

# Fate of the bacterial cell envelope component, lipopolysaccharide, that is sequentially mediated by viruses and flagellates

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**Abstract**—Transformation of a cell envelope component of bacteria, lipopolysaccharide (LPS), from bacterial cells into a dissolved form was investigated during time course experiments using seawater culture in conjunction with determining population dynamics of three microbial groups, which are flagellates, bacteria, and viruses. The surface seawater collected from a coastal site was enriched with glutamic acid, and then incubated. LPS concentration was determined using the *Limulus* amoebocyte lysate assay. In the early period of incubation, LPS in the particulate fraction ( $>0.2\ \mu\text{m}$ ) increased to the maximum in association with bacterial abundance; thereafter, both decreased together. During the fluctuation of LPS in the particulate fraction, LPS in the dissolved fraction ( $<0.2\ \mu\text{m}$ ) also increased with viral abundance, but then decreased after flagellate abundance began to increase. However, in a culture condition without flagellates, LPS in both dissolved and particulate fractions increased continuously in association with viral abundance. This increase in LPS in the two fractions associated with viral abundance continued while bacterial abundance was maintained at a similar level between the middle and late incubation periods. The temporal change in LPS in the two fractions and its coupled microbial dynamics indicate that LPS in bacterial cells was transformed into nonliving dissolved and particulate forms by viral lysis and that thereafter the LPS was removed through ingestion by flagellates. Thus, our results suggest that the fate of the bacterial cell envelope component, LPS, that is sequentially mediated by viruses and flagellates in the sea.

**Key words:**

## Introduction

Dissolved organic matter (DOM) in seawater is one of the largest active reservoirs of organic carbon on Earth (Hedges 1992), and its importance in marine geochemical cycles and microbial food webs has been widely accepted (Ducklow and Carlson 1992, Williams 2000, Hedges 2002). However, the chemical nature, source, formation and removal processes of DOM are not yet fully elucidated (Benner 2002, Carlson 2002, Ogawa and Tanoue 2003). Characterizations of DOM in this decade have revealed that specific biomolecules of bacteria that are derived from the cell envelope, including the outer membrane protein, peptidoglycan, and lipopolysaccharide (LPS), are ubiquitously distributed in a dissolved form in the ocean (Tanoue et al. 1995, McCarthy et al. 1998, Wakeham et al. 2003, Benner and Kaizer 2003, Zou et al. 2004). In addition, recent studies reported that a major

fraction of peptidoglycan in particulate organic matter (POM) could not be accounted for in terms of intact bacterial cells (Benner and Kiser 2003, Shibata et al. 2006). Hence, considerable amounts of the cell envelope components of bacteria might exist in a nonliving form within both the dissolved and particulate fractions in seawater.

The transformation of the cell envelope components in bacterial cells into a nonliving dissolved form has been demonstrated, particularly focusing on constituents of peptidoglycan (Ogawa et al. 2001, Pérez et al. 2003, Middelboe and Jørgensen 2006, Kawasaki and Benner 2006, Kitayama et al. 2007). However, to our knowledge, the transformation into nonliving dissolved as well as particulate forms has not been documented with regard to the other cell envelope components. LPS is found exclusively in the outer membrane of gram-negative bacteria, and its concentration in seawater has been determined using the *Limulus* amoebocyte lysate (LAL) assay (Watson et al. 1977, Maeda and Taga 1979). The LPS

data that were acquired using this assay showed that a significant portion of LPS was present in a  $<0.3 \mu\text{m}$  size fraction in seawater or was liberated from intact bacterial cells in freshwater (Evans et al. 1978, Maeda and Taga 1979). These results are in line with that of a recent study reporting that short-chain  $\beta$ -hydroxy acid, which is attributed to LPS, is a possible source of marine DOM (Wakeham et al. 2003). Thus, investigating the transformation of LPS into both non-living dissolved and particulate forms would elucidate why LPS is distributed in a form freed from intact bacterial cells in the environment.

