled pools. We used an independent method (enzymatic hydrolysis) against which to compare the chemical hydrolysis method in field samples. The two methods agreed very well (less than 17 % and 11 % discrepancy between the methods for $^{32}P_i$ and ^{3}H-TdR, respectively). From the close agreement between the two methods we conclude that chemical hydrolysis can measure labeled DNA in field samples with reasonable accuracy. The specificity of nucleases for hydrolyzing nucleic acids makes the enzymatic method an excellent standard for comparison. It might, in fact, be used instead of chemical hydrolysis for routine processing of samples. In its present form, however, it does not represent a savings in effort compared to the chemical hydrolysis protocol and it is more expensive.

In tests with cultures (using electrophoretic purification of DNA as an independent standard method) chemical hydrolysis of $^{32}P_i$ incorporation samples has also shown the potential to measure labeled viral DNA with great accuracy (PHS Ø21/50; Fig. 4). However, the agreement between the methods was poor with PHS Ø21/43. The bacterium of PHS Ø21/43 produces large amounts of mucus in culture which could have adversely affected the assay by errors of the sort noted above (under *Choice of Isotope*). We note also that the culture experiments were conducted with bacteria concentrations two orders of magnitude higher than commonly found in the ocean. Any further studies with cultures should include a larger number of PHS and use more natural bacteria concentrations.

Conversion Factors

Empirical conversion factors are the most reliable basis for converting label incorporation in viral DNA into virus production. It would be important to determine the spatial and temporal variability of the conversion factors and to find out whether or

not it is necessary to determine a calibration factor for each environment and sampling period. The range of genome sizes of phages produced in seawater samples is potentially quite large (perhaps an order of magnitude) and this could make it inappropriate to apply a single conversion factor in all environments. Further, the intracellular isotope dilution of the tracer may vary (below). At present, we recommend measuring empirical calibration factors in each environment being studied. Only by determining conversion factors in a variety of environments will we be able to resolve, or at least to better formulate, this problem.

To examine the difference between conversion factors derived from cultures *vs.* field samples, we compared our field-derived factor (5×10^{19} viruses mole-P_i^{-1}) to one derived from $^{32}P_i$-incorporation experiments with three marine phage isolates in culture (Wikner *et al.*, submitted). For the culture experiments, a mean conversion factor of 3.8×10^{19} viruses mole-P_i^{-1} was derived from the observed phage specific activities and the phosphate content of the media. The culture-derived factor and the field-derived factor differ by less than 32 %. The culture-derived factor is expected to be underestimated since virus numbers were based on PFU rather than TEM counts (the plaquing efficiency for one isolate is approximately 70 %). If all three isolates are assumed to have plaquing efficiencies of 70 % then the field derived factor becomes 5.4×10^{19} which is in excellent agreement with the field factor.

Due to varying recoveries of the different protocols we have presented, it may be necessary to apply a correction if a conversion factor is obtained using a protocol different from that used to obtain the data being converted. For instance, the ^{32}P conversion factor for the unmanipulated sample (5×10^{19} viruses mol^{-1}) was determined by the chemical hydrolysis procedure without $(NH_4)_2SO_4$ precipitation and no carrier addition. This factor will underestimate virus production if applied to data obtained by the hydrolysis procedure as it was originally developed and first used in the field (Steward *et al.*, 1992). The protocol used in that field study has a yield which is 40 % of that used to determine the conversion factor (Fig. 3b). For converting the data in that study, the conversion factor was, therefore, accordingly corrected to 1.25×10^{20} viruses mol^{-1}.

Whether the conversion factors provide an accurate estimate of virus production depends primarily upon the accuracy with which gross virus production is measured by TEM. As mentioned, TEM counts provide net virus production which will underestimate the gross production to a greater or lesser degree depending on the relative magnitude of simultaneous virus decay. If viruses are counted accurately, and assuming significant virus turnover, the conversion factors should be underestimated (*i.e.*, fewer viruses than expected for the label incorporated) and thus provide underestimates of virus production. Manipulating samples to enhance virus production (but not decay) is recommended to minimize the underestimation.

