



Title	Significant contribution of lytic mortality to bacterial production and DOC cycles in Funka Bay, Japan
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Citation	Journal of oceanography, 72(2), 177-187 <a href="https://doi.org/10.1007/s10872-015-0316-2">https://doi.org/10.1007/s10872-015-0316-2</a>
Issue Date	2016-04
Doc URL	<a href="http://hdl.handle.net/2115/64938">http://hdl.handle.net/2115/64938</a>
Rights	The final publication is available at Springer via <a href="http://dx.doi.org/10.1007/s10872-015-0316-2">http://dx.doi.org/10.1007/s10872-015-0316-2</a>
Type	article (author version)
File Information	Kudo-Kakuta.pdf



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Significant contribution of lytic mortality to bacterial production and DOC cycles in Funka

Bay, Japan

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## Abstract

A modified dilution experiment was conducted to evaluate the relative contribution of viral lysis and protozoan grazing to the mortalities of heterotrophic bacteria in Funka Bay, a subarctic coastal bay. The experiment included the stepwise dilution of the original seawater with virus-free seawater (10 kDa ultrafiltered) to change the encounter rate of both virus and protozoa to heterotrophic bacteria, incubation for 48 hours and monitoring the change in the abundance of heterotrophic bacteria. In a parallel experiment, the original seawater was replaced by 1.0  $\mu\text{m}$  fractionated seawater to eliminate protozoa and the same dilution was conducted with the virus-free seawater to estimate only lytic mortality. The viral lysis and protozoan grazing rates in the surface water ranged from 0.40 to 1.19  $\text{d}^{-1}$  and 0.08 to 0.27  $\text{d}^{-1}$ , respectively. Viral lysis was the main cause for the bacterial mortality (79.8 $\pm$ 3.2%). The net (in situ) growth rate of heterotrophic bacteria was about 0.15  $\text{d}^{-1}$ . In the bottom water (90 m), both mortalities were lower than those at the surface and the net growth rate was mostly a negative value. The contribution of released dissolved organic matter (DOM) through lysis to the bacterial carbon demand (BCD) was evaluated. The lysed bacterial cells might release DOM to the ambient environment, in which bacterial organic matter is recycled in the subsequent bacterial production. The potential contribution was estimated ranging from 25 to 27% in the surface water and 31% in the bottom water, suggesting the lytic mortality

significantly fueled DOM to the subsequent bacterial production.

**Keywords:** heterotrophic bacteria, virus lysis, protozoa grazing, C budget, microbial loop

## 1. Introduction

Heterotrophic bacteria play an important role in material cycle and transferring organic matter to higher trophic consumers in aquatic food web. Viral lysis and protozoan grazing are two major mortalities of bacteria in a pelagic system. Whether bacterial cells are lysed or grazed has different implications in terms of the ecological and biogeochemical points of view. Grazed production is transferred to the 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 release dissolved organic matter (DOM), in a closed loop in which bacterial organic matter is fueled back to the following bacterial production in the euphotic zone (Proctor and Fuhrman 1991; Bratbak et al. 1993; Wilhelm et al. 2002). Additionally, macro- and micronutrients are supplied to bacteria and phytoplankton through lysis (Middelboe et al. 1996; Gobler et al. 1997; Noble and Fuhrman 1998). Thus, it is important to determine the magnitude of grazing and lytic pressures on bacterial production to understand the biogeochemical cycles and energy flows within the microbial loop.

The relative importance of lytic and grazing pressures was studied in either micro- or mesocosms, and in the field. Several studies indicated that lysis is comparable to grazing pressure in the coastal environments (Fuhrman and 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 phytoplankton bloom. Weinbauer and Höfle (1998) found that the lytic mortality is dominant in the anaerobic hypolimnion layer of Lake Plüsee, Germany, where protists are less due to oxygen depletion. The ecological role and evolutionary point of view of viruses are reviewed in several papers (Weinbauer 2004; Weinbauer and Rassoulzadegan 2004).

The dilution technique described by Landry and Hassett (1982) is used to estimate grazing pressure of micro-zooplankton on phytoplankton and later applied to protozoan bacterivory (Tremaine and Mills 1987). The technique involves stepwise dilution of whole micro-plankton communities with the particle-free seawater diluents (0.2  $\mu\text{m}$  filter pore-size) to create gradients in the predator-prey encounter rates, wherein changes in the abundance of prey are closely monitored from a 12 to 48 h incubation period. Taira et al. (2009) developed a modified dilution method to estimate the bacterial mortality due to viral infection and protozoan grazing simultaneously. The method includes the dilution of different fractions of the original seawater (non-filtered) and the 1.0  $\mu\text{m}$ -filtered seawater (grazer-free) with the 10 kDa filtered seawater (virus-free) to set up four gradients of predator-prey interaction, and monitoring the change in bacterial abundance during the incubation.

Funka Bay is located in a subarctic region, southwest of Hokkaido, and has an area of 2,300 km<sup>2</sup> and a maximum depth of approximately 100 m. Spring phytoplankton bloom occurs prominently in March and produces one-third of the annual primary production (Kudo and Matsunaga 1999). Dominant phytoplankton are micro-sized centric diatoms such as *Chaetoceros* spp and *Thalassiosira* spp. (Shimada 2000). Much of the produced C during the spring bloom is not consumed by meso-zooplankton (Copepods) due to a low biomass in spring and flows into a microbial loop (Odate and Maita 1988; Ban et al., 2000). Bacterial production in the euphotic zone is enhanced just after the spring bloom (Lee et al. 2001a). The annual bacterial production was estimated as 140 g C m<sup>-2</sup> yr<sup>-1</sup>, which was a similar magnitude to the primary production (100-170 g C m<sup>-2</sup> yr<sup>-1</sup>) (Lee et al. 2001a). Assuming a BGE (Bacterial Growth Efficiency) as 30% for coastal area (del Giorgio and Cole 2000), the annual BCD (Bacterial Carbon Demand) would be 467 g C m<sup>-2</sup> yr<sup>-1</sup>, exceeding the annual primary production. None of the other possible organic C sources could explain this deficiency. However, they only evaluate micro-zooplankton grazing as mortality of bacteria. Taira et al. (2009) reported that viral lysis accounts for more than 60% of the total mortality of bacteria in two coastal areas of Hokkaido. If the viral lysis has a significant contribution to the bacterial mortality in the bay, DOC supply through lysis could be expected to refuel the following BCD. In this study, the dilution method by Taira et al. (2009) was applied to

estimate the virus lysis and grazing mortality on heterotrophic bacteria in Funka Bay. The organic carbon flow including the virus lysis was also evaluated in the bay. We conducted the dilution experiments monthly from April to October for the surface samples representing euphotic zone and the bottom samples because active regeneration process by heterotrophic bacteria takes place near the sediment-water interface (Kudo et al. 2007).

## 2. Materials and methods

### 2.1 Sampling

Oceanographic observation was conducted monthly from April to October in 2008 aboard the *T/S Ushio Maru*. The sampling station was Stn. 30 (42°16.2'N, 140°36.0'E, depth 92 m, Fig. 1). Salinity and temperature were monitored with a Sea-Bird 19 plus CTD sensor. Water samples for Chlorophyll *a* (Chl *a*) and nutrients were taken vertically with a CTD-CMS. Discrete samples for incubation were obtained with teflon-coated lever-action 10 L Niskin samplers attached to a Kevlar wire.

### 2.2 Chl *a* and nutrients

An aliquot of sample for Chl *a* analysis was filtered through a Whatman GF/F filter. The

filter was stored frozen in *N, N*-dimethylformamide to extract Chl *a* (Suzuki and Ishimaru, 1990). The extracted Chl *a* was measured with a Hitachi F-2000 spectrofluorometer. The samples for nitrate (NO<sub>3</sub>), nitrite (NO<sub>2</sub>), ammonium (NH<sub>4</sub>), phosphate (PO<sub>4</sub>) and silicic acid (Si(OH)<sub>4</sub>) analysis were stored frozen for the subsequent analysis with an auto-analyzer (QuAAtro, Bran+Luebbe).

### *2.3 Dissolved Organic Carbon (DOC)*

The sample for DOC analysis was dispensed directly from the Niskin sampler into a borosilicate glass bottle through an in-line filtration cartridge (Advantec) with a GF/F filter and then 20 mL of subsample was transferred to a glass ampoule using a glass pipette, flame-sealed and stored frozen at -30°C until analysis. All glassware and GF/F filters used were pre-combusted at 450°C for 5 h prior to use. DOC concentration was determined by the high-temperature catalytic oxidation method with a Shimadzu TOC 5000A (Cauwet 1999).

