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Regulation of Bacterioplankton Production and Cell Volume in a Eutrophic Estuary†

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During three periods of 16 to 25 days, bacterioplankton production, bacterial cell volume, chlorophyll *a*, CO_2 assimilation, and particulate organic carbon were measured in enclosures situated in the eutrophic estuary Roskilde Fjord, Denmark. The enclosures were manipulated with respect to sediment contact and contents of inorganic nutrients, planktivorous fish, and suspension-feeding bivalves. Nutrient enrichment, the presence of suspension feeders, and sediment contact induced pronounced changes in bacterial production, as well as minor changes in bacterial cell volume; however, these effects seemed to be indirect, transmitted via phytoplankton. Bacterial production, measured as [^{3}H]thymidine incorporation, closely followed changes in phytoplankton biomass and production, with time lags of 5 to 10 days. Good correlations of mean bacterioplankton production to chlorophyll *a* concentration and CO_2 assimilation suggested phytoplankton to be the dominating source of bacterial substrate, apparently independent of nutrient stress. Zooplankton >140 μm, bivalves, and sediment seemed to provide insignificant, if any, substrate for bacterioplankton, and benthic suspension feeders seemed not to act as direct competitors for dissolved organic carbon. The bacterioplankton mean cell volume, measured by image analysis, changed seasonally, with the smallest cells during the summer. Within each period, the bacterial cell volume correlated positively to growth rate and negatively to temperature.

Phytoplankton is likely to be the major autochthonous source of dissolved organic carbon (DOC) in pelagic environments, as it constitutes a relatively large biomass with a fast turnover. Substantial release of DOC has been demonstrated from decaying algae (8) as well as from actively growing phytoplankton (9, 22, 25). Zooplankton also release DOC by excretion and losses during grazing (2, 10, 19, 31, 41). In shallow waters, the benthic community might act both as a sink for DOC, mainly through the potential uptake by suspension-feeding bivalves (17, 26, 38), and as a source of DOC through the possible release during decay of sedimented particles and by benthic primary producers. Finally, DOC might be supplied from allochthonous inputs, both natural and synthetic (43).

Thus, several sources contribute to the sustainment of a high secondary production of bacterioplankton, creating an environment with great spatial and temporal variability in the amount and composition of utilizable DOC (35). Planktonic bacteria possess a physiological potential for meeting this challenge. They account for most of the pelagic biosurface (3, 42), and their fast turnover rates allow rapid community responses to a changing environment. The bacterioplankton cell volume can also undergo rapid changes (27, 32).

The purpose of this study was to examine the response of bacterioplankton activity to changes in the supply of carbon substrates in a eutrophic estuary. Enclosed water columns of approximately 6 m^3 were manipulated with respect to sediment contact and contents of inorganic nutrients, planktivorous fish, and suspension-feeding bivalves. These manipulations were intended to create different carbon substrate regimes for the bacterioplankton. Thus, the addition of inorganic nutrients was envisaged to induce changes in the biomass, production, and physiological state of the phytoplankton. The addition of planktivorous fish was presumed to selectively reduce the biomass of mesozooplankton and thereby eliminate this possible source of DOC. Finally, the inclusion of sediment contact aimed at studying the overall role of the sediment as a source of or sink for DOC, while the addition of suspension-feeding bivalves to enclosures without sediment contact was intended to reveal the isolated role of these benthic populations.

MATERIALS AND METHODS

Enclosure experiments were set up in a eutrophic estuary, Roskilde Fjord, Denmark, near the city of Frederikssund, on 7 April, 18 June, and 9 September 1986. The temperatures, irradiances, and initial concentrations of inorganic nutrients are summarized in Table 1. Twenty-four transparent polyethylene bags (diameter, 1.5 m; approximate depth, 3.5 m) were filled with water from the estuary and fixed to a pontoon bridge 200 m from shore. The flexible polyethylene walls allowed tidal currents to induce mixing of the enclosed water.

