

# Simultaneous estimation of viral lysis and protozoan grazing on bacterial mortality using a modified virus-dilution method

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**ABSTRACT:** A modified dilution method designed to simultaneously estimate bacterial mortality due to viral infection and protozoan grazing was developed and compared with the standard dilution protocol. Various fractions of original seawater (non-filtered) and 1.0  $\mu\text{m}$ -filtered seawater (grazer-free) collected from coastal waters in Hokkaido, Japan, were diluted with 10 kDa filtered seawater (virus-free) to set up 4 gradients of predator–prey interaction, and monitored every 12 h for bacterial abundance during a 48 h incubation. In more diluted fractions, bacterial abundance increased rapidly, and a good linear fit with a negative slope (as mortality) was found between the apparent growth rate and fractions of original water. The resulting slopes of the regression in samples prepared from the original seawater were significantly higher than grazer-free samples, which denotes both viral lysis and protozoan grazing in the former, and only viral lysis in the latter. Ranges of specific lytic and grazing rate were 0.53 to 0.98 and 0.05 to 0.13  $\text{d}^{-1}$ , respectively, and lytic pressure accounted for 87 to 91 % of the total mortality. Comparison of our method (using virus-free diluent) with the standard dilution protocol (using 0.2  $\mu\text{m}$  diluent) showed significant differences in slope and  $y$ -intercept (potential growth rate without mortality) found at 0.26  $\text{d}^{-1}$  and 0.88  $\text{d}^{-1}$ , respectively. The above results suggest that using the standard dilution protocol might underestimate instantaneous growth rate, particularly in environments where lytic pressure is relatively high.

**KEY WORDS:** Bacterial mortality · Virus infection · Protozoan grazing

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## INTRODUCTION

It is well recognized that both viral lysis and protozoan grazing are important agents of bacterial mortality in pelagic systems. Whether bacterial cells are lysed or grazed has different ecological and biogeochemical implications because of the different fates of bacterial production in microbial food webs. Grazed production is transferred to higher trophic levels to some extent, and eventually lost from the euphotic zone in the form of sinking particles (Azam et al. 1983, Pace 1988). Lysed bacterial cells, however, release dissolved organic carbon (DOC), in a closed loop in which bacterial carbon is recycled as bacterial production in the euphotic zone (Proctor & Fuhrman 1991, Bratbak et al. 1993, Wilhelm et al. 2002). In addition, macro- and

micronutrients are supplied to bacteria and phytoplankton through lysis (Middelboe et al. 1996, Gobler et al. 1997, Noble & Fuhrman 1999). It is therefore important to determine the magnitude of grazing and lytic pressures on bacterial production to better understand the biogeochemical cycles and energy flows via microbial food webs.

Over the past decade, evaluations of the relative importance of lytic and grazing pressures have been studied in micro- and mesocosms and in the field. Several studies in coastal environments showed that levels of lytic and grazing pressure are similar (Fuhrman & Noble 1995, Steward et al. 1996, Almeida et al. 2001). Guixa-Boixereu et al. (1999) suggested that viruses are the dominant agents of bacterial mortality especially in a non-steady-state situation such as in the event of

phytoplankton bloom. Weinbauer & Höfle (1998) found that lytic mortality is strongly dominant in the anaerobic hypolimnion layer of Lake Plüsee, Germany, where protists are scarce due to oxygen depletion. The ecological role and evolutionary biology of prokaryotic viruses have been reviewed by Weinbauer (2004) and Weinbauer & Rassoulzadegan (2004).

In the literature, there is no single technique available to simultaneously estimate grazing pressure and lytic mortality. To estimate grazing mortality, one technique involves monitoring the time-course change in bacterial abundance either through size fractionation (Wright & Coffin 1984, Weisse & Scheffel-Möser 1991), dilution (Tremaine & Mills 1987), or metabolic inhibitors (Newell et al. 1983, Fuhrman & McManus 1984). Another technique quantifies labeled analogues incorporated or ingested into the protozoan food vacuoles or cytoplasm, such as fluorescent-labeled beads or bacterial cells (FLB, FLBC; Børsheim 1984, Sherr et al. 1987), or radio-labeled bacterial cells (Hollibaugh et al. 1980, Nygaard & Hessen 1990).

Lytic mortality estimations are based on the burst-size or the number of viruses released from one lysed bacterium) examined with transmission electron microscopy (TEM), or derived from viral production rate (Heldal & Bratbak 1991, Wilhelm et al. 2002), [<sup>3</sup>H]-thymidine incorporation (Steward et al. 1992), or incubation with fluorescently labeled viruses (Noble & Fuhrman 2000). It can also be determined using the frequency of infected bacterial cells which can be converted from the frequency of visibly infected bacteria (Proctor et al. 1993, Binder 1999).

