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FACTORS CONTROLLING BACTERIAL ABUNDANCE, BIOMASS AND GROWTH AT THE BERMUDA ATLANTIC TIME-SERIES SITE

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ABSTRACT

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FACTORS CONTROLLING BACTERIAL ABUNDANCE, BIOMASS AND GROWTH AT THE BERMUDA ATLANTIC TIME-SERIES SITE

by Kjell Gundersen

The Bermuda Atlantic Time-series Study (BATS) site represents a unique hydrographical oceanic environment to study the factors controlling bacterial growth dynamics. The region was sampled at monthly intervals from 1991 to 1996. A typical annual cycle was defined by a deep winter mixing, followed by an increasing stratification of the mixed layer through summer and fall. There were striking seasonal patterns in phytoplankton productivity with a strong maximum immediately following the deep winter mixing and the intrusion of inorganic nutrients. Bacterial growth rates showed a similar pattern but had a secondary peak in late summer/fall of the same magnitude as the spring bloom. Bacterial abundance showed only slightly elevated concentrations in spring.

A number of time course storage experiments showed that bacterial abundance decreased by 24-50 % within 7-29 days in samples preserved with 2.5 % glutaraldehyde. By adding a protease inhibitor prior to the addition of glutaraldehyde, the loss of bacterial cells was reduced to 17-18 % over a 30 day period. These findings lead to the recommendation that samples for bacterial abundance should be processed immediately for epifluorescence enumeration.

An average of 47 % of all bacterial cells passed the pore size of a Whatman GF/F filter and these viable cells should be included in biomass estimates. An annual average of 26 % of estimated C settled below the spigot of a Niskin water sampler. Consequently, C measurements made on GF/F filters must account for the particles settling below the spigot of a water sampler as well as the number of bacteria lost during the process of filtration.

This study is the first to present single cell elemental C, N and P measurements from natural bacteria in the Sargasso Sea. A wide range in elemental content was found between single cells and this could be expressed as a function of the cell size. By applying an average cell volume, an annual average of 10 fg C, 1.9 fg N and 0.28 fg P was calculated per bacterial cell.

The average percentage integrated stocks of C in the upper 250 m of the water column, was 20 (phytoplankton), 18 (microheterotrophs) and 62 (other non-living detrital matter). Bacterial biomass was higher than phytoplankton outside the spring bloom period, but non-living carbon showed an overall dominance through out the year.

Phytoplankton generation time was relatively constant over the season. Bacterial generation time was ten times longer and showed a greater seasonal variation, but largely followed the changes in primary production. Assuming that 50-70 % of the bacterial cells were non-living, the mean bacterial generation time was estimated to be 7 times (0-60 m) and 1.4 times (80-140 m) longer than phytoplankton generation time. During the spring bloom event, an average of 85 % of the bacterial growth rate was removed by grazing and viral lysis. This was the only noted decoupling between growth and removal of bacteria at BATS. During the remainder of the year bacterial growth was balanced by the loss rate, due to grazing and viral lysis.

A linear relationship was found between net DOC accumulation and primary production in natural surface waters at BATS. Phytoplankton net DOC excretion constituted 42 % of the primary production rate, while *Trichodesmium* colonies only excreted 12 % (puffs) and 23 % (tufts). By using a conservative estimate of the bacterial growth efficiency (14 %) and the net DOC accumulation rate from this study, gross DOC excretion was equivalent to the rate of primary particulate C production. Results from this study suggests that bacteria at the BATS site are using the majority of the DOC generated by primary production.

Prior to the spring bloom and the associated increase in DOC excretion, bacteria appeared to be C limited in the surface waters at BATS. Following the peak in primary production and coinciding with the depletion of inorganic nutrients in the euphotic zone, the bacterial cells became less C starved, but never reached a true N or P limitation. Regenerated nutrients from grazing and viral lysis of bacteria and new production by diazotrophic *Trichodesmium* colonies and trichomes, may support the bacteria with N and P in the euphotic zone in summer and fall. The substrate dependent growth and increase in biomass of bacteria exhibited at BATS in spring, is indicative of a bottom-up controlled system, whereas the bacteria appeared to be top-down controlled by grazing and viral lysis for the remainder of the year.

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ACKNOWLEDGEMENTS

Thanks to Dr. A.H Knap for giving me the opportunity to work on the role of bacteria in a novel environment such as the Sargasso Sea. My first year in the BATS program at the BBSR, when 'everyone did everything', was shared with R.L. Dow and R.J. Johnson. With Dr. A.F. Michaels and the time-share program, we started specialising and expanding the work load. I do like to thank all the BATS technicians that came later, for helping out in sampling and analysis of CTD temperature and salinity, inorganic nutrient analysis, O_2 analysis, primary production and pigments, that was used in this thesis.

In addition to sediment trap samples and CHN analysis, I got the opportunity to start up the BATS side of the bacterial abundance estimates and the bacterial growth (TdR incorporation) measurements, in conjunction with my thesis study. F. Bahr provided the numerous contour plots from the BATS core data base used in this study.

I have also enjoyed several collaborative efforts during my time at the BBSR. The idea behind the bacterial storage experiments came up during a morning coffee session at the Department of Microbiology and my collaborative effort with M. Heldal and G. Bratbak was the first overseas experience since I left the University of Bergen in 1989. The single cell elemental content study of bacteria was the next in line and would not have been possible without the help and guidance from M. Heldal and S. Norland at the Department of Microbiology.

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Finally, a very special thanks to Karen, my personal advisor and partner in life, for helping me through some difficult times. Your support and encouragement has been invaluable and there is no doubt, that is why I managed the finish line.

DEFINITIONS AND ABBREVIATIONS

ANOVA	Analysis of variance
BATS	Bermuda Atlantic Time-series Study site, 27 nautical miles SE off Bermuda
BC	Bacterial Carbon
B:P ratio	Ratio between bacterial and phytoplankton C biomass
C	Carbon
Chl-*a*	Chlorophyll- *a*
CHN	Carbon, Hydrogen and Nitrogen elemental analyzer
C:N	Ratio between carbon (C) and nitrogen (N)
C:P	Ratio between carbon (C) and phosphorous (P)
CTD	Conductivity, Temperature and Density sensor instrument
C:V ratio	Ratio between carbon (C) and volume (V)
D	Depth (m)
DAPI	4',6-diamidino-2-phenylindole
DIN	Dissolved Inorganic Nitrogen
DIP	Dissolved Inorganic Phosphate
Dregs	Particles that settle below the spigot in a Niskin bottle
EFM	Epifluorescence Microscope
GF/F filter	Whatman GF/F glassfiber filter (nominal poresize 0.5-0.7 μm)
GM	Geometric Mean
GoFlo bottle	Trace metal clean water sampling bottle with spring mechanism outside
GT	Generation time (ln 2 / μ), where μ is the specific growth rate
HC	Heterotrophic Carbon; Bacteria, Nanoplankton and Microzooplankton C
LAP	Leucine aminopeptidase
LC	Living Carbon; organic carbon accounted for by living organisms
LOG	Biggs power (10^x) logarithm
N	Nitrogen
Niskin bottle	Gas proof water sampling bottle with a spring mechanism inside
N:V ratio	Ratio between nitrogen (N) and volume (V)
OC	Other Carbon, not accounted for as phytoplankton or bacterial carbon
P	Phosphorous
PC	Phytoplankton Carbon
PMSF	Phenolmethylsulfonylfluoride
POC	Particulate Organic Carbon
Puff	Spherically arranged trichomes in a *Trichodesmium* colony

DEFINITIONS AND ABBREVIATIONS (CONT.)

P:V ratio	Ratio between phosphorous (P) and volume (V)
SE	Standard Error (degrees of freedom = n-1) of the sample mean
Station 'S'	Hydrostation 'S', 15 nautical miles SE off Bermuda
TdR	Methyl ^{3}H-thymidine
TEM	Transmission Electron Microscope
Tuft	Cylindrically arranged trichomes in a *Trichodesmium* colony
V	Volume
Whatman GF/F	Whatman GF/F glassfiber filter (nominal poresize 0.5-0.7 μm)

Chapter 1: An introduction to the BATS site and the Sargasso Sea

1.1 INTRODUCTION

A wide variation in time and space scales, of physical and biological properties, have been reported in open ocean ecosystems (Dickey 1991). Seasonal patterns of ocean stratification and primary production in the Sargasso Sea, controlled by physical forcing and biological response, was already reported almost 40 years ago by Menzel & Ryther (1960, 1961). The BATS site in the Sargasso Sea, is situated in a meridional transition between two distinct areas (Siegel et al. 1990); North of BATS the upper ocean is characterised by a strong seasonality, with winter mixing and ventilation of 18 °C nutrient rich mode water into the euphotic zone (Talley & Raymer 1982, Ebbesmeyer & Lindstrøm 1986). South of the BATS site, the upper ocean is stratified all year around and nutrient levels are low (Siegel et al. 1990).

Continuos profiles of temperature, salinity and oxygen, as well as water samples of inorganic nutrients, particulate organic carbon and pigments, are here reported from monthly BATS cruises in 1991-1996. Some of these data from the early years, have previously been published by Lohrenz et al. (1992) and Michaels et al. (1994), but are included here to give an introduction to the BATS site. Three N-S transects through the BATS site, ranging from 26-35 °N, showing temperature distribution and oxygen concentrations, are also reported in order to show the meridional transition between the two distinct areas of the Sargasso Sea.

1.2 MATERIALS AND METHODS

The water samples analysed in this study, were collected at discrete depths between the surface and 1000 metres at the BATS site (31° 50' N, 64° 10' W) in the Sargasso Sea (Figure 1-1), in 12 l Niskin bottles attached to a 12 position General Oceanics Model 1015 rosette. In July 1993 the CTD rosette was replaced with a 24 position model. For the primary production incubations and bacterial incorporation of methyl tritiated-thymidine (TdR), seawater was collected using *Kevlar* wire and 10 l *GoFlo* bottles at 8 depths down to 140 meter. Continuous temperature and oxygen profiles down to 500 m depth, were recorded on discrete stations along the three N-S transect cruises (Figure 1-2). The methods presented in this chapter, are core methods commonly used in the BATS program. A detailed description of each method can be obtained from the BATS Method Manual Version 3 (1993).

Continuous profiles of temperature, salinity and oxygen

The *Sea-Bird* CTD instrument was mounted on a 12 (or 24) position *General Oceanics* Model 1051 rosette. The CTD system consisted of an SBE 9 underwater CTD unit and an SBE 11 deck unit, with four principle components; Pressure sensor, temperature sensor, flow through conductivity sensor and a pump for the conductivity and oxygen electrode. In order to calibrate and quality control the Sea-Bird CTD sensors, water samples for salinity and dissolved oxygen were taken from discrete depths through the water column, using the *Niskin* bottles attached to the *General Oceanics* rosette. An ordinary BATS cruise, included at least one CTD profile down to 4200 m. Temperature, salinity and oxygen are here only reported down to 1000 m depth at the BATS site and down to 500 m along the N-S transect cruises.

Seawater salinity was sampled in borosilicate glass bottles sealed with a polypropylene lined cap. Salinity was determined on a *Guildline* model 8400a Autosal Salinometer, calibrated with IAPSO Standard Seawater.

Oxygen samples were collected in 115 ml BOD bottles. Great care was taken to avoid air bubbles from being present during sampling. Immediately after obtaining the seawater sample, 1 ml of manganese chloride, followed by 1 ml of alkaline sodium iodide, was added. The stopper was carefully placed in the bottle and the sample vigorously mixed. The sample was remixed 5-10 min later when the precipitate had settled to the bottom. The samples were analysed after at least 8 h and within 24 h of storage. Chemical determination of oxygen was based on the method of Winkler (1888), later modified by Strickland & Parsons (1972). The sample was added 1 ml of 50 % sulphuric acid and titrated with 0.1 N thiosulphate, using a *Metrohm* 655 Dosimat Oxygen Auto-titrator. Burette, endpoint detector and A/D converter was controlled by a PC computer system software, developed by Williams & Jenkinson (1982).

Analysis of nitrate + nitrite (DIN) and inorganic phosphate (DIP)

DIN and DIP were sampled at discrete depths from surface down to 1000 m (0.8 µm Nuclepore *in-line* filtration during sampling), collected in acid-cleaned amber bottles and stored at -20 °C. Before analysis, the samples were thawed to room temperature. DIN was determined on a *Technicon* Auto Aanalyzer II System using the methods of Oudot & Montel (1988) and Raimbault et al. (1990). DIP was analysed simultaneously with DIN on the autoanalyser, using a modified method from Strickland & Parsons (1972).

Primary production, pigments (Chl-*a*) and particulate organic carbon (POC)

Samples for the primary production assay, were collected from GoFlo bottles at surface down to 140 m depth. The 8 GoFlo bottles were attached to a Kevlar line at 20 m intervals. The primary production method is a modification of the trace metal clean procedure described by Fitzwater et al. (1982); A ^{14}C-sodium bicarbonate solution was diluted with a carbonate solution to a final specific activity of 80 µCi per ml. Under low light conditions 20 µCi was added to each polycarbonate bottle (250 ml). Powder-free

polyethylene gloves were worn during all stages of the primary production assay. The bottles were deployed *in-situ* attached to a floating array, at the same depths as sampled, from dusk to dawn. An aliquot of 50 ml was drawn from each bottle and filtered onto 25 mm Whatman GF/F filters. The filters were acidified (0.5 N HCl) and dried overnight. A 10 ml aliquot of liquid scintillation cocktail (Aquasol) was added and the samples were counted on a *Packard* Tricarb 2000 CA Liquid Scintillation Analyzer, using the external channel ratio method.

Chl-*a* and POC samples were taken from the CTD Niskin bottles. Chl-*a* was sampled at discrete depths from surface down to 250 m, filtered onto 47 mm Whatman GF/F filters (4 l per depth) and stored in liquid nitrogen. The filters were extracted in 90 % acetone for 24-48 h at -20 °C and analysed on a *Turner Design* 110 Fluorometer. The fluorometer was calibrated with a chlorophyll-*a* standard (*Anacystis nidulans*, Sigma Chemical Company) and calculated according to the method of Strickland & Parsons (1972).

POC was sampled at discrete depths between surface and 1000 m, filtered onto 25 mm Whatman GF/F filters (2 l per depth) and stored at -20 °C. Prior to analysis, the filters were dried, acidified by HCl fumes and dried again. The samples were analysed on a *Control Equipment Corporation* (CEC 240-XA) Elemental Analyzer, using acetanilide (71.09 % C, 10.36 % N) as a standard.

1.3 RESULTS

The BATS site (32° 50' N, 64° 10' W) is situated 45 nautical miles south east of Bermuda (Figure 1-1). A detailed study of the ocean biogeochemistry was published by Michaels et al. (1994). Hence, only a brief description of the seasonality in the upper 140 m of the water column, is given here for a few selected parameters measured in the BATS program. To date, three out of a total of nineteen BATS validation cruises have been

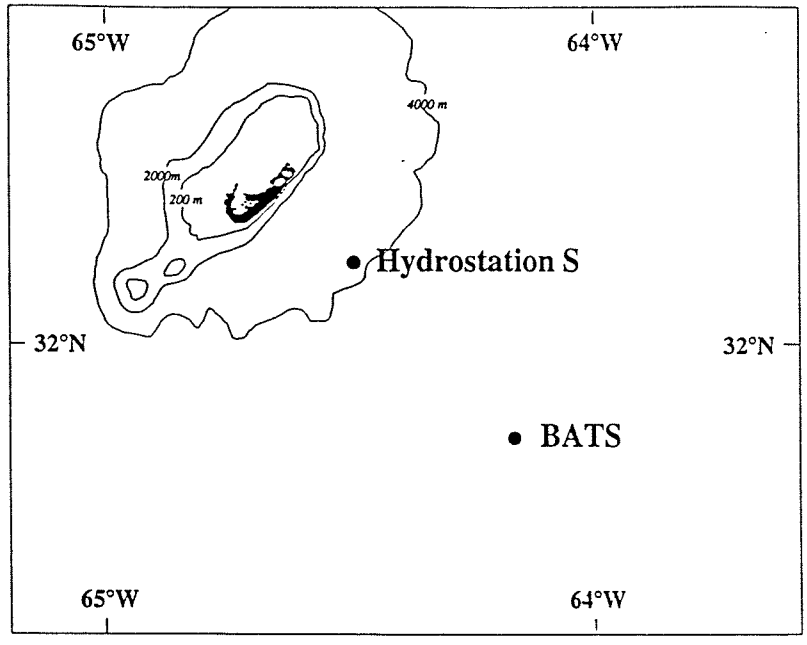

Figure 1-1
Map showing the depth contours in the vicinity of Bermuda, Hydrostation 'S' and the BATS site.

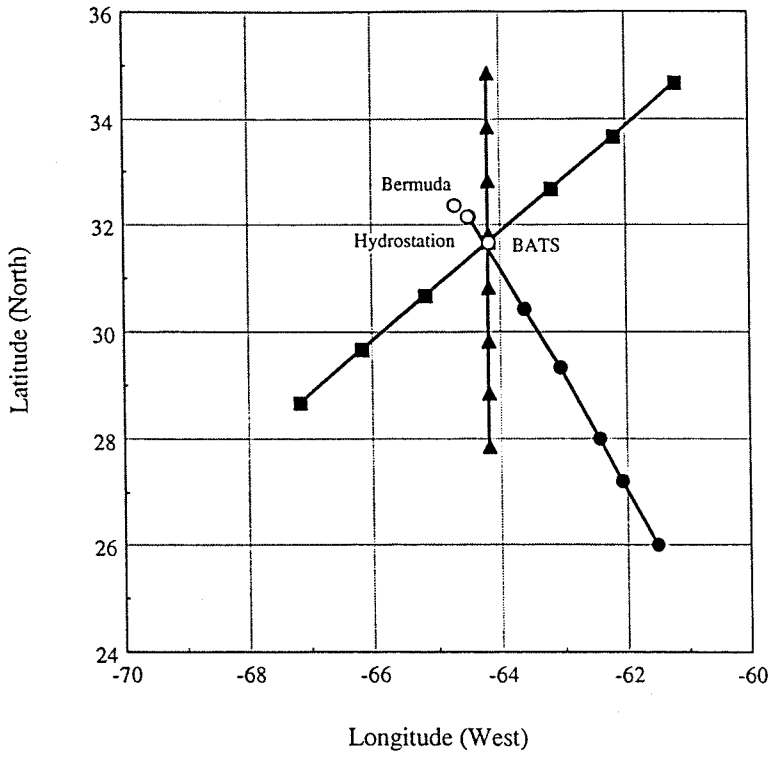

Figure 1-2
Map showing positions and tracks of N-S transect cruises through the BATS site in February 1994 (closed triangles), September/October 1994 (closed circles) and July 1995 (closed squares). The open circles are the positions of Bermuda, Hydrostation 'S' and the BATS site.