Treatments were conducted in duplicate according to the scheme shown in Table 2. Eight enclosures (designated S) were open to the sediment, while the rest were closed. In eight of these enclosures without sediment contact (designated M), 345 g (wet weight) of *Mytilus edulis* (15- to 22-mm shell length) was distributed in three cages at 1-, 2-, and 3-m depths. Half of the enclosures (N) were loaded with 0.67 g of NO_3^--N and 0.1 g of PO_4^{3-}-P every second to fourth day, giving increases in enclosure concentrations of 7 μM NO_3^- and 0.7 μM PO_4^{3-}. Finally, to half of the enclosures (F) was

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TABLE 1. Temperature ranges, mean irradiances, and initial concentrations of inorganic nutrients

Mo of expt	Temp range (°C)	Irradiance (MJ m^{-2} day^{-1})	Concn (μM) of[b]:	
			DIN	DIP
April	0–9[a]	10	30.7	2.4
June	17–22	25	4.4	12.3
September	11–13	12	5.7	12.3

[a] 0 to 4°C until the last two sampling dates.
[b] DIN, Dissolved inorganic nitrogen; DIP, dissolved inorganic phosphorus.

added *Gasterosteus aculeatus* (three-spined sticklebacks, 1 to 2 years old, approximately 10 g [wet weight] per enclosure). The chosen intensities of the treatments were based on a priori knowledge of the study area, in an attempt to achieve effective but realistic manipulations. Thus, additions of inorganic nutrients were intended to equalize phytoplankton consumption estimated from measured primary production in the estuary (L. M. Jensen et al., personal communication). The addition of fish corresponded to the naturally occurring biomass of planktivorous fish, and the number of bivalves was adjusted to mimic the filtration capacity of the natural benthic community, taking the natural size distribution into account (33).

Samples were taken at 2- to 4-day intervals at 1000 to 1100 h for 16 to 25 days. Water was sampled from 0.5-, 1.5-, and 2.5-m depths with a 5-liter bottle sampler and was integrated before subsampling. Bacterioplankton net production and chlorophyll *a* were measured in all enclosures, while particulate organic carbon and autotrophic CO_2 assimilation were measured in only half of the enclosures, one of each combination of manipulations. Differences between the enclosures were analyzed in an attempt to elucidate the quantitative importance of various substrate sources for bacterioplankton.

Bacterioplankton net production. Net production was determined from [^{3}H]thymidine incorporation into cold trichloroacetic acid precipitate (12, 29). Samples (15 ml) were incubated with 5 nM methyl-[^{3}H]thymidine for 20.00 or 30.00 min and then fixed in 1% Formalin and filtered onto 0.45-μm cellulose nitrate filters. The filters were washed with 5% ice-cold trichloroacetic acid and radioassayed by liquid scintillation counting (29). To calculate bacterial net production in carbon units, [^{3}H]thymidine incorporation rates were multiplied by 1,100 cells per fmol of [^{3}H]thymidine (30), by the actual mean cell volume measured by image analysis, and by 0.35 pg of C μm^{-3} (5). The factor used to convert incorporated thymidine into bacterial cell production is consistent with the theoretical factor originally stated by Fuhrman and Azam (12); however, considerable variability of this factor has often been reported from empirical calibrations (references in reference 30). An intensive calibration study on freshwater bacterioplankton (40) demonstrated significant variability of the conversion factor only when the bacterial generation time was less than 20 h. A similar study, which was carried out at the location of our enclosure experiments in Roskilde Fjord (30), showed no variation in conversion factor (standard error of the mean <5%) among 45 batch calibration experiments at different temperatures and medium enrichments and bacterial generation times of 5 to 200 h. The conversion from biovolume to carbon biomass was also intensively calibrated on bacterioplankton assemblages from the study area (5).

TABLE 2. Combinations of enclosure manipulations[a]

Enclosure type (treatment)	Without added nutrients		With added nutrients	
	− Fish	+ Fish	− Fish	+ Fish
Open to the sediment	S	SF	SN	SNF
Closed	C	F	N	NF
Closed (+ mussels)	M	MF	MN	MNF

[a] Abbreviations: S, open to sediment; C, control; M, mussels; F, fish; N, nutrients.