The dilution technique described by Landry & Hassett (1982) was initially used to estimate grazing pressure of micro-zooplankton on phytoplankton, and later applied to protozoan bacterivory (Tremaine & Mills 1987). The technique involves progressive dilution of whole microplankton communities with particle-free seawater diluents (0.2 µm filter pore size) to create gradients in predator–prey encounter rates, wherein changes in the abundance of prey are closely monitored during a 12 to 48 h incubation period. In the standard dilution protocol, the net growth ( $k$ ) of bacteria in the incubated sample is expressed as the difference between instantaneous growth ( $\mu$ ) and mortality loss ( $n$ ). It is assumed however that  $\mu$  remains constant with the level of dilution ( $D$ ) in which grazing mortality ( $m_g$ ) is considered to be dependent and can be mathematically expressed as:

$$k = \mu - m_g \times D \quad (1)$$

However, lytic pressure is not accounted for in the above expression resulting in underestimation of  $\mu$ , because 0.2 µm filtered diluent retains most of the viruses. Murray & Jackson (1992) define lytic mortality

as the contact rate (CR) of viruses with bacteria, which depends on abundances and encounter rates, and is calculated as:

$$CR = (Sh \times 2\pi \times d \times DF) \times B \times V \quad (2)$$

where  $Sh$  is the Sherwood number (dimensionless),  $d$  is the average diameter of bacterial cells,  $D$  is the diffusivity of viruses, and  $B$  and  $V$  are the abundance of bacteria and viruses, respectively. Specific CR is calculated from the correction of CR for bacterial abundance to estimate the number of contacts per bacterium with time (Wilhelm et al. 1998). When only bacterial abundance is diluted with 0.2 µm diluents, specific CR is constant (as is lytic pressure), whereas grazing pressure is reduced. Therefore, in order to account for the mortality rates due to lysis ( $m_v$ ), Eq. (1) can be rewritten as:

$$k = (\mu - m_v) - m_g \times D \quad (3)$$

Nonetheless, if virus-free seawater is used as a diluent, the net growth rate of bacteria ( $k_1$ ) should now be calculated as the difference between instantaneous growth rate and mortality due to lysis and grazing which is a function of  $D$ :

$$k_1 = \mu - (m_v + m_g) \times D \quad (4)$$

The net growth of bacteria without protozoan grazing ( $k_2$ ) can be measured separately from a paralleled dilution series in which protozoa are eliminated beforehand as given by:

$$k_2 = \mu - m_v \times D \quad (5)$$

Grazing pressure is finally calculated from the difference of  $k_1$  and  $k_2$ .

Thus, the objective of this study is to develop a modified dilution protocol designed to estimate both lytic and grazing pressures simultaneously, and to verify influence of the absence of lytic parameter in the standard protocol and compare that with the technique in this study.

## MATERIALS AND METHODS

**Sample collection.** Two oceanographic research cruises in Stns T31, T35, and E9 (Table 1, Fig. 1) took place during the 53rd (May 6 to 13, 2006) and 59th (July 11 to 22, 2006) cruises of the RV 'Ushio-Maru' of Hokkaido University. Temperature and salinity measurements were monitored using a CTD 911 system (Seabird). In each station, seawater samples for various experiments were collected at 2 m depth using acid-cleaned, teflon-coated 10 l lever-action Niskin bottles attached to a Kevlar line. Water samples (2 l) were carefully transferred into acid-cleaned polycarbonate Nal-

Table 1. Stations and their positions and date of sampling

Station	Position	Date
T31	42° 39.2' N, 143° 41.2' E	May 8, 2006 July 13, 2006
T35	42° 26.0' N, 143° 59.5' E	July 12, 2006
E9	45° 19.9' N, 143° 39.9' E	July 16, 2006

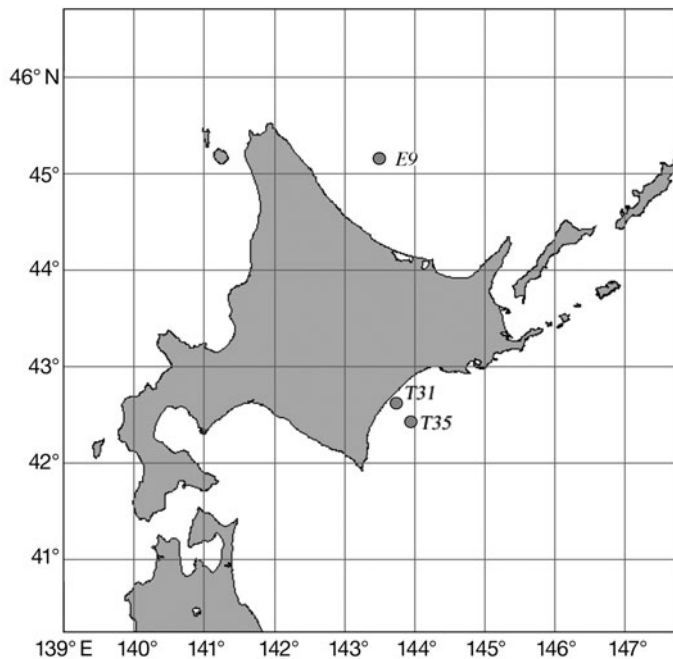


Fig. 1. Hokkaido, Japan: locations of sampling points

gene bottles and processed immediately as follows. Samples for enumeration of microbial abundances were dispensed in autoclave-sterilized 50 ml polypropylene tubes. For bacteria and viral abundance determination, aliquots of 2 to 5 ml of samples were fixed with electron microscopy-grade glutaraldehyde at 2% final concentration and immediately frozen in liquid nitrogen while still onboard, and then stored at  $-80^{\circ}\text{C}$  until preparation of sample slides. Samples for heterotrophic nanoflagellate (HNF) abundance was also fixed with glutaraldehyde and stored at  $4^{\circ}\text{C}$ , and sample slide preparation was made 3 to 4 d after collection.