N-S transects (Figure 1-2), of which the hydrographical parameters, temperature and oxygen, are reported in this study.

Hydrography

The depth of the mixed layer at the BATS site showed a winter maximum every January-March, followed by rapid stabilisation to shallow summer mixed layer (Figure 1-3 and 1-4). Nutrient concentrations (DIN and DIP) in the euphotic zone were uniformly low during most of the years (Figure 1-5 and 1-6), only interrupted by elevated concentrations during the winter mixing (Figure 1-5). Detectable DIP concentrations during the winter mixing, were only present in 1993 and 1994 (Figure 1-6). Oxygen concentrations were above atmospheric saturation in the euphotic zone throughout the period, with slightly lower positive anomalies during the winter mixing and elevated values during spring and summer (Figure 1-7).

The N-S cruises showed a permanent thermal stratification of the mixed layer south of BATS, between 26 and 29.5 °N (Figure 1-8 A and B). The mixed layer stratification weakened and deepened northwards from 29.5 °N in February 1994 (Figure 1-8 A). A further strengthening of the mixed layer in September/October 1994 (Figure 1-8 B) was evident south of 29.5 °N. Oxygen concentrations were above atmospheric saturation in the euphotic zone along all transects (Figure 1-9) and strongly associated with seawater temperatures in the range of 20-27 °C (Figures 1-8 and 1-9).

Primary production, Chl-*a* and POC concentrations

Following the winter mixing events in spring, there were distinct peaks in primary productivity (Figure 1-10). POC and Chl-*a* maxima were often associated with the production peaks (Figure 1-11 and 1-12). The rate of primary production and the particle concentrations became less pronounced through summer and fall.

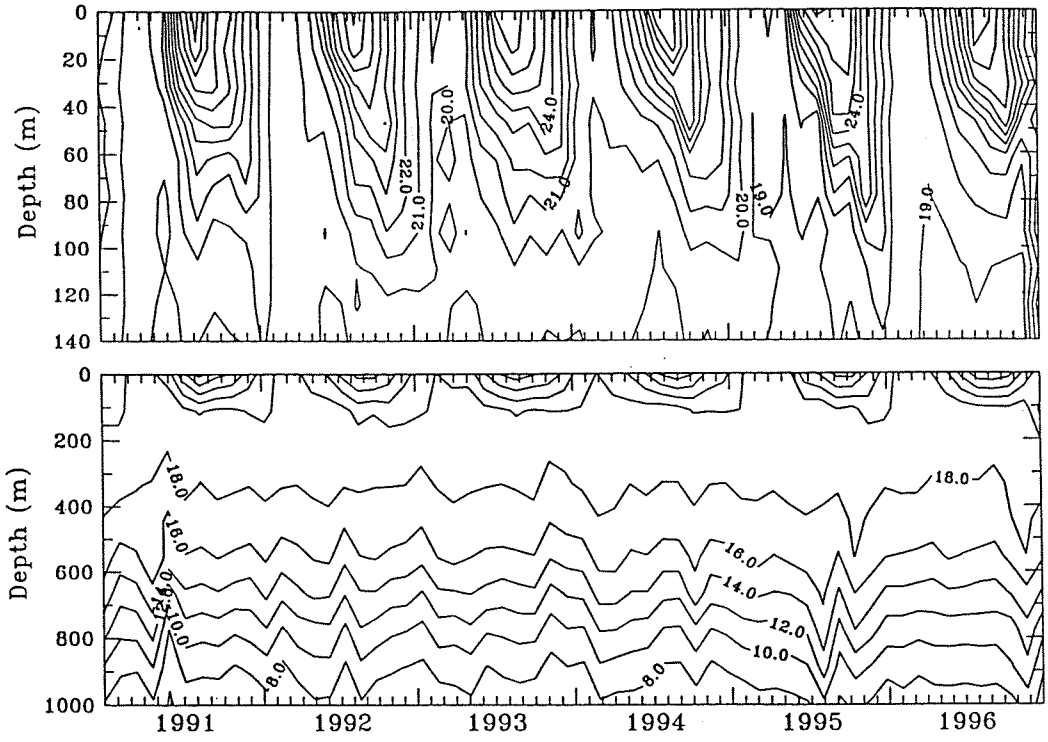

Figure 1-3
Contour plot of seawater temperature (°C) at BATS in 1991-1996. Contour interval is 1 °C (upper panel) and 2 °C (lower panel).

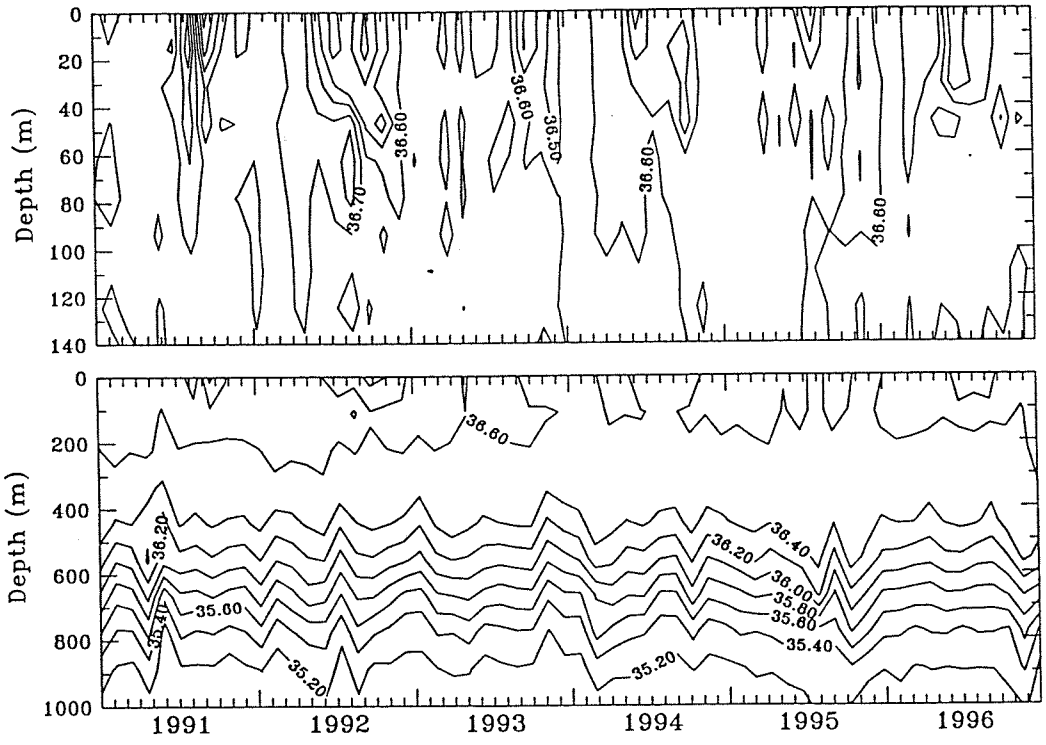

Figure 1-4
Contour plot of salinity (relative units) at BATS in 1991-1996. Contour interval is 0.1 (upper panel) and 0.2 (lower panel).

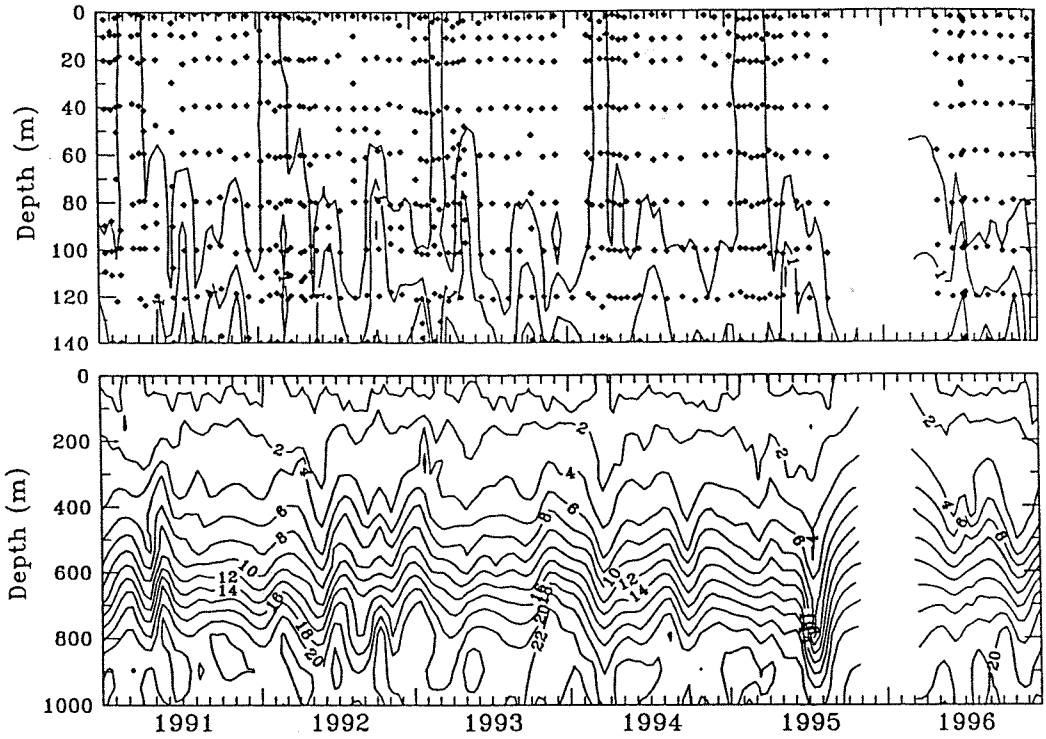

Figure 1-5
Contour plot of DIN (μmol kg^{-1}) at BATS in 1991-1996. Contour interval is 0.2 and 1 μmol kg^{-1} (upper panel) and 2 μmol kg^{-1} (lower panel). Black diamonds shows sampling depths and sampling frequency.

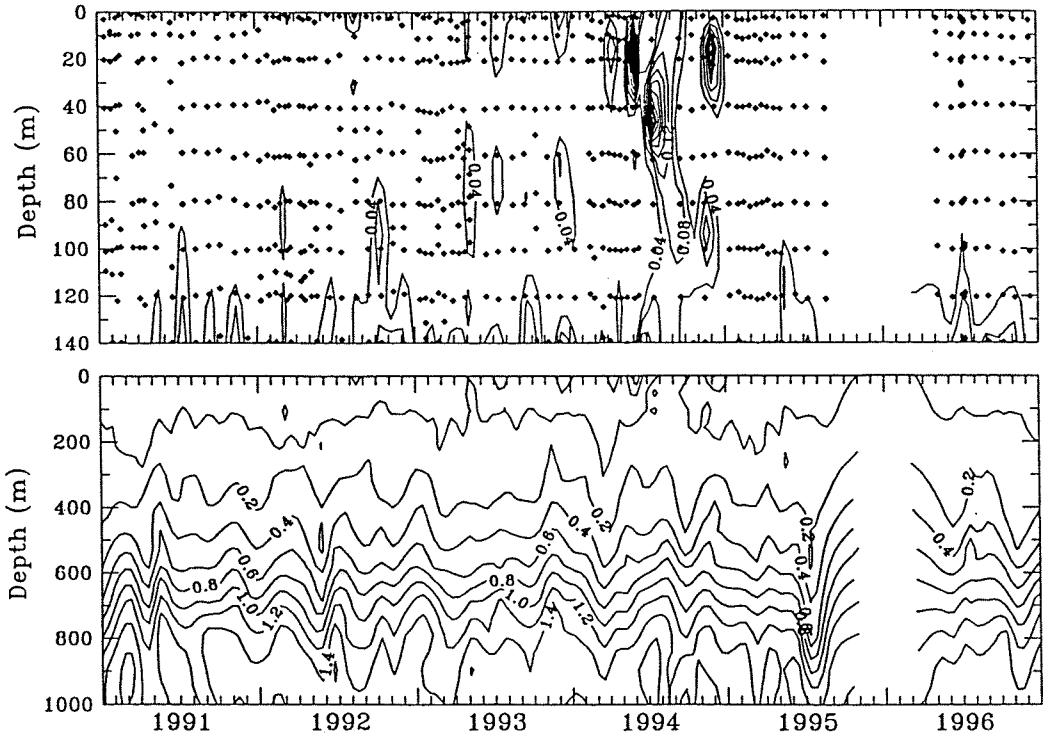

Figure 1-6
Contour plot of DIP (μmol kg^{-1}) at BATS in 1991-1996. Contour interval is 0.04 μmol kg^{-1} (upper panel) and 0.2 μmol kg^{-1} (lower panel). Black diamonds shows sampling depths and sampling frequency.

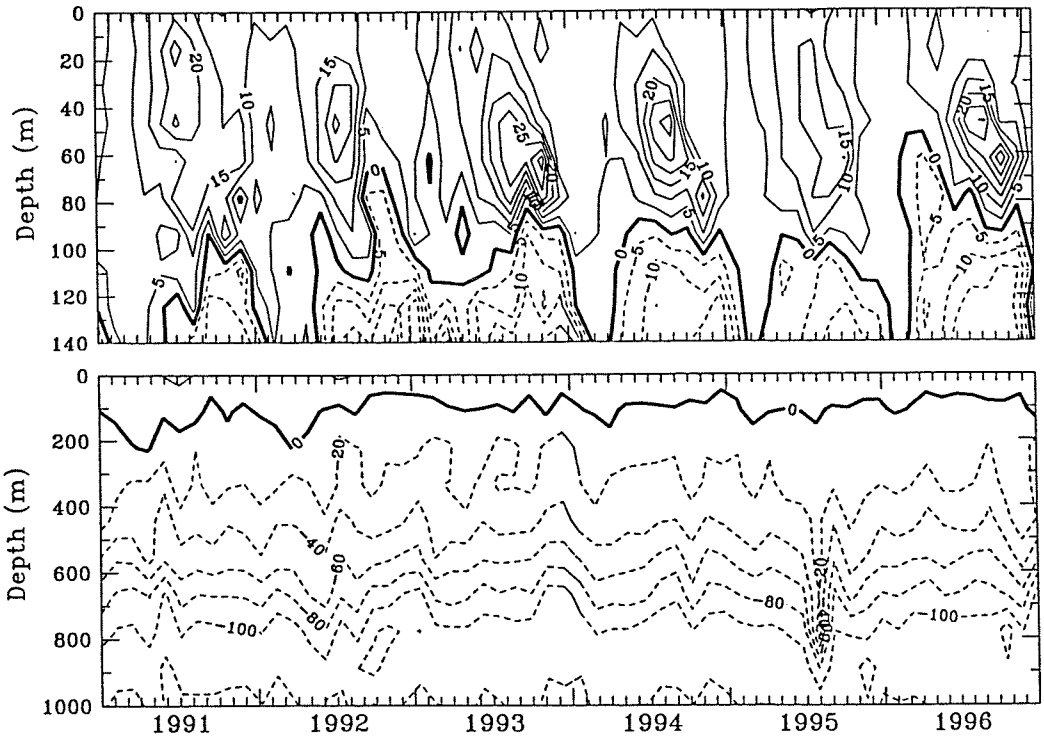

Figure 1-7
Contour plot of oxygen anomaly (μmol kg^{-1}) at BATS in 1991-1996. The anomaly is defined as the difference between the measured oxygen concentration and the theoretical oxygen saturation at *in situ* temperature and salinity. Dotted lines are negative values. Contour interval is 10 μmol kg^{-1} (upper panel) and 20 μmol kg^{-1} (lower panel).