### *2.4 Bacteria, HNF and Virus abundances*

Samples for enumeration of microbial abundances were dispensed into autoclave-sterilized 50 mL polypropylene tubes. For bacteria abundance (BA) and viral abundance (VA) 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 on board and then stored at  $-80^{\circ}\text{C}$  in an ultrafreezer until preparation of slides. Samples for heterotrophic nanoflagellates (HNF) abundance also were fixed with glutaraldehyde and stored at  $4^{\circ}\text{C}$ , and sample slide preparation was made within 3 to 4 d after collection. An aliquot of 0.5 to 1 mL subsample for BA and VA counting was filtered on Anodisc filters ( $0.02\ \mu\text{m}$  pore size, Whatman). These were then placed on drops of SYBR Gold (Molecular probes) solution diluted at 1:400 in TE buffer (10 mM Tris-HCl, 1 mM EDTA, 10 mM  $\text{NaH}_2\text{PO}_4$ , pH 7.5) and stained for 15 min in the dark (Chen et al 2001). The filters 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). For HNF, An aliquot of 25 mL subsample was filtered on blackened Nuclepore filter (type PC,  $1.0\ \mu\text{m}$  pore size), and dual-stained with DAPI (4'6-diamidino-2-phenylindole) for 5 min followed by primulin (Direct Yellow 59, 250 mg  $\text{L}^{-1}$  in Tris-HCl buffer, pH 4.0) for another 15 min (Martinussen and Thingstad 1991). Filters were then mounted on glass slides with non-fluorescent immersion oil and frozen until enumeration. Viruses, bacteria, and HNF were counted using an Olympus BX-51 epifluorescence microscopy at 1000 x magnification. SYBR Gold 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 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 >200 viruses or bacteria and >100 of HNFs counted. An image with red fluorescence under green excitation was eliminated from counting due to autotrophs with photosynthetic pigments. The coefficient of variation (CV) for counting was less than 10%.

### *2.5 Dilution experiments*

Water samples taken from the surface (0 m) and the bottom (90 m) were processed within 6 h after collection. 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 using a Q0100 grade ultrafilter (MW cut off 10,000; 10 kDa). Four-point dilution series consisting of 10, 40, 70 and 100% of the original seawater (unfiltered) was prepared diluting with the virus-free 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 filtration through 1.0  $\mu\text{m}$  Nuclepore filter (type PC) prior to dilution were incubated. Subsamples for enumerating BA were collected into autoclave-sterilized centrifuge tubes (1.5 or 5 mL) every 12 h, and preserved as described above. Filter holders and incubation bottles were acid-cleaned with 10% HCl and rigorously rinsed with Milli-Q water.

## 2.6 Potential growth rate, lysis and grazing mortality

Apparent growth rate was obtained from the slope of the regression line for time vs natural log of BA for each dilution bottle. Then, apparent growth rates obtained from the unfractionated and 1.0  $\mu\text{m}$  fractionated series were plotted separately against the dilution factor. The slope values of the regression lines for the unfractionated and 1.0  $\mu\text{m}$  fractionated series indicated lysis plus grazing mortality rate and only lysis mortality rate, respectively. The difference between the two slope values was grazing mortality rate. The y-intercept values of these regression lines represent a potential growth rate without lytic and grazing pressure. If these values are not identical, the average value was used for the potential growth rate. Growth rate at 1.0 of dilution factor was the net (*in situ*) growth rate under the original lytic and grazing pressure. Bacterial production ( $\text{mg C m}^{-3} \text{ d}^{-1}$ ) was calculated by multiplying potential growth rate ( $\text{d}^{-1}$ ) by bacterial C biomass ( $\text{mg C m}^{-3}$ ). Bacterial C biomass was estimated by multiplying BA ( $\text{cells L}^{-1}$ ) by bacterial cell quota ( $\text{g C cell}^{-1}$ ). In this study, bacterial cell quota was used at  $30.2 \text{ fg C cell}^{-1}$  for coastal samples (Fukuda et al., 1998). While incubation was conducted single for each setting, the calculation of lysis and grazing mortality rates for each sample were based on regression analysis at four point dilution series at  $p < 0.01$ .

### 3. Results

#### 3.1 Hydrography

Surface temperature was 6°C in April and increased to 20°C in August and September (Fig. 2a). Temperature below 80 m was almost constant at <5°C throughout the observation period. Salinity ranged from 32.0 to 33.5 at the surface and did not change at 33.5 below 80 m (Fig. 2b). Thermocline developed around 30 to 50 m depth from June to September. Following the definition by Ohtani (1971), Oyashio water occupied in spring and Tsugaru warm water (TWW) entered in the mid- and deeper-layer after August, evidenced by the increase in salinity. Hydrographical condition in 2008 was similar to the other years in the bay (Kudo et al. 2007).

#### 3.2 Nutrients and DOC

NO<sub>3</sub>, a limiting nutrient in the bay (Kudo et al. 2000) was almost depleted at the surface during the observation period (Fig. 2c). Accumulation of NH<sub>4</sub> was found in the deeper layer (below 60 m) (Fig. 2d). NH<sub>4</sub> concentration reached >8 μmol L<sup>-1</sup> near the bottom in May and July. Then, NH<sub>4</sub> concentration decreased and NO<sub>3</sub> increased due to nitrification at this depth (Kudo et al. 2007). PO<sub>4</sub> and Si(OH)<sub>4</sub> showed a similar pattern of seasonal change in NO<sub>3</sub> (not shown).

DOC concentration ranged from 40 to 110  $\mu\text{mol L}^{-1}$  (Fig. 3). From April to June, concentration was relatively uniform in the water column with a variation of about 10  $\mu\text{mol L}^{-1}$ . A high concentration ( $>100 \mu\text{mol L}^{-1}$ ) was found in the surface mixed layer with a maximum at 40 m in September. In the deeper layer (60-90 m), concentration decreased to 40  $\mu\text{mol L}^{-1}$  after August.

### *3.3 Chl a, heterotrophic bacteria, HNF and virus abundance*

Chl *a* concentration was below 1.0  $\mu\text{g L}^{-1}$  throughout the water column during the observation period except 40 m in August where Chl *a* was at 1.2  $\mu\text{g L}^{-1}$  (Fig. 4a). Heterotrophic bacterial abundance (BA) was generally higher in the upper layer (0-30 m) than in the deeper layer (60-90 m)(Fig. 4b). The highest BA occurred in the upper layer of May and June at 1-1.5  $\times 10^9$  cells  $\text{L}^{-1}$ . The BA in the deeper layer ranged from 0.3 to 0.7  $\times 10^9$  cells  $\text{L}^{-1}$ . HNF abundance in the upper layer of April was at 0.75 – 0.9  $\times 10^6$  cells  $\text{L}^{-1}$  (Fig. 4c). The abundance decreased in this layer of July and August and increased again in September. The abundance was low in the deeper layer at less than 0.1  $\times 10^6$  cells  $\text{L}^{-1}$  except in April when it was at 0.2  $\times 10^6$  cells  $\text{L}^{-1}$ . The virus abundance (VA) in the upper layer was in the order of 10 – 25  $\times 10^9$  particles  $\text{L}^{-1}$  and that in the deeper layer was relatively uniform at 10 – 14  $\times 10^9$  particles  $\text{L}^{-1}$  (Fig. 4d).

### *3.4 Bacterial mortality and growth rate*

As an example for the dilution experiment, the result at 0 m in June was shown in Fig. 5. The initial BA increased linearly with increasing the fraction of the original seawater (Fig. 5a), suggesting that dilution with 10 kDa diluents successfully produced the gradients of BA. The time course change in BA during 48 h incubation showed an exponential increase in each bottle (Fig. 5b). A correlation between time and natural log of BA was significant ( $p < 0.01$ ) with the coefficient ( $r$ )  $> 0.95$ , indicating the growth of heterotrophic bacteria was maintained at exponential growth phase and balanced with mortality for each setting. The apparent growth rate was the highest in the 10% of the original seawater bottle and the lowest in the 100% bottle. The apparent growth rate decreased linearly with the fraction of the original seawater or HNF free seawater (Fig. 5d). These correlations were significant ( $p < 0.01$ ) with the coefficients ( $r$ )  $> 0.99$ . The slopes of  $< 1.0 \mu\text{m}$  fractionated seawater,  $0.45 \text{ d}^{-1}$  and unfractionated seawater,  $0.56 \text{ d}^{-1}$  gave a lytic mortality rate and the sum of lytic mortality and HNF grazing mortality rates, respectively. The difference in the two slopes,  $0.11 \text{ d}^{-1}$  indicated the HNF grazing mortality rate. The y-intercepts of these regression lines,  $0.71$  and  $0.69 \text{ d}^{-1}$  were the potential growth rates in the absence of lytic and grazing pressure. Theoretically these y-intercepts should be identical, but due to the experimental artifact the average value was used if it is not the case. The growth rate for 100% unfractionated seawater,  $0.13$

$d^{-1}$  indicated the net growth rate with the *in situ* lytic and grazing pressure. The correlation analysis for the other dates indicated that the correlations between incubation time and natural log of BA for each bottle and between the apparent growth rate and the fraction of original seawater for each treatment were significant at  $p < 0.01$ .

