

Effects of glutamate and glucose on N cycling and the marine plankton community

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ABSTRACT: Phosphate-enriched natural coastal seawater was incubated with the addition of glutamate and glucose, and the effects on coastal bacteria were studied, as well as subsequent effects on phytoplankton and heterotrophic nanoflagellates (HNF). Based on the changes in bacterial and HNF density, chlorophyll *a* concentrations and ¹⁵N dilution, the addition of glucose resulted in bacteria out-competing phytoplankton for available nitrogen. Glutamate addition resulted in the enhancement of both bacterial and phytoplankton populations. However, the increased bacterial population returned to background level rapidly; this may have been caused by tight coupling between bacterial production and grazing on bacteria. In spite of adding the same amount of organic carbon in both treatments (about 90 $\mu\text{mol l}^{-1}$), enhancement of the net particulate organic carbon production by the entire planktonic community was considerably higher when glutamate was added. These results suggest that the biochemical basis of the bacterial substrate controls not only bacteria but also phytoplankton and HNF, through trophic interactions such as competition for nitrogen and predator–prey relationships.

KEY WORDS: Bacteria · Labile DOM · Phytoplankton · Heterotrophic nanoflagellates

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INTRODUCTION

In aquatic environments, bacteria play a central role in ecological and biogeochemical cycles. Dissolved free amino acids (DFAA) and monosaccharides, principal components of the cell, are an important part of labile dissolved organic carbon (DOC), and marine bacteria promptly consume these molecules (Fuhrman 1990, Rich et al. 1996). Many studies have evaluated the uptake rates of these compounds in seawater (e.g. Keller et al. 1982, Fuhrman 1987, Glibert et al. 1991, Rich et al. 1996, Kirchman et al. 2001). In spite of low concentrations, these compounds sustain a considerable part of bacterial growth; this is possible through the high turnover rates of these compounds (Kirchman 2003).

To utilize monosaccharides for growth, bacteria require nitrogenous nutrients; thus, bacterial growth is

directly influenced by N availability (Kirchman 1990, Goldman & Dennett 1991, Skoog et al. 2002). This, in turn, might affect the production of phytoplankton and heterotrophic nanoflagellates (HNF), through trophic interactions, such as competition for nitrogen, and through predator–prey relationships. To our knowledge, however, no studies have yet compared the effects of DFAA with those of monosaccharides on bacteria and on their competitors and grazers.

To evaluate these effects, we conducted incubation experiments in coastal waters with the addition of glutamate or glucose, 2 compounds naturally found in the ocean, under phosphate-enriched conditions. Observations of the changes in density of bacteria and HNF and in chlorophyll *a* (chl *a*) concentrations with organic and inorganic nutrients offered insight into how the addition of these compounds influences bacterial growth and, ultimately, their competitors and grazers.

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MATERIALS AND METHODS

Sampling site and pre-incubation. Surface seawater was collected with a bucket at a pier in Sagami Bay (35° 54' N, 139° 20' E), Japan, on 27 June 2001. Seawater was poured into a 20 l carboy, kept in the dark and brought to the Ocean Research Institute within a few hours. In the laboratory, seawater was filtered with a 94 μm mesh net to remove large zooplankton, and was then pre-incubated for 1 d with cool-white fluorescent illumination of 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ (14:10 h light:dark) to acclimatize to experimental conditions. The temperature was kept at around 22°C.

Incubation and ^{15}N isotope dilution experiments. After the pre-incubation period, the seawater was dispensed into six 2 l polycarbonate bottles. Three treatments were employed in this experiment (+P: no additional organic matter and added PO_4^{3-} [3 $\mu\text{mol l}^{-1}$, final conc.], +CP: addition of glucose [16 $\mu\text{mol l}^{-1}$, final conc.] and PO_4^{3-} [3 $\mu\text{mol l}^{-1}$, final conc.] and +CNP: addition of glutamate [17 $\mu\text{mol l}^{-1}$, final conc.] and PO_4^{3-} [3 $\mu\text{mol l}^{-1}$, final conc.]). Because we used laboratory-grade reagents, organic contamination was negligible. For instance, glucose and glutamate additions both led to an expected increase in DOC concentrations, and glucose addition did not influence dissolved organic nitrogen (DON) concentrations in +CP (see Fig. 3). Duplicate bottles were used for each treatment, and these bottles were incubated as above. Incubation experiments were started in the dark cycle, and samples were collected in the dark on Days 0, 1 and 4. At each sampling, seawater (200 ml) was filtered through a GF/F filter (47 mm), and this filtrate was frozen for later analyses in a 100 ml polyethylene bottle (for determination of NH_4^+ , NO_3^- , NO_2^- , PO_4^{3-} , DOC and DON). The filter with particulate organic carbon (POC) was frozen until analysis for its carbon content. For chl *a* analysis, a subsample (100 ml) was filtered through a GF/F filter (25 mm) and the filter was frozen. For bacterial and HNF counts, subsamples (10 and 50 ml, respectively) were fixed with formalin (for bacteria, final conc. 1%) or glutaraldehyde (for HNF, final conc. 2%) and stored at 4°C. On Day 0, analyses for POC, bacteria and HNF were carried out with pre-incubated seawater with no additives, and the same values were assumed for the 3 treatments.