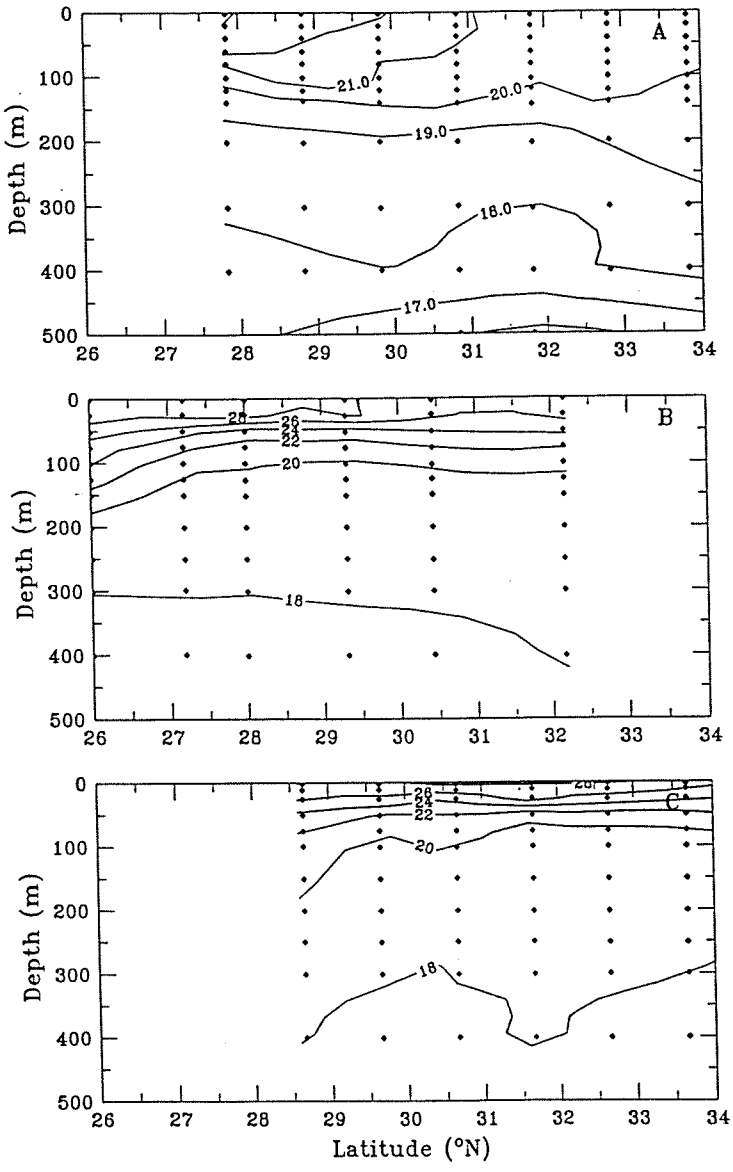

Figure 1-8
Contour plot of seawater temperature (˚C) from transect cruises 1-4 February 1994 (A), 26 September-2 October 1994 (B) and 25-29 July 1995 (C). Contour interval is 1 ˚C (A) and 2 ˚C (B and C). Black diamonds shows sampling depths and sampling frequency.

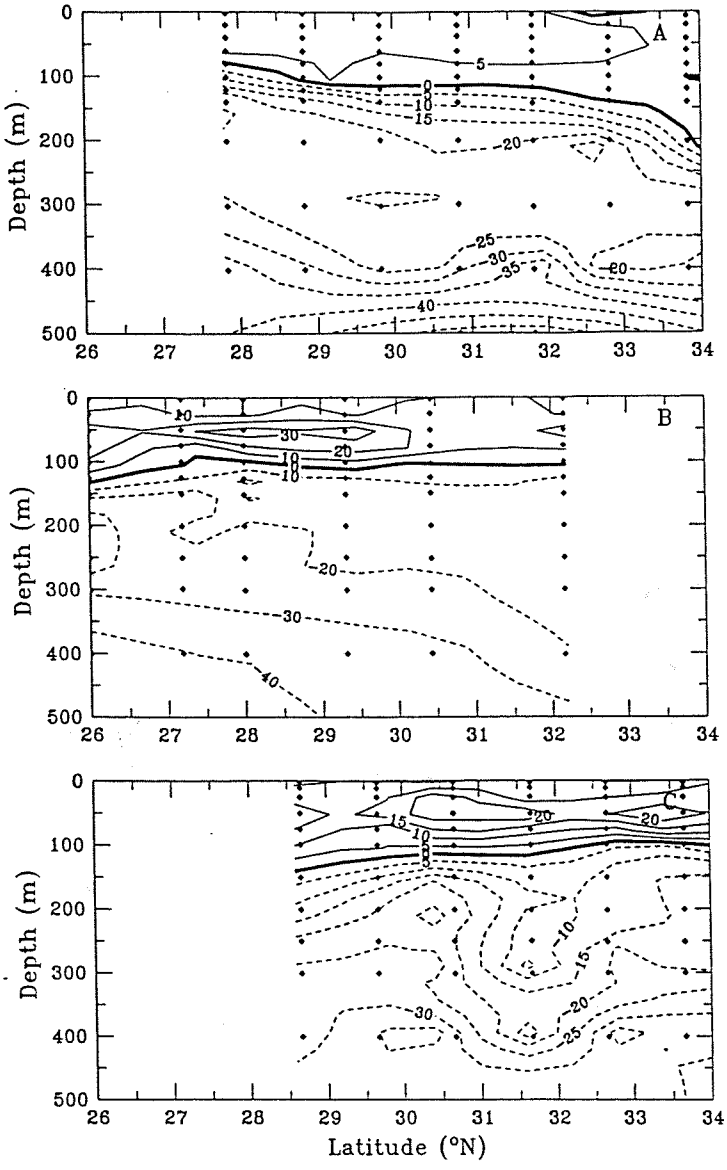

Figure 1-9
Contour plot of oxygen anomaly (μmol kg^{-1}) from transect cruises 1-4 February 1994 (A), 26 September-2 October 1994 (B) and 25-29 July 1995 (C). Contour interval is 5 μmol kg^{-1} (A and C) and 10 μmol kg^{-1} (B). Black diamonds shows sampling depths and sampling frequency.

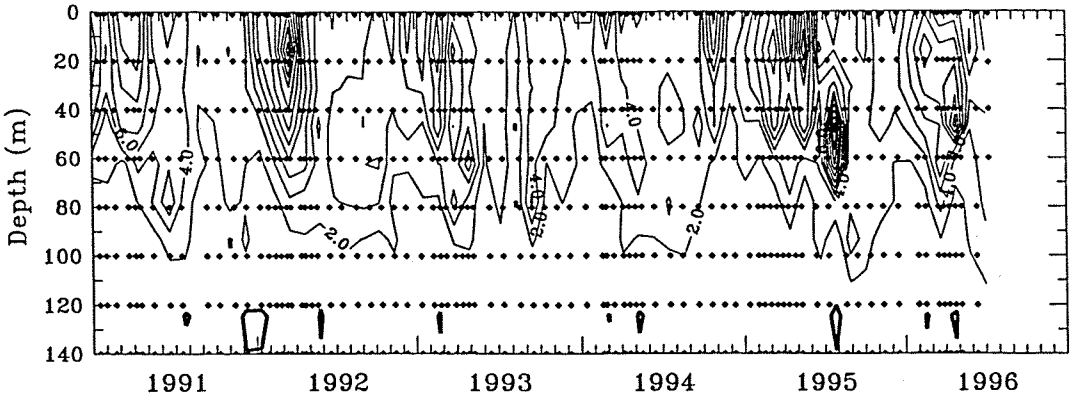

Figure 1-10
Contour of primary production rates (mg C $m^{-3}d^{-1}$) at BATS in 1991-1996. Contour interval is 2 mg C $m^{-3}d^{-1}$.

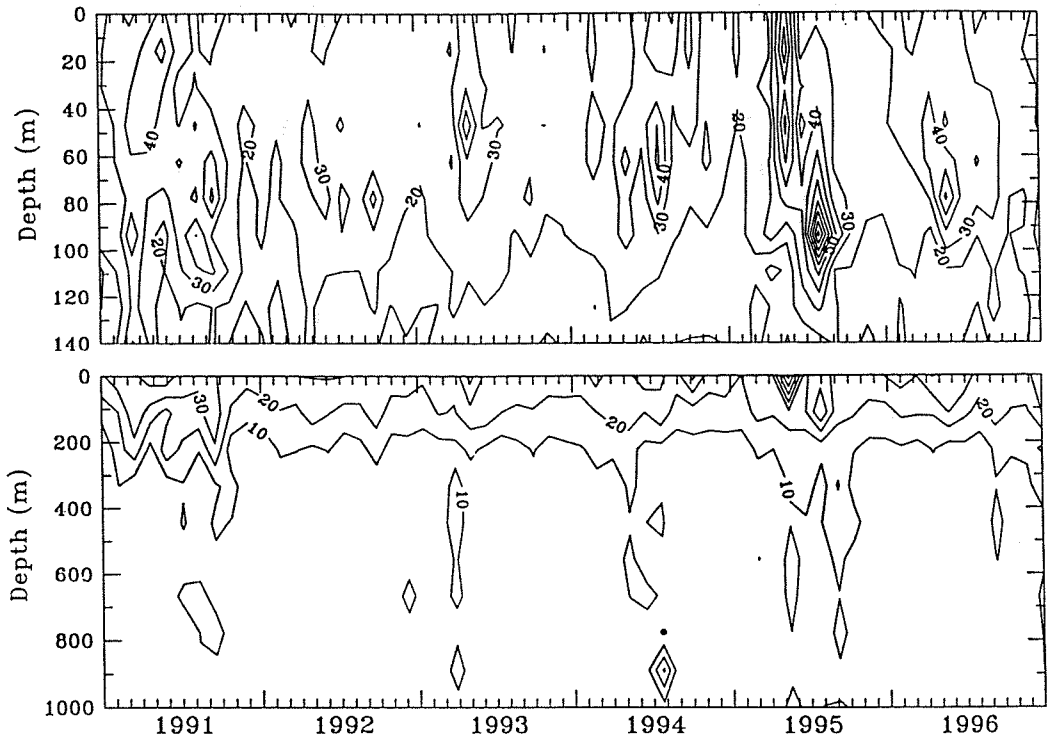

Figure 1-11
Contour plot of POC (μg kg^{-1}) at BATS in 1991-1996. Contour interval is 10 μg kg^{-1} (upper and lower panel).

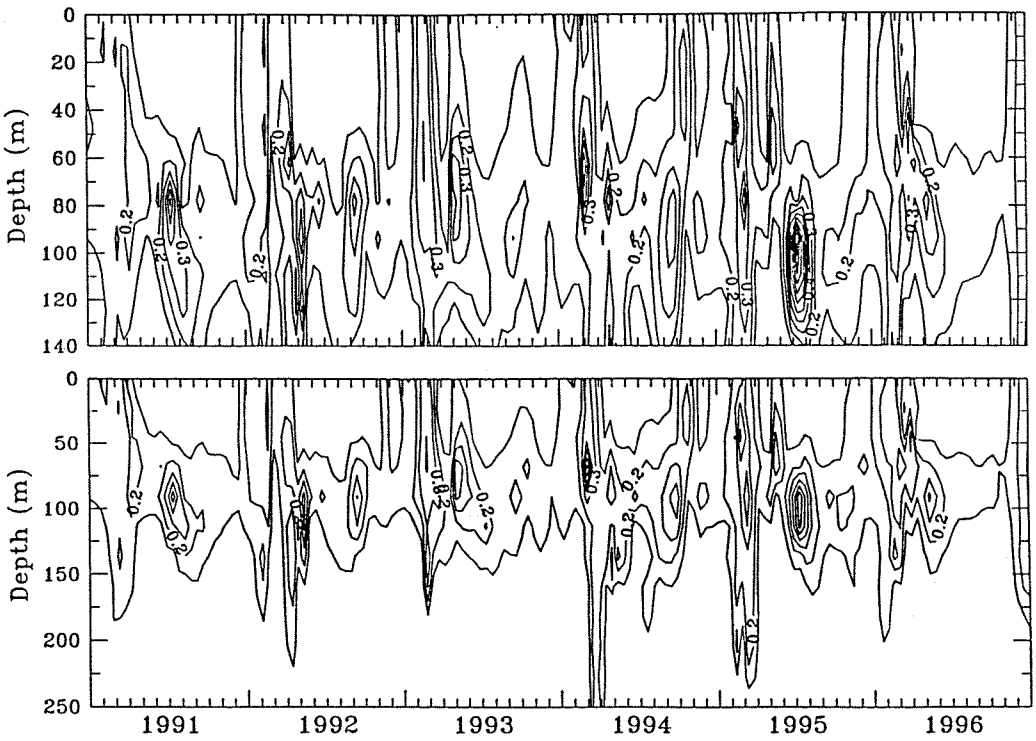

Figure 1-5
Contour plot of Chl-*a* (μg kg^{-1}) at BATS in 1991-1996. Contour interval is 0.1 μg kg^{-1} (upper and lower panel).

1.4 DISCUSSION

The seasonality illustrated in the hydrography at the BATS site (Figures 1-3 to 1-6), primary production (Figure 1-10) and particle distributions (Figure 1-11 to 1-12) is similar to what was described by Menzel & Ryther (1960, 1961) from the same area of study. The seasonal patterns observed at the BATS site were recently re-evaluated by Michaels et al. (1994). This part of the Sargasso Sea has a complex mesoscale physical structure; Cold core rings, mode water eddies and rich fine-scale eddy structures are all present in this region (Lai & Richardson 1977, Cornillion et al. 1986, Ebbesmeyer & Lindstrøm 1986) and Michaels et al. (1994) cautioned in the interpretation of these timeseries data. However, strong seasonal patterns were observed both in this study, in the Michaels et al. (1994) study and in the historical record (Menzel & Ryther 1960, 1961).

Menzel & Ryther (1960, 1961) first reported a seasonal pattern in stratification, production and biomass at Hydrostation 'S'. A similar trend was also observed in this study; surface cooling and wind mixing in winter and spring creates a deepening of the mixed layer (Figure 1-3 and 1-4). As the vertical mixing extends down into the nutrient rich layers, nutrients are introduced to the euphotic zone (Figure 1-5 and 1-6). Increased irradiance and lower wind speed, characterising the spring, lead to rapid stratification of the mixed layer. The newly introduced inorganic nutrients stimulate a primary production bloom (Figure 1-10) that can last for 1-3 months. This study and Michaels et al. (1994) show a pronounced spring bloom similar to the Menzel & Ryther (1960, 1961) study. However, the primary production rates are 2-4 times higher than those measured by Menzel & Ryther (1960, 1961). Michaels et al. (1994) suggested that the difference in primary production rates, compared to the historical record, was due to the trace-metal clean techniques applied in the BATS program, yielding higher primary production rates under similar nutrient and light conditions.

The increase in Chl-*a* and POC often associated with the primary production peak (Figure 1-11 and 1-12) showed fluctuations between cruises, which are probably a result from a combination of mesoscale variability and the seasonal succession of phytoplankton during the bloom period (Michaels et al. 1994).

Siegel et al. (1990) observed a spatial evolution of (18 °C) mode water along the 70 °W longitude in April 1985, similar to the N-S gradient found in February 1994 along 64 °W (Figure 1-8 A). The observed meridional changes in hydrography, controlling phytoplankton growth and community structure, were also suggested to be a reflection of the seasonal hydrography and phytoplankton successions in the area (Siegel et al. 1990). The summer (July 1995) and fall (September/October 1994) transects in this study, saw a strengthening of the mixed layer stratification and an increase in the oxygen anomaly, reconfirming the conclusions drawn from the Siegel et al. (1990) study regarding seasonal successions in both time and space.

Chapter 2: Bacterial abundance estimates*)

2.1 INTRODUCTION

Bacteria are considered the main heterotrophic link between particles and dissolved organic matter in open ocean and coastal ecosystems (Pomeroy 1974, Azam et al. 1983). Although still widely discussed (Li et al. 1992, Caron et al. 1995, Roman et al. et al. 1995), bacterial biomass has also been suggested to be a major part of the particulate organic carbon pool in open ocean and coastal water communities (Dortch & Packard 1989, Fuhrman et al. 1989, Cho & Azam 1990). Therefore, an accurate assessment of the bacterial biomass is important in the study of aquatic biogeochemical cycling of carbon, nitrogen and phosphorus. Direct microscopic counting of bacteria stained with fluorescent dyes (Zimmerman & Meyer-Reil 1974, Hobbie et al. 1977 and Porter & Feig 1980) has been a standard procedure during the last two decades for enumerating bacteria in natural water samples. The relative ease of handling and low cost of epifluorescence microscopy has made it the most widely used technique for the enumeration of bacteria. Bacterial samples preserved with formaldehyde or glutaraldehyde, are routinely stored at +4 °C for weeks, months and sometimes years before counting.

Questions have been raised about the preservation of bacteria in stored samples. In a comparative study of bacterial preservatives, Nishino (1986) found Lugol's iodine to be superior to glutaraldehyde preservation of marine bacteria, but the number of bacteria declined in both treatments as a function of storage time. A loss of 45 % and 69 % of the initial bacteria concentration was found in seawater samples preserved with glutaraldehyde and stored at +4 °C for 1 and 6 months (Nishino 1986). Spinrad et al. (1989) noted a loss of bacteria in formaldehyde preserved samples stored for two months

*) *A major part of this chapter was published as: Gundersen, K., Bratbak, G. & M. Heldal. 1996. Factors influencing the loss of bacteria in preserved seawater samples. Mar. Ecol. Prog. Ser. 137:305-310 (Appendix 5).*

and applied a logarithmic function to correct the bacterial cell concentration. Indeed, a loss of bacteria has been observed with most commonly used poisons or preservatives, such as Lugol's solution (Nishino 1986), formaldehyde (Spinrad et al. 1989) and glutaraldehyde (Nishino 1986, Turley & Hughes 1992).

There are several potential processes that may cause a loss of bacteria in preserved samples. These include: 1) attachment of cells to the bottle surface and other surfaces (aggregates) in the sample (Turley & Hughes 1992); 2) cell shrinkage, which may cause an increased loss by filtration and preparation of the sample; 3) virus infected bacteria in the lytic cycle may lyse and disintegrate cells in the preserved sample; 4) continued enzymatic activity and cell dissolution in glutaraldehyde preserved samples.

In one extensive study, Turley & Hughes (1992) investigated and discussed the possible causes of loss of bacteria in preserved seawater samples. They suggested that cells adhering to the wall of the sample bottle could account for 41-48 % of the original bacterial concentration in samples stored for about 11 months. However, 27-51 % of the total loss could not be accounted for. Bacterial DNA and RNA is not preserved by aldehydes (Hayat 1981) and enzymatic breakdown activity may also have a deleterious effect on the nucleotides present in bacteria. Continued enzymatic activity in glutaraldehyde and formaldehyde preserved organic matter has been reported in several studies (Fahimi & Drochmans 1968, Kolb-Bachofen 1977, Synowiecki et al. 1982, Shepard et al. 1983).