Bacterial cell volumes and cell numbers. Cell volumes and numbers were measured in selected samples by image analysis applied to epifluorescence microscopy (56). Samples (0.5 to 2 ml) were fixed by Formalin (1% final concentration), stained with acridine orange (0.02% final concentration), and filtered onto black 0.2-μm Nuclepore filters. Dry filters were mounted in Cargile B immersion oil and examined under a Zeiss Universal epifluorescence microscope equipped with an ultrasensitive video camera. Video images were digitized and treated numerically to reveal binary images of white objects on a black background. The bacterial cells in 20 microscope fields (corresponding to an average of 200 to 400 cells) were counted and measured. The cell volumes were estimated from measurements of object area and convex perimeter, and the data were divided into 20 logarithmic size classes within the range of 0.01 to 1 μm^3.

Chlorophyll a. Samples (25 to 100 ml) were filtered onto Whatman GF/C filters. Chlorophyll *a* was extracted in 96% ethanol for 20 h and measured fluorometrically. Calibrations to spectrophotometric measurements were done one to two times during each experiment (33).

Particulate organic carbon. Samples (10 to 25 ml) were filtered onto Whatman GF/F filters, which prior to use had been wet oxidized in 0.1 M $K_2Cr_2O_7$–60% H_2SO_4 at 140°C for 3 h to reduce background. After filtration, filters were rinsed with 1 ml of distilled water with NaCl (15 g $liter^{-1}$) and dried at 60°C. The dried filters were combusted at 650°C in an oxygen flow, and the released CO_2 was measured by infrared gas analysis (model 225MK3; Analytical Development Company, Ltd.). Measurements were corrected for filter background and calibrated against glucose standards.

CO_2 assimilation rate. A relative measure of phytoplankton photosynthesis was obtained from in situ incubation of 10-ml samples with 1 μCi of sodium [^{14}C]bicarbonate for 2 h at the surface and at a 70-cm depth. Incubation was stopped by $HgCl_2$ (5 ppm [5 μg ml^{-1}]). Unassimilated ^{14}C was removed by acidification to pH 3 and bubbling for 30 min. Samples were shaken with 8 ml of Dynagel (Beckman) and radioassayed by liquid scintillation. Time zero incubations were subtracted as blanks. The concentration of dissolved inorganic carbon was determined by infrared gas analysis.

RESULTS AND DISCUSSION

The data set displayed bacterioplankton production rates and chlorophyll *a* concentrations ranging over more than an order of magnitude. Deviations between duplicate enclosures covaried with means of duplicates on log-log plots (Fig. 1), revealing relative deviations averaging 17% in bacterial production and 18% in chlorophyll *a* concentration. These mean deviations between duplicate enclosures and the standard error ranges (Fig. 2 and 3) indicated that the observed differences in enclosure development were actually effects of the manipulations and not merely caused by random fluctuations. Spatial heterogeneity, causing differ-

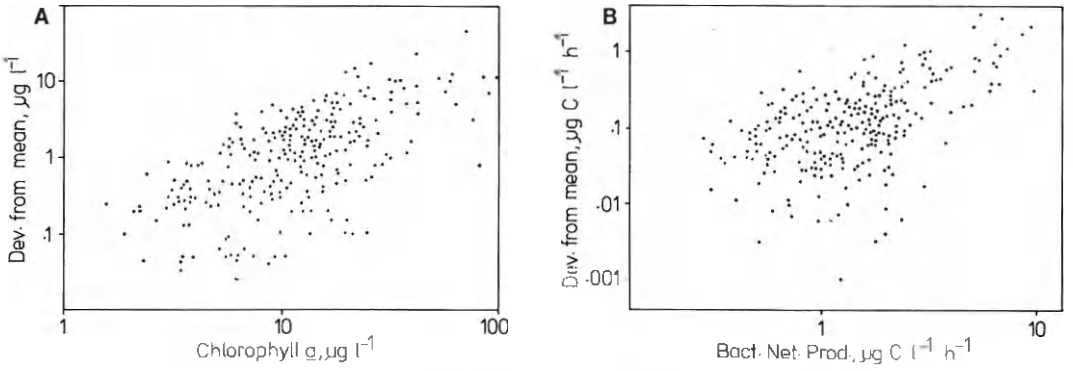

FIG. 1. Deviations between measurements of chlorophyll *a* (A) and bacterioplankton production (B) in duplicate enclosures, plotted against the respective means of duplicates.

ences between enclosures even at the starts of the experiments, might have been a major contributor to the observed deviations.