Samples for DOC analysis were dispensed from the Niskin bottles into borosilicate glass bottles through an in-line filtration cartridge (Advantec) after which 20 ml subsamples were transferred to glass ampoules using glass pipettes, flame-sealed and stored frozen at  $-25^{\circ}\text{C}$  until analysis. All glasswares and GF/F filters used were precombusted at  $450^{\circ}\text{C}$  for 5 h prior to use. DOC concentration was determined by high-temperature

catalytic oxidation with a Shimadzu TOC 5000A (Cauwet 1999). Chlorophyll *a* (chl *a*) concentration was determined fluorometrically after extraction in *N,N*-dimethylformamide using a Hitachi F2000 fluorescence spectrophotometer (Suzuki & Ishimaru 1990).

**Dilution experiments.** Filter holders and incubation bottles were acid-cleaned with 10% HCl and rigorously rinsed with Milli-Q water. After pre-filtration of sample through 47 mm Nuclepore filter (type PC, pore size of  $0.2\ \mu\text{m}$ ), virus-free diluents were prepared by  $\text{N}_2$  pressurized tangential filtration with an ultrafilter unit using a Q0100 grade ultrafilter (Molecular weight cut off 10 kDa). A 4-point dilution series consisting of 10, 40, 70 and 100% of the original or unfiltered seawater sample was prepared with the diluents in 125 ml polycarbonate bottles, and incubated for 48 h in the dark at *in situ* temperatures using thermo-controlled incubators. Similarly, another dilution series in which protozoa was eliminated by sample filtration onto  $1.0\ \mu\text{m}$  Nuclepore filter (type PC) prior to dilution and incubated. In the same manner, the standard dilution protocol ( $0.2\ \mu\text{m}$  diluents) was used to analyze samples collected at Stn 35 in July. Subsamples for enumerating bacterial abundances were collected into autoclave-sterilized centrifugation tubes (1.5 or 5 ml) every 12 h, and preserved as described above.

**Enumeration of microbes with epifluorescence microscopy.** Viruses, bacteria, and HNF were stained with fluorochromes and counted using an Olympus BX-51 epifluorescence microscopy at  $1000\times$  magnification. Viruses were processed with a slightly modified protocol described by Noble & Fuhrman (1998). Samples from 0.5 to 1 ml were filtered on Anodisc filters ( $0.02\ \mu\text{m}$  pore size, Whatman) backed by Millipore nitrocellulose filter (type AA,  $0.45\ \mu\text{m}$  pore size). These were then placed on drops of SYBR Green I (Molecular Probes) solution diluted at 1:400 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and stained for 15 min in the dark. The membranes were placed on glass slides and added with  $25\ \mu\text{l}$  of 50% glycerol/ 50% PBS buffer (120 mM NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.5) containing 0.1% *p*-phenylenediamine as antifade and mounting agents. For bacteria, 2 ml samples were filtered on Millipore track-etched membranes (type GTBP,  $0.2\ \mu\text{m}$  pore size), stained with  $5\ \mu\text{g ml}^{-1}$  (final concentration) of 4'-6-diamidino-2-phenylindole (DAPI) for at least 7 min (Kepner & Pratt 1994). For HNF, 25 ml of water sample was filtered on blackened Nuclepore filter (type PC,  $1.0\ \mu\text{m}$  pore size), and dual-stained with DAPI for 5 min followed by primulin (Direct Yellow 59,  $250\ \text{mg l}^{-1}$  in Tris-HCl buffer, pH 4.0) for another 15 min (Martinussen & Thingstad 1991). Filters for bacteria and HNF samples were mounted on glass slides with non-fluorescent immersion oil and frozen until enumeration.

SYBR Green I was detected under blue excitation with an Olympus filter cassette (excitator 470 to 490 nm, dichroic mirror 500 nm, 520 nm barrier filter), while DAPI and primulin were detected under UV excitation (excitator 330 to 385 nm, dichroic mirror 400 nm, 420 nm barrier filter). For each slide, at least 10 fields were randomly selected with a total of >400 viruses or bacteria and >100 HNFs counted. The coefficient of variation (CV) for counting was <10%.

**Statistical analysis.** Least-square regression analysis was performed to determine the relationship between bacterial growth rate and fraction of whole or original water. Significance of the regression analysis and the difference in slopes of the regression lines between the unfractionated and 1.0  $\mu\text{m}$  fractionated dilution series were tested using analysis of variance (ANOVA) (Sokal & Rohlf 1972). Statistical analyses were performed with StatView software (SAS).

## RESULTS

### Station characteristics

The temperature in July (12.8 to 13.5°C) was higher than in May (2.5°C) (Table 2). Stn T31 was located in the Tokachi River estuary, characterized by a lower salinity in comparison to the offshore station (T35). Chlorophyll *a* (chl *a*) showed spatial and temporal changes, with the lowest concentration (0.8  $\mu\text{g l}^{-1}$ ) at Stn T35 in July, and the highest at Stn T35 in May (17.8  $\mu\text{g l}^{-1}$ ). DOC concentration was 85  $\mu\text{M}$  at Stn T31, which was higher than observed in the offshore station (T35), indicating the terrestrial influence at Stn T31. In July, a bacterial abundance of  $1.05 \times 10^6$  cells  $\text{ml}^{-1}$  at Stn T31 was the highest among the stations studied. Viral and HNF abundances were also high in the same station at  $10.67 \times 10^6$  viruses  $\text{ml}^{-1}$  and  $4.01 \times 10^3$  cells  $\text{ml}^{-1}$ , respectively. VBR (virus to bacteria ratio) was 7.3:10.2. In May, the lowest virus abundance and VBR were found at Stn T35 at  $4.75 \times 10^6$  viruses  $\text{ml}^{-1}$  and 7.3, the site also showing the lowest bacterial abundance of  $0.65 \times 10^6$  cells  $\text{ml}^{-1}$ .