The lytic mortality rate at the surface was the highest at  $1.14 d^{-1}$  in April and moderate at  $0.4 - 0.5 d^{-1}$  during May and October (Table 1, Fig. 6a). The protozoan grazing rate was  $0.27 d^{-1}$  in April and  $0.08 - 0.15 d^{-1}$  in the other months. The lytic mortality accounted for  $80.6 \pm 2.5\%$  of the total mortality where protozoan grazing was  $19.4\%$  of the total (Fig. 6c). The potential growth rate ranged from  $0.53$  to  $1.57 d^{-1}$  (Table 1). The *in situ* (net) growth rate ranged from  $0.03$  to  $0.16 d^{-1}$ .

At the bottom (90 m), the lytic mortality was  $0.23 - 0.35 d^{-1}$  and protozoan grazing ranged from  $0.08$  to  $0.19 d^{-1}$  (Table 1, Fig. 6b). The lytic mortality at 90 m was one half to one fourth of that at the surface. The lytic mortality accounted for  $68.2 \pm 5.3\%$  of the total mortality (Fig. 6d). The potential growth rate ranged from  $0.30$  to  $0.39 d^{-1}$  where the net growth rate was negative values ( $-0.1 - -0.2 d^{-1}$ ) except in May ( $0.08 d^{-1}$ ).

## 4. Discussion

### 4.1 Bacterial growth and mortality

In April, the bacterial production at the surface was the highest during the study period. Bacterial growth is usually limited by resource (DOM) availability, grazing, temperature and virus lysis (Sherr and Sherr 1984; Fuhrman and Noble 1995; Felip et al. 1996). The main flow to DOM pool seems the supply from primary production. However, *in situ* Chl *a* was low at  $0.21 \mu\text{g L}^{-1}$ . The massive diatom bloom takes place from late February to March in Funka Bay (Kudo and Matsunaga 1999). Primary production during the spring bloom accounts for one-third of the annual production in the bay. Bacterial growth and production are stimulated just after the spring bloom or artificially induced bloom (Lee et al. 2001b; Kudo et al. 2009). The highest bacterial production in April seemed the result of increased DOM supply after the bloom through exudation, viral lysis or zooplankton grazing. In summer, bacterial production at the surface also increased due to the elevated temperature (Lee et al. 2001a).

In the present study, the potential growth rate of bacteria ranged from  $0.53$  to  $1.57 \text{ d}^{-1}$  at the surface which was a similar range to the previous study ( $0.62 \pm 0.54 \text{ d}^{-1}$ ) conducted in Funka Bay (Lee et al 2001a) with a similar pattern of seasonal change. Lee et al. (2001a) estimated the growth rate from  $^3\text{H}$ -thymidine based bacterial production measurement. We did not measure the bacterial carbon production by the alternative methods ( $^3\text{H}$ -thymidine or leucine incorporation) in parallel with the dilution experiment. However, the potential growth rates of bacteria in the present study

were well agreed with those estimated from other method ( $^3\text{H}$ -thymidine method).

Lee et al (2001a, b) examined the temporal variation of bacterial abundance and production in Funka Bay. The reported ranges for bacterial abundance ( $2.6 - 9.1 \times 10^8$  cells  $\text{L}^{-1}$ ) and production ( $0.1 - 22.9 \mu\text{g C L}^{-1} \text{d}^{-1}$ ) are the same order of magnitude as the present study. However, they only considered HNF grazing and temperature as factors controlling the bacterial abundance and production. HNF grazing accounted for only 20% at the surface and 30% at the bottom of the total mortality in the present study. We observed a relatively high lytic mortality, about 80% of the total mortality at the surface and 70% at the bottom in Funka Bay.

In order to remove protozoa from the original seawater 1.0  $\mu\text{m}$  filtration was conducted, but there seems possibility not all protozoa were eliminated using 1.0  $\mu\text{m}$  filters. About 20% of protozoa passed through a 1.0  $\mu\text{m}$  filter off Tokachi coastal water (Taira et al. 2009). They discussed the potential overestimation of virus lysis as 10% caused by grazing of protozoa  $<1.0 \mu\text{m}$  in size while it is uncertain that small protozoa can graze heterotrophic bacteria with the same size range. We did not verify the proportion of protozoa which passed through a 1.0  $\mu\text{m}$  filter in the present study.

Filtration by a 1.0  $\mu\text{m}$  filter might remove particle-attached bacteria. The initial BA of HNF free seawater showed a lower number (0 to 30%) than that of the original seawater in the present

study. Lee et al. (2001a) reported the contribution of attached bacteria on the total bacterial production accounted for ~50%. This indicated that the attached bacteria had a higher production rate per cell. In the dilution experiment, growth rate and mortality was estimated by the relative change in BA for each setting, thus the difference in the initial BA between HNF free seawater and the original seawater may not influence the obtained results.

A significant correlation between BA and VA was found for all data ( $p < 0.01$ )(Fig. 7a). However, no significant relationship between BA and HNF abundance was found (Fig. 7b). A strong relationship between BA and HNF abundance was reported in waters where significant grazing control of bacteria takes place (Berninger et al. 1991; Sanders et al. 1992; Boras et al. 2009). The result in the present study suggests that a close coupling presents between bacteria and viruses, providing host for virus and controlling BA by lysis although VA in the present study included viruses infecting not only heterotrophic bacteria, but also eukaryotes. Most marine bacteriophages sustain their populations by the cycles of infection, multiplication in the host, then lysis (Fuhrman 2000). VA is closely coupled to the trophic status of the system with lower abundance in the open ocean and much higher abundance in the estuarine water (Cochlan et al. 1993; Weinbauer and Suttle 1997). The range of VA in the present study ( $1.0-2.5 \times 10^{10}$  particles  $L^{-1}$ ) fell within the range reported for coastal waters (Cochlan et al. 1993; Cully and Welschmeyer 2002; Wilhelm et al.

2002).

Viral infection and protozoan grazing balanced with the major fractions of bacterial growth, thus the net growth was nominal at the surface or slightly negative at the bottom (Fig. 6). Both of these routes would return bacterial intracellular constituents to the surrounding water. However, protozoan consumption supplies DOM to food webs via zooplankton grazing to pelagic fish to some extent while lysed bacterial cells release DOM directly to the ambient environment in which bacterial carbon is recycled as bacterial production (Proctor & Fuhrman 1991; Bratbak et al. 1993; Wilhelm et al. 2002). Furthermore, nutrients are also supplied to phytoplankton and bacteria through lysis (Middelboe et al. 1996; Gobler et al. 1997; Noble and Fuhrman 1998).

#### *4.2 Carbon flow in microbial loop*

Based on the result of the dilution experiment, the bacterial production at the surface ( $25 \text{ mg C m}^{-3} \text{ d}^{-1}$ ) was transferred to protozoa at  $4.3 \text{ mg C m}^{-3} \text{ d}^{-1}$  and to DOC pool at  $18.2 \text{ mg C m}^{-3} \text{ d}^{-1}$  through viral lysis (Fig. 8a). Only a small fraction,  $2.5 \text{ mg C m}^{-3} \text{ d}^{-1}$  of the production, resulted in the net growth (Biomass increase). Assuming the average BGE is 37.5% because a half of heterotrophic bacterial production in the bay is conducted by particle-attached (Lee et al. 2001a) and BGEs for attached and free-living bacteria are 45% and 30%, respectively (del Giorgio and

Cole 2000), BCD was calculated at  $66.6 \text{ mg C m}^{-3} \text{ d}^{-1}$ . The C flow through viral lysis accounted for 27% of the BCD. The deficiency of BCD was  $48.4 \text{ mg C m}^{-3} \text{ d}^{-1}$ . Exudation rate of DOC from phytoplankton was not measured in the present study. The percent extracellular release (PER) of DOC by phytoplankton varies greatly from 0 to 80% with the average at 10-20% depending on local environmental conditions and species compositions (Nagata 2000). High PER tends to be observed during the declining phase of phytoplankton blooms accompanied with nutrient depletion (Larsson and Hagström 1982; Lancelot 1983). The spring bloom in the bay was terminated by  $\text{NO}_3$  depletion (Kudo et al. 2000) and the primary production during the bloom ranged from 90 to  $137 \text{ mg C m}^{-3} \text{ d}^{-1}$  (Kudo et al. 2015). If the supply of DOC from primary production during the bloom compensates the deficiency, 35 to 54% of the primary production should flow to bacteria.