The experiment in April was carried out during the decay of a bloom dominated by *Skeletonema costatum*. The temperature remained below 4°C until the last two sampling dates. No responses to the manipulations were observed (data not shown). In June, phytoplankton were dominated by dinoflagellates. In enclosures with mussels, however, unicellular cyanobacteria surpassed densities of 0.5×10^6 ml^{-1} and constituted 70 to 93% of the phytoplankton biovolume at the end of the experiment. In September, diatoms dominated the phytoplankton, reaching high densities in some enclosures (Fig. 3).

Time courses of bacterioplankton production, chlorophyll *a* concentration, and CO_2 assimilation in June and September are shown in Fig. 2 and 3. Data from enclosures with and without fish were pooled, since the effects of fish on the three parameters were minor in both periods. Additions of inorganic nutrients and mussels, however, induced pronounced changes. As expected, nutrient additions increased chlorophyll *a* concentrations and CO_2 assimilation rates, except in September for enclosures with mussels. Enclosures with mussels generally showed lower concentrations of chlorophyll *a* and volumetric rates of CO_2 assimilation than their parallels without mussels. Enclosures open to the sediment responded differently in the two periods. In June, chlorophyll *a* concentration and CO_2 assimilation in enclosures with sediment contact exceeded the values from the other enclosures, while in September, the values were between those found in closed enclosures with and without mussels (Fig. 2 and 3). Qualitative changes in phytoplankton species composition between different enclosures were not observed. Quantitative changes, however, included marked differences in the distribution of autotrophic picoplankton (33) and smaller changes among other groups of phytoplankton (unpublished data).

Responses of bacterioplankton (net production) to the different manipulations closely mimicked the changes in chlorophyll *a* concentration and CO_2 assimilation but with a time lag of several days (Fig. 2 and 3). The peaks of bacterial production at the ends of the experiments could have been due to decay of phytoplankton. Leakage of up to 30% of the carbon biomass within 3 days has been demonstrated in decaying freshwater phytoplankton (8, 15). Increased bacterioplankton production was, however, also found in enclo-

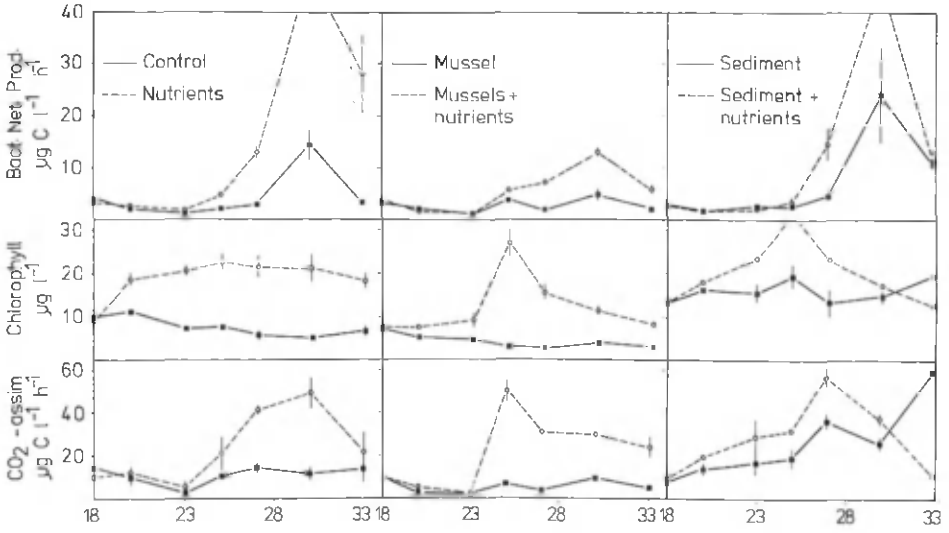

FIG. 2. Bacterioplankton production, chlorophyll *a* concentration, and CO_2 assimilation in June. Values from enclosures with and without fish were pooled. Bars indicate standard errors of the means (bacterial production and chlorophyll concentration, $n = 4$; CO_2 assimilation, $n = 2$).