### Dilution experiments

Bacterial abundance measured in the original seawater collected at Stn T31 in July was  $1.05 \times 10^6$  cells  $\text{ml}^{-1}$ . Virus and HNF abundance were  $1.06 \times 10^7$   $\text{ml}^{-1}$  and  $4.0 \times 10^3$  cells  $\text{ml}^{-1}$ , respectively. Initial abundance of bacteria, virus and HNF increased linearly with fraction of the original seawater (Fig. 2), suggesting that dilution with 10 kDa diluents successfully produced gradients with bacterial, viral and HNF abundance. Bacterial abundance in 100% fraction showed little change in the entire incubation period (Fig. 3). As the fraction of diluents increased, bacterial abundance also increased rapidly with time and the apparent bacterial growth rate (slope of the least-squares regression of natural log of bacterial abundance versus time) increased in both unfractionated and fractionated samples through the 1.0  $\mu\text{m}$  dilution series (Fig. 3). A significant ( $p < 0.05$ ) linear relationship was obtained with a coefficient of determination ( $r^2$ ) ranging from 0.80 to 0.97 in both dilution series, except the 100% fraction, where no apparent increase was observed. Apparent bacterial growth rates for the 10% dilution series in May were 0.55 and 0.56  $\text{d}^{-1}$  for unfractionated and 1.0  $\mu\text{m}$  fractionated samples, respectively.

Least-square regression analysis was used to test whether an increase in apparent growth rate is directly proportional to the dilution factor (Fig. 4). A good linearity with negative slope was found in both dilution series. The regression coefficients (slope) at Stn T31 in May were  $-0.58$  and  $-0.53$   $\text{d}^{-1}$  for the unfractionated and 1.0  $\mu\text{m}$  fractionated series, respectively (Fig. 4A) and there was significant difference between 2 slopes ( $p < 0.01$ ). The resulting slopes of the regressions represent both viral lysis and protozoan grazing for the unfractionated series whereas only viral lysis is explained for 1.0  $\mu\text{m}$  fractionated series. The difference between the 2 slope values of 0.05  $\text{d}^{-1}$  indicated bacterial mortality due to grazing. The  $y$ -intercepts of these regression lines were 0.63 and 0.64  $\text{d}^{-1}$  for unfractionated and 1.0  $\mu\text{m}$  fractionated series, respectively. These values represent the instantaneous growth rate in the absence of lytic and grazing pressure. In July, the regression co-

Table 2. Temperature and salinity, bacteria, virus and heterotrophic nanoflagellates (HNF) abundances, chlorophyll *a* and dissolved organic carbon (DOC)

Station	Temp. (°C)	Salinity	Bacterial abundance ( $10^6$ cells $\text{ml}^{-1}$ )	Viral abundance ( $10^6$ viruses $\text{ml}^{-1}$ )	HNF abundance ( $10^3$ cells $\text{ml}^{-1}$ )	Chl <i>a</i> ( $\mu\text{g l}^{-1}$ )	DOC ( $\mu\text{M}$ )
T35 May	2.5	32.83	0.65	4.75	1.35	17.8	58.6
T31 July	13.3	31.55	1.05	10.67	4.01	5.5	84.9
T35 July	12.8	32.76	0.80	5.93	1.47	0.8	63.4
E9 July	13.5	32.35	0.92	7.75	1.55	0.3	70.0