The transformation of LPS from bacterial cells into a dissolved form appears to occur through bacterial mortality, which is mainly due to grazing of flagellates and/or viral lysis (Nagata 2000, Fuhrman 2000). This is because flagellates ingest unassimilated prey bacteria (Nagata and Kirchman 1992, Ferrier-Pages et al. 1998) and viral lysis of bacteria results in the release of cellular solutes and structural components (Weinbauer and Peduzzi 1995, Middelboe and Jørgensen 2006). In addition to the transformation through mortality, LPS could be released directly from bacteria (Heissenberger and Herndl 1994, Kawasaki and Benner 2006). LPS also would be transformed into a nonliving particulate form, as its direct release from bacteria (Passow 2002) and viral lysis of bacteria (Shibata 1997) lead to formation of particles larger than  $0.2 \mu\text{m}$  in diameter. However, in previous studies, the transformation into DOM or POM derived from bacteria has been demonstrated under culture conditions where bacteria, flagellates, and viruses were or were not present together but not when temporal changes in the abundance of these three microbes were monitored throughout incubation. Therefore, examining the temporal change in LPS concentration that is present in both dissolved and particulate forms in conjunction with the dynamics of three microbes would offer insight into the fate of cell envelope components mediated by microbes in the sea.

We investigated the transformation of LPS into dissolved and particulate forms and its relationship to the abundance of microbes in time course experiments using seawater culture. The experiments were conducted using surface seawater enriched with glutamic acid in which natural microbial assemblages of flagellates, bacteria, and viruses were included, but in which only growth of the flagellate assemblage was artificially controlled. The results of our study provide insights into the role of the three microbes in controlling abundance of the bacterial cell envelope component in a non-living form in the sea.

## Materials and Methods

### Sample collection

Seawater samples were collected using Niskin bottles at

a depth of 10 m on 11 October 2002 (for Experiment 1) and 25 January 2003 (for Experiment 2) at a site 2 km off the Manazuru Peninsula in Sagami Bay, Japan ( $35^{\circ}09'N$ ,  $139^{\circ}10'E$ ). Polycarbonate bottles (4 L; Nalgene Nunc Int.) that were prewashed with HCl and Milli-Q water were washed with the sample seawater 3 times and then filled. These samples were stored in the dark at 4°C until later treatment.

### Seawater culture experiments

For Experiment 1, a seawater sample was filtered through a  $10 \mu\text{m}$  pore-size Nuclepore filter. A portion of the filtrate was then further filtered through a  $0.8 \mu\text{m}$  pore-size Nuclepore filter to remove bacterial grazers to the maximum extent. Incubation in seawater culture was separately conducted using the  $<10 \mu\text{m}$  and  $<0.8 \mu\text{m}$  filtrates. For Experiment 2, a seawater sample was filtered through a  $10 \mu\text{m}$  pore-size Nuclepore filter. Then a portion of the filtrate was further filtered separately through a GF/F glass fiber filter (nominal pore-size,  $0.7 \mu\text{m}$ ) and a  $0.2 \mu\text{m}$  pore-size Nuclepore filter. Both the  $10 \mu\text{m}$  and GF/F filtrates were diluted by the prepared  $0.2 \mu\text{m}$  filtrate at a ratio of 1 : 100. Incubation was separately conducted in the seawater culture using the two diluted filtrates ( $<10 \mu\text{m}$  and GF/F filtrates). The combination of filtration through the GF/F filter that had a slightly smaller pore size than the Nuclepore filter (pore size,  $0.8 \mu\text{m}$ ) and dilution of the GF/F filtrate with another filtrate obtained through a  $0.2 \mu\text{m}$  Nuclepore filter was utilized to prevent an increase in flagellate abundance to the maximum extent in one culture condition in Experiment 2 (see Discussion). For the two culture conditions of Experiments 1 and 2, each prepared filtrate was placed in a single polycarbonate container (volume, 10 L; Nalgene Nunc Int.). For stimulation of bacterial production, glutamic acid was added to all cultures, producing a final concentration of  $300 \mu\text{g L}^{-1}$ . Those cultures were then incubated in the dark at room temperature ( $20 \pm 2^{\circ}\text{C}$ ).