A number of storage experiments were conducted in order to investigate the effect on bacterial seawater samples preserved in 2.5 % glutaraldehyde (final conc.). Specific studies of attachment of cells to the inside surface of a bottle, cell shrinkage caused by preparation and filtration, virus infected cells that may lyse and disintegrate and the effect of continued enzyme activity and cell dissolution, were performed in order to evaluate the loss of bacterial cells in glutaraldehyde preserved seawater samples. By applying the recommendations derived from this chapter, bacterial abundance was enumerated on monthly cruises at the BATS site from 1991 to 1996.

2.2 MATERIALS AND METHODS

In order to investigate the effect of storage of bacteria preserved in glutaraldehyde, a number of experiments were conducted with coastal seawater (Bergen Harbour and Raunefjorden, Norway) and seawater collected at BATS and Hydrostation 'S'. Unbuffered, unfiltered glutaraldehyde (25 % v/v *Baker* Analyzed Reagent Grade in Bermuda and 25 % v/v *Merck* Electron Microscope Grade in Norway) was used to preserve the seawater samples. Both transmission electron microscopy (TEM) and epifluorescence microscopy (EFM) was used in order to investigate the storage problem.

Preparations for the TEM analysis was made by centrifuging algae, bacteria and viral particles in the water sample onto electron microscope grids with carbon stabilised *formvar* film (Bratbak & Heldal 1993). Bacteria were counted using a *JEOL* 100CX TEM at 20,000 X magnification. Similar preparations were used for counting of bacteria containing mature virus particles. From every TEM sample, a minimum of 100 bacterial cells were counted per sample, which results in a counting error of less than 10 % of the calculated mean.

For the EFM analysis, 20 ml of open ocean seawater sample and 5 ml of coastal and estuarine water samples were filtered and prepared according to the method of Porter & Feig (1980). The filtered bacteria were stained using a concentration of 25 µg 4',6-diamidino-2-phenylindole (DAPI) per ml of Milli-Q water. Since the time course samples were all closely related in time and distance (Kirchman et al. 1983), only one filter per one sample per time point was enumerated by the EFM method. A replication study with surface Sargasso seawater was done to evaluate the precision of the EFM enumeration method, using a two-level nested ANOVA (Sokal & Rohlf 1969). The replication study of the EFM enumeration method showed that the main variance component was found between squares counted on a filter (Table 2-1). There was no significant difference ($P > 0.05$) between bottles and samples within bottles. A minimum of 900 bacterial cells or 15 subsample squares were counted per filter sample, which results in a counting error of less than 20 % of the calculated mean.

Table 2-1
Nested ANOVA of bacterial cells enumerated by EFM; n = number of replicates between bottles, filters and subsample squares counted on a filter; df = degrees of freedom; MS = means of squares, F = the variance ratio; Var.comp. (%) = the relative source of variation.

Source	n	df	MS	F	Var. comp. (%)
Bottle	2	1	1.36	5.86	14.9
Filter	2	2	0.23	0.30	0.1
Squares	17	67	0.77		85.0

Loss of bacteria caused by cell shrinkage and by attachment of cells to the inside of the sample container

Bacterial samples for the time course experiments were collected at 1 m depth from Bergen Harbour in January 1993. One sample was preserved with glutaraldehyde (2.5 % final conc.) and aliquots were distributed into a series of 20 ml polyethylene scintillation vials (1-3 for each time point). In addition, aliquots of 100 and 1000 ml were stored in glass bottles to check for any effects of volume to surface ratio of the sample container. All samples were stored at +4 °C in the dark. Bacteria were enumerated over a time course of 94 days by the EFM and the TEM method. On the last day of the experiment, bacteria were also counted in the 100 ml and the 1000 ml samples.

Bacterial adhesion to the inside surface of a sample container with surface seawater from Raunefjorden, Norway, was studied by direct observation using both the EFM and the TEM technique. The glass sample bottle containing a one year old glutaraldehyde fixed seawater sample was emptied without agitation and the bottle was broken into pieces. Unwashed pieces from the side of the broken bottle was stained with DAPI as described above, mounted in liquid paraffin on a glass slide with the inside surface up and with a coverslip on the top. More than 40 fields of view were inspected by the EFM, each with an area of 400 μm^2 at 1,000 X magnification. Replicates for TEM of

the inside surface of the bottle was made by coating unwashed pieces (3-6 mm^3) of the crushed bottle with platinum and carbon in a *Balzers* BAF 400 Freezetcher: platinum at 45 °C, 1900 V, 80 mA (2nm) and carbon at 90 °C, 2400 V, 120 mA (20 nm). The replicas were transferred to electron microscope grids and inspected in the TEM. More than 200 fields of view, with an area of 50 μm^2 at 20,000 X magnification, were inspected by the TEM technique. The inside area of the glass bottle that was covered by the seawater sample, was calculated to be 100 cm^2.

Disappearance of cells with mature viral particles

During a mesocosm experiment conducted in May 1993 at the Marine Biological Field Station at Raunefjorden 20 km south of Bergen, the fraction of bacteria containing mature viral particles varied from < 1 to about 30 % (Heimdal et al. 1994). In order to check for a loss of bacterial cells, selected samples from these experiments were preserved with glutaraldehyde (2.5 % final conc.), stored for 72 days at +4 °C and then recounted in the TEM.

Enzyme activity and lysis of bacterial cells

In a first attempt to inhibit the impact of enzymatic activity on cell lysis and disintegration of bacteria, water was collected from 100 m depth in November 1992 at Hydrostation 'S', quick frozen in liquid nitrogen and stored at -20 °C. During a time course of 43 days, quick frozen samples of natural seawater and seawater preserved in glutaraldehyde (2.5 % final conc.), were compared to samples preserved in 2.5 % glutaraldehyde and stored at +4 °C. The frozen samples were thawed in cold tap water and processed immediately. The number of bacteria was counted by the EFM method.

A protease inhibitor, phenolmethylsulfonylfluoride (PMSF) (Turini et al. 1969, James 1978, Moss & Fahrney 1978), was added to bacteria bottles with Sargasso Sea water from 80 m depth at Hydrostation 'S' in September 1993 and from 80 m depth at the

BATS site in April 1994. PMSF is not readily soluble in aqueous solutions and final concentrations higher than 1 μM precipitated out of the seawater. Stock solutions of PMSF, stored in pure alcohol, were stable for at least a month (James 1978). A 100 μM stock solution of PMSF was prepared fresh in 95 % ethanol a couple of days prior to use. To one series of seawater samples, PMSF was added to a final concentration of 1 μM in the September 1993 time course experiment. After 5 minutes at room-temperature, glutaraldehyde was added to a final concentration of 2.5 %. Another series of seawater samples was preserved in 2.5 % glutaraldehyde. All the sample bottles were stored at +4 °C. A time zero sample was filtered, stained with DAPI and prepared immediately from both treatments. All stained preparations were stored at -20 °C. At the end of the time course, all samples were thawed and enumerated by the EFM method. One sample, designating a time point, was prepared and counted from each bottle.

In the April 1994 time course experiment, Sargasso Sea water with two different concentrations of PMSF (0.5 and 1.0 μM final conc.) and 2.5 % glutaraldehyde, was compared to seawater samples preserved in 2.5 % glutaraldehyde only. The samples were prepared and enumerated by the EFM method.

Bacterial abundance at the BATS site

Bacterial abundance was measured in a time-series at BATS, from 1991 to 1996. Bacteria were sampled at 20 discrete *Niskin* bottle depths from surface down to 1000 m. Glutaraldehyde was added to each of the 90 ml seawater samples (2.5 % final conc.) and stored at +4 °C. The samples were filtered and the DAPI stained cells were enumerated by the EFM method (Porter & Feig 1980) as described above. The glutaraldehyde preserved seawater samples were processed after no longer than three days of storage at +4 °C. The DAPI stained *Nuclepore* filter samples were mounted in oil on glass slides and stored at -20 °C for no longer than 6 months, before enumeration.

2.3 RESULTS

Loss of bacteria caused by cell shrinkage and by attachment of cells to the inside of the sample container

A comparison shows that the number of bacteria decreases on average by 43 % (SE = 1.2) with both the EFM and the TEM technique (Figure 2-1). The basic difference between these two methods is that the preparation for EFM is based on filtration and staining of cellular DNA with the fluorescent dye DAPI, while preparation for TEM is based on centrifugation onto a *formvar* film and contrast staining of the cells with uranyl acetate.

Less than 0.6 % of the original number of bacteria from Bergen Harbour, enumerated by the TEM replica technique ($2 \cdot 10^9$ l^{-1}), were found to be attached to the inside surface of the sample bottle after one year of storage time. The figures obtained with DAPI staining and the EFM method were less than 0.4 %. Moreover, there was no significant difference ($P > 0.05$) in bacterial numbers in samples stored in bottles with volumes ranging from 20-1000 ml (Figure 2-1) although the surface to volume ratio of these sample containers differed by a factor of four.

In an attempt to recover 30 % of the bacteria lost in 4 week old glutaraldehyde fixed seawater samples from the Sargasso Sea, no detectable increase was observed after 0.5 and 1 min sonication prior to counting the samples by EFM (Figure 2-2).

Disappearance of bacterial cells with mature viral particles

A slightly negative correlation of cells in lysis and loss of bacteria per sample was observed in glutaraldehyde preserved seawater samples from Raunefjorden (Figure 2-3). The linear regression however, was weak ($p < 0.25$) suggesting that the cell losses were not significanly lower in bacterial samples where the number of cells in lysis (bacteria containing mature viral particles) were most frequent.

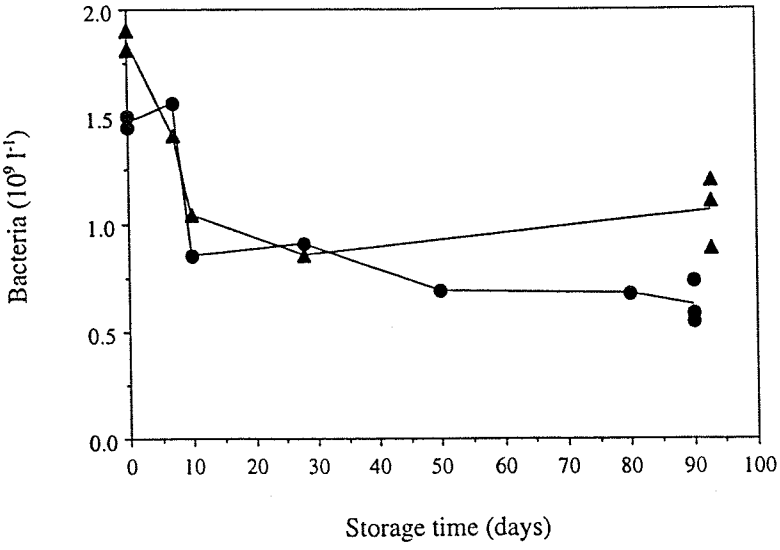

Figure 2-1
Bacterial number as a function of storage time, in seawater samples preserved with glutaraldehyde (2.5 % final conc.) from Bergen Harbour in January 1993. The samples were stored in 20 ml scintillation vials. Bacteria were enumerated by the EFM (closed circles) and TEM (closed triangles) method. The triplicates at the end of the time course are bacteria samples stored in 20, 100 and 1000 ml glass bottles.

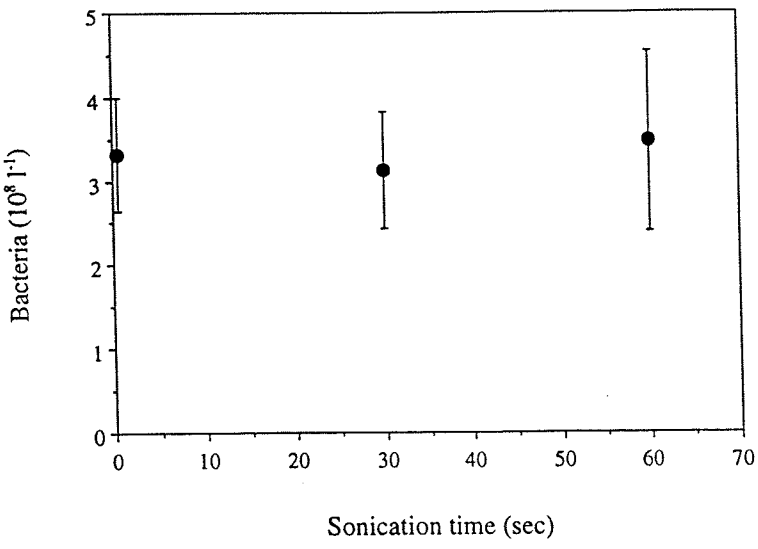

Figure 2-2
Bacterial number as a function of sonication time (sec) in surface seawater samples from BATS in February 1991. Vertical bars are standard error (SE) of the sample mean and n = 3 for each time point. The samples were stored 4 weeks and a loss of 30 % of the initial abundance was recorded, prior to the sonication.

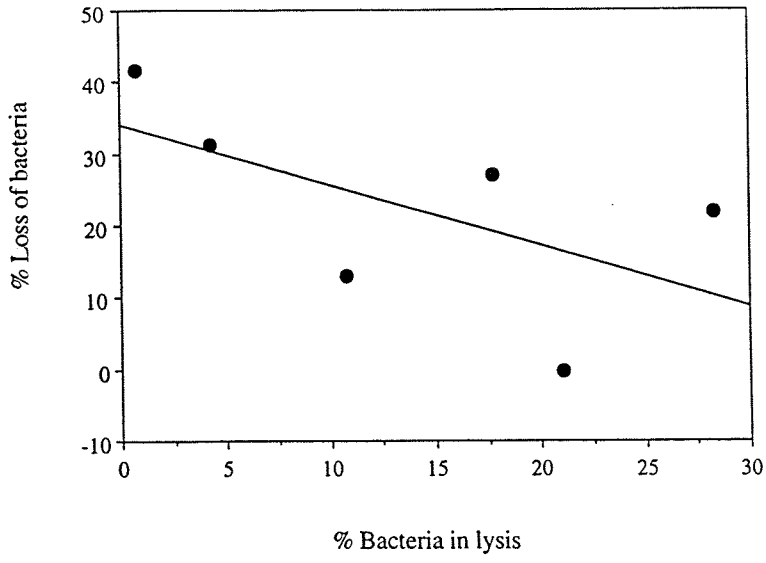

Figure 2-3
Loss of bacteria in seawater samples preserved with glutaraldehyde (2.5 % final conc.) and stored for 72 d, as a function of the percentage of cells containing mature viral particles at the time of sampling ($r^2 = 0.361$, $n = 6$, $F_{[1,4]} = 2.26$, $p < 0.25$).

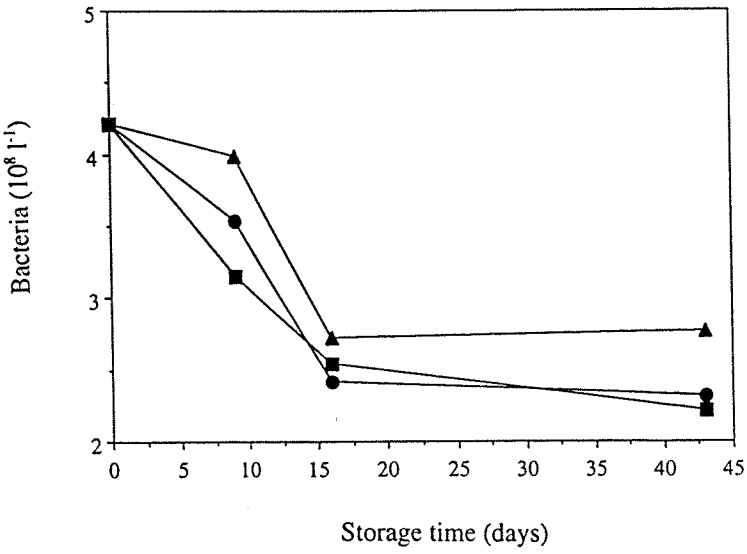

Figure 2-4
Bacterial number as a function of storage time in seawater samples taken from 100 m depth at Hydrostation 'S' in the Sargasso Sea in September 1992. Closed circles: Unpreserved seawater, quick frozen and stored at -20 °C. Closed triangles: Seawater preserved in glutaraldehyde (2.5 % final conc.), quick frozen in liquid nitrogen and stored at -20 °C. Closed squares: Seawater preserved in 2.5 % glutaraldehyde and stored at +4 °C.

Enzyme activity and lysis of bacterial cells

Bacterial numbers in seawater samples preserved with glutaraldehyde, quick frozen and stored at -20 °C declined by only 5 % after 9 days of storage (Figure 2-4). Bacteria in the preserved samples stored at +4 °C and in the unpreserved frozen samples, had at this time point reached 72-81 % of the initial concentration. After 16 days the bacterial numbers were reduced to 56-62 % in all the treatments.

By adding 1.0 μM PMSF to Sargasso seawater samples prior to the addition of 2.5 % glutaraldehyde, only 17 and 18 % of the bacterial cells were lost after 21 and 36 days of storage time (Figure 2-5). During the same time course, seawater samples preserved only with glutaraldehyde, decreased by 50 and 25 % (Figure 2-5). By using 1.0 μM PMSF prior to the addition of glutaraldehyde, the loss was only 7 and 13 % of the initial concentration of bacteria in both experiments, after 9 and 8 days respectively (Figure 2-5). Although the difference between the treatments were less pronounced in the latter experiment the same trend in the time course was observed: Seawater samples preserved with 1.0 μM PMSF and glutaraldehyde had a higher number of bacteria than seawater preserved with only glutaraldehyde (Figure 2-5). In the second time course experiment, final concentrations of 0.5 and 1.0 μM PMSF were used and a positive linear correlation was found between number of bacterial cells preserved and the concentration of PMSF used after 9 and 21 days of storage ($r^2 = 0.676$, $n = 6$, $F_{[1,4]} = 8.35$, $p < 0.05$). PMSF is unstable in aqueous solutions and a precipitate was formed in seawater samples at concentrations higher than 1.0 μM.