To estimate the rates of NH_4^+ uptake and regeneration, the ^{15}N isotope dilution experiment (Blackburn 1979, Caperon et al. 1979) was done. In the ^{15}N isotope dilution experiments, 2 size-fractionated subsamples of seawater were prepared from each treatment: one from whole seawater and the other from the <1 μm fraction (Nuclepore). From each incubation bottle, subsamples of the whole fraction (200 ml) and the <1 μm fraction (200 ml) were poured into 250 ml polycarbon-

ate bottles, $^{15}\text{NH}_4^+$ was added to a final concentration of about 0.5 $\mu\text{mol l}^{-1}$, and subsamples were then incubated for 2 h in the dark. At the beginning and the end of the ^{15}N dilution experiments, seawater was filtered through a GF/F filter (25 mm), and this filtrate was frozen for later analyses in a 100 ml polyethylene bottle for determination of the concentration and nitrogen isotopic ratio of NH_4^+ . These experiments were done in the dark on Days 0, 1 and 4. During the Day 0 experiments, uptake and regeneration rates of ammonium were estimated from pre-incubated seawater with no additions, and the same values were assumed for the 3 treatments. From concentrations and isotopic ratios of NH_4^+ at the beginning and the end, the rates of NH_4^+ uptake and regeneration were calculated (Blackburn 1979, Caperon et al. 1979).

The GF/F filters were pre-combusted at 450°C for 3 h, and all bottles were acid washed and rinsed thoroughly with Milli-Q water prior to use. Vacuum-filtration onto a GF/F filter was done at <50 mm Hg to minimize cell disruption.

Chemical, isotopic, bacterial and HNF analyses. Concentrations of inorganic nutrients were determined with a BRAN + LUEBBE Autoanalyzer III, using a modified method of Strickland & Parsons (1972). DOC and DON concentrations were measured using a high-temperature catalytic oxidation method with a modified Shimadzu TOC-5000 unit after Ogawa et al. (1999). POC concentrations were measured using a CHN analyzer (NA-1500, Fisons Instruments). Before this analysis, the POC on the GF/F filter was acidified with HCl vapor to remove carbonate.

The isotopic ratios of NH_4^+ were analyzed using a continuous flow mass spectrometer (Tracermass, Europa Scientific) equipped with a CN analyzer (Roboprep-CN, Europa Scientific). Details of the sample preparation and the analysis were described elsewhere (Hasegawa et al. 2000). Chl *a* concentrations were determined using the fluorometric method of Strickland & Parsons (1972), as modified by Suzuki & Ishimaru (1990), using a Turner Designs fluorometer. Bacteria and HNF were counted directly by epifluorescence microscopy after staining with DAPI for bacteria (Porter & Feig 1980) or with DAPI and FITC for HNF (Sherr & Sherr 1983), with some modification. Because we counted both non-pigmented and pigmented nanoflagellates as HNF, our HNF also included autotrophic nanoflagellates.