In summer, bacterial production at the surface decreased to  $14.0 \text{ mg C m}^{-3} \text{ d}^{-1}$  and resulting C flow through viral lysis was  $9.2 \text{ mg C m}^{-3} \text{ d}^{-1}$  (Fig. 8b). Assuming the BGE at 37.5%, BCD was  $37.3 \text{ mg C m}^{-3} \text{ d}^{-1}$ . The C flow through viral lysis accounted for 25% of the BCD. A typical primary production in this season is about  $20 \text{ mg C m}^{-3} \text{ d}^{-1}$  (Kudo et al. 2015). The organic carbon supply from primary production was estimated from 2 to  $16 \text{ mg C m}^{-3} \text{ d}^{-1}$  presuming the PER at 10 – 80%. The C supply from the two pathways could not satisfy the BCD with  $12.1\text{-}26.1 \text{ mg C m}^{-3} \text{ d}^{-1}$  deficiency. Some other C source should make up the deficiency. In the C flow, the supply of

DOC from protozoa and meso-zooplankton was not measured in the present study. In coastal areas, the contribution of zooplankton to the DOC flux is potentially large when these grazers consume a large fraction of primary production (White and Roman 1992; Dagg 1993). Meso-zooplankton biomass of the bay, which consisted of copepods, increased during May and September at the maximum of  $140 \text{ mg C m}^{-3}$  in June (Odate and Maita 1988, Ban pers. comm.). The release of DOC by zooplankton represents about 10-20% of ingestion (Copping and Lorenzen 1980; Strom et al. 1997). The food requirements by meso-zooplankton were estimated at  $20\text{-}30 \text{ mg C m}^{-3} \text{ d}^{-1}$  in this period (Ban pers. comm.). Thus, the release of DOC by zooplankton ranged from 2 to  $6 \text{ mg C m}^{-3} \text{ d}^{-1}$ , accounting for 8 to 50% of the deficiency.

At the bottom in April, bacterial production was  $4.7 \text{ mg C m}^{-3} \text{ d}^{-1}$ , one-third to one-fifth of the surface values (Fig. 8c). The carbon return flow through viral lysis was  $3.9 \text{ mg C m}^{-3} \text{ d}^{-1}$ . The BCD was calculated at  $12.5 \text{ mg C m}^{-3} \text{ d}^{-1}$ . The C flow through viral lysis accounted for 31% of the BCD. The deficiency of  $8.6 \text{ mg C m}^{-3} \text{ d}^{-1}$  would be compensated from the organic carbon supply from the surface as sinking particle or mixing with other water mass. After the spring bloom an increase in sinking particle flux which was equivalent to 40% of the primary production during the bloom was observed (Miyake et al. 1998). The integrated organic carbon flux was  $25 \text{ g C m}^{-2}$  in April. Then an active regeneration process of organic matter took place near the bottom until

August (Kudo et al. 2007). Another potential source of DOC was the inflow of Tsugaru warm water (TWW) which replaced the bottom water after summer. However, DOC concentration in TWW was much lower than the existing bottom water (Fig. 3). Thus, this inflow was not a source of DOC. The greater part of DOC was refractory, a fraction that is not consumed by heterotrophic bacteria (Williams and Druffel 1987). Although there is a pool of labile DOC that has a turnover time of less than a few days that support bacterial production, it does not represent even 1% of total DOC (Carlson and Ducklow 1995). Thus, bioavailable (labile DOC) fractions that turn over quickly within the microbial loop may not appear as the change in the bulk DOC concentration.

## 5. Conclusion

The contribution of viral lysis and protozoan grazing to the mortalities of heterotrophic bacteria was evaluated monthly from spring to autumn in a subarctic coastal bay. Viral lysis accounted for about 80% of the bacterial mortality and protozoan grazing showed a smaller contribution (20%). As a consequence of lysis, DOC was released to the surrounding water, fueling the subsequent bacterial production. The potential contribution of this released DOC to the BCD was estimated as 25 to 27% at the surface and 31% at the bottom. Thus, virus lysis plays an important role in controlling bacterial abundance and labile DOC cycles in the microbial loop.

## Acknowledgments

We thank the Captain, officers and crew of the T/S *Ushio Maru* for their helpful assistance.

We are also grateful to Dr. M. Uchimiya for technical instruction for the study.

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## Figure legends

Fig. 1 Sampling station in Funka Bay.

Fig. 2 Seasonal change in temperature (a), salinity (b), nitrate (NO<sub>3</sub>) (c) and ammonium (NH<sub>4</sub>) (d).

Fig. 3 Seasonal change in dissolved organic carbon (DOC).

Fig. 4 Seasonal change in Chl *a* (a), bacterial abundance (BA) (b), heterotrophic nanoflagellate abundance (HNF) (c) and virus abundance (VA)(d).

Fig. 5 Plot of bacterial abundance (BA) versus fraction of original seawater using 10 kDa diluents at 0 m in June. Linear regression:  $y = 0.025 + 2.17x$ ,  $r = 0.999$  (a). Growth curves of BA for the different fraction of original water in two dilution series. Both grazing and lytic pressures were included in the original seawater (b) and only lytic pressure was included in the HNF free seawater (c). Correlation between specific growth rate and the fraction of original seawater and HNF free seawater (d). Linear regressions of the original seawater:  $y = 0.69 - 0.56x$ ,  $r = 0.998$  and the HNF free seawater:  $y = 0.714 - 0.45x$ ,  $r = 0.994$ .

Fig. 6 Lysis, grazing, net growth and potential growth rates at 0 m (a) and bottom (b), the relative percentage of lysis and grazing to the total mortality at 0 m (c) and bottom (d).

Fig. 7 Plots of BA versus VA (a) and BA versus HNF (b) for all data.

Fig. 8 Carbon flow in spring (April) at the surface (0 m) (a), in summer (July) at the surface (0 m) (b) and in spring (April) at the bottom (90 m)(c). A, V, B, P and Z denote autotrophs (phytoplankton), viruses, heterotrophic bacteria, protozoa (HNF) and meso-zooplankton (copepods), respectively. Numerical numbers indicate carbon flow indicated by the arrow and productivity in the box. All units are in  $\text{mg C m}^{-3} \text{ d}^{-1}$ .

Table 1 Potential and in situ (net) growth rates ( $\mu$ ) of heterotrophic bacteria, and lytic and grazing mortalities.

Depth	Month	Temperature (°C)	Potential $\mu$ (d <sup>-1</sup> )	in situ $\mu$ (d <sup>-1</sup> )	Lysis (d <sup>-1</sup> )	Grazing (d <sup>-1</sup> )	Lysis %
0 m							
	April	6.0	1.57	0.16	1.14	0.27	80.7
	June	15.7	0.70	0.13	0.45	0.11	80.3
	July	18.2	0.78	0.10	0.52	0.15	77.2
	September	20.1	0.60	0.09	0.40	0.10	80.3
	October	14.4	0.53	0.03	0.42	0.08	84.3
90 m							
	April	4.0	0.34	-0.15	0.31	0.17	64.4
	June	4.0	0.39	0.07	0.23	0.08	74.9
	September	4.0	0.39	-0.11	0.32	0.19	63.6
	October	5.0	0.30	-0.20	0.35	0.15	70.0