Intracellular isotope dilution can also influence conversion facors. We refer to *intracellular* isotope dilution, because a correction for extracellular isotope dilution of $^{32}P_i$ is made by measuring the phosphate concentration (however, see below) and that for ^{3}H-TdR is assumed to be negligible. Isotope dilution will be included in an empirically determined factor, but errors could result in applying a factor if the

intracellular isotope dilution varies in different environments. Based on experiments with three marine phage-host systems, Wikner *et al.* (submitted) have modeled the effect of intracellular isotope dilution of $^{32}P_i$ on the incorporation of tracer into phage DNA. The model predicts that the degree of isotope dilution will depend on whether phage rely on the host genome as a source of nucleotides. If so, the specific activity is expected to increase slowly with time at a rate dependent on the specific growth rate of the host. The model can be applied to ^{3}H-TdR as well, but isotope dilution for TdR could be even higher due to *de novo* synthesis of TdR contributing to the unlabeled pool. This may explain why the apparent incorporation of P_i relative to TdR (ratio of the slopes in Fig. 5) is three times higher than the expected ratio of 4. The expected ratio assumes an average G+C content of 50 % for phage genomes in the assemblage, which is the average of the range (27 to 72 %) for almost all bacterial viruses which have been analyzed (Ackermann and DuBow, 1987). Variation in the actual mean G+C content of natural viral assemblages could also contribute to deviations from the expected P_i to TdR ratio. Another possible explanation for such deviation is error in determining P_i concentration. P_i incorporation could have been overestimated due to overestimation of biologically-available P_i by the molybdenum-blue assay (see *Choice of Isotope* discussion).

Our conversion factor (unmanipulated sample) for the ^{3}H-TdR incorporation method (6.17×10^{20} viruses mol^{-1}) can be compared with the conversion factor for bacteria (2×10^{18} bacteria mol^{-1}; Fuhrman and Azam, 1982). The ratio of the conversion factors is ~ 300. If we assume that phage and bacteria DNA syntheses involve equal isotope dilution then the ratio of bacteria genome to phage genome should also be ~ 300. If we assume the DNA content of marine bacteria to be 3 fg (Fuhrman and Azam, 1982) or 1.8×10^{9} Da, then the average genome of the phage produced in our sample would have been 6×10^{6} Da or the equivalent of 9 kbp. This is roughly 20 % of the median size of all bacterial viruses for which the genome size has been determined (2.9×10^{7} Da; calculated from data in Ackermann and DuBow, 1987).

A better comparison is between virus and bacteria factors determined by similar methods in the same sample during the same time period. In the manipulated sample (Fig. 6), a bacteria conversion factor of 3.4×10^{18} bacteria mol^{-1} is calculated for the period between 0 and 12 hours. This is not far from the bacteria factor commonly used (above). However, for the period during which the virus factor was calculated (12 to 18 h), the bacteria factor increases to 1.1×10^{19} bacteria mol^{-1}. The ratio of the bacteria and virus conversion factors for this period is about 180. By the same argument followed above, the implied virus genome size is approximately 15 kbp. This is roughly 30 % of the median phage genome size. In either case, it appears likely that either isotope dilution is somewhat higher for incorporation into virus than bacteria DNA or that, in these samples, the genomes of viruses produced during the incubation were smaller than average. The isotope dilution model presented in Wikner *et al.* (submitted) is consistent with the former conclusion.

Direct estimation of isotope dilution in field samples may be possible by combining field calibration experiments, as described above, with electrophoretic and autoradiographic analyses of purified viral DNA from parallel samples. This should allow direct measurement of both the size and activity of the genomes of actively

replicating viruses which can then be compared with the implied genome size from the conversion factor. The difference should reflect the isotope dilution. Comparison of expected and observed activity in each distinguishable band may reveal the variability in isotope dilution between species. Such electrophoretic analysis has been used sucessfully with single phage-host systems in culture (Wikner *et al.*, submitted), but may be more challenging with field samples. More extensive DNA purification will be necessary prior to electrophoresis, since field samples will contain numerous viral genomes of unknown sizes, some of which could be obscured by co-migration with other labeled contaminants.

The detection limit of the assay depends primarily on the precision of the replicates, which may vary. In practice, the lowest significant rate for $^{32}P_i$ incorporation into viral DNA which we have measured in a field sample is 9 DPM h^{-1}. This was converted to a production rate of 6.1 x 10^5 viruses ml^{-1} d^{-1} (Steward *et al.*, 1992). This results in a turnover time of 30 d for the virus pool in that sample.