RESULTS AND DISCUSSION

Ambient dissolved inorganic nitrogen (DIN) and DON concentrations in the seawater were 2.0 and 7.6 $\mu\text{mol l}^{-1}$, respectively, which were within the ranges

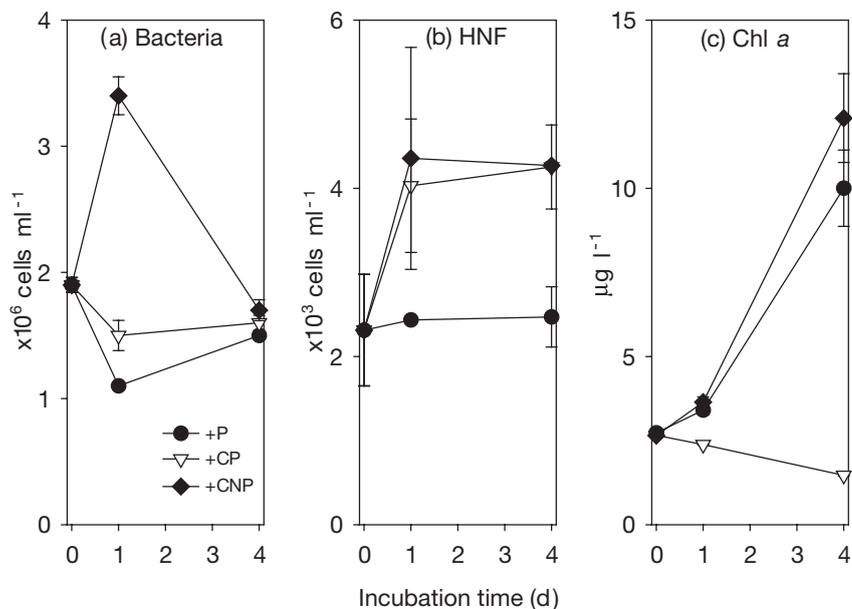


Fig. 1. Changes in (a) number of bacteria, (b) heterotrophic nanoflagellates (HNF) and (c) chlorophyll *a* (chl *a*) concentrations, and the ranges of duplicate samples. On Day 0, analyses for bacteria and HNFs were done on pre-incubated seawater with no additives, and the same values were assumed for the 3 treatments (+P: no additional organic matter and added PO₄³⁻ [3 µmol l⁻¹, final conc.], +CP: addition of glucose [16 µmol l⁻¹, final conc.] and PO₄³⁻ [3 µmol l⁻¹, final conc.] and +CNP: addition of glutamate [17 µmol l⁻¹, final conc.] and PO₄³⁻ [3 µmol l⁻¹, final conc.]

(0 to 57 and 3 to 10 mol l⁻¹, respectively) of concentrations commonly found in coastal waters (Sharp 1983). The ambient DOC concentration was 95 µmol l⁻¹, and was thus somewhat higher than the normal range of DOC concentrations (60 to 90 µmol l⁻¹) in the seawater of temperate and tropical regions (Benner 2003). Thus, inorganic and organic nutritional conditions in seawater might not deviate considerably from typical coastal waters. Although some phytoplankton, especially in highly eutrophic environments, utilize DON (Gobler & Sañudo-Wilhelmy 2001, Berg et al. 2003), such species would be minor here, because we did not use highly eutrophic seawater.

In the present study, we added large amounts of glucose (16 µmol l⁻¹, final conc.) and glutamate (17 µmol l⁻¹, final conc.) to the seawater, to make the effect of labile organic compounds clear. Because only duplicate incubations were done for each treatment, inter-treatment comparisons cannot be made statistically, and we did not identify the species composition of the plankton. However, our observations on chl *a*, bacteria, HNF, and organic and inorganic nutrients allow us to discuss some direct effects of amino acids and monosaccharides on bacterial metabolism and the subsequent effects on phytoplankton and bacterivores.

In +P treatments, bacterial cell numbers decreased by half from initial levels by Day 1, and then slightly

increased to 1.5 × 10⁶ cells ml⁻¹ by Day 4 (Fig. 1a). A similar trend was seen in +CP treatments, but the magnitude of the changes was less. In contrast, by Day 1, bacterial numbers in +CNP increased to 3.4 × 10⁶ cells ml⁻¹. Because bacterial cell numbers on Day 1 were greater in +CP and +CNP than in +P treatments, the addition of organic substances promoted bacterial growth; this effect was very pronounced in +CNP (Fig. 1a). In spite of the 3 different treatments, bacterial numbers were almost at the same level on Day 4 (Fig. 1a). Chl *a* concentrations in +P and +CNP gradually increased up to 10 and 12 µg l⁻¹, respectively, by Day 4 (Fig. 1c). Conversely, chl *a* concentrations in +CP continuously decreased during incubation, and fell to a minimum value of 1.5 µg l⁻¹.