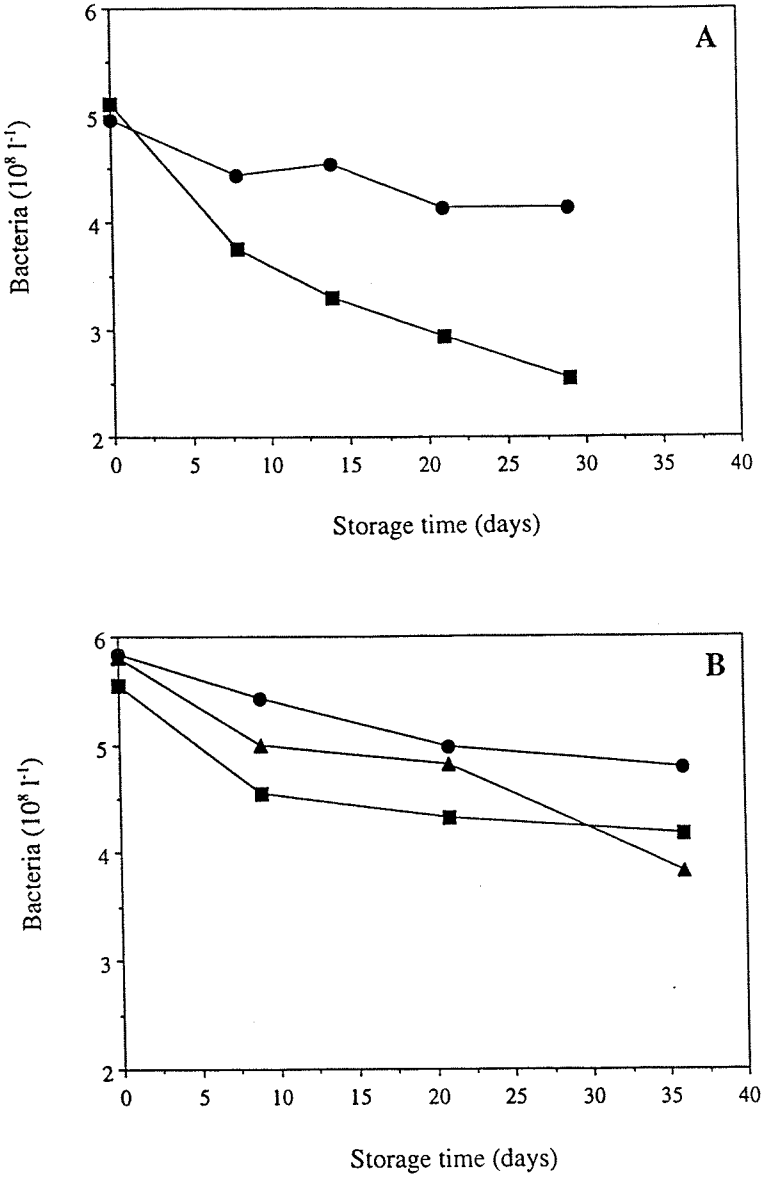

Figure 2-5
Bacterial number as a function of storage time in seawater samples taken from 80 m depth (A) at Hydrostation 'S' in September 1993 and (B) at BATS in April 1994. Closed circles: Seawater preserved in 1.0 µM PMSF and glutaraldehyde (2.5% final conc.). Closed triangles: Seawater preserved in 0.5 µM PMSF and 2.5 % glutaraldehyde. Closed squares: Seawater preserved in 2.5% glutaraldehyde. All samples were stored at +4 °C during the time course.

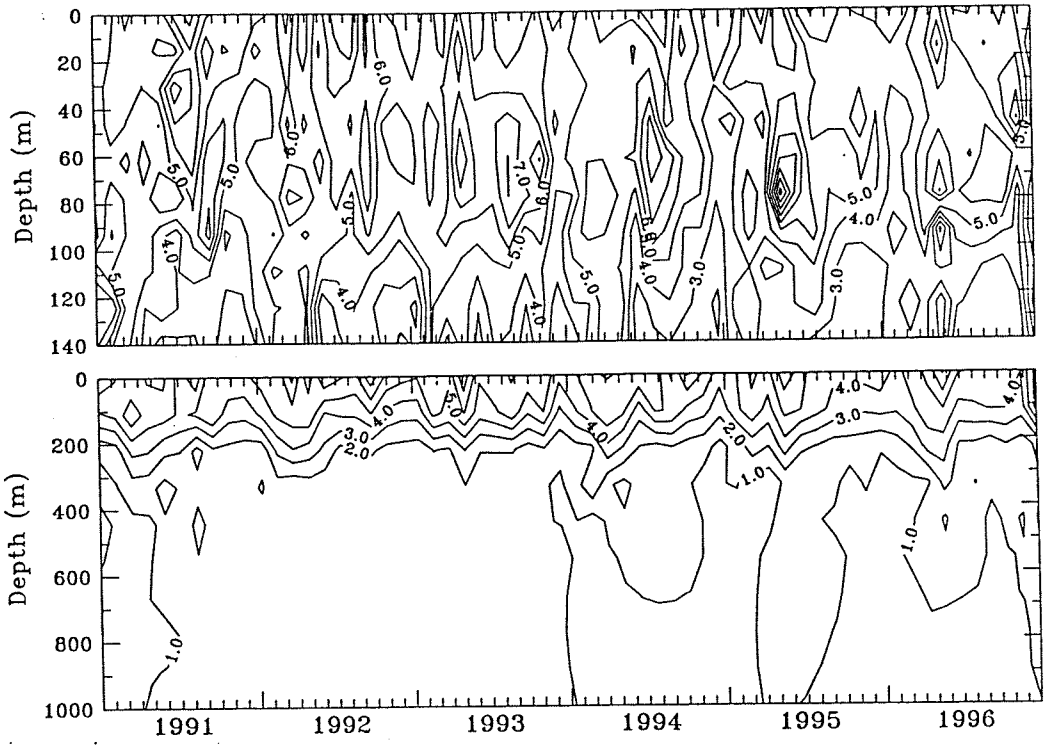

Figure 2-6
Contour plot of bacterial abundance (10^8 cells kg^{-1}) at BATS in 1991-1996. Contour interval is 1 • 10^8 cells kg^{-1} (upper and lower panel).

Bacterial abundance at the BATS site

Bacterial abundance was estimated from routine BATS cruises between 1991 and 1996, from the surface down to 1000 m depth (Figure 2-6). Peak abundances (6-10 • 10^8 cells kg^{-1}) followed the winter mixing and primary production peaks. The bacterial abundance peaks were associated with elevated Chl-*a* and POC concentrations (Figures 1-11 and 1-12).

2.4 DISCUSSION

Loss of bacteria caused by cell shrinkage and by attachment of cells to the inside of the sample container

The basic difference between the two bacteria enumeration techniques used in this study, is that the preparation for EFM is based on filtration and staining of cellular DNA with the fluorescent dye DAPI, while preparation for TEM is based on centrifugation onto a formvar film and contrast staining of the cells with uranyl acetate. Hence, if bacterial cells did shrink during storage and got lost during the filtration processes for the EFM method, they should be retained on the membrane used for enumerating cells by the TEM . Consequently, the loss of cells could not be caused by increased filtrational loss due to cell shrinkage as a function of storage time and the EFM method of enumerating bacteria gave comparable numbers of cells to that using the TEM method (Figure 2-1).

Less than 0.6 % of the original number of bacteria from Bergen Harbour, enumerated by the TEM replica technique (2 • 10^9 l^{-1}), were found to be attached to the inside surface of the sample bottle after one year of storage time. The figures obtained with DAPI staining and the EFM method were less than 0.4 %. Moreover, there was no significant difference in bacterial abundance detected in samples stored in bottles with a surface to volume ratio varying by a factor of four. Turley & Hughes (1992) were able to

recover 37-70 % (percentage of initial number of bacteria) by a 4 minute sonication of samples stored for 338-340 days. In this study, 4 week old glutaraldehyde fixed seawater samples from the Sargasso Sea were sonicated in an attempt to recover the missing 30 % of the bacteria. No detectable numbers of bacteria were recovered after 1 minute of sonication prior to counting the samples by EFM (Figure 2-2). In comparison, Turley & Hughes (1992) found an increase of 1.5-2.5 times the initial cell number after only 1 minute sonication of samples stored for 338-340 days.

These results suggests that the loss of bacteria in this study cannot be explained by attachment to the walls of the sample container. Turley and Hughes (1992) also found that 30-90 % of bacteria remained unaccounted for and concluded that other factors as well as attachment must play a significant role in cell loss with time. Although there is a considerable difference in storage time between these two sonication studies, the reason for the discrepancy between our observations is not readily explained. Different properties of the bacterial communities used in the experiments may be one possibility, since Turley and Hughes (1992) used bacteria from the North Atlantic, whereas the collections in this study were from the Sargasso Sea. Wiik (1984) noted that sonicating cultured bacteria for longer than 2 minutes damaged and disrupted the cells. However, Turley & Hughes (1992) studied the effect of sonication on fresh seawater samples and found 4 x 1 min sonication with cooling and shaking in between, resulted in no decrease in bacterial cell numbers (C. Turley, pers. com.). Different surface properties of the sample containers could be another possible explanation, since Turley & Hughes (1992) used polystyrene tissue culture flasks, whereas glass bottles and polyethylene scintillation vials were used in this study. More recently, however, Turley & Hughes (1994) found no significant difference in bacterial cell numbers between polystyrene tissue culture flasks and *Nalgene* bottles.

Disappearance of cells with mature viral particles

The potential loss of cells in preserved samples due to lysis and disintegration of cells in the late stage of the lytic cycle was not supported by the results in this study (Figure 2-3). The slightly negative correlation observed was only significant on the $p < 0.25$ level, suggesting that the cell losses were not less in samples with more frequently occuring cells in lysis. Only bacteria containing mature viral particles were detected in this study. If it had been possible to include cells at any stage in the lytic cycle (i.e. all cells that were bound for lysis at the time the sample was preserved) the results may have been different.

Enzyme activity and lysis of bacterial cells

This study suggest that the loss of bacterial cells can be delayed several days by freezing seawater samples at -20 °C and that the addition of a protease inhibitor (PMSF) can slow down the loss significantly (Figures 2-4 and 2-5). Furthermore, the findings indicate that a considerable part of the loss of bacteria must be related to enzymatic breakdown of cells in the preserved sample. Addition of glutaraldehyde (2.5 % final conc.) may thus not sufficiently penetrate the cells in order to inactive the enzymatic activity associated with the bacteria, leading to cell lysis and disintegration. PMSF is unstable in aqueous solutions and a precipitate was formed in seawater samples at concentrations higher than 1 μM. Thus, other protease inhibitors may be better suited for inhibiting protease activity in preserved seawater. James (1978) found that PMSF inhibited enzymes are not reactivated when the inhibitor is deactivated. However, a minor reduction of bacterial cells ($3 \cdot 10^6$ $l^{-1}d^{-1}$) in samples pre-treated with 1 μM PMSF was observed in this study (Figure 2-5). This may suggest that PMSF insufficiently inhibited the enzymatic breakdown of bacterial cells or that other factors as well as proteolytic activity, are responsible for the loss of bacteria as a function of storage time.

Bacterial abundance at the BATS site

The multitude of bacteria present in open ocean and coastal waters, was clearly visualised by the introduction of the EFM technique using acridine orange (AO) (Hobbie et al. 1977), and DAPI (Porter & Feig 1980). Bacterial abundance at BATS in 1991-1996 (Figure 2-6) did not show any striking seasonal patterns. The numbers generated in this study, are of the same order as reported in previous studies from the Sargasso Sea (Fuhrman et al. 1989, Carlson 1994). Slightly elevated abundances and increased bacterial growth at the time of the spring bloom, most likely triggered an increase in bacterivore nano and micro-plankton (Fuhrman & McManus 1984, Landry et al. 1984, Wright & Coffin 1984, Andersen & Fenchel 1985) that effectively grazed down and controlled the abundance of bacteria (see also Chapter 5). More recently, Bergh et al. (1989) and Børsheim et al. (1990) were able to show that the concentration of marine phage (virus) was one order of magnitude higher than the bacterial abundance and much higher than previously reported. They concluded that viral parasitism as well as grazing, may effect bacterial mortality (Børsheim et al. 1990).

Chapter 3: Elemental C, N and P content of individual bacterial cells

3.1 INTRODUCTION

Despite the importance of bacteria in most biogeochemical cycles, very little attention has been focused on how to measure the accurate amount of individual elements in single cell bacteria. In their review of bacterial growth measurements, Ducklow and Carlson (1992) found it ironic that so much attention has been focused on thymidine conversion factors, when choices about bacterial carbon (C) per cell seems to be made almost arbitrarily.

Until recently, most estimates of the elemental composition of individual bacteria was done by combining epifluoresecence microscopy (EFM) for bacterial sizing and conventional carbon and nitrogen (CHN) elemental bulk analysis. This approach has been most successful in cultures, since debris and other plankton interfere with the analysis from natural seawater. Earlier studies showed a great variation in bacterial C conversion factors (Table 3-1). During the last two decades, the elemental nitrogen (N) content in natural bacterial populations have only been reported twice, ranging from 8-41 fg N μm^{-3} (Nagata 1986, Fagerbakke et al. 1996), while the elemental phosphorous (P) content has only been reported once (Fagerbakke et al. 1996: 2-5 fg P μm^{-3}). It is interesting to note that estimates from early studies such as Ferguson and Rublee (1976) and Watson et al. (1977), using natural seawater and applying theoretical assumptions about bacterial cell density (Dry Wet^{-1} ratio and C Dry^{-1} ratio), were closer to more recent estimates from studies such as Norland et al. (1995), Tuomi et al. (1995) and Fagerbakke et al. (1996), who used x-ray detection of the elemental composition of single cell bacteria. Heldal et al.

Table 3-1
Elemental C conversion factors in studies of aquatic bacteria; SW = Seawater; NA = Information not available; EFM = Epifluoresecence Microscope; TEM = Transmission Electron Microscope; CHN = Carbon, Hydrogen and Nitrogen analysis; FA = Formaldehyde; GA = Glutaraldehyde; AO and DAPI = EFM detection of bacteria using acridine orange (AO) or DAPI (4',6-diamidino-2-phenylindole); XRMA = x-ray microanalysis.

Reference	Sample source	Conv. Factor fg C μm^{-3} mean (range)	Cell volume range, μm^3	Fixation	Detection
Ferguson & Rublee 1976	Coastal SW	[a] 122 (NA)	0.04-12.0	5% FA	EFM, AO
Watson et al. 1977	Coastal SW	[b] 121 (NA)	0.04-1.10	0.1% GA	TEM
Bratbak & Dundas 1984	Culture	220 (160-290)	NA	Airdry	^{14}C-Inulin, CHN
Bratbak 1985	Culture	560 (240-930)	0.11-0.71	5% GA	EFM, AO
Heldal et al. 1985	*Escherichia coli*	90 (NA)	1.25	Airdry	TEM, XRMA
Nagata 1986	Lake water	106 (39-188)	0.10-0.25	2% FA	EFM, AO, CHN
Bjørnsen 1986	Culture	350 (180-730)	0.08-0.34	1% FA	EFM, AO
Lee & Fuhrman 1987	Culture	380 (210-600)	0.04-0.07	5% FA	EFM, AO, CHN
Norland et al. 1987	Coastal SW	NA (50-100)	0.01-7.00	Airdry	TEM
Kogure & Koike 1987	Culture	195 (83-354)	0.17-2.02	2% FA	Electronic
Scavia & Laird 1987	Culture	154 (59-207)	NA	2% FA	EFM, AO
Simon & Azam 1989	Culture	112 (NA)	0.26-0.40	NA	EFM [c], AO
Nagata & Watanabe 1990	Lake water	120 (59-252)	0.17-1.80	1% GA	EFM, AO, CHN
Kroer 1994	Culture	720 (210-1610)	0.07-0.15	2% FA	EFM, AO, CHN
Norland et al. 1995	*Escherichia coli*	[d] 126 (85-168)	0.70-4.60 [e]	Airdry	TEM, XRMA
Tuomi et al. 1995	Coastal SW	[d] 81 (63-127)	0.15-0.60 [e]	Airdry	TEM, XRMA
Fagerbakke et al. 1996	Coastal SW	[d] 59 (30-82)	0.10-0.36 [e]	Airdry	TEM, XRMA
Fagerbakke et al. 1996	Brackish	[d] 103 (94-112)	0.22-0.40 [e]	Airdry	TEM, XRMA
Fagerbakke et al. 1996	Lake water	[d] 53 (51-55)	0.38-0.44 [e]	Airdry	TEM, XRMA
Fagerbakke et al. 1996	Culture	[d] 105 (62-180)	0.60-4.20 [e]	Airdry	TEM, XRMA

a) Assuming a bacterial density of 1.1 g cm^{-3} (Doetsch & Cook 1973), a dry to wet ratio of 0.23 (Jordan 1919, Roberts et al. 1957) and a carbon to dry weight ratio of 0.344 (Ferguson *unpubl.*).
b) Assuming a bacterial density of 1.1 g cm^{-3} (Lamanna et al. 1973), a dry to wet weight ratio of 0.22 (Luria 1960) and carbon to dry weight ratio of 0.50 (Luria 1960).
c) Photomicrograph detection of cell size. Carbon was reconstituted from measured macromolecules.
d) Average calculated from growth and stationary phase. The range was calculated from the two means of the stationary and growth phase using the standard deviation.
e) Size range calculated from two averages of growth and stationary phase using the standard deviations.