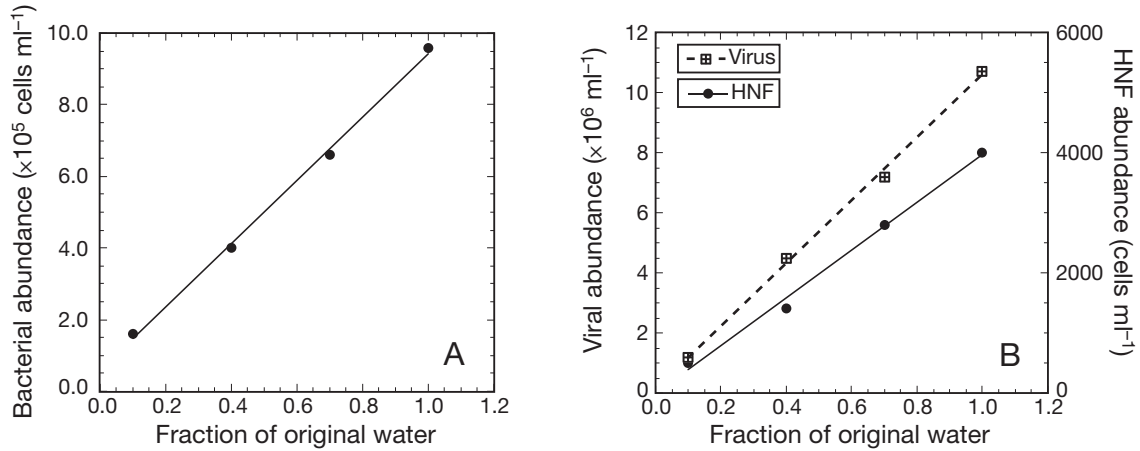


Fig. 2. Plots of (A) bacterial abundance and (B) viral and HNF abundances versus fraction of original water using 10 kDa diluents at Stn T31 in July. Solid line in upper panel: linear regression of fraction of original water vs. bacterial abundance ( $y = 0.573 + 8.87x$ ,  $r^2 = 0.996$ ). Solid and broken line in lower panel: linear regression for viral abundance ( $y = 0.18 + 10.4x$ ,  $r^2 = 0.996$ ) and heterotrophic nanoflagellate (HNF) abundance ( $y = -6.6 + 3966x$ ,  $r^2 = 0.992$ ), respectively

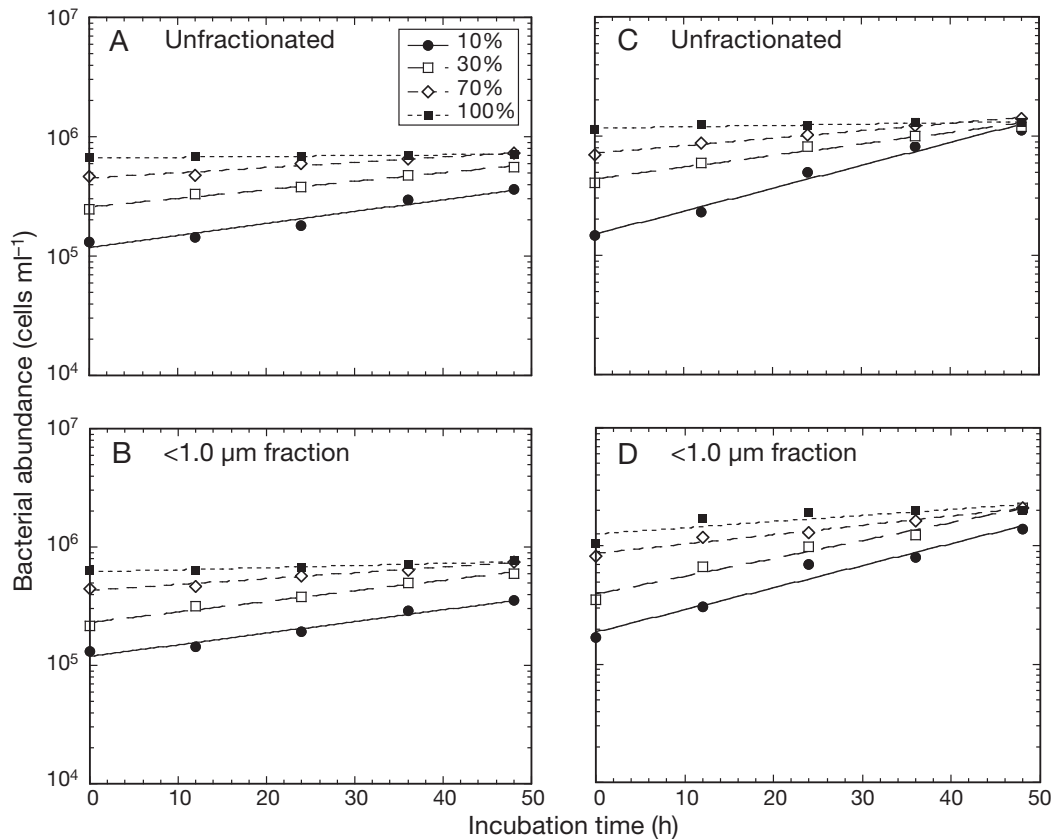


Fig. 3. Growth curves of bacterial abundance in 2 dilution series obtained at Stn T31 in (A) May and (B) July. Both grazing and lytic pressures were included in the unfractionated samples (upper panels). Only lytic pressure was included in the  $<1.0 \mu\text{m}$  samples (lower panels), since protozoan grazing was eliminated by filtration

efficients (slope) at Stn T31 were  $-1.11$  and  $-0.98 \text{ d}^{-1}$  for the unfractionated and  $1.0 \mu\text{m}$  fractionated series, respectively (Fig. 4B). The difference between the 2 slope values of  $0.13 \text{ d}^{-1}$  indicates grazing pressure.

Linear relationships were obtained in both dilution series in all experiments with  $r^2$  ranging from 0.964 to

0.998 (Table 3). The lytic pressure obtained from the  $1.0 \mu\text{m}$  fractionated series was  $0.53 \text{ d}^{-1}$  (Stn T35 in May) to  $0.98 \text{ d}^{-1}$  (Stn T31 in July), and the grazing pressure was  $0.05 \text{ d}^{-1}$  (Stn T35 in May) to  $0.13 \text{ d}^{-1}$  (Stn T31 in July) (Table 4). Lytic mortality accounted for was 87.2 to 91.3% of the total mortality (Table 4).