In +P, concentrations of NH₄⁺ were constant for 1 d, and then decreased to 0.14 µmol l⁻¹ by Day 4; the NO₃⁻ + NO₂⁻ concentrations gradually decreased to 0.29 µmol l⁻¹ (Fig. 2a,b). NH₄⁺ concentrations in +CP were 0.05 µmol l⁻¹ on Day 1 and undetectable (<0.05 µmol l⁻¹) on Day 4; NO₃⁻ + NO₂⁻ were also undetectable (<0.05 µmol l⁻¹) from Days 1 to 4, suggesting conditions of N depletion. In +CNP, NH₄⁺ continuously increased, and reached 8.4 µmol l⁻¹ by Day 4. In contrast, NO₃⁻ + NO₂⁻ concentrations were almost constant during incubation. PO₄³⁻ concentrations were never depleted in any of the treatments during the incubation period (Fig. 2c).

During the 4 d incubation experiments, DOC concentrations in +P decreased from 95 to 88 µmol l⁻¹ (Fig. 3a). In the +CP and +CNP treatments, DOC concentrations decreased to the same level as in +P by Day 4. In +P and +CP, DON concentrations were constant (ca. 7 µmol l⁻¹), during the incubation period (Fig. 3b). In +CNP, DON concentrations rapidly decreased to 1/3 of initial levels. Thereafter, DON concentrations increased up to 9.6 µmol l⁻¹ by Day 4.

The addition of glucose and glutamate enhanced POC production by Day 1, and the magnitude of this is higher for glutamate (Fig. 3c). After 1 d, POC concentrations in +CP and +CNP gradually decreased; in +P and +CP they were almost at the same level on Day 4. In +CNP, POC concentration on Day 4 was about 1.3 times higher than in the other treatments (Fig. 3c).

In the ¹⁵N isotope dilution experiment, most of the NH₄⁺ uptake and regeneration rates were low

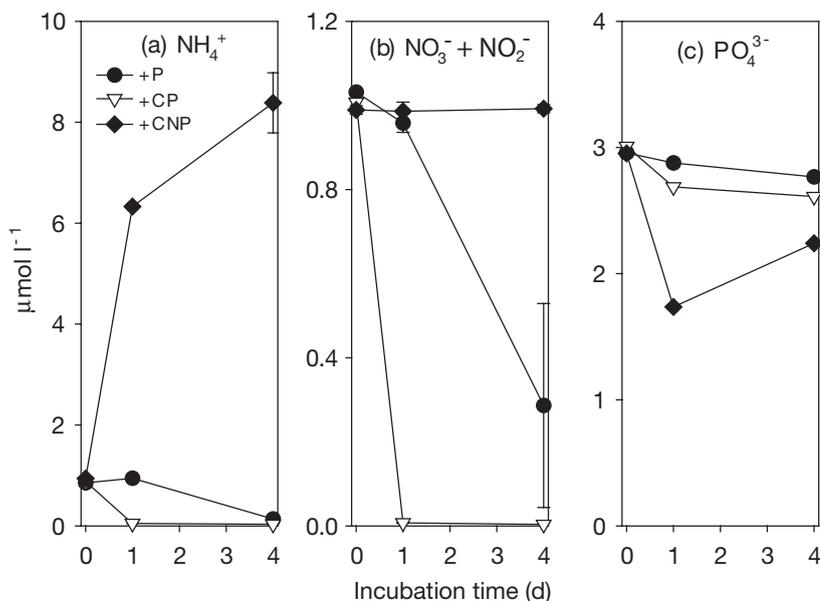


Fig. 2 Changes in (a) ammonium, (b) nitrate + nitrite and (c) phosphate concentrations, and the ranges of duplicate samples. See Fig. 1 for treatment abbreviations

(<50 $\text{nmol l}^{-1} \text{h}^{-1}$, Table 1), and some rates were negative (-45 to $-1.5 \text{ nmol l}^{-1} \text{h}^{-1}$) by calculation; these are denoted as dashes in Table 1, because values never theoretically become negative. Within a short incubation time (2 h), changes in NH_4^+ concentrations and isotopic ratios were small in the seawater with low activities (data not shown), and rate estimations were sensitive to small errors in analytical procedure.