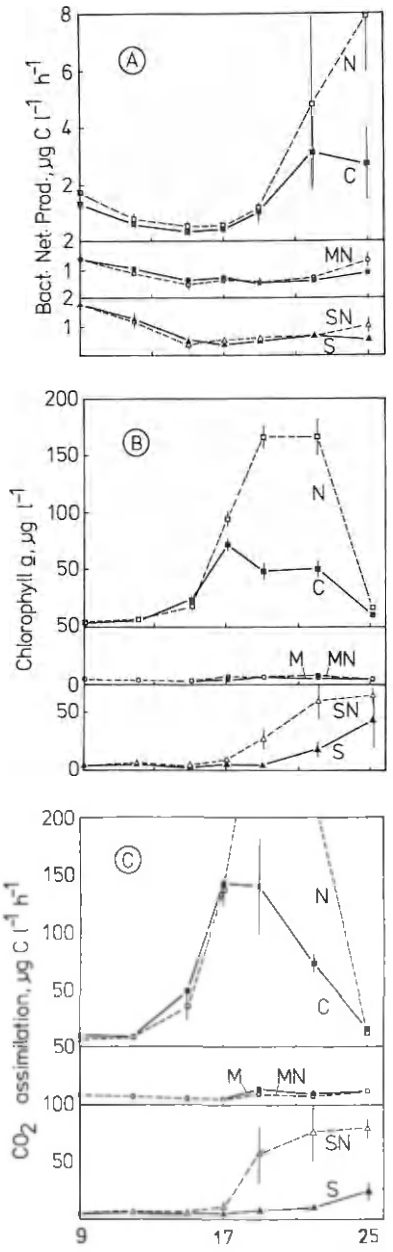

FIG. 3. Bacterioplankton production, chlorophyll *a* concentration, and CO_2 assimilation in September. Values from enclosures with and without fish were pooled. Bars indicate standard errors of the means (bacterial production and chlorophyll concentration, $n = 4$; CO_2 assimilation, $n = 2$). Abbreviations: N, nutrients added; C, control; M, mussels added; S, open to sediment.