(1985) first introduced the x-ray detection method to measure single cell elemental components of bacteria. Absorption of low energy x-ray photons, due to the window thickness of the original energy dispersive x-ray detector, made it only possible to measure elements down to a molecular weight of 23 [Na] (Heldal et al. 1985). Recently, a more sensitive detector was installed and light elements such as C and N could be measured accurately and with adequate precision (Heldal 1993, Norland et al. 1995).

Bacterial seawater samples from the BATS site in May 1992, were collected from 5 selected depths, using Niskin water bottles and preserved (2.5 % final conc. glutaraldehyde) in order to investigate the C and N elemental content in single cell bacteria. In 1995, seawater samples were collected at 10 m depth in February, March, May, July and September, using a GoFlo bottle. These samples were prepared fresh, in order to avoid sample bias possibly caused by using a fixative and the elemental content of C, N and P was investigated. The samples were analysed using the TEM and x-ray detector facility at the University of Bergen, Norway.

3.2 MATERIALS AND METHODS

In order to measure the elemental C and N content of individual bacterial cells, seawater samples were collected at 20, 60, 100, 110 and 140 m depth at BATS in May 1992, using Niskin bottles. Glutaraldehyde was added to each of the 90 ml seawater samples (2.5 % final conc.) and stored at +4 °C. The preserved seawater samples were processed at the University of Bergen, Norway, one week after sampling took place.

In February, March, May, July and September of 1995, surface seawater samples were collected at 10 m depth from the BATS site, using a GoFlo bottle, in order to study the C, N and P content of individual cells. These samples were stored at +4 °C and processed 6-12 hours after sampling, using the ultracentrifuge facility (Sorvall Combi Plus Ultracentrifuge) at the Bermuda Biological Station for Research Inc., Bermuda.

The bacterial cells were centrifuged at 20.000 x g, onto an aluminium grid coated with a carbon-stabilised formvar polymer membrane. Details of the preparation method is given by Heldal (1993). The supernatant was removed and the aluminium grid with the cells was rinsed with 0.2 µm filtered Milli-Q water and stored dry until analysis. The samples were analysed in a JEOL 100CX TEM. Cell volume (V) was calculated by assuming that all bacteria are essentially cylindrical shapes, with two half spherical ends. Hence, only the width (W) and the length (L) of the bacteria was measured;

$$V = \pi / 4 \bullet W^2 \bullet [(L - W) / 3]$$

The Tracor Z-MAX 30 energy-dispersive detector was calibrated using latex beads with a known carbon content, Coenzyme A, Ca-ß-glycerophosphate, KNO_3 and $K_4[Fe(CN)_6]$, as described by Norland et al. (1995).

3.3 RESULTS

All samples for TEM analysis (with the exception of 140 m) were taken above the nutricline at BATS in May 1992 and in February, March, May, July and September 1995. The geometric mean (GM) of the elemental C, N and P content of bacteria, tabulated as a function of depth in 1992 (Table 3-2) and as a function of time in 1995 (Table 3-3), showed the range of the elemental content in discrete cells varying by a factor of 10-220 times for C, 14-147 times for N and 23-300 times for P. The elemental content per bacterial cell volume however (Table 3-4 and 3-5), ranged only by a factor of 6-13 times for C, 2-23 times for N and 5-10 times for P. There was a linear relationship between the elemental C, N and P content per cell and the cell volume (Figure 3-1).

A weak power function relationship was found between the volume and the elemental ratios of C:V ($r^2 = 0.049$, $n = 163$, $F_{[1,161]} = 8.46$, $p < 0.005$), N:V ($r^2 = 0.039$, $n = 163$, $F_{[1,161]} = 6.33$, $p < 0.005$) and P:V ($r^2 = 0.014$, $n = 83$, $F_{[1,81]} = 1.21$, $p < 0.5$). The amount of elemental C, N and P per cell (fg $cell^{-1}$) was plotted as a function of the cell volume (μm^{-3}) in Figure 3-1. The elemental content, expressed as a linear

Table 3-2
Geometric means of the elemental C and N content (fg cell^{-1}) of individual bacterial cells from the upper 140 m of the water column at BATS in May 1992.

Depth (m)	fg C cell^{-1}	min - max	fg N cell^{-1}	min-max	n
20	20.4	3.2 - 263.0	3.0	0.3 - 44.2	16
60	12.9	1.7 - 104.0	2.8	0.3 - 15.9	18
100	17.4	3.0 - 105.0	2.8	0.7 - 11.7	17
110	12.9	2.9 - 58.5	2.6	0.5 - 10.1	17
140	6.0	1.8 - 25.5	1.7	0.5 - 7.1	9

Table 3-3
Geometric means of the elemental C, N and P content (fg cell^{-1}) of individual bacterial cells sampled from 10 m depth at BATS in 1995.

	fg C cell^{-1}	min - max	fg N cell^{-1}	min - max	fg P cell^{-1}	min - max	n
February	6.5	0.6 - 84.0	1.7	0.2 - 20.1	0.3	0.04 - 2.5	17
March	4.0	1.3 - 12.4	0.9	0.2 - 3.7	0.2	0.01 - 0.8	16
May	5.9	1.3 - 154.3	1.0	0.2 - 25.5	0.1	0.03 - 9.0	24
July	1.6	0.1 - 22.2	0.3	0.1 - 4.2	0.1	0.01 - 0.7	16
September	4.0	0.5 - 47.0	0.7	0.1 - 11.3	0.1	0.03 - 0.7	15

Table 3-4
Geometric means (GM), the range of elemental C and N conversion factors (fg μm^{-3}) and the C:N ratio (mw) of bacterial cells from the upper 140 m of the water column at BATS in May 1992. n = number of bacteria measured per depth.

Depth (m)	fg C μm^{-3}	min - max	fg N μm^{-3}	min - max	C:N	n
20	177.8	61.7 - 360.4	25.7	4.9 - 115.1	7.1	16
60	83.2	47.3 - 149.9	17.8	8.6 - 29.2	4.8	18
100	234.4	118.9 - 396.5	37.2	10.7 - 67.5	6.3	17
110	234.4	111.2 - 424.4	46.8	24.2 - 81.2	5.0	17
140	120.2	61.6 - 307.4	33.9	26.3 - 48.8	3.6	9
GM_{Grand}	158.5	47.3 - 424.4	30.2	4.9 - 115.1		77

Table 3-5
Geometric means (GM), the range of elemental C, N and P conversion factors (fg μm^{-3}) of bacterial cells from surface waters at BATS in 1995. n = number of bacteria measured.

	fg C μm^{-3}	min -max	fg N μm^{-3}	min - max	fg P μm^{-3}	min - max	n
February	109.7	31.0 - 193.7	28.2	13.0 - 52.6	5.6	2.2 - 10.8	17
March	128.8	45.7 - 278.4	30.2	12.2 - 70.4	6.5	1.8 - 17.6	16
May	144.5	48.8 - 357.8	25.1	13.5 - 66.5	4.4	1.3 - 11.4	24
July	100.0	24.1 - 327.0	17.0	4.0 - 51.3	3.0	1.1 - 11.4	16
September	208.9	75.0 - 537.1	33.9	8.5 - 71.5	5.8	2.0 - 13.6	15
GM_{Grand}	138.5	24.1 - 537.1	26.8	4.0 - 71.5	4.9	1.1 - 17.6	88

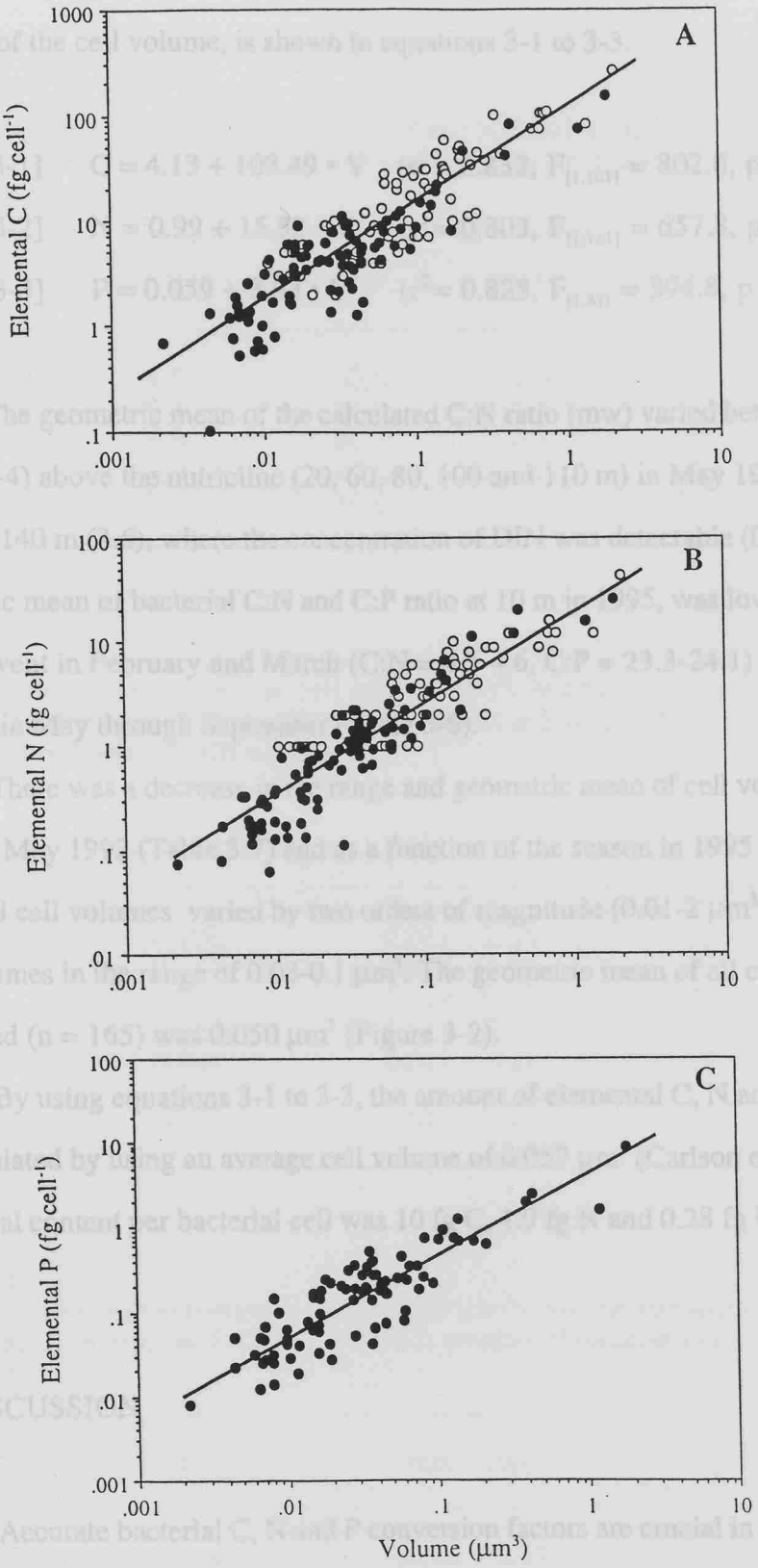

Figure 3-1
Elemental content of carbon (A), nitrogen (B) and phosphorus (C) in bacteria (fg $cell^{-1}$), as a function of the cell volume (μm^3) in bacterial cells at the BATS site. Open circels are data from May 1992 and closed circles are from February, March, May, July and September 1995. Note the LOG scale.

function of the cell volume, is shown in equations 3-1 to 3-3.

[3-1] $C = 4.13 + 103.49 \cdot V$ ($r^2 = 0.832$, $F_{[1,161]} = 802.6$, $p << 0.001$)

[3-2] $N = 0.99 + 15.52 \cdot V$ ($r^2 = 0.803$, $F_{[1,161]} = 657.8$, $p << 0.001$)

[3-3] $P = 0.059 + 3.93 \cdot V$ ($r^2 = 0.828$, $F_{[1,81]} = 394.8$, $p << 0.001$)

The geometric mean of the calculated C:N ratio (mw) varied between 4.8 and 7.1 (Table 3-4) above the nutricline (20, 60, 80, 100 and 110 m) in May 1992, but was even lower at 140 m (3.6), where the concentration of DIN was detectable (0.8 μM). The geometric mean of bacterial C:N and C:P ratio at 10 m in 1995, was low during the spring bloom event in February and March (C:N = 4.5-4.6, C:P = 23.3-24.1) but almost doubled in May through September (Table 3-6).

There was a decrease in the range and geometric mean of cell volume sizes with depth in May 1992 (Table 3-7) and as a function of the season in 1995 (Table 3-8). Bacterial cell volumes varied by two orders of magnitude (0.01-2 μm^3), dominated by cell volumes in the range of 0.03-0.1 μm^3. The geometric mean of all cell volumes measured (n = 165) was 0.050 μm^3 (Figure 3-2).

By using equations 3-1 to 3-3, the amount of elemental C, N and P per cell could be calculated by using an average cell volume of 0.057 μm^3 (Carlson et al. 1995). The elemental content per bacterial cell was 10 fg C, 1.9 fg N and 0.28 fg P.

3.4 DISCUSSION

Accurate bacterial C, N and P conversion factors are crucial in the assessment of bacterial biomass calculations. Since the importance of bacterial biomass in the oceans was first reported by Hobbie et al. (1977), several attempts have been made to accurately assess bacterial elemental conversion factors. By the beginning of the nineteen eighties marine bacteria were routinely counted and sized by the TEM and EFM techniques, but no direct

Table 3-6
Geometric means of the elemental C:N, C:P and N:P ratios (mw) in individual bacterial cells collected at 10 m at BATS in 1995. n = number of bacterial cells measured.

Month	C:N	C:P	N:P	n
February	4.5	50.3	5.0	17
March	5.1	54.9	4.7	16
May	6.8	96.3	6.3	24
July	6.9	87.5	6.0	16
September	7.3	89.6	5.5	15

Table 3-7
Geometric mean of the measured cell volume (μm^3) and the size range of bacteria in the upper 140 m of the water column at BATS in May 1992. n = number of bacterial cells measured.

Depth (m)	GM (μm^3)	min - max	n
20	0.120	0.011 - 2.04	16
60	0.150	0.021 - 1.35	18
100	0.074	0.013 - 0.72	17
110	0.055	0.014 - 0.26	17
140	0.049	0.017 - 0.18	9
GM_{Grand}	0.086	0.011 - 2.04	77

Table 3-8
Geometric mean of the measured cell volume (μm^3) and the size range of bacteria at 10 m depth at BATS in 1995. n = number of bacterial cells measured.

	GM (μm^{-3})	min - max	n
February	0.060	0.010 - 0.43	17
March	0.030	0.008 - 0.18	16
May	0.041	0.044 - 1.83	24
July	0.016	0.002 - 0.14	16
September	0.019	0.006 - 0.22	15
GM_{Grand}	0.029	0.002 - 1.83	88

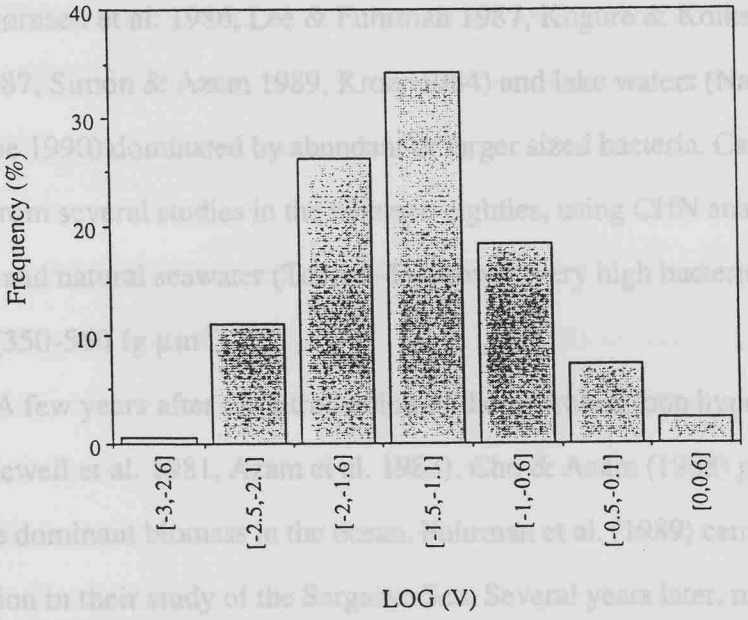

Figure 3-2
Frequency of occurrence (%) of bacterial cell volumes (LOG V) measured on bacteria sampled from a depth profile at BATS in May 1992 and at surface on five BATS cruises in 1995. GM = 0.050 μm^3 (n = 154).

method was available to accurately measure the individual single cell elemental content.

Prior to 1995, all measurements of bacterial C and N were generated from bacterial cultures (Table 3-1). A few studies (Ferguson & Rublee 1976, Watson et al. 1977) applied theoretical conversion factors or factors derived from cultured bacteria, on natural assemblages of bacteria. Others like Nagata (1986), Lee & Fuhrman (1987) and later Nagata & Watanabe (1990) and Kroer (1994), determined bacterial C and N content by filtration and CHN analysis. The main problem with this approach, is that CHN analysis does not discriminate between non-living debris, autotrophs and bacterial cells. Hence, this approach worked best on cell cultures (Bratbak & Dundas 1984, Bratbak 1985, Bjørnsen et al. 1986, Lee & Fuhrman 1987, Kogure & Koike 1987, Scavia & Laird 1987, Simon & Azam 1989, Kroer 1984) and lake waters (Nagata 1986, Nagata & Watanabe 1990) dominated by abundant or larger sized bacteria. Carbon conversion factors from several studies in the nineteen eighties, using CHN analysis of filtered cultures and natural seawater (Table 3-1), showed very high bacterial C conversion factors (350-560 fg μm^{-3}).