However, rate estimations were relatively robust for high activities, and some features of NH_4^+ uptake and regeneration can be seen in Table 1. In +CP on Day 1, NH_4^+ uptake rates in the $<1 \mu\text{m}$ fraction were as fast as in the whole fraction, suggesting that most NH_4^+ uptake activities were within the $<1 \mu\text{m}$ fraction, which almost completely contained the bacterial populations (on average, 105% of that in the whole fraction; data not shown). Most NH_4^+ uptake activities by phytoplankton are likely to be reflected in the $>1 \mu\text{m}$ fraction, since few phytoplankton are tiny (about 10%), e.g. cyanobacteria, and would be included in the small size-fraction (Kirchman et al. 1989). Thus, most of the uptake within the $<1 \mu\text{m}$ fraction can probably be attributed to bacteria.

In +CNP, NH_4^+ regeneration in the $<1 \mu\text{m}$ fraction was 67% of that in the whole seawater on Day 1; this was caused by bacterial dissimilation of glutamate simultaneously with assimilation (Tupas et al. 1994). A part of this may be attributed to small ($<1 \mu\text{m}$) bacterivores (Fuhrman & McManus 1984, Cynar et al. 1985). The rest of the NH_4^+ regeneration in whole seawater (33%) was likely regenerated by the activities of HNF (Fenchel 1982), because the steep decrease in bacteria from Days 1 to 4 implied intense grazing on bacteria and, consequently, NH_4^+ regeneration (Strom 2000). A part of the NH_4^+ regeneration in the larger fraction ($>1 \mu\text{m}$) was attributed to phytoplankton, because some phytoplankton could oxidize amino acids extracellularly (Palenik & Morel 1990a,b).

The C:N ratios of the available organic substrates affect whether bacteria act as net regenerators or consumers on DIN (Fenchel et al. 1988, Goldman & Dennett 2000). Glutamate appears to stimulate

bacteria directly and phytoplankton indirectly through regeneration. When glucose was added, bacteria utilized most of the available inorganic nitrogen in their surroundings to satisfy their stoichiometrical demand (Table 1). Thus, bacteria may deprive phytoplankton of NH_4^+ and suppress their growth (Fig. 1c).

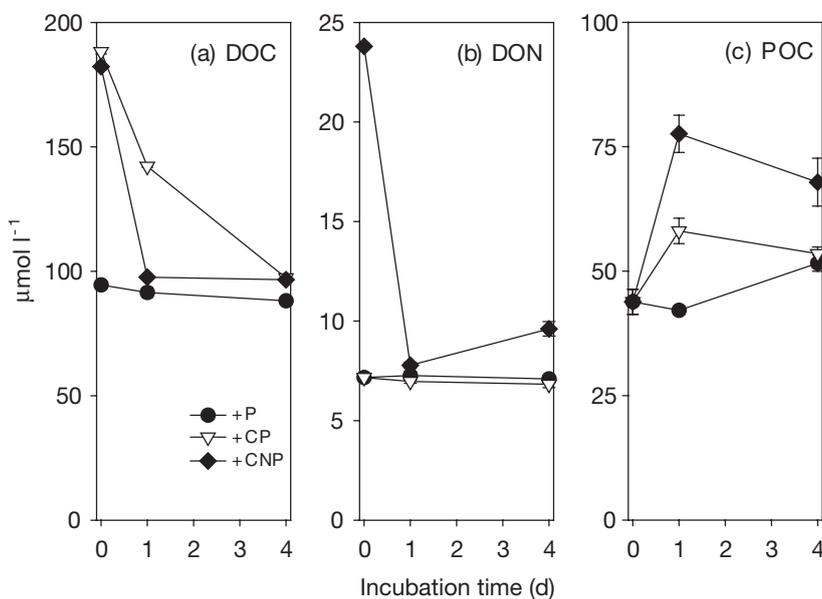


Fig. 3. Changes in (a) dissolved organic carbon (DOC), (b) dissolved organic nitrogen (DON) and (c) particulate organic carbon (POC) concentrations, and the ranges of duplicate samples. On Day 0, analyses for POC were done on pre-incubated seawater with no additives, and the same values were assumed for the 3 treatments. See Fig. 1 for treatment abbreviations

Table 1. Uptake and regeneration rates of NH_4^+ ($\text{nmol l}^{-1} \text{h}^{-1}$) in 3 treatments (+P, +CP, +CNP) on Days 0, 1, and 4. Values are averages (\pm ranges) of duplicate samples. Analyses on Day 0 were done on pre-incubated seawater with no additives, and the same values were assumed for the 3 treatments (–: negative values by calculation, see 'Results and discussion' for explanation; see Fig. 1 for treatment abbreviations)