During a time course observation, subsamples were taken from the cultures and placed in a polypropylene tube (volume, 15 ml; Corning, Inc.) at each time point for determination of LPS concentration. A portion of the subsample was gently filtered through a  $0.2 \mu\text{m}$  pore-size Acrodisc syringe filter (HT Tuffryn; Pall Co., USA). The filtered ( $<0.2 \mu\text{m}$ ) and unfiltered fractions were immediately used for LPS determination. Subsamples were also taken and subsequently placed in a polypropylene tube (volume, 50 ml; Corning, Inc.), and then fixed with formaldehyde for enumerating bacteria and viruses (final concentration, 0.74%) or in a brown polyethylene bottle (volume, 250 ml; Nalgene Nunc Int.) with glutaraldehyde for enumerating flagellates (final concentration, 1%). All fixed samples were stored at 4°C until slide preparation and enumeration.

### Enumeration of microbes

Bacteria and viruses from subsamples were counted using the original SYBR Green I staining protocol (Noble and Fuhrman 1998a). Seawater samples (volume, 2 to 20 ml) were filtered through a 0.02  $\mu\text{m}$  pore-size Anodisc filter after which the filter was stained with SYBR Green I in the dark for 15 min. Glass slides were prepared with an anti-fade mounting reagent consisting of 50% glycerol and 50% phosphate buffered saline with 0.1% *p*-phenylenediamine. A minimum of 20 randomly selected fields were examined, producing a count of at least 400 bacterial cells and viral particles. On the other hand, flagellates were counted using the 4',6-diamidino-2-phenylindole (DAPI) and fluorescein isothiocyanate (FITC) double staining protocol (Sherr and Sherr 1983). Samples were stained with the two reagents for 20 min, and then filtered through a 0.8  $\mu\text{m}$  pore-size Nuclepore filter (volume, 15 to 100 ml). A minimum of 20 randomly selected fields were examined, producing a count of at least 50 flagellate cells; however, when no cell was observed in 50 fields, further enumeration was not done. One slide was prepared for each subsample. A BX50 epifluorescence microscope (Olympus Co.) was used for counting microbes on filters mounted on glass slides and covered with a coverslip.

### Determination of LPS concentration

LPS concentration was determined using the colorimetric LAL assay (Limulus Color KY Test; Wako Pure Chemical Industries, Ltd.). The assay was done immediately after sample preparation of the filtered ( $<0.2 \mu\text{m}$ ) and unfiltered fractions for the determination of LPS in dissolved and total fractions, respectively.

The LAL reagent and standard LPS purified from *Escherichia coli*, UKT-B (Code No, 291-53101; Wako Pure Chemical Industries, Inc.), were dissolved with 3 and 5 ml of LPS-free physiological saline (NaCl, 0.9%; Ohtuka Pharmaceutical Co. Ltd.), respectively. 50  $\mu\text{l}$  of each seawater sample, LPS-free physiological saline (50  $\mu\text{l}$ ), and the LAL reagent (50  $\mu\text{l}$ ) were placed in wells of a 96-well microtiter plate, and then the plates were incubated for 30 min at 37°C. During the incubation period, absorbance at 405 and 650 nm was continuously monitored by a microplate reader, Sunrise Rainbow Thermo (Tecan Group Ltd.), with the assistance of a commercially available software program (LS-PLATEmanager 2000; Wako Pure Chemical Industries, Ltd.).