Table 3. Linear regression analysis between unfractionated and 1.0  $\mu\text{m}$  fractionated dilution series. Parentheses: SD

Period	Station	Fraction	Regression coefficient ( $\text{d}^{-1}$ )	y-intercept ( $\text{d}^{-1}$ )	$r^2$	Significance of slopes (p)
May	T35	unfractionated	0.58 (0.03)	0.63 (0.02)	0.995	0.0164
		<1.0 $\mu\text{m}$	0.53 (0.04)	0.64 (0.03)	0.989	
July	T31	unfractionated	1.11 (0.06)	1.16 (0.02)	0.995	0.0002
		<1.0 $\mu\text{m}$	0.98 (0.03)	1.16 (0.04)	0.998	
	T35	unfractionated	0.86 (0.10)	0.86 (0.06)	0.982	<0.0001
		<1.0 $\mu\text{m}$	0.75 (0.04)	0.87 (0.02)	0.998	
	E9	unfractionated	0.79 (0.11)	0.75 (0.07)	0.964	0.0008
<1.0 $\mu\text{m}$	0.70 (0.04)	0.75 (0.03)	0.989			

Instantaneous growth rate was lowest at  $0.63 \text{ d}^{-1}$  at Stn T35 and highest at  $1.16 \text{ d}^{-1}$  at Stn T31 in July (Table 4). In fact, the sum of the lytic and grazing mortality rates was almost equal to the instantaneous growth rate. The ratio of the lytic mortality rate to the instantaneous growth rate exceeded 80%, with the highest rate (93.3%) found at Stn E9 in July.

### Comparison to the standard protocol

Samples collected at Stn T35 in July were analyzed using the standard dilution protocol and the protocol developed in the present study and comparison of results is shown in Fig. 5. A significant correlation between the apparent growth rate and the fraction of

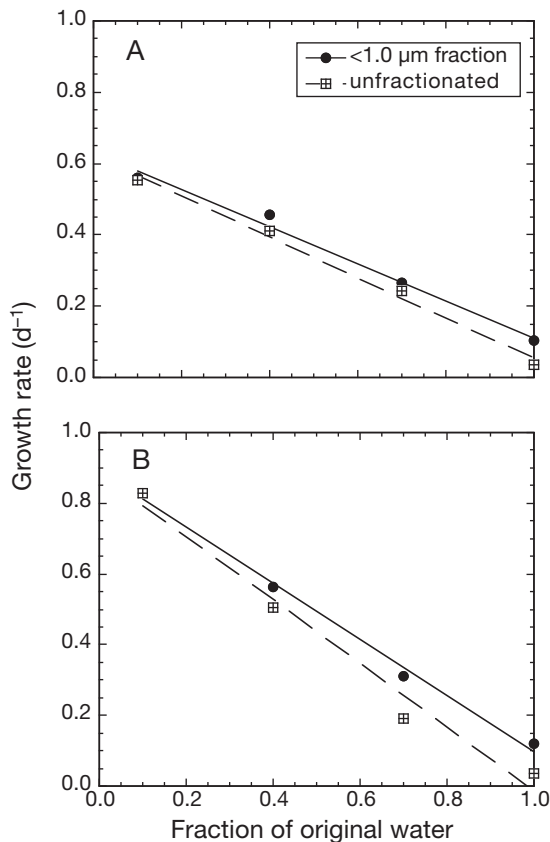


Fig. 4. Correlation between specific growth rate and fraction of original sample for the unfractionated (lysis + grazing) and 1.0  $\mu\text{m}$  fractionated (lysis) incubations at Stn T31 in (A) May and (B) July. Solid and broken lines: linear regression of <1.0  $\mu\text{m}$  fraction ( $y = 0.891 - 0.792x$ ,  $r^2 = 0.994$ ) and unfractionated seawater ( $y = 0.883 - 0.897x$ ,  $r^2 = 0.976$ ), respectively

Table 4. Instantaneous bacterial growth and mortality rates due to viral infection and protozoan grazing obtained from the virus-dilution experiments and ratio of lysis to total mortality (viral infection + protozoan grazing)

Station	Growth rate ( $\text{d}^{-1}$ )	Lysis rate ( $\text{d}^{-1}$ )	Grazing rate ( $\text{d}^{-1}$ )	Ratio of lysis (%)
T35 May	0.63	0.53	0.05	91.3
T31 July	1.16	0.98	0.13	88.2
T35 July	0.86	0.75	0.11	87.2
E9 July	0.75	0.70	0.09	88.6

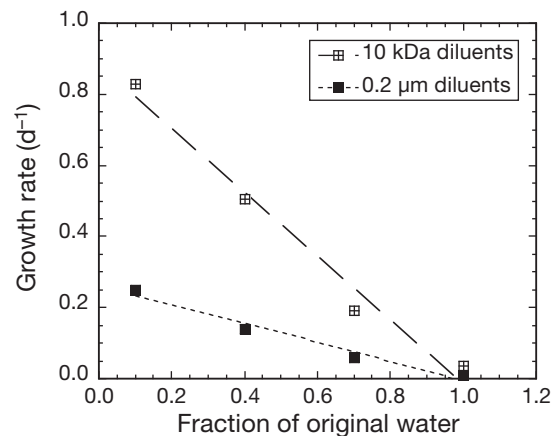


Fig. 5. Correlation between growth rates and relative mortal pressures using the standard protocol (0.2  $\mu\text{m}$  diluents) and new protocol (10 kDa diluents) at Stn 35 in July. Dashed and dotted lines: linear regression of 10 kDa diluents ( $y = 0.882 - 0.896x$ ,  $r^2 = 0.976$ ) and 0.2  $\mu\text{m}$  diluents ( $y = 0.260 - 0.266x$ ,  $r^2 = 0.970$ ), respectively

original water was found for both dilution series ( $r^2 = 0.989$ ). However, the slope of 0.2  $\mu\text{m}$  diluent ( $0.27 \text{ d}^{-1}$ ) was significantly ( $p < 0.01$ ) lower than that of 10 kDa diluent ( $0.90 \text{ d}^{-1}$ ).