A few years after the introduction of the microbial loop hypothesis (Pomeroy 1974, Newell et al. 1981, Azam et al. 1983), Cho & Azam (1988) proposed that bacteria were the dominant biomass in the ocean. Fuhrman et al. (1989) came to the same conclusion in their study of the Sargasso Sea. Several years later, more extensive studies of the entire size spectrum of auto and heterotrophs, using a flow-cytometer (Li et al. 1992) showed that the dominance of bacterial biomass was rather circumstantial and based on arbitrary conversion factors. Hence, there was a crucial need for accurate conversion factor estimates from natural populations of both phytoplankton and bacteria.

Studies of the elemental composition of single bacterial cells was published as early as 1985 (Heldal et al.). However, due to the low sensitivity of the first x-ray detectors, the earliest C-conversion factors derived directly from cultured and natural single cell bacteria have only appeared during the last four years (Gundersen et al. 1994, Norland et al. 1995, Tuomi et al. 1995, Fagerbakke et al. 1996). Interestingly, these more recently reported estimates of bacterial C conversion factors are in the same range as

the earliest estimates (Ferguson & Rublee 1976, Watson et al. 1977) based on volume and purely experimental estimates (Table 3-1).

This study shows a wide range of elemental C, N and P per bacterial cell observed at BATS in May 1992 and in February, March, May, July and September 1995 (Table 3-2 and 3-3), but there was a good correlation between the elemental content and the size of the bacterial cells (Figure 3-1, Equations 3-1 to 3-3). In order to derive a representative conversion factor of C, N and P per bacterial cell, it was necessary to make some general assumptions; In their study of the cell volume by image analysis, Carlson et al. (1995) found that bacteria at BATS were on average 0.057 μm^3 in the upper 200 m of the water column. This estimate is similar to the geometric average of the volume calculated in this study (0.050 μm^3). Since Carlson et al. (1995) analysed more than 500 cells per sampled depth in the upper 200 m of the water column, it was assumed that an average volume of 0.057 μm^3 was a more accurate representation of the bacterial population in the surface waters at BATS. By using an average volume of 0.057 μm^3 and equations 3-1 to 3-3, a grand average of 10 fg C, 1.9 fg N and 0.28 fg P was calculated per bacterial cell.

Several studies have noted that the ratio of elemental C content per bacterial cell volume (C:V) increases as a function of the reciprocal of the cell volume (Simon & Azam 1989, Lee & Fuhrman 1987) indicating that the elemental content per cell does not change with the size of the bacteria. This study showed a good linear correlation between the elemental C, N and P content and the cell volume (Figure 3-1), suggesting that larger cells have a relatively higher elemental content than smaller bacteria. This may also explain the weak power function relationship found in this study, between elemental content per cell volume (C:V, N:V and P:V) and the cell volume (V).

This x-ray microanalysis study was done on air-dried cells and bacteria fixed with glutaraldehyde. Glutaraldehyde preserved, cultured *Escherichia coli* cells have been found to shrink as much as 40 % relative to nonpreserved, airdried ones (Fagerbakke et al. 1996). Cell shrinkage and reduction of the cell volume, may therefore have occurred with the cells fixed in glutaraldehyde in May 1992. However, the average size of the cultured *E. coli* cells were 3.8 μm^3, which is 44 times larger than the geometric mean of the

glutaraldehyde preserved and airdried bacteria analysed in May 1992 (Table 3-7). Simon & Azam (1989) found an almost constant amount of protein in a larger size range of bacteria (0.026 - 0.4 μm^3). They suggested that smaller bacteria are more compact than larger ones, due to a certain minimum of space required for the DNA pool in each cell (Simon & Azam 1989). If this is true, bacteria in this study may have shrunk less than the cultured *E. coli* cells (40 %) and hence, cell shrinkage may have been of minor importance. Yet, we cannot fully rule out that our preserved bacteria may have shrunk during the week-long storage in glutaraldehyde. If cell shrinkage did occur, the actual C:V ratio should be smaller than the one measured in this study. However, the C:V ratio in unpreserved bacterial cells at BATS in May 1995 (GM = 163.5) was not significantly different ($P > 0.05$) from the glutaraldehyde preserved samples from May 1992 (GM = 158.5). Although insignificant, the calculated difference between the two average surface C:V ratios from 1992 and 1995, shows that glutaraldehyde preservation may have caused the bacterial cells to shrink no more than 23 %. Consequently, glutaraldehyde preservation may not have had such a detrimental effect on the bacterial cell volume in cells from the Sargasso Sea, as for the cultured *E. coli* reported by Fagerbakke et al. (1996).

The process of airdrying, may also have caused the cells to shrink. Woldringh et al. (1977) however, noted in their study of bacterial preparations for SEM and TEM, that the cells would flatten during the process of drying and the length and width of the cell (parameters used to calculate V) remained unchanged. The same findings were also reported by Fagerbakke et al. (1996).

Heldal et al. (1985) noted that formaldehyde fixed bacterial cells, lost nearly all potassium and chlorine while other elements remained in the cells. Hence, low molecular weight elements such as C and N may have leaked out of the cells during the week long storage of the seawater samples preserved in glutaraldehyde prior to being prepared for TEM analysis. Organic cellular C and N in bacteria however, are predominantly associated with proteins and nucleic acids (Norland et al. 1995) and hence, only minor

amounts of organic C and N were expected to leak out of the bacteria prior to the airdrying.

Bacteria for the x-ray microanalysis, were sampled immediately after the primary production bloom in May 1992 (Figure 1-10). The mixed layer, at that time, was depleted of DIN (Figure 1-5). The lack of free N in the mixed layer, was also reflected in the C:N ratio of single bacterial cells in May 1992 (Table 3-4). There was a distinct difference in the C:N ratio from the upper 110 m of the water column (average 5.8) and 140 m depth (3.6) (Table 3-4). Marine bacterial C:N ratios from the literature, range from 14.3 (Tezuka 1990) to 3.6 (Kroer 1994). Gräzer-Lempart et al. (1986) found an average bacterial C:N ratio of 3.4 from chemostat cultured *Hyphomicrobium* with excess N. Low N and excess C available in the chemostat medium, produced C:N ratios around 5.3 (Gräzer-Lempart et al. 1986). Zweifel et al. (1993) reported an average C:N ratio of 4.5 in the Baltic Sea, which is an area known to be N-limited (Lignell et al. 1992), suggesting that bacteria at BATS in May 1992, were N-limited above the nutricline (C:N = 5.8). Lower C:N ratios (3.4) have been found at high growth rates in cultures of *E. coli* (Fagerbakke 1995). Therefore, the C:N ratio of 3.6 of bacteria below the nitracline at 140 m depth, associated with the primary production peak and DIN concentrations in the order of 1 μM (Figures 1-10 and 1-5), suggests that the cells were growing in an N-unlimited environment. The C:N and C:P ratio of bacteria at 10 m depth in February and March 1995, also showed that the cells were not limited of neither N nor P during the winter mixing and the sequential bloom event (Table 3-6). From May on however, the nutrient concentration and the primary production activity declined (Figures 1-5, 1-6, 1-10) and the C:N and C:P ratio increased almost twofold (Table 3-6), indicating that the cells were close to both N and P limitation at the surface for the remainder of the year.

Table 3-9 gives an overview of the average elemental C content per cell volume in bacteria analysed by the TEM x-ray microanalysis method. In general, bacterial cells with high C content also had high C:P ratio. The majority of cells in a growth phase with excess nutrients and bacteria from coastal and brackish waters had lower C:N and C:P ratios and hence, were less starved for N and P (Table 3-9). Coastal and brackish waters

Table 3-9
Elemental C content per cell volume (fg μm^{-3}) and elemental C:N and C:P ratios (by weight), in bacteria reported from the literature, analysed by the TEM x-ray microanalysis method.

	fg C μm^{-3}	C:N	C:P	Source
Cell cultures:				
Echerichia coli (growth)	86	3	10	Norland et al. 1995
E. coli (growth)	92	3	11	Fagerbakke et al. 1996
Vibrio natriegens (growth)	100	4	20	Fagerbakke et al. 1996
E. coli (stationary)	165	4	16	Norland et al. 1995
E. coli (stationary)	160	5	16	Fagerbakke et al. 1996
V. natriegens (stationary)	67	4	18	Fagerbakke et al. 1996
Fresh water:				
Kalandsvatn, Norway (Oct'92)	53	5	20	Fagerbakke et al. 1996
Brackish water:				
Tvärminne, Finland (Jul'93)	103	6	23	Fagerbakke et al. 1996
Coastal seawater:				
Raunefjord, Norway (Oct'93)	68	4	18	Tuomi et al. 1995
Knebel Vig, Denmark (Jun'92)	32	4	14	Fagerbakke et al. 1996
Knebel Vig, Denmark (Jul'94)	60	5	25	Fagerbakke et al. 1996
Raunefjord, Norway (Jun'93)	78	4	18	Fagerbakke et al. 1996
Open ocean:				
Sargasso Sea (Feb'95)	110	5	23	This study
-- (Mar'95)	129	5	24	--
-- (May'95)	145	7	45	--
-- (Jul'95)	100	8	52	--
-- (Sep'95)	209	7	43	--

are closer to and more frequently influenced by terrestrial nutrient sources and hence, generally show higher inorganic nutrient concentrations. Open oceanic environments however, are characterised by random seasonal inputs of nutrients into the euphotic zone (Figures 1-5 and 1-6). In comparison with the recent literature, data from this study (Table 3-9) suggest that open ocean bacteria have a higher C:V ratio may be more frequently nutrient limited, relative to bacteria in coastal and brackish waters. Additionally, the C:N and C:P ratios found after the spring bloom period 1995, using the x-ray microanalysis technique (Tables 3-6 and 3-9), are the highest recorded for natural samples reported in the literature.

Chapter 4: Rapidly sinking particles (dregs) and the biomass of primary producers and bacteria

4.1 INTRODUCTION

Several studies in recent years have made an attempt to calculate the relative importance of bacterial and phytoplankton biomass in open oceans (Fuhrman et al. 1989, Caron et al. 1995, Roman et al. 1995). A number of factors crucial in determining the relative distribution of POC are: 1) sampling bias of POC by particles settling below the spigot (dregs); 2) the entire size spectre of bacteria may not be accounted for due to loss of small cells during filtration; 3) the choice of accurate bacterial and phytoplankton conversion factors in order to calculate bacterial C (BC) and phytoplankton C (PC) and 4) the influence by rare particles, such as diatom cells and cyanobacterial colonies (*Trichodesmium*) that are usually not included in ordinary POC samples collected in Niskin bottles. Past open ocean studies have made arbitrary assumptions about one or several of the factors pointed out above. Hence, previous studies of open ocean biomass distribution may not have been as exhaustive as was necessary to accurately estimate the dominant source or sources of biomass.

Gardner (1977) first noted that rapidly sinking particles were not accounted for in seawater samples collected through the spigot of a Niskin bottle. Even though Calvert & McCartney (1979) came to the same conclusion in their study two years later, the problem with rapidly sinking particles in water collection bottles has been poorly addressed. Gardner (1977) concluded that the percentage loss of particles in Niskin bottles was greater in areas with low particle concentrations throughout the water column and hence, the impact on particulate samples in oligotrophic areas such as the Sargasso Sea may have a great potential.

Glassfibre filters such as Whatman GF/F filters, are commonly used to collect suspended particles for CHN analysis because they are chemically inert. Due to their nominal poresize (0.5-0.7 μm), an unfortunate side effect is that they may not collect smaller particles such as bacteria very efficiently (Lee & Fuhrman 1987). Open ocean bacteria attached to particles and other living organisms, may potentially underestimate the total abundance if only free-living cells are enumerated. Bacteria attached to particles have been addressed in several studies (Pomeroy & Diebel 1980, Hoppe 1981, Fukami 1985, Davoll & Silver 1986) and Borstad & Borstad (1977) found that bacteria were the most numerous organisms associated with *Trichodesmium* colonies off the Barbados. Choice of conversion factors is crucial in order to calculate BC from bacterial abundance. During the last two decades, several studies have attempted to accurately measure the C content in bacteria (Table 3-1). Until recently, these studies were frequently hampered by inadequate equipment and the inability to discern bacterial cells from detrital matter and eukaryotes. In Chapter 3 a novel technique is presented that allows an accurate measure of the elemental content of single cell bacteria, suggesting that 10 fg C $cell^{-1}$ is a more appropriate conversion factor than the more commonly used 20 fg C $cell^{-1}$ (Lee & Fuhrman 1987).

For a number of years, Chl-*a* has been used to derive phytoplankton C biomass (PC) (Eppley et al. 1977, Cullen & Eppley 1981, Redalje & Laws 1981, Cullen 1982, Cullen et al. 1982, Redalje 1983, Malone et al. 1993). Early studies by Mullin et al. (1966) showed an apparent lack of linearity in the C:Chl-*a* ratio. Strathmann (1967) reported different species of phytoplankton that exhibited different C:Chl-a ratios and Redalje & Laws (1981) were able to demonstrate a negative correlation between the C:Chl-*a* ratio and the phytoplankton growth rate. Redalje & Laws (1981) were the first to introduce the use of ^{14}C-labelled Chl-*a* and this greatly improved the accuracy of these measurements. Malone et al. (1993) most recently derived the C:Chl-*a* ratio as a power function of the depth at BATS, ranging from 20-100 by weight using the ^{14}C-labelled Chl-*a* technique.

Niskin bottles commonly used to sample suspended particles in the ocean, may discriminate against the relative importance of rare particles such as large diatom cells and cyanobacterial colonies in water column biomass estimates. Mats of diatoms such as *Rhizosolenia sp.* have been reported from the Atlantic Ocean, but are rare as far north as the BATS site in the Sargasso Sea (Carpenter et al. 1977). Other rare single phytoplankton cells reported are the dinoflagellate *Pyrocystis* and the prasinophyte *Halosphaera*, but the single diatom *Ethmodiscus* is more abundant and the largest by two to three orders of magnitude relative to the other cell species (Villareal & Lipschultz 1995). Studies of buoyancy and the internal nitrogen pool, have confirmed a migratory pattern in *Pyrocystis* (Rivkin et al. 1984, Ballek & Swift 1986), *Rhizosolenia* (Villareal et al. 1993) and *Ethmodiscus* (Villareal & Carpenter (1994). Active buoyancy regulation and accumulation at shallow depths in open ocean waters have also been reported for cyanobacterial colonies of *Trichodesmium* (Carpenter & Price 1977, Romans et al. 1994). Due to their significance in abundance and size, *Trichodesmium* and *Ethmodiscus* were enumerated and the C biomass measured in surface waters at BATS in 1995-1996 (*Trichodesmium*) and 1996-1997 (*Ethmodiscus*).

In order to assess the total POC content in seawater samples collected at the BATS site, specific experiments on 7 BATS cruises in 1991 and 1992 were performed to evaluate the amount of particles rapidly sinking below the spigot of a Niskin bottle. The amount of bacterial cells passing a GF/F filter was investigated on several cruises in 1995. The calculated loss of POC on GF/F filters, due to particles sinking below the spigot during sampling and bacteria passing a GF/F filter, was corrected in the calculation of total POC. PC calculated from Chl-*a* using published conversion factors (Malone et al. 1993), and BC calculated from bacterial abundance estimates using a conversion factor of 10 fg C $cell^{-1}$, were compared to total POC. The seasonal impact and importance of *Ethmodiscus* and *Trichodesmium* C biomass in surface waters at BATS, was evaluated by seasonal and depth profile abundance estimates and discrete C content analysis.

4.2 MATERIALS AND METHODS

In order to evaluate the carbon speciation at BATS, 12 litre Niskin seawater samples from below the spigot (dregs) were compared with POC samples collected above the spigot. Settling of POC below the spigot of a Niskin bottle was investigated on seven BATS cruises in the period of November 1991 to July 1992, between surface and 250 m. Bacterial abundance (Chapter 2), Chl-*a* and POC (Chapter 1), was sampled at discrete depths from surface down to 250 m. Following the routine sampling (1 h settling period), the Niskin bottles were left to drain leaving only seawater below the spigot. The remaining seawater below the spigot (0.25-0.5 l) was collected in a polycarbonate bottle connected to a funnel (20 cm diameter) through the bottom of the Niskin bottle. Volume was measured in a 500 ml graduated cylinder and the sample was filtered onto a pre-combusted GF/F filter and processed as described for the POC analysis. Bacterial cells passing a Whatman GF/F filter were studied by estimating bacterial abundance (Chapter 2) in two size fractions; whole seawater and particles that passed a GF/F filter. Bacterial abundance estimates from natural seawater and GF/F filtrate samples, were done in January, February and March 1992 and in September 1993. In order to determine the abundance of bacteria attached to *Trichodesmium* colonies, two sets of the cyanobacterial colonies sampled in September 1993 (as described below), were preserved in 10 ml of seawater and glutaraldehyde (2.5 % final conc.). The colonies were sonicated for 60 s and filtered onto an Irgalan Black stained 0.2 μm Nuclepore filter. The bacteria were enumerated according to Porter & Feig (1980) as described in Chapter 2.