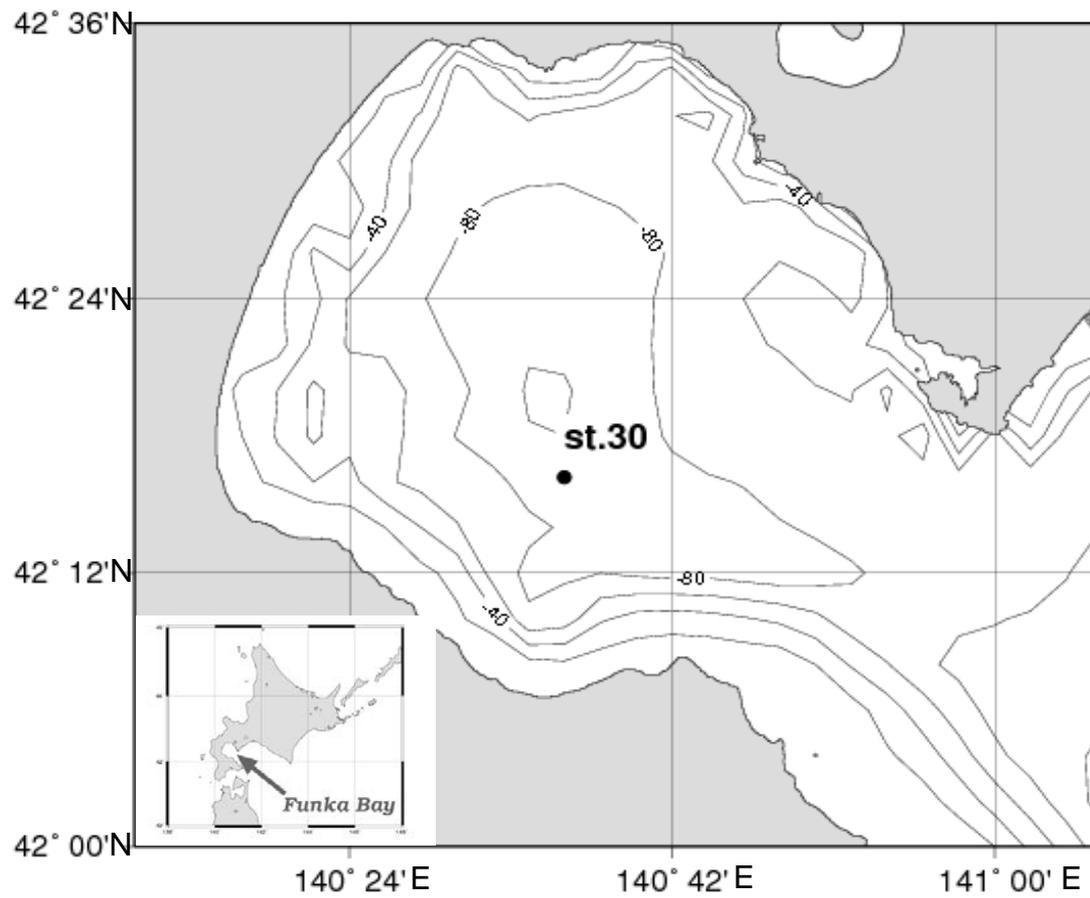


Fig. 1 Kakuta & Kudo

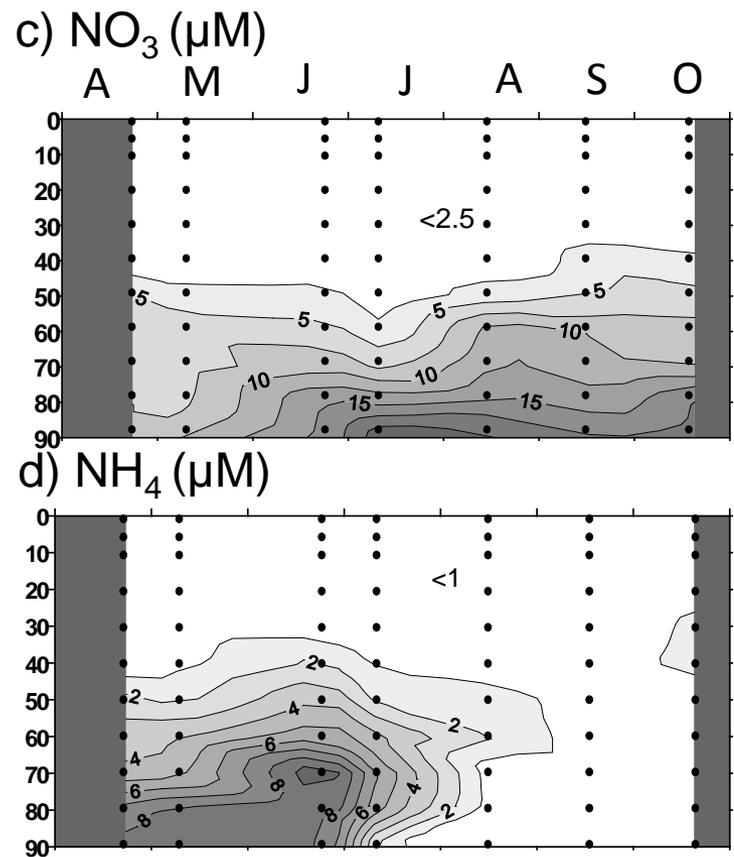
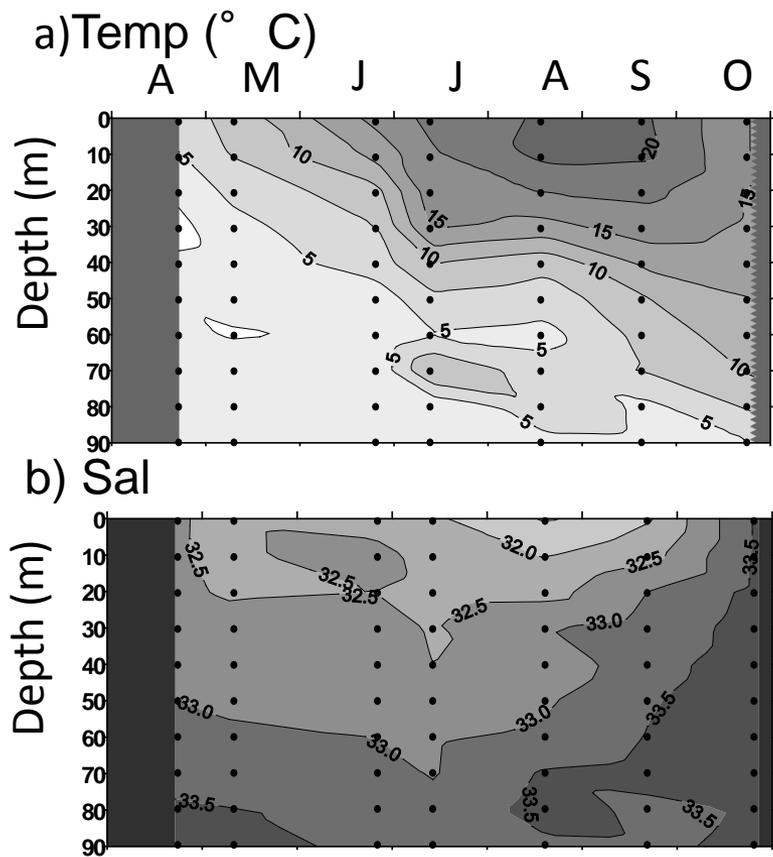