LPS concentration was determined using a logarithmic plot calibration curve of the standard LPS versus onset time, which was defined as the period of time the reaction continued until the difference in absorbance between 405 nm and 650 nm reached 0.015. The LPS concentration was corrected by the recovery rate of the internal LPS standard (concentration, between 0.05 to 2.5 ng in 50  $\mu\text{l}$  of physiological saline); the standard was placed in wells of the microtiter plate replacing the LPS-free physiological saline. The LPS concen-

tration in two fractions for each subsample was measured in triplicate. The coefficient of variation in the three measurement values is usually within 10%. LPS concentration in the particulate fraction was calculated by subtracting the concentration in the dissolved fraction from that in the total fraction.

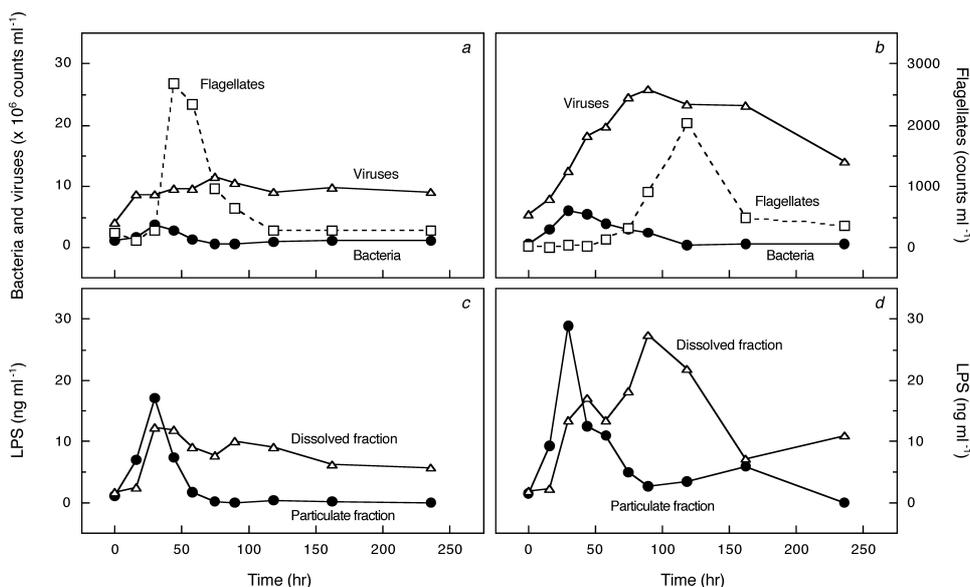
## Results

### Experiment 1

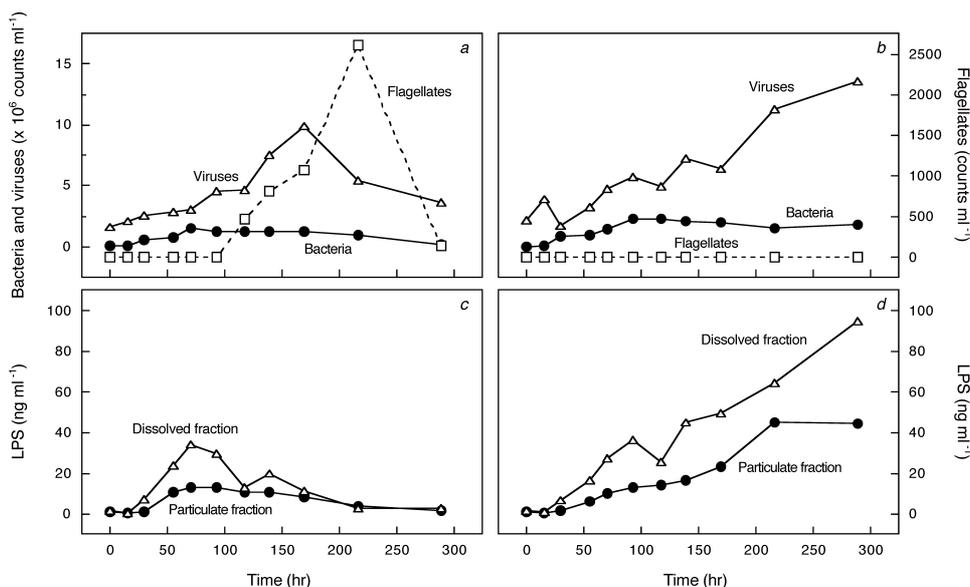
In Experiment 1, LPS in the particulate fraction ( $>0.2 \mu\text{m}$ ) and bacterial abundance increased to maximum values at 30 h in the  $<0.8 \mu\text{m}$  and  $10 \mu\text{m}$  filtrates and then decreased (Fig. 1). LPS in this fraction was significantly correlated with bacterial abundance during incubation in the  $<10 \mu\text{m}$  ( $n=10$ ,  $r=0.95$ ,  $p<0.001$ ) and  $<0.8 \mu\text{m}$  ( $r=0.83$ ,  $p<0.01$ ) filtrates. While the LPS in the particulate fraction fluctuated in the early period of incubation, LPS in the dissolved fraction also increased with viral abundance in both filtrates, and, subsequently, became more abundant than that in the particulate fraction from 43 h onward. However, LPS in the dissolved fraction was significantly correlated with viral abundance in only the  $<0.8 \mu\text{m}$  filtrate ( $n=10$ ,  $r=0.79$ ,  $p<0.01$ ). The maximum concentration of dissolved LPS was observed at 30 and 89 h in the  $<10$  and  $<0.8 \mu\text{m}$  filtrates, respectively, and that value in the latter filtrate was 2.7-fold higher than in the former filtrate. Although flagellate abundance belatedly started to increase at 57 h in the  $<0.8 \mu\text{m}$  filtrate compared with that at 30 h in the  $<10 \mu\text{m}$  filtrate, the LPS in the dissolved