Method Application

In consideration of the above points, we recommend that *in situ* bacteriophage production be measured by the ^{3}H-TdR incorporation method (without $(NH_4)_2SO_4$ precipitation or carrier addition), except where production is very low. In samples with very low productivity, the $(NH_4)_2SO_4$ step and carrier should be included. Measuring virus production by $^{32}P_i$ incorporation may prove useful in some situations (*e.g.*, measuring production of both bacteriophage and phytoplankton viruses and including both DNA- and RNA-containing viruses), but exploiting its full potential will require further method development. With the current limitation of $^{32}P_i$ to DNA virus measurements, ^{3}H-TdR may be preferable to $^{32}P_i$ for most applications at present.

In conclusion, we have presented results demonstrating the feasibility of estimating virus production in aquatic samples using a radiotracer approach. Although further investigation is needed to establish its accuracy, the method we have developed provides a precise, sensitive means of estimating bacteriophage production in seawater and is a promising new tool for studying phage ecology in the ocean. Development of such techniques is critical to eliminating the technical barriers which impede the study of viruses in marine microbial food webs and their impact on the ocean's biogeochemistry.

References

Ackermann H.-W. and DuBow M.S., 1987. Viruses of prokaryotes. Volume II: Natural groups of bacteriophages. Boca Raton, CRC Press, Inc., 242 p.

Adams M., 1959. Bacteriophages. New York, Interscience, XVIII + 592 p.

Ahrens R., 1971. Untersuchungen zur Verbreitung von Phagen der Gattung *Agrobacterium* in der Ostsee. *Kieler Meeresforsch.*, **27**, 102-112.

Anthony R.S. and Spector L.B., 1972. Phosphorylated acetate kinase. *J. Biol. Chem.*, **247**, 2120-2125.

Bergh Ø., Børsheim K.Y., Bratbak G. and Heldal M., 1989. High abundance of viruses found in aquatic environments. *Nature*, **340**, 467-468.

Børsheim K., Bratbak G. and Heldal M., 1990. Enumeration and biomass estimation of planktonic bacteria and viruses by transmission electron microscopy. *Appl. Environ. Microbiol.*, **56**, 352-356.

Bratbak G., Heldal M., Norland S. and Thingstad T.F., 1990. Viruses as partners in spring bloom microbial trophodynamics. *Appl. Environ. Microbiol.*, **56**, 1400-1405.

Chamberlain W. and Shapiro J., 1969. On the biological significance of phosphate analysis; comparison of standard and new methods with a bioassay. *Limnol. Oceanogr.*, **14**, 921-927.

Cochlan W.P., Wikner J., Steward G.F., Smith D.C. and Azam F., in press. Spatial distribution of viruses, bacteria, and chlorophyll-*a* in neritic, oceanic and estuarine environments. *Mar. Ecol. Prog. Ser.*, **92**.

Cunningham L., Catlin B.W. and Privat de Garilhe M., 1956. A deoxyribonuclease of *Micrococcus pyrogenes*. *J. Am. Chem. Soc.*, **78**, 4642.

Faust M.A. and Correll D.L., 1976. Comparison of bacterial and algal utilization of orthophosphate in an estuarine environment. *Mar. Biol.*, **34**, 15-162.

Fuhrman J.A. and Azam F., 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica and California. *Appl. Environ. Microbiol.*, **39**, 1085-1095.

Fuhrman J.A. and Azam F., 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Mar. Biol.*, **66**, 109-120.

Harold F.M., 1966. Inorganic polyphosphates in biology: structure, metabolism, and function. *Bact. Rev.*, **30**, 772-794.

Harrison W.G., Azam F., Renger E.H. and Eppley R.W., 1977. Some experiments on phosphate assimilation by coastal marine plankton. *Mar. Biol.* **40**, 9-18.

Hayat M.A. and Miller S.E., 1990. Negative Staining. New York, McGraw-Hill Publishing Company, 253 p.

Heldal M. and Bratbak G., 1991. Production and decay of viruses in aquatic environments. *Mar. Ecol. Prog. Ser.*, **72**, 205-212.

Hollibaugh J.T., 1988. Limitations of the [^{3}H]thymidine method for estimating bacterial productivity due to thymidine metabolism. *Mar. Ecol. Prog. Ser.*, **43**, 19-30.

Holm-Hansen O. and Riemann B., 1978. Chlorophyll a determination; Improvements in methodology. *Oikos*, **30**: 438-447.