Fig. 2 Kakuta & Kudo

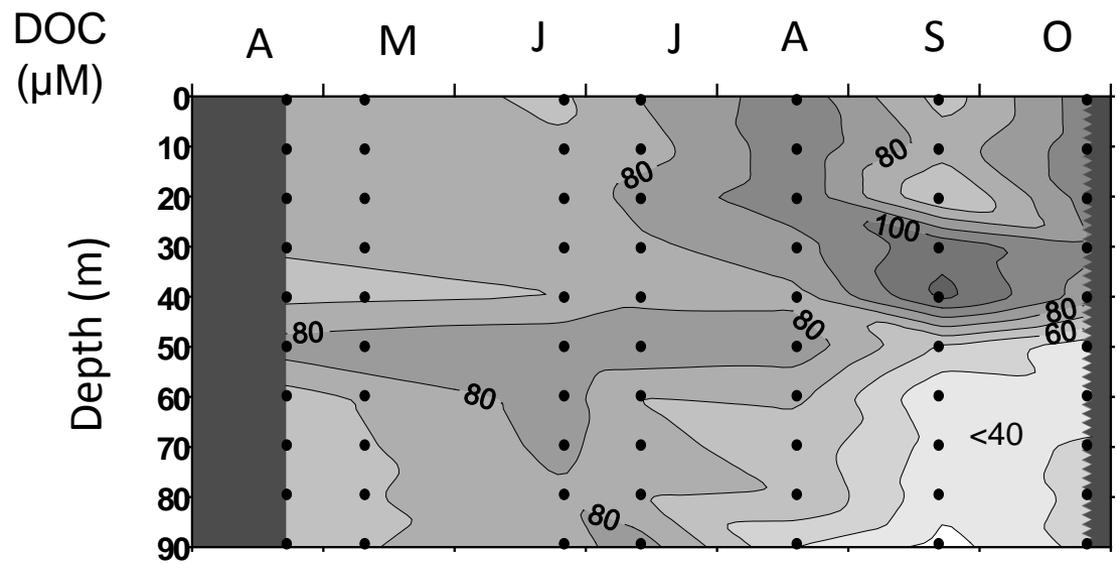
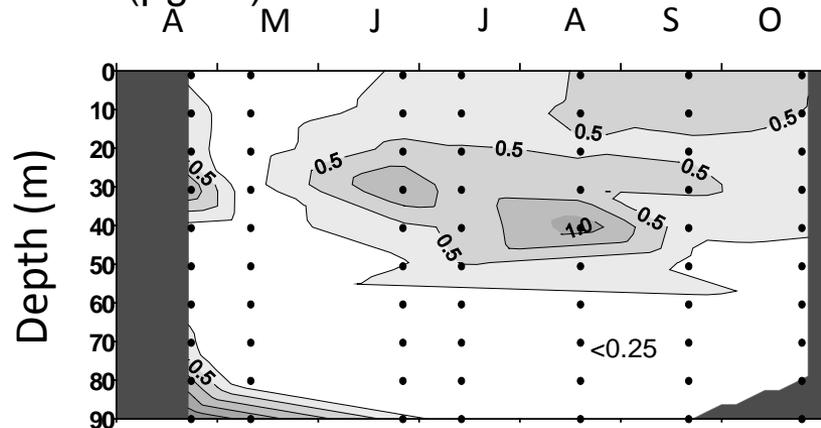
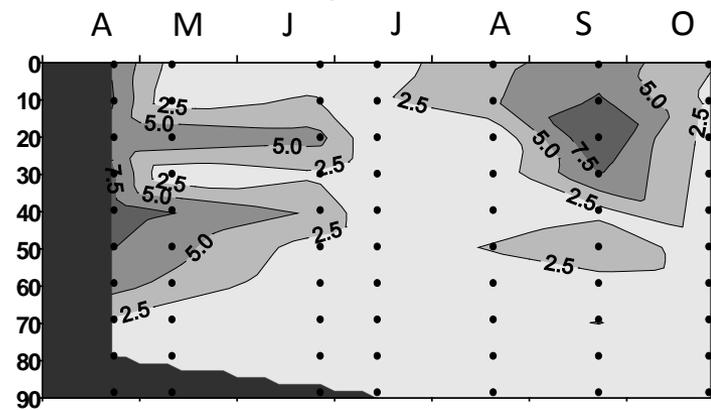


Fig. 3 Kakuta & Kudo

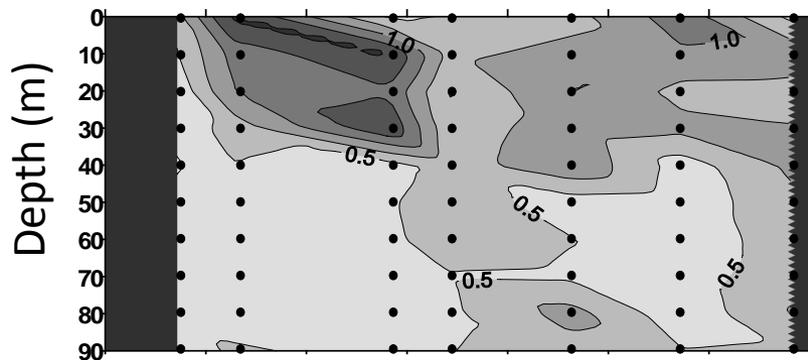
a) Chl. a ( $\mu\text{g L}^{-1}$ )



c) HNF ( $\times 10^5 \text{ cells L}^{-1}$ )



b) BA ( $\times 10^9 \text{ cells L}^{-1}$ )



d) VA ( $\times 10^9 \text{ L}^{-1}$ )

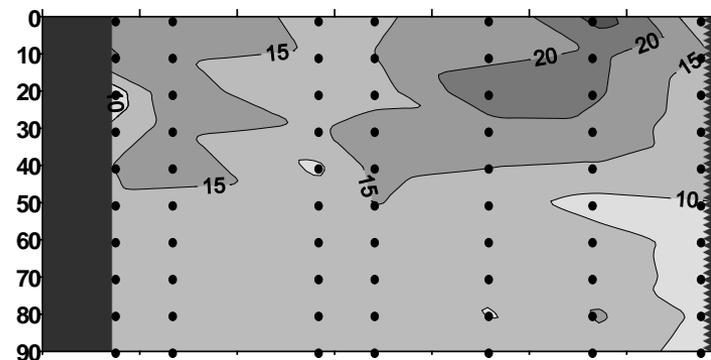


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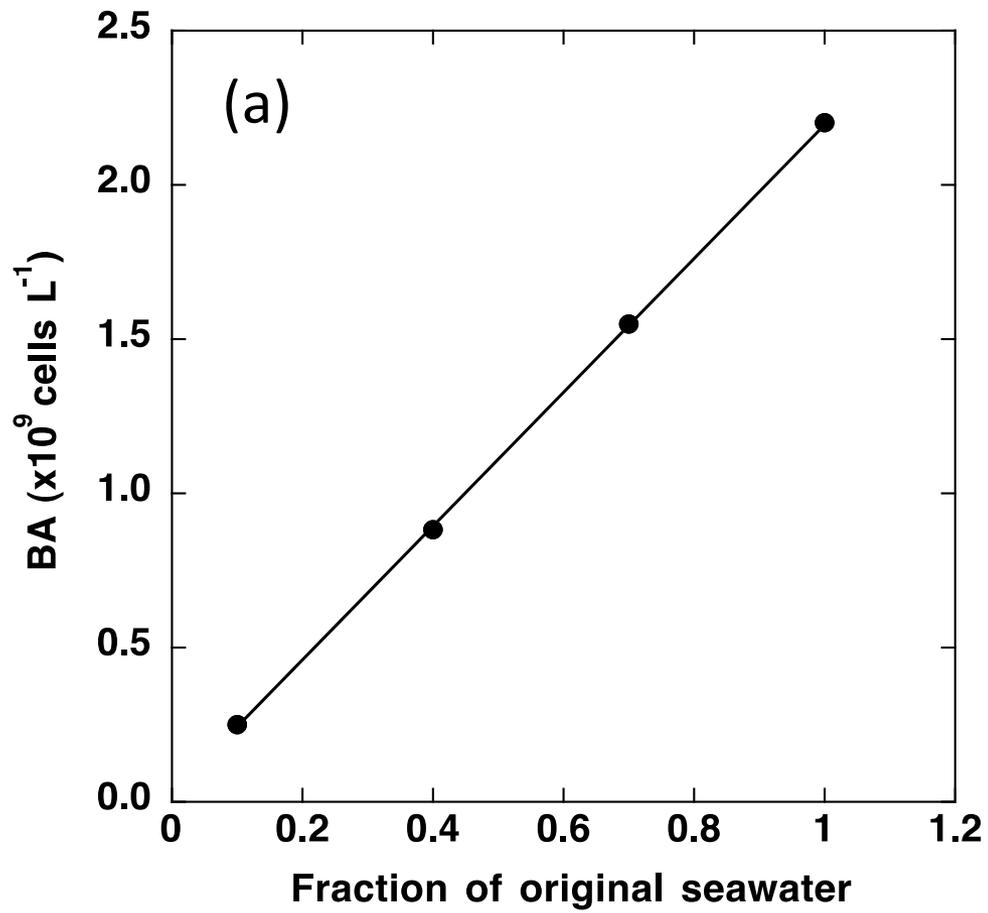


Fig. 5a Kakuta & Kudo

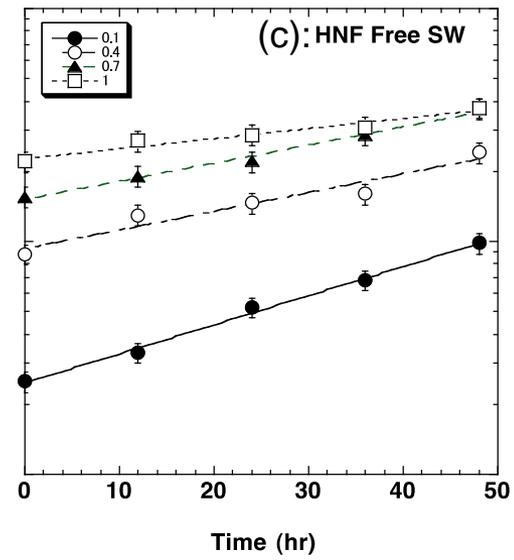
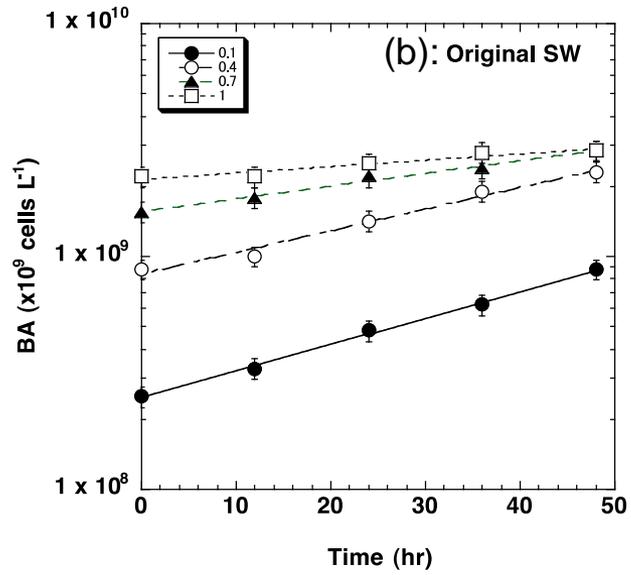