fraction decreased in both filtrates after the start of the increase in flagellate abundance. Viral abundance also decreased after flagellate abundance reached a maximum at 43 and 118 h in the  $<10$  and  $<0.8 \mu\text{m}$  filtrates, respectively. In the  $<10 \mu\text{m}$  fraction, the peak abundance of bacteria, flagellates, and viruses was observed in that order with incubation time, whereas the sequence was bacteria, viruses, and flagellates in the  $<0.8 \mu\text{m}$  filtrate.

### Experiment 2

In the  $<10 \mu\text{m}$  filtrate of Experiment 2, LPS in the particulate fraction gradually increased with bacterial abundance, and then reached a maximum value at 70 h (Fig. 2). During incubation, LPS in the particulate fraction was significantly correlated with bacterial abundance ( $n=11$ ,  $r=0.88$ ,  $p<0.001$ ). Along with the increase in LPS in the particulate fraction, LPS in the dissolved fraction also increased with viral abundance, and then the LPS became more abundant than in the particulate fraction. However, the LPS in the dissolved fraction decreased while flagellate abundance increased from 93 h to reach a maximum value at 217 h, and became comparable to that in the particulate fraction in the late period of incubation. In addition, viral abundance also started to decrease from 169 h. A peak abundance of bacte-



**Fig. 1.** Temporal changes in microbial abundance and LPS concentration in  $<10 \mu\text{m}$  (a, c) and  $<0.8 \mu\text{m}$  (b, d) filtrates during incubation for Experiment 1. Symbols for flagellates (□), bacteria (●), and viruses (△) indicate the abundance of each microbe in the upper portion of the figure. LPS in dissolved (△) and particulate (●) fractions are shown in the lower portion of the figure.



**Fig. 2.** Temporal changes in microbial abundance and LPS concentration in  $<10 \mu\text{m}$  (a, c) and GF/F (b, d) filtrates during incubation for Experiment 2. Symbols for flagellates (□), bacteria (●), and viruses (△) indicate the abundance of each microbe in the upper portion of the figure. LPS in dissolved (△) and particulate (●) fractions are shown in the lower portion of the figure.

ria, viruses, and flagellates was observed in that order with incubation time.