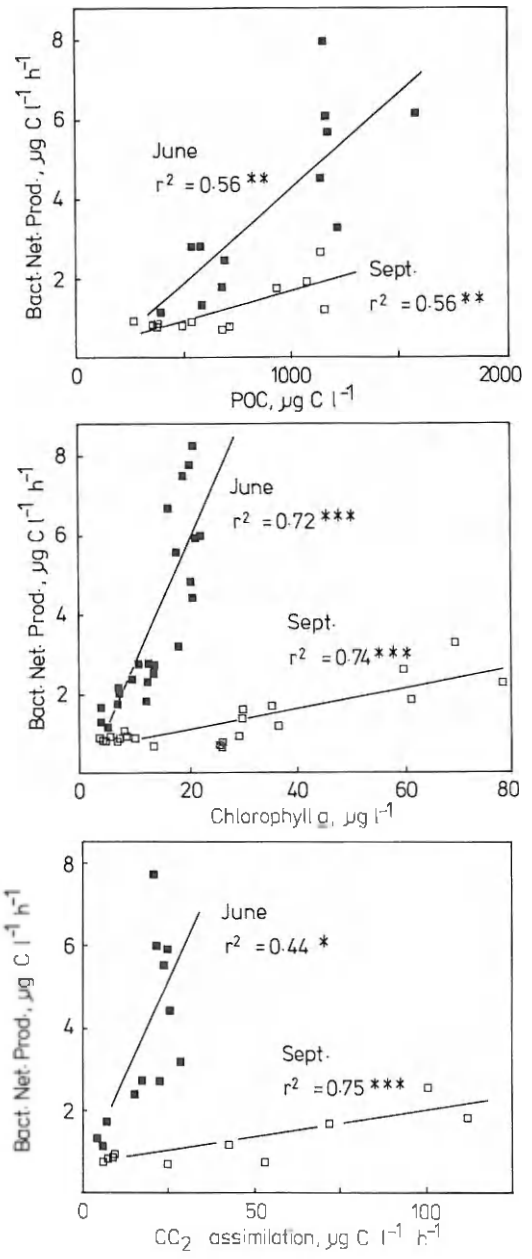

FIG. 4. Correlation analyses of integrated values from experimental periods. Significance levels of correlation (5, 1, and 0.1%) are indicated (*, **, and ***, respectively). POC, Particulate organic carbon.

sures without indications of a decay of phytoplankton (e.g., enclosures S and M in June, Fig. 2). A time lag in bacterial response to changes in phytoplankton might reflect an adaptation to alterations in substrate composition. Time lags of 10 to 15 days in bacterioplankton response to spring blooms have been reported for marine (21) and freshwater (23) environments. As a consequence of the apparent delay in bacterioplankton response, further consideration of data was based on integrated values over the experimental periods.

The average bacterioplankton production covaried significantly ($P < 5\%$) with particulate organic carbon, chlorophyll *a*, and CO_2 assimilation in both June and September (Fig. 4). The slopes of the regression lines were, however, significantly ($P < 5\%$) higher in June than in September in all three regressions. One explanation for this difference could be that the two periods differed markedly with respect to temperature and phytoplankton composition. Furthermore, the bacterial response was probably not completely covered within the experimental period in September (Fig. 3). A close correlation was found between chlorophyll *a* concentration and CO_2 assimilation ($r^2 = 0.93$ for June and September experiments pooled). A tight coupling of bacterioplankton production to phytoplankton biomass and production has previously been demonstrated during spring blooms in fresh water (4, 23, 34) and in the sea (21). As a further

TABLE 3. Bacterioplankton production in proportion to chlorophyll *a* and to CO_2 assimilation[a]

Treatment	June expt		September expt	
	BP/Chl[b]	BP/PP[c]	BP/Chl	BP/PP (ppt)
Nutrients				
Without	262	216	81	50
With	281	225	110	78
Fish				
Without	268	207	89	65
With	274	233	102	64
Mussels				
Without[d]	308	261	38	20
With[d]	266	194	170	109
Sediment	239	207	78	63

[a] Mean values of manipulated enclosures compared with mean values of unmanipulated controls. BP, Bacterioplankton production; Chl, chlorophyll *a*.

[b] Determined as micrograms of carbon per liter per hour per milligram of chlorophyll *a*.

[c] Determined as parts per thousand (micrograms per milligram).

[d] Enclosures either were (with) or were not (without) in contact with sediment.

examination of the coupling between bacteria and phytoplankton, proportions of bacterial production to chlorophyll *a* (Table 3, BP/Chl) and to CO_2 assimilation (Table 3, BP/PP) in manipulated and unmanipulated enclosures were compared.

Additions of inorganic nutrients clearly stimulated phytoplankton growth in both periods (Fig. 2 and 3) and, especially in June, counteracted a strong nitrogen limitation (unpublished data). If phytoplankton release of DOC was particularly related to nutrient stress, as often suggested (e.g., references 20 and 37), we should expect decreased proportional bacterioplankton production in the nutrient-loaded enclosures. On the contrary, increases in bacterioplankton production were found in response to nutrient additions (Table 3), suggesting that, rather, "extracellular release is a normal function of healthy cells" (reference 25, p. 262). This increase could be explained as a direct stimulation by the added inorganic nutrients, if bacterioplankton growth was limited by the availability of these nutrients rather than by the supply of carbon substrates. In that case, however, we should expect a more immediate response in bacterial production than was actually observed (Fig. 2 and 3). The observed increase might represent recycling of DOC from an increased flow through the microbial food web (6). Thus, Andersson et al. (2) estimated a release of amino acids from feeding nanoflagellates corresponding to 7% of their ingestion.