## DISCUSSION

### Dilution experiments

The dilution method was originally developed by Landry & Hassett (1982) to estimate the grazing impact of microzooplankton on phytoplankton. The dilution of original seawater with filtered seawater resulted in the change in the rate of encounter of consumers with prey cells, i.e. a change in the grazing rate while the growth rate of phytoplankton may not change (though the cell density changes with dilution factor). As a consequence, the observed rate of change in phytoplankton abundance at different dilutions has a linear relationship with the dilution factor. The negative slope of the relationship is the grazing coefficient while the  $y$ -axis intercept is the phytoplankton instantaneous growth rate ( $\mu$ ) without grazing.

A linear relationship was found between the apparent bacterial growth rate and the fraction of whole water, and significant differences ( $p < 0.05$ ) in regression slopes were found between the original seawater and 1.0  $\mu\text{m}$  fractionated dilution series (Fig. 4). These results suggest that the dilution technique with virus-free diluents can be applied to directly determine the bulk lytic pressure on bacteria, and at the same time, size-fractionated incubation enabled us to estimate both lytic pressure and grazing pressure. There are only few studies which have applied the dilution method to estimate the impact of viruses on microorganism. Evans et al. (2003) estimate viral lysis and micro-zooplankton grazing on picoeukaryote *Micromonas* by diluting original seawater with either 0.2  $\mu\text{m}$  filtrate (grazer-free) or 10 kDa filtrate (virus- and grazer-free). Their method has similarities with the method developed in the present study, except that they diluted the original water with 0.2  $\mu\text{m}$  filtrate (grazer-free) in different proportions. As both water samples contained viruses, no change in mortality rate due to viral lysis was observed. In the present study, 1.0  $\mu\text{m}$  filtrate (grazer-free) was diluted with 10 kDa filtrate (virus- and grazer-free), which produced the changes in encounter rates of virus on bacteria. Jacquet et al. (2005) reported protozoan and viral-mediated mortality of bacteria in Lake Bourget. They performed dilution of 2  $\mu\text{m}$  filtrate (grazer-free) with 10 kDa filtrate (virus- and grazer-free) in 4 dilution series, and obtained viral-mediated mortality similar to our findings. However, they measured protozoan grazing rate separately using FLBs; this was dif-

ferent from our method, which was designed to estimate lytic and grazing pressure simultaneously by a simple dilution experiment.

Comparison between the standard protocol with 0.2  $\mu\text{m}$  diluents and the present protocol using 10 kDa diluents (Fig. 5) suggest that under conditions where lytic pressure is relatively high, the instantaneous growth rate obtained from the standard protocol might have been underestimated because almost all viruses could remain in the 0.2  $\mu\text{m}$  diluents. Consequently, previous studies using the standard protocol might have reported underestimated values. Results from laboratory and field studies suggest the importance of viral infection on phytoplankton growth (Suttle et al. 1990, Bratbak et al. 1993, Cottrell & Suttle 1995). Since the dilution technique is more commonly applied to phytoplankton community studies, previous estimates of biogeochemical fluxes and carbon budgets as well as nutrients and other relevant components such as the cloud-nucleation agent dimethyl sulphide (DMS), might be modified by the new dilution approach which takes into account viral infection.

The virus-dilution technique developed in the present study has several advantages similar to the standard dilution technique described by Landry (1993):

- (1) The protocol is capable of estimating both growth and lytic mortality rates in a single incubation.
- (2) Simultaneous study of the dynamics of host organism components originating from lytic mortality (e.g. DMS) is possible. In fact, Hill et al. (1998) demonstrated that viral infection causes the total release of the intracellular DMS precursor (DMSP) from the common Prasinophyte *Micromonas pusilla*. Since viruses are also known to infect major DMSP-containing bloom organisms such as *Emiliania huxleyi* (Brussaard et al. 1996) and *Phaeocystis pouchetii* (Malin et al. 1998), *in situ* DMS production due to phytoplankton lysis—as well as grazing—can be estimated in field studies.
- (3) The method does not require expensive or sophisticated equipments such as TEM, or complicated procedures such as preparations of FLBC or viruses to determine lytic mortality.
- (4) Since it does not employ radio-isotopes such as [ $^3\text{H}$ ]-thymidine, there are no difficulties and constraints on its application in the field.

An additional point is that bacterial abundance was counted with epifluorescence microscopy, but use of flow-cytometry may improve counting time and precision.

Based on the obtained lytic mortality rates, other viral parameters could be indirectly estimated using conversion factors. Given an appropriate burst size, viral production rate could be estimated from bacterial mortality due to viral infection. In addition, theoretical

burst size could be estimated when viral production is determined simultaneously with a direct method such as [<sup>3</sup>H]-thymidine incorporation.