Fig. 5b Kakuta & Kudo

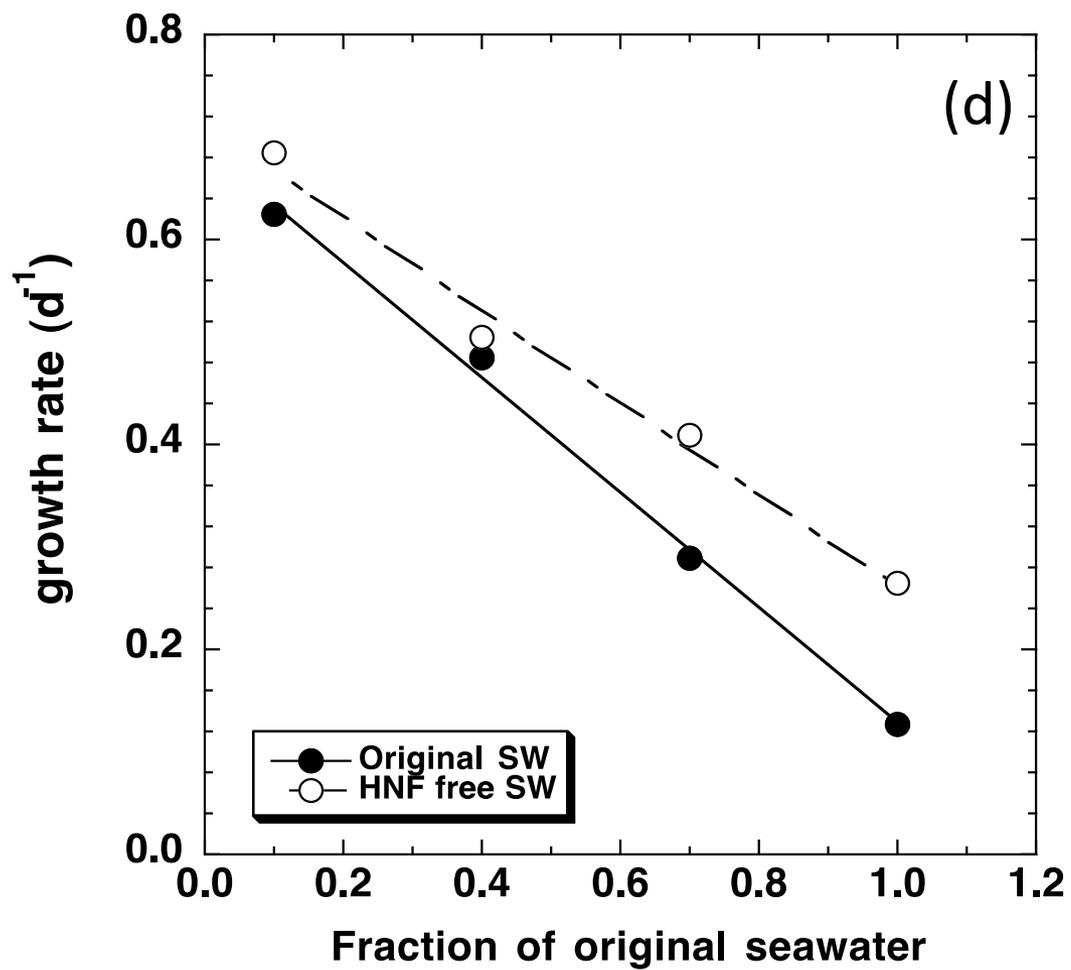


Fig. 5c Kakuta & Kudo

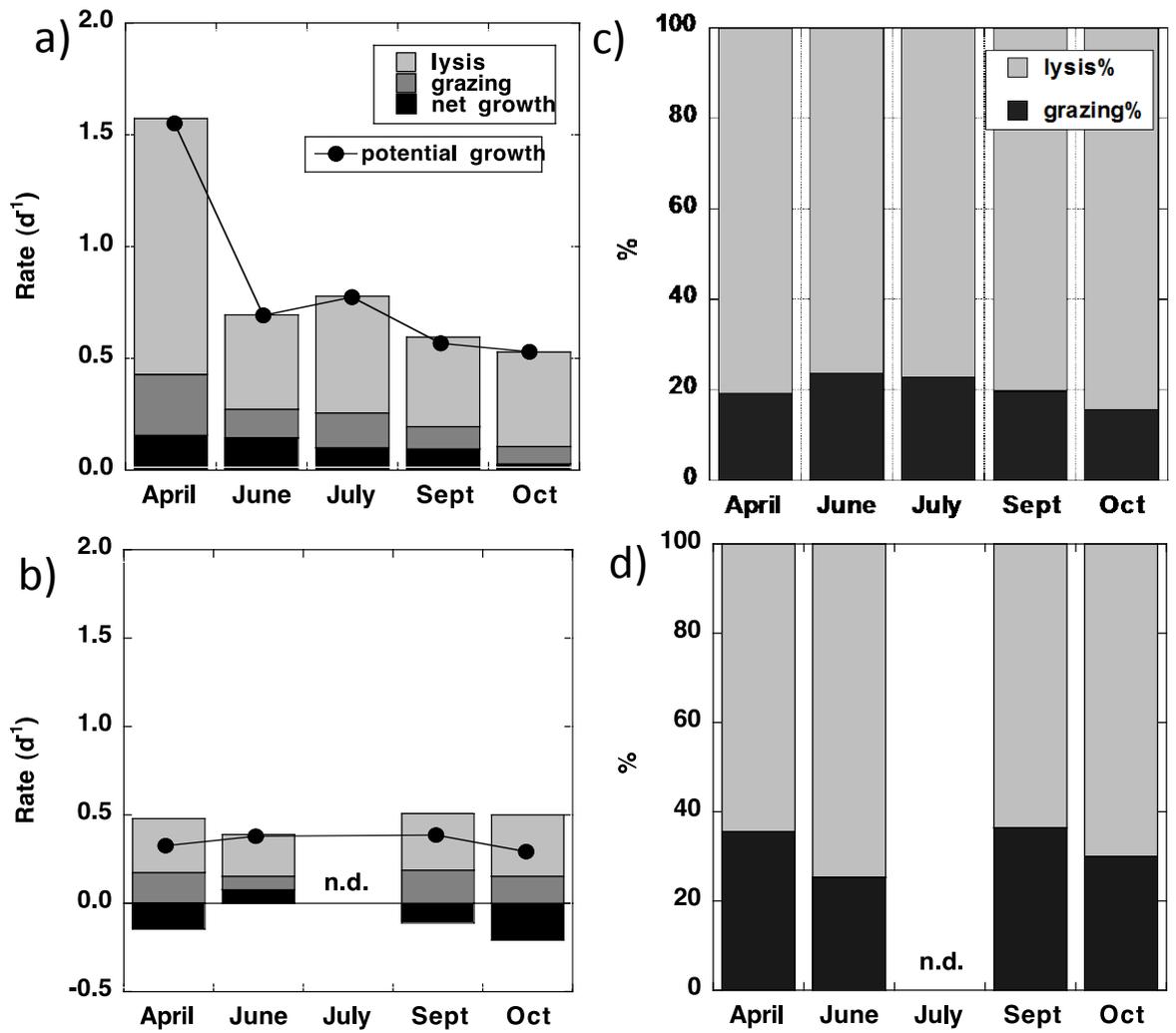


Fig. 6 Kakuta & Kudo

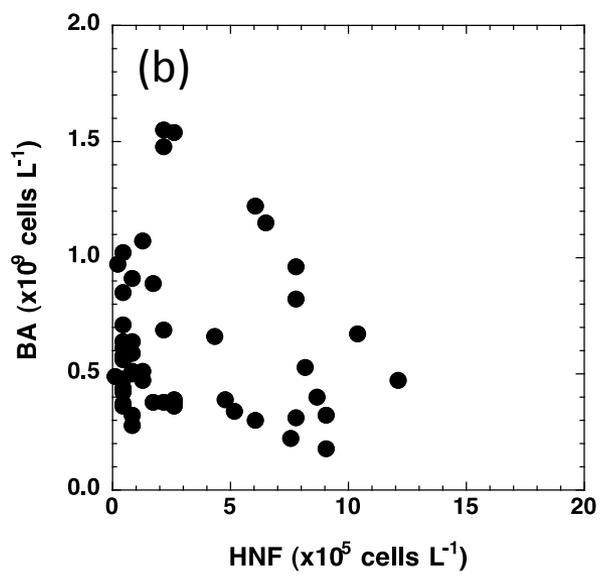
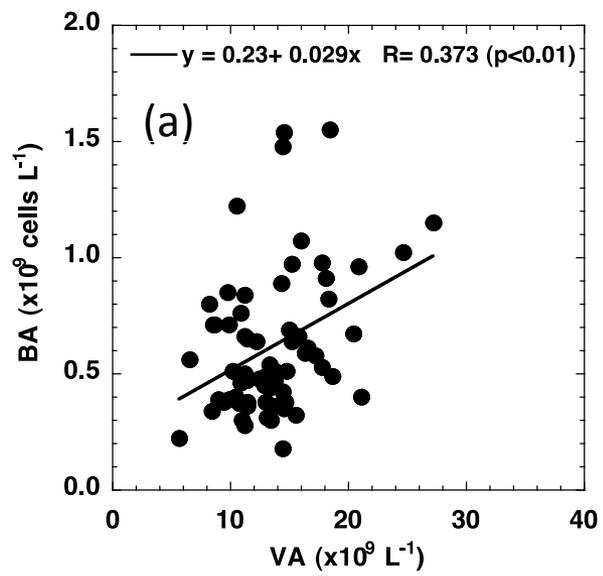


Fig. 7 Kakuta & Kudo

(a) Spring 0 m

mg C m<sup>-3</sup> d<sup>-1</sup>