Junowicz E. and Spencer J.H., 1973. Studies on bovine pancreatic deoxyribonuclease. I. General properties and activation with different bivalent metals. *Biochem. Biophys. Acta*, **312**, 72-84.

Kennell D., 1967. Use of filters to separate radioactivity in RNA, DNA and protein, 686-693. *In: Methods in Enzymology*. Academic Press.

Kirchman D., K'Nees E. and Hodson R., 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. *Appl. Environ. Microbiol.*, **49**, 599-607.

Moebus K., 1991. Preliminary observations on the concentration of marine bacteriophages in the water around Helgoland. *Helgoländer Meeresunters.*, **45**, 411-422.

Nomizu T., Mizuike A., 1986. Electron microscopy of submicron particles in natural waters-specimen preparation by centrifugation. *Mikrochim. Acta*, **1**, 65-72.

Parsons T., Maita Y. and Lalli C., 1984. A Manual of Chemical and Biological Methods for Seawater Analysis. Oxford, Pergamon Press, 173 p.

Paul J.H., Jiang S.C. and Rose J.B., 1991. Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration. *Appl. Environ. Microbiol.*, **57**, 2197-2204.

Pollard P.C. and Moriarty D.J.W., 1984. Validity of the tritiated thymidine method for estimating bacterial growth rates: Measurement of isotope dilution during DNA synthesis. *Appl. Environ. Microbiol.*, **48**, 1076-1083.

Porter K.G. and Feig Y.S., 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr.*, **25**, 943-948.

Proctor L.M. and Fuhrman J.A., 1990. Viral mortality of marine bacteria and cyanobacteria. *Nature*, **343**, 60-62.

Proctor L.M. and Fuhrman J.A., 1991. Roles of viral infection in organic particle flux. *Mar. Ecol. Prog. Ser.*, **69**, 133-142.

Richardson K.J., Margolin A.B. and Gerba C.P., 1988. A novel method for liberating viral nucleic acid for assay of water samples with cDNA probes. *J. Virol. Methods*, **22**, 13-21.

Simon M. and Azam F., 1989. Protein content and protein synthesis rates of planktonic marine bacteria. *Mar. Ecol. Prog. Ser.*, **51**, 201-213.

Smith D.C. and Azam F., 1992. A simple, economical method for measuring bacterial protein synthesis rates in seawater using ^{3}H-leucine. *Mar. Microb. Food Webs*, **6** (2), this issue.

Steward G.F., Wikner J., Cochlan W.P., Smith D.C. and Azam F., 1992. Estimation of virus production in the sea: II. Field results. *Mar. Microb. Food Webs*, **6** (2), this issue.

Tarapchak S.J., Bigelow S.M. and Rubitschun, C., 1982. Overestimation of orthophosphorus concentrations in surface waters of southern Lake Michigan: effects of acid and ammonium molybdate. *Can. J. Fish. Aquat. Sci.*, **39**, 296-304.

Wikner J., Vallino J.J., Steward G.F., Smith D.C. and Azam F., submitted. Nucleic acid of host bacteria as a major source of nucleotides for three marine bacteriophages.

Wommack K.E., Hill R.T., Kessel M., Russek-Cohen E. and Colwell R.R., 1992. Distribution of viruses in the Chesapeake Bay. *Appl. Environ. Microbiol.*, **58**, 2965-2970.

Yoon W.B. and Rosson R.A., 1990. Improved method of enumeration of attached bacteria for study of fluctuation in the abundance of attached and free-living bacteria in response to diel variation in seawater turbidity. *Appl. Environ. Microbiol.*, **56**, 595-600.

Ziai M.R., Giordano A., Armandola E. and Ferrone S., 1988. Purification by ammonium sulfate precipitation of bacteriophage λgt11 DNA for restriction analysis of cloned cDNA inserts. *Anal. Biochem.*, **171**, 192-196.

Errata

In the Materials and Methods Section under the subheading CHEMICAL HYDROLYSIS, please note the following clarifications:

° Page 61: For the nuclease treatment of samples, the final concentrations were 5 U ml^{-1} each of DNase and RNase and 25 U ml^{-1} of micrococcal nuclease.

° Page 63: The volume of PB used to resuspend pellets following $(NH_4)_2SO4$ precipitation was not indicated. The volume used was as 500 μl.