In the same experiment, with the GF/F filtrate, no increase in flagellate abundance was observed during incubation. Because LPS continuously increased in both dissolved and particulate fractions in parallel with viral abundance, LPS in dissolved ( $n=11$ ,  $r=0.96$ ,  $p<0.001$ ) as well as particulate ( $r=0.96$ ,  $p<0.001$ ) fractions correlated significantly with viral abundance during the incubation period. On the other hand, bacterial abundance increased until 93 h but then

maintained that level until the end of the incubation. Thus, the difference between the LPS in both fractions and bacterial abundance became more pronounced with time, and, therefore, LPS in the two fractions did not significantly correlate with bacterial abundance during the incubation period (each correlation;  $n=11$ ,  $r=0.53$ ,  $p>0.05$ ). Compared with the LPS concentrations in each fraction at the final time point in the  $<10 \mu\text{m}$  fraction, those in the GF/F filtrate became much higher in the dissolved and particulate fractions (38- and 34-fold, respectively). During the incubation period, only

a simultaneous increase in bacterial and viral abundance was observed.

## Discussion

### Effect of pretreatment on growth of microbes in seawater culture

Our results from Experiment 1 showing that flagellate abundance was belatedly increased in the culture using the  $<0.8\ \mu\text{m}$  filtrate compared with that using the  $<10\ \mu\text{m}$  filtrate indicate that filtration with the  $0.8\ \mu\text{m}$  pore size filter resulted in removal of a significant portion of, but not all, flagellates from seawater. On the other hand, in Experiment 2, flagellate abundance increased in the  $<10\ \mu\text{m}$  filtrate, but not in the  $<\text{GF/F}$  filtrate during the incubation period. This lack of increase in the latter filtrate is attributed to the removal of a large number of flagellates by filtration with the  $<\text{GF/F}$  filter and to death of flagellates from starvation due to the reduction of prey bacterial abundance to less than the representative grazing threshold of marine flagellates (Eccleston-Parry and Leadbeater 1994) by dilution of the  $\text{GF/F}$  filtrate with a filtrate obtained through a  $0.2\ \mu\text{m}$  Nuclepore filter. Thus, by taking into account these differences in the growth or presence of microbes observed between the two conditions in each experiment, the impact of the three microbial groups on the transformation of LPS into dissolved and particulate forms is discussed in the sections below.

### Transformation of LPS into a dissolved form

We found that abundance of LPS in the dissolved fraction became greater than in the particulate fraction during the incubation period. This is consistent with results of studies done in natural environments (Evans et al. 1978, Maeda and Taga 1979) showing that a significant portion of LPS existed in the dissolved fraction of seawater (11–75%) and was not associated with intact bacterial cells in freshwater (29–92%). Similarly, the transformation of LPS into a dissolved form that was observed in our study is supported by a report showing that short-chain  $\beta$ -hydroxy acid, which is attributed to LPS, is a possible source of marine DOM (Wakeham et al. 2003). Thus, our results confirmed that transformation of LPS from bacterial cells into the dissolved fraction took place regardless of culture conditions.

The facts that LPS in the dissolved fraction increased with viral abundance indicate that intact bacterial cells were broken down by viral lysis, and, consequently, LPS associated with the cells was transformed into a dissolved form. This pathway for transformation of LPS is supported by results of a monoxenic culture experiment that included viruses and host bacteria (Middelboe and Jørgensen 2006), which showed that the other component of the cell envelope, peptidoglycan, was transformed into a dissolved form by viral

lysis of host cells. Because viruses are responsible for 10 to 60% of bacterial mortality (Fuhrman 2000, Wommack and Colwell 2000, Weinbauer 2004, Suttle 2005), their contribution to the transformation of LPS into a dissolved form is potentially large.