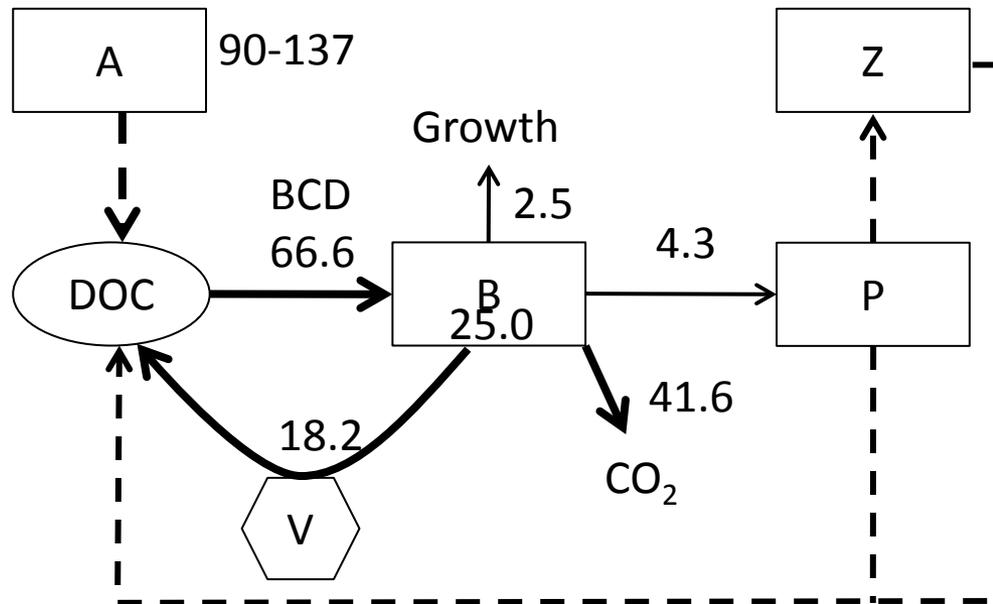


Fig. 8a Kakuta & Kudo

(b) Summer 0 m

mg C m<sup>-3</sup> d<sup>-1</sup>

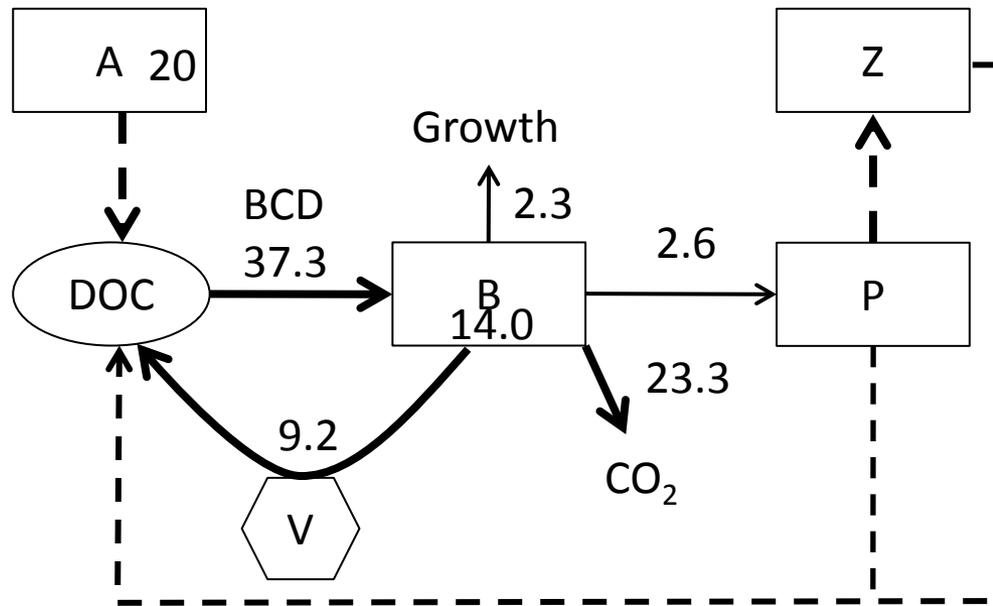


Fig. 8b Kakuta & Kudo

(c) Spring 90 m

mg C m<sup>-3</sup> d<sup>-1</sup>

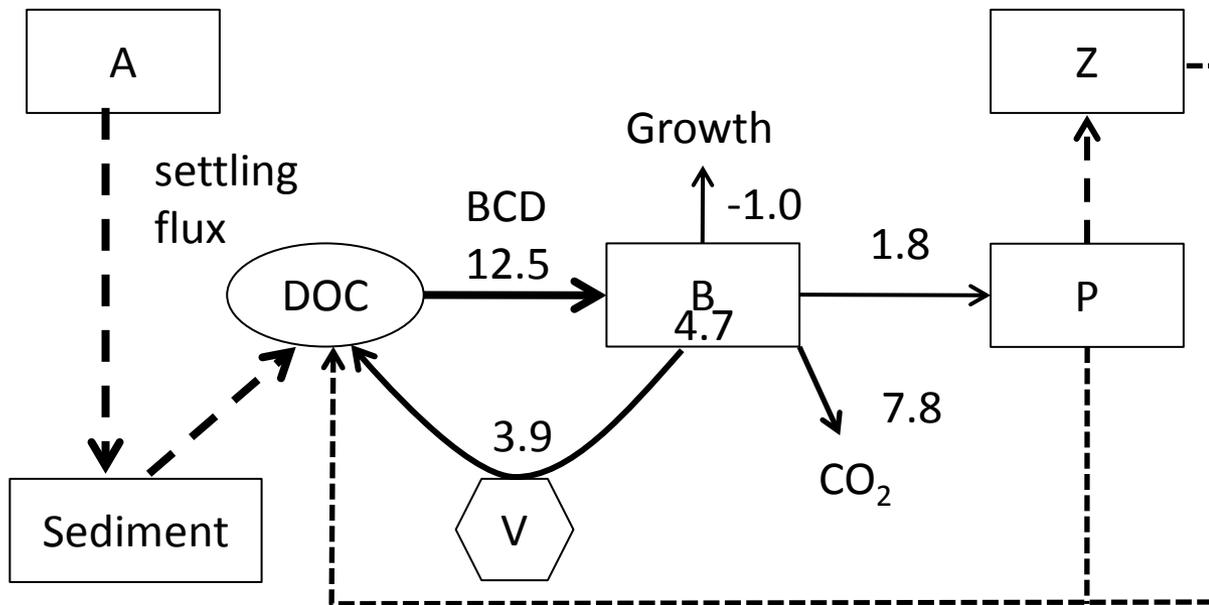


Fig. 8c Kakuta & Kudo