The added fish effectively removed zooplankton >140 μm (dominated by crustaceans) but had little effect on zooplankton in the size class of 45 to 140 μm (mainly tintinnids and rotifers; data in reference 16). Minor increases in proportional bacterioplankton production were recorded in response to additions of fish (Table 3), suggesting that zooplankton >140 μm did not significantly provide substrate to bacterioplankton in the estuary. This is in contrast to the model of Williams (42) suggesting that mesozooplankton contribute almost half of the bacterial carbon substrate through sloppy feeding and excretion. In freshwater lakes, mesozooplankton has been shown to sustain a major part of bacterial production (31, 34).

Laboratory studies have suggested that suspension-feeding bivalves are potentially significant utilizers of dissolved organic carbon (17, 26, 38). This possibility seems realistic, considering the large flow of water passing the large gill surface area. In June, enclosures with added mussels showed approximately 20% lower proportional bacterioplankton production compared with enclosures without mussels (Table 3). This minor decrease might be interpreted as an effect of direct competition for DOC. The decrease could, however, also reflect reduced recycling from the microbial food web, since the mussel additions in June significantly reduced the biomass of micro- and nanozooplankton (data in references 6 and 16). In September, the added mussels reduced chlorophyll *a* concentration and CO_2 assimilation to less than 10% of the levels of enclosures without mussels. During this period, a significant increase in proportional bacterioplankton production was found in response to mussel addition (Table 3). Thus, neither of the two experiments provided indications of a significant competition for DOC between bacterioplankton and suspension-feeding bivalves.

The sediment might affect the pelagic system both through the activity of benthic suspension feeders and by release of inorganic and organic nutrients. In June, large autotrophic biomass and high activity were recorded in enclosures with sediment contact (Fig. 2), indicating a release of inorganic nutrients from the sediment. In September, results for enclosures with sediment contact were similar to those for the closed enclosures with mussels for 1 week, and then the phytoplankton production and biomass started to increase (Fig. 3). The enclosures open to the sediment showed values of proportional bacterioplankton production between those of the closed enclosures with and without mussels (Table 3). The most pronounced effect was found in the September experiment, which also showed the strongest effect of the added mussels on chlorophyll *a* (33). These results do not indicate any significant release of readily utilizable DOC from the benthos. It cannot be excluded, however, that the sediment may release more recalcitrant DOC with a turnover time that exceeds the duration of our experiments.

Bacterial cell volumes showed unimodal distributions within the interval of 0.01 to 1 μm^3 (Fig. 5). The size distributions at the start of the experiments differed significantly ($P < 0.01$), with a decrease by a factor of 3 in mean cell volume from April to June (Fig. 5 and Table 4). Similar seasonal trends have been recorded in other studies and attributed to changes in temperature and flagellate biomass (2, 7, 39). In this study, the flagellate biomass was almost the same in April and June (6), suggesting that temperature is a major factor controlling cell volume, with roughly a doubling in cell volume at a temperature decrease of 10°C. It remains unanswered whether these seasonal changes in cell volume were due to a change in bacterioplankton species composition or a change of cell volume within the same species. Hagström and Larsson (13) found experimental evidence for increasing cell volume at decreasing temperatures in continuous bacterioplankton cultures kept at a fixed growth rate.

The bacterial cell volume increased significantly during the experiments in June and September (Table 4), perhaps because of a containment effect (11). Compared with that of the unmanipulated control enclosure, nutrient enrichment and mussel addition shifted the size distribution toward larger and smaller cells, respectively. This effect of the manipulations, which was minor compared with the seasonal changes and detectable only by the large number of cells measured, could be either direct or indirect via the changes in substrate flow described above. Cell volume has been demonstrated to correlate positively with growth rate (μ) in