However, there are disadvantages to the technique. The modified dilution technique requires a relatively longer incubation period, which can lead to ambiguous or imprecise rate estimates and which comes with potential risks of contamination or enrichments in the diluted fractions, which could affect the estimation of growth rates in the diluted samples. We recommended shortening the incubation period with more frequent samplings to avoid these potential problems, even though the present study adopted 48 h of incubation time. Since the virus-dilution technique employs 1.0 µm size fractionation in order to eliminate protozoa and thus estimate only lytic pressure, it is inevitable that lytic pressure will be overestimated if some protozoa pass through the 1.0 µm filter.

Not all protozoa were eliminated using 1.0 µm filters. About 20% of protozoa passed through a 1.0 µm filter in our samples, thus this may cause an underestimation of grazing mortality if the grazing rate of protozoa is a linear function of their abundance. To evaluate this underestimation, Eq. (5) was modified to

$$k_2 = \mu - (m_v + r \times m_g) \times D \quad (5)'$$

where *r* is the percent of protozoa in the 1.0 µm filtrate. By solving Eq. (4) and (5)', the grazing rate at Stn T31 in July (Table 4) was corrected from 0.13 to 0.16 d<sup>-1</sup>, assuming that 20% of protozoa remained in the filtrate and grazing rate is a linear function of abundance. The lytic mortality was also corrected from 0.98 to 0.95 d<sup>-1</sup>. The ratio of lysis to total mortality changed from 88 to 82%. It is uncertain whether protozoa <1.0 µm (small flagellates) can graze heterotrophic bacteria with the same size range. Use of a smaller pore-size filter (<1.0 µm) may also eliminate heterotrophic bacteria as well as small flagellates. Size spectra or composition of protozoa may differ for samples examined, therefore one should check the abundance of protozoa in the 1.0 µm fractionated samples.

Another potential source of error is the disregarding or discounting of production and lytic mortality of particle-associated bacteria, colonizing and harvesting organic particles such as cell debris and aggregates, and degrading polymers with extracellular enzymes. In environments receiving high suspended matter loading, such as estuaries, particle-associated bacteria are known to dominate, and have relatively high activity, compared to free-living bacteria (Bell & Albright 1981, Crump et al. 1998). Bioavailable DOC supplied from the organisms with >1.0 µm size—such as phytoplankton-derived DOC associated with trophic processes and metabolic exudation—could be interrupted. In the event of phytoplankton blooms, where

autotrophic and heterotrophic processes are strongly coupled and enhanced, the instantaneous bacterial growth rate derived from the 1.0 µm fractionated dilution series can be underestimated. The enrichment of DOM and nutrients through filtration (1.0 µm and ultra filtration) was nominal (within 10% increase from the original) in the present study. However, Anodisc filters may cause noticeable PO<sub>4</sub> enrichment in the filtrate, so they should not be used for preparation of virus-free diluents (I. Kudo unpubl.).

### Incubation conditions

It is important that an appropriate incubation period is determined to obtain a significant slope of the regression curve. Incubation time is dependent not only on bacterial growth rate, but also in the duration of viral latent period, which is defined as the time between viral contact and bacterial lysis. In this study, sub-sampling was performed every 12 h for 48 h in all experiments. However, since the incubation period is relatively long, alteration of conditions may have occurred during incubation, and this could cause substrate limitation on bacterial growth, and changes in bacterial and viral abundances. Therefore, it would be better to sample at shorter intervals, i.e. every 6 to 8 h, strictly monitor changes in bacterial abundance and check the outliers in the regression plot in order to obtain an appropriate slope of the regression curve.

In virus dilution experiments, cell lysis might be the initial result of infections that occurred prior to dilution. Although the length of lytic cycle (turnover time) is usually tightly coupled with the growth rate of the bacteria, viral turnover time in coastal waters is ~0.5 d (Wilhelm et al. 2002, Weinbauer 2004). Thus, if any such influence is detected in the previously infected cells, it will likely be limited to the early period of incubation.

### Magnitude of viral lysis to bacterial mortality

Lytic mortality accounted for >80% of the instantaneous growth rate in all sampling sites, although the environmental characteristics of each site are clearly different from one site to another. A number of studies showed that viruses are responsible for 10 to 50% of total bacterial mortality in surface waters (Fuhrman & Noble 1995, Steward et al. 1996, Almeida et al. 2001, Wilhelm et al. 2002), and 50 to 100% in environments which have unfavorable conditions to protozoans, such as the anaerobic hypolimnion layer (Weinbauer & Höfle 1998, Guixa-Boixereu et al. 1999). The percentage of total bacterial mortality in the present study, however,



was higher than what was obtained in coastal surface waters. Therefore, further studies are recommended to assess the wider applications of the dilution protocol developed in this study for other areas and seasons.

**Acknowledgements.** The authors thank the captain and crew of the TS 'Ushio Maru' for assistance in sampling. We also appreciate C. W. Lee and F. A. Aya for reading and correcting the manuscript. This study was funded by the Grant-in-Aid for Scientific Research No. 15310019 of the Japan Society for the Promotion of Science.

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*Editorial responsibility: Matthias Seaman, Oldendorf/Luhe, Germany*

*Submitted: September 2, 2008; Accepted: November 6, 2008  
Proofs received from author(s): March 17, 2009*