### Transformation of LPS into a particulate form

In both of our experiments with flagellates, we found that LPS in the particulate fraction was significantly correlated with bacterial abundance under three culture conditions. This is in line with previous results of an LPS assay showing that LPS bound to bacterial cells significantly correlated with bacterial abundance in natural seawater samples (Watson et al. 1977). In contrast, LPS in the particulate fraction did not significantly correlate with bacterial abundance in the  $\text{GF/F}$  filtrate in Experiment 2, which employed culture conditions without flagellates. The profound increase in LPS in the particulate fraction without an increase in bacterial abundance in the  $\text{GF/F}$  filtrate indicates that a non-living form of LPS accounts for this increase. A similar result was reported by Kawasaki and Benner (2006). They found that glucosamine and galactosamine, which can be found in LPS, simultaneously increased as POM without an increase in bacterial abundance during incubation of seawater culture. Although spontaneous assembly or abiotic aggregation of LPS in a dissolved form (Chin et al. 1998, Kerner et al. 2003, Engel et al. 2004) is possible, it is yet to be clarified how the LPS in a nonliving particulate form was produced.

### Role of flagellates in removing processes of nonliving LPS from seawater

Of importance is our finding that the increase in LPS in dissolved and particulate fractions was dramatically suppressed in the presence of flagellates under the culture conditions. This indicates that biological activity of flagellates was responsible for the removal of the LPS from seawater. A number of studies have demonstrated that flagellates ingest various types of organic substrates, such as high molecular weight DOM and colloids (Sherr 1988, Marchant and Scott 1993, Tranvik et al. 1993, Tranvik 1994). Tranvik et al. (1993) demonstrated that LPS extracted from *Escherichia coli* was ingested by a natural assemblage and isolates of flagellates. However, to our knowledge, ingestion by flagellates of bacteria-derived DOM formed through interactions among the three microbes (viruses, bacteria and flagellates) has not been documented. Thus, it is strongly suggested that the ingestion by flagellates serves as a removal mechanism of LPS in the nonliving forms from seawater.

It is also known that flagellates ingest viral particles in seawater (Gonzalez and Suttle 1993). In our study, we found that viral abundance was suppressed in the presence of flagellates. This is consistent with the observation in a lake water culture experiment by Tranvik (1994). Thus, it is very likely

that the ingestion of flagellates was responsible for the decrease in viral abundance in our seawater culture experiments. The suppression of viral abundance by flagellates should also suppress LPS production in the dissolved form due to the reduction of the frequency of new viral infections in bacteria.

### Pathway for transformation and removal of LPS and its implications

That viral lysis of bacteria results in the release of cellular solutes and structural components after which lysis products are utilized by bacteria has been hypothesized (Bratbak et al. 1990, Proctor and Fuhrman 1990) and demonstrated (Middelboe et al. 1996, Noble and Fuhrman 1998b, Middelboe et al. 2003). On the other hand, the results of our study indicated that LPS in bacterial cells was transformed into nonliving dissolved and particulate forms by viral lysis, followed by removal through ingestion by flagellates. Thus, this suggests another fate of the cell envelope component of bacteria, a part of the biomass in a nonliving form as transformed by viral lysis.

We found that an increase in nonliving LPS in the particulate and/or dissolved fraction is pronounced when viral abundance is increased following an increase in bacterial abundance but prior to flagellate abundance, or when only bacteria and viruses appear in that order over time. These facts suggest that the quantitative change in specified organic matter reflected the sequence of peak abundance of microbes, which was determined by dynamics among the activities of bacteria, viruses, and flagellates. Hence, the temporal increase in abundance of the cell envelope component would occur more prominently in an environment that includes (1) bacterial production that is temporally but drastically enhanced, and (2) viral production (a frequency of viral lysis of bacteria) that is increased following an increase in bacterial production but prior to flagellate ingestion of the nonliving LPS, preferably when that activity of flagellates is suppressed for any reason. This hypothesis should be confirmed by field observations in future studies.

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