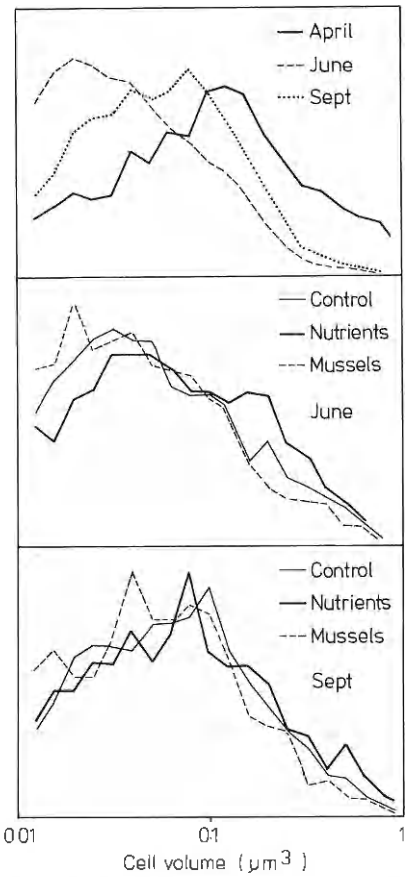

FIG. 5. Frequency distributions of bacterioplankton cell volume determined by digital image analysis. (Top) Size distributions from the first sampling date of each experiment (all enclosures pooled). (Middle and bottom) Size distributions in three different enclosures in June and September experiments, respectively (last three sampling dates pooled). The numbers of measured cells and mean cell volumes are given in Table 4.

bacterial cultures (22, 24, 36). We found significant correlations of cell volume to ln(μ) in all three enclosure experiments, with different slopes and intercepts of the regression lines (Table 5). The explained variation was low (15 to 17%), but a major part of the residual variation might be due to imprecise determination of volumes and turnover rates. The residuals correlated to flagellate biomass only in September ($P < 0.05$), with a very low slope of the regression line (0.00020 μm^3 [μg of flagellate C $liter^{-1}]^{-1}$).

Larsson and Hagström (22) found that the cell volume (Vol) correlated to the frequency of dividing cells (FDC, in percent) in mixed marine bacterioplankton cultures grown at 15°C. The regression equation was Vol = 0.011 ln(μ) + 0.11 ($n = 4$), which together with the relation ln(μ) = 0.163 FDC − 3.749 at 15°C (calculated by Newell and Christian [28] from data in reference 14) gives Vol = 0.0675 ln(μ) + 0.253. The slope of that regression is significantly higher than that obtained in this study.

TABLE 4. Mean cell volumes of bacterioplankton[a]

Mo of expt	Start[b]	End[c] with treatment:		
		Mussels	Control	Nutrients
April	0.158 (0.013)			
June	0.057 (0.002)	0.071 (0.008)	0.084 (0.007)	0.097 (0.012)
Sept	0.076 (0.003)	0.078 (0.003)	0.091 (0.004)	0.109 (0.007)

[a] In cubic micrometers. Data in parentheses are standard error of the mean (n = 3 to 12). Volumes correspond to the size distributions shown in Fig. 5.
[b] First sampling date, all enclosures pooled (number of measured cells ranging from 1,577 to 3,970).
[c] Last three sampling dates pooled (number of measured cells ranging from 736 to 1,888).

TABLE 5. Linear regression of bacterioplankton cell volume to turnover rates[a]

Mo of expt	Regression line equation	r^2	n	P
April	Vol = 0.0204 ln (μ) + 0.219	0.15	34	<0.05
June	Vol = 0.0077 ln (μ) + 0.109	0.17	60	<0.01
Sept	Vol = 0.0117 ln (μ) + 0.142	0.15	52	<0.05

[a] The explained variation (r^2), number of observations (n), and significance level (P) are given for each of the three enclosure experiments. Volumes are in cubic micrometers; turnover rates (μ, growth per hour) were calculated as production divided by biomass.

Bacterial cell volume estimates are required for the determination of biomass and production rates. In many studies, cell volumes measured in a few samples have been extrapolated to the entire data set. This procedure is dubious, since cell volume is a dynamic parameter, as is also illustrated by this study. On the other hand, a precise measurement of cell volume in all samples is tedious, even when automated by image analysis. Further development of empirical models for the prediction of changes in bacterial cell volume from already-measured parameters (e.g., temperature, turnover, and flagellate abundance) might prove helpful to studies, in which a precise measurement of cell volume in all samples is not feasible.

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