Treatment	Day 0		Day 1		Day 4	
	Whole	<1 μm	Whole	<1 μm	Whole	<1 μm
Uptake						
+P	27 \pm 22	–	–	–	80 \pm 49	5.0 \pm 0.1
+CP	27 \pm 22	–	340 \pm 7.4	340 \pm 17	170 \pm 8.9	46 \pm 1.7
+CNP	27 \pm 22	–	93 \pm 7.3	12 \pm 33	60 \pm 41	3.9 \pm 44
Regeneration						
+P	43 \pm 14	–	0.1 \pm 63	–	17 \pm 12	–
+CP	43 \pm 14	–	85 \pm 10	140 \pm 29	31 \pm 11	11 \pm 9.4
+CNP	43 \pm 14	–	310 \pm 11	210 \pm 8	140 \pm 130	16 \pm 14

In contrast, bacteria were tightly controlled by grazing. The lack of increase in bacteria with a DOC decrease of $7 \mu\text{mol l}^{-1}$ in the +P treatment over 4 d supports this hypothesis (Figs. 1a & 3a). Further, bacterial numbers were almost at the same level on Day 4 in spite of different treatments (Fig. 1a). This also suggests tight coupling between production and grazing control. Grazing pressure on bacteria was greater in +CP and +CNP treatments than in +P, since HNF in +CP and +CNP were twice the level found in +P from Days 1 to 4 (Fig. 1b).

As a consequence of trophic interactions, and in spite of the same amount of added organic carbon in +CP and +CNP (about $90 \mu\text{mol l}^{-1}$), the enhancement of net POC production (which corrected net POC production in +P) was 9 times higher in +CNP than in +CP. The increased bacterial production was offset by this decreased primary production within the system (Fig. 1); thus, glucose addition had no effect on the total POC production (Fig. 3c).

During and/or after the productive season, DOC accumulations of up to $30 \mu\text{mol l}^{-1}$ were generally observed in surface seawaters (Williams 2000). Thingstad et al. (1997) explained these accumulations as a mechanism whereby the predation and shortage of inorganic nutrients reduce bacterial biomass and growth, respectively, and result in the accumulation of labile DOC. The shortage of inorganic nutrients is also the result of competition with phytoplankton. The delay in glucose degradation compared to glutamate (Fig. 3a) might be caused by the depletion of nitrogenous nutrients, because bacteria consumed glucose faster than glutamate under NH_4^+ -enriched conditions (Goldman & Dennett 1991). Thus, the combined effects restricting DOC utilization are likely to be stronger than grazing alone. However, the result of the ^{15}N dilution experiment (Table 1) and the changes in bacteria and chl *a* concen-

trations (Fig. 1a,c) showed that even if bacterivores exist, bacteria were successful competitors for inorganic nitrogen.

In mesocosm studies, bacteria also out-competed phytoplankton for inorganic nutrients after addition of glucose (Parsons et al. 1981, Jacquet et al. 2002, Joint et al. 2002) and appeared to consume added glucose ($106 \mu\text{mol C l}^{-1}$) during a 10 d experiment (Havskum et al. 2003). Further, during a 6 d mesocosm experiment, about half of the added glucose was consumed, although the model predicted that most of the glucose would remain (Thingstad et al. 1999). Unknown mechanisms might release bacteria from the restriction

caused by grazing and N shortage. Thus, other organic compounds that are less labile than monomers such as glutamate and glucose might explain most of the observed seasonal DOC accumulation in surface seawater.

While our data showed that DFAA enhanced both bacterial and phytoplanktonic production, a review by Kirchman (2003) reported that the percentage of bacterial production supported by DFAA decreased from coastal regions to oceanic ones. This implies that bacteria compete more actively with phytoplankton for nitrogenous nutrients in oligotrophic regions. In general, bacterial biomass increases relative to phytoplankton in oligotrophic regions, in contrast to in eutrophic ones (e.g. Cole et al. 1988); a cause of this trend might be the superiority of bacteria in the competition for nitrogen. Our data suggested whether or not the bacterial N demand (or other nutrients) was satisfied by the supply of organic compounds has a great influence on primary production and, accordingly, on marine carbon cycles. Thus, it is ecologically and biogeochemically important to assess not only bacterial production, but also which compounds sustain it.

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