The large diatom *Ethmodiscus* and colonies of *Trichodesmium sp.* were collected on monthly cruises in surface net tows in 1995, 1996 and 1997 using a 1 m Nylon plankton net (mesh size 335 μm). While the ship was drifting, the plankton net was towed at surface for 15-20 min. Current flow through the net was calculated using a flow meter (General Oceanics Inc.) at the entrance of the net. A depth profile (5, 20, 50, 75 and 100 m depth) was sampled on three occasions (May, August and October) in 1995 and in October 1996. Two nets at a time were lowered as fast as they could sink to the

desired depth, left for 15 minutes and then hauled up and immediately retrieved at surface. A retrieved net tow sample was carefully resuspended in an 8 litre white polypropylene bucket and subsamples were taken using a 400 ml polypropylene beaker. A minimum of 10 subsamples were counted for the abundance estimates of *Ethmodiscus* cells and *Trichodesmium* colonies. *Trichodesmium* appears as two morphologically different shaped colonies; the *puff* colonies have radially shaped trichomes and the *tufts* consists of cylindrically shaped trichomes tapered off in each end. No distinction was made between the two morphologies in the abundance estimates in this study. The diatom cells and the *Trichodesmium* colonies were collected using a polypropylene inoculation loop (1 µl) and placed on precombusted punch-hole sized Whatman GF/C filters for the CHN analysis. Every punch-hole GF/C contained 3-4 cells of *Ethmodiscus* and 2 colonies of *Trichodesmium* per filter. *Ethmodiscus* was separated into single and double (dividing) cells and *Trichodesmium* was separated into puffs and tufts for the CHN analysis. The filters were processed and analysed as ordinary CHN samples (Chapter 1).

Total POC content corrected for dregs and the loss of bacterial cells from GF/F filters, was compared to PC and BC collected above the spigot. PC was calculated from Chl-*a* (Chapter 1) using the least square regression model for the C:Chl-*a* ratio, derived from the BATS site by Malone et al. (1993). BC was calculated from the bacterial abundance estimates (Chapter 2) and an elemental biomass of 10 fg C $cell^{-1}$.

4.3 RESULTS

POC settling below the sampling spigot in a Niskin bottle was investigated at BATS on seven cruises from November 1991 to July 1992 (Table 4-1). The percentage of total POC in a Niskin bottle that sank below the spigot varied little with both depth (18-46 %) and season (15-34 %). A total average of 26 % (Table 4-1) was used to correct for

Table 4-1
Percentage of total POC that sinks below the spigot in a 12 litre Niskin bottle. Mean and standard error (SE) was calculated per depth (right hand columns), per cruise (bottom of each column) and from the total number of measurements (lower right corner). Average settling time, including CTD sampling time in the water, was approximately 3 hours.

Depth (m)	Nov'91	Dec'91	Jan'92	Feb'92	Mar'92	Apr'92	Jul'92	**Mean**	**SE**
1	16.8	8.7	15.8	31.1	26.2	25.4	ND	**20.6**	**8.3**
20	11.9	10.9	20.8	24.5	26.6	20.3	29.7	**20.7**	**7.1**
40	21.1	12.2	14.6	29.4	14.6	8.4	22.0	**17.5**	**7.1**
60	24.3	11.4	36.0	45.3	17.0	5.4	25.2	**23.5**	**13.9**
80	14.9	13.5	15.5	39.3	11.3	15.3	17.1	**18.1**	**9.5**
100	22.9	20.1	47.5	47.6	14.6	27.1	29.0	**29.8**	**13.0**
110	14.1	18.6	17.4	36.9	18.8	7.3	21.9	**19.3**	**9.1**
120	34.6	36.3	13.5	26.6	30.6	9.8	26.3	**25.4**	**10.1**
140	33.9	14.3	26.7	16.0	23.8	12.8	36.6	**23.4**	**9.6**
160	34.9	28.7	18.3	42.2	48.6	10.8	ND	**30.6**	**14.3**
200	66.0	47.3	17.0	ND	34.8	11.8	45.2	**37.0**	**20.2**
250	51.4	71.8	23.3	35.3	46.2	21.8	72.7	**46.1**	**20.9**
Mean	**28.9**	**24.5**	**22.2**	**34.0**	**26.1**	**14.7**	**32.6**	**25.9**	
SE	**16.4**	**19.0**	**10.2**	**9.6**	**12.2**	**7.3**	**16.2**		**14.4**

Table 4-2
The C:N ratio of particulate organic matter that sinks below the spigot in a 12 litre Niskin bottle. Mean and standard error (SE) was calculated per depth (right hand columns), per cruise (bottom of each column) and from the total number of measurements (lower right corner). Average settling time, including CTD sampling time in the water, was approximately 3 hours.

Depth (m)	Nov'91	Dec'91	Jan'92	Feb'92	Mar'92	Apr'92	Jul'92	**Mean**	**SE**
1	14.1	10.6	7.4	11.4	13.5	14.7	ND	**11.9**	**2.7**
20	11.0	17.7	6.2	7.7	12.8	10.8	11.7	**11.1**	**3.7**
40	13.3	16.0	7.2	7.0	8.1	7.3	11.6	**10.1**	**3.6**
60	7.7	10.8	6.9	6.7	7.7	8.2	10.5	**8.4**	**1.6**
80	9.0	11.2	7.9	7.5	8.5	8.5	10.7	**9.1**	**1.4**
100	8.7	10.5	8.5	6.2	8.9	11.5	10.1	**9.2**	**1.7**
110	16.2	16.2	5.4	7.0	8.1	8.2	12.6	**10.5**	**4.5**
120	8.3	13.6	8.7	8.0	9.3	6.3	9.8	**9.1**	**2.3**
140	10.2	16.7	5.9	8.9	9.5	6.2	9.4	**9.5**	**3.6**
160	13.3	17.2	7.1	8.9	6.7	6.6	ND	**10.0**	**4.4**
200	9.8	12.8	12.9	ND	9.6	8.2	13.4	**11.1**	**2.2**
250	10.5	12.8	8.8	12.1	9.1	9.0	11.6	**10.5**	**1.6**
Mean	**11.0**	**13.8**	**7.7**	**8.3**	**9.3**	**8.8**	**11.1**	**10.0**	
SE	**2.6**	**2.8**	**2.0**	**1.9**	**2.0**	**2.5**	**1.3**		**2.9**

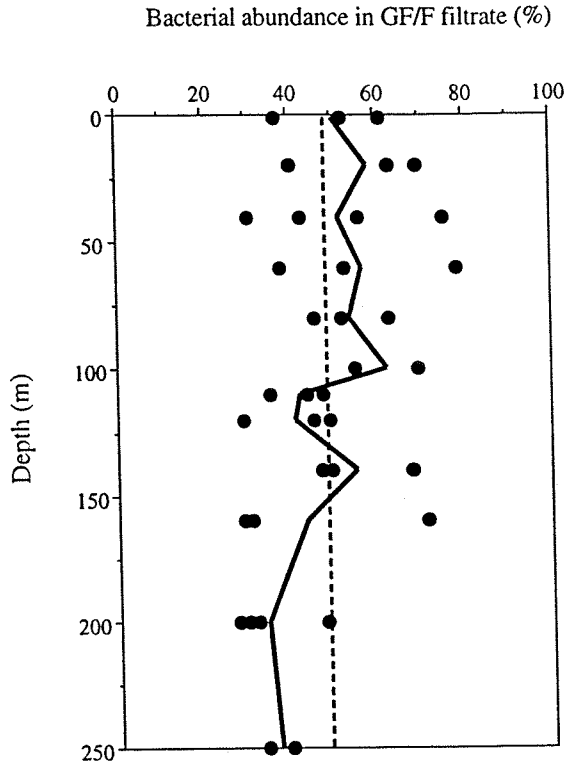

Figure 4-1
Bacterial abundance in GF/F filtrate, calculated as a percentage of unfiltered natural seawater samples, from the upper 250 m of the water column, collected at BATS in January, February and March 1992 and in September 1993.

the calculated total POC in the Niskin bottle samples. The C:N ratio (mw) of dregs collected from Niskin bottles, was slightly higher than Redfield ratio (Table 4-2), but varied seasonally and with the depth. The lowest C:N ratios recorded, were in the mixed layer in January/February 1992 and associated with particle maximum in March/April 1992 (Table 4-2).

An average of half the number of Sargasso Sea bacteria (range 28-79 %) passed a Whatman GF/F filter (Figure 4-1). Number of bacteria attached to *Trichodesmium* colonies, were 10^3-10^4 times less abundant than natural seawater estimates (Table 4-3).

Malone et al. (1993) found a log relationship between the C:Chl-*a* ratio and the depth at BATS. The C:Chl-*a* relationship derived for August was used to calculate the phytoplankton C for the years 1991-1996, even though this equation was systematically higher than the one from March-April 1990 (Malone et al. 1993). Bacterial C was calculated by using a conversion factor of 10 fg C per cell (Chapter 3). The remaining (Other C) was estimated by subtracting bacterial and phytoplankton C from total POC. All particulates were estimated from integrated numbers (0-250 m, 0-65 m, 65-135 m, 135-250 m) calculated from BATS cruises in 1991-1996. The relative distribution (% of total POC) of phytoplankton C showed distinct seasonal peaks in late winter/early spring when phytoplankton exceeded bacterial C (Figure 4-2). Bacterial C integrated over the upper 250 m of the water column, did not show any striking seasonal pattern, but was higher than phytopankton after the spring bloom each year (Figure 4-2 B). The ratio between

Table 4-3
Number of bacteria (10^8 l^{-1}) associated with *Trichodesmium* puffs and tufts at BATS in September 1993. Number of bacteria attached to *Trichodesmium* per volume, was calculated by assuming an average of 50 col m^{-3}.

	Seawater	Puff	Tuft
Bact col^{-1}	--	0.026	0.023
Bact l^{-1}	4.8	0.010	0.009
% of SW	--	0.22	0.19

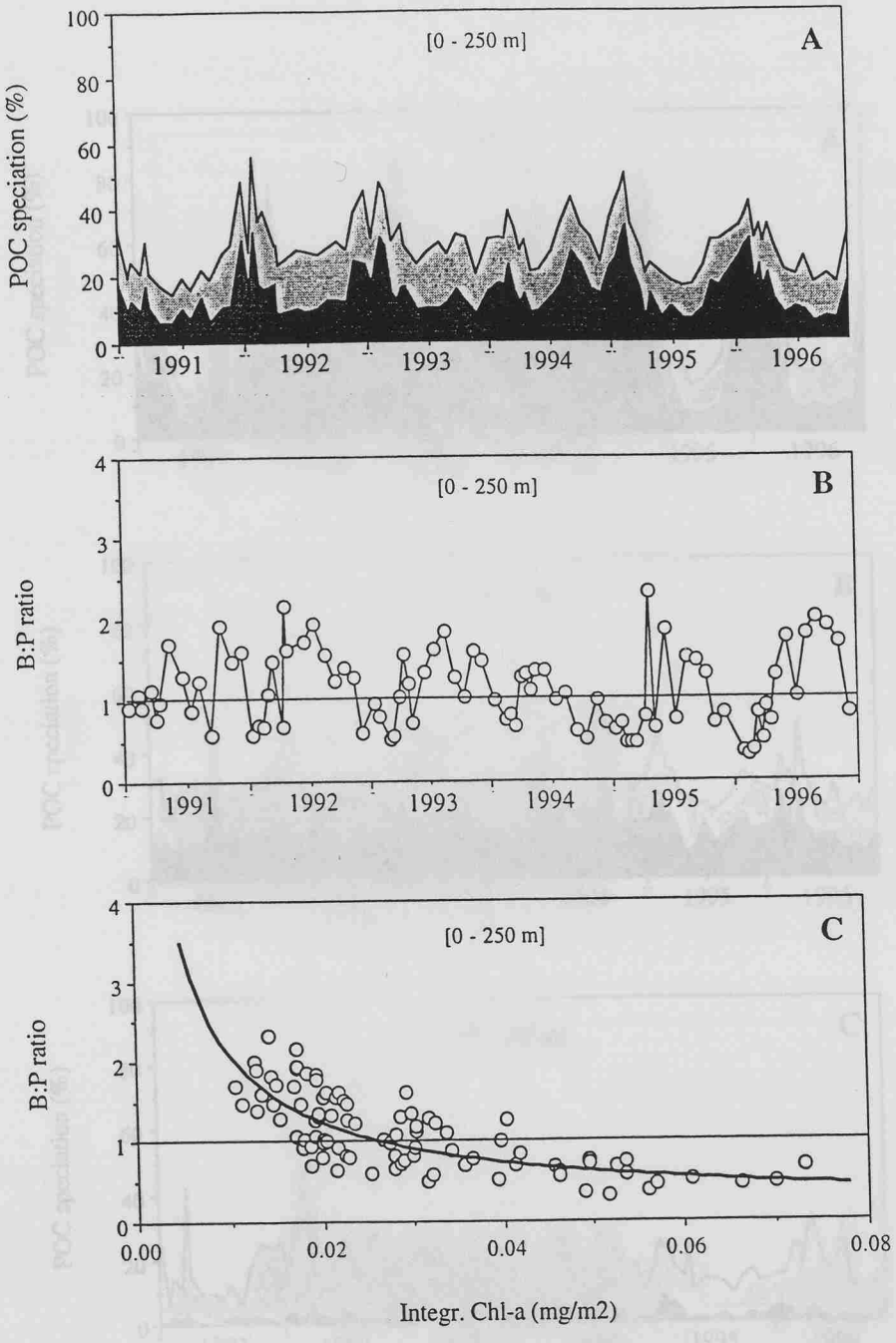

Figure 4-2
A: Relative distribution of POC (%) integrated from the upper 250 m of the water column at BATS in 1991-1996. PC (dark shade) was calculated using the least-squares regression equation of C:Chl-*a* in August, from Malone et al. (1993). BC (grey shade) was calculated using 10 fg C $cell^{-1}$. Total POC was corrected for particles sinking below the spigot of a Niskin bottle. OC (white area) was calculated as total POC minus PC and BC. B: The ratio of the integrated biomass of BC and PC (B:P-ratio), integrated from the upper 250 m of the water column in 1991-1996. Horizontal bar is BC = PC. C: The B:P-ratio was plotted as a function of Chl-*a* (mg m^{-2}), integrated from the upper 250 m of the water column. The log shaped curve was fitted as a negative power function of the integrated Chl-*a* ($y = 0.066 \cdot x^{-0.750}$).

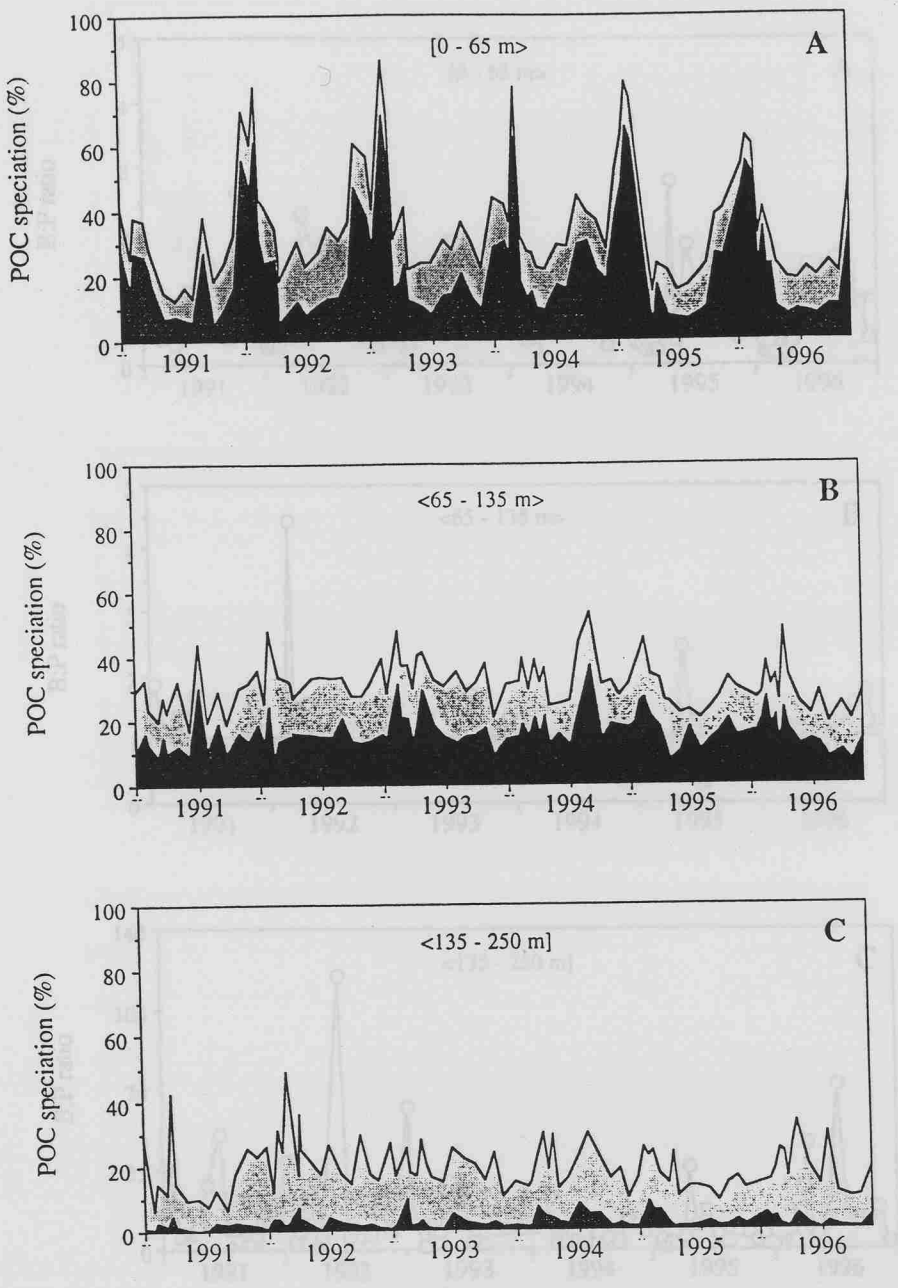

Figure 4-3
Relative distribution of POC (%) integrated from the upper 0-65 m (A), 65-135 m (B) and 135-250 m (C) of the water column at BATS in 1991-1996. PC (dark shade) was calculated using the least-squares regression equation of C:Chl-*a* in August, from Malone et al. (1993). BC (grey shade) was calculated using 10 fg C $cell^{-1}$. Total POC was corrected for particles sinking below the spigot of a Niskin bottle. OC (white area) was calculated as total POC minus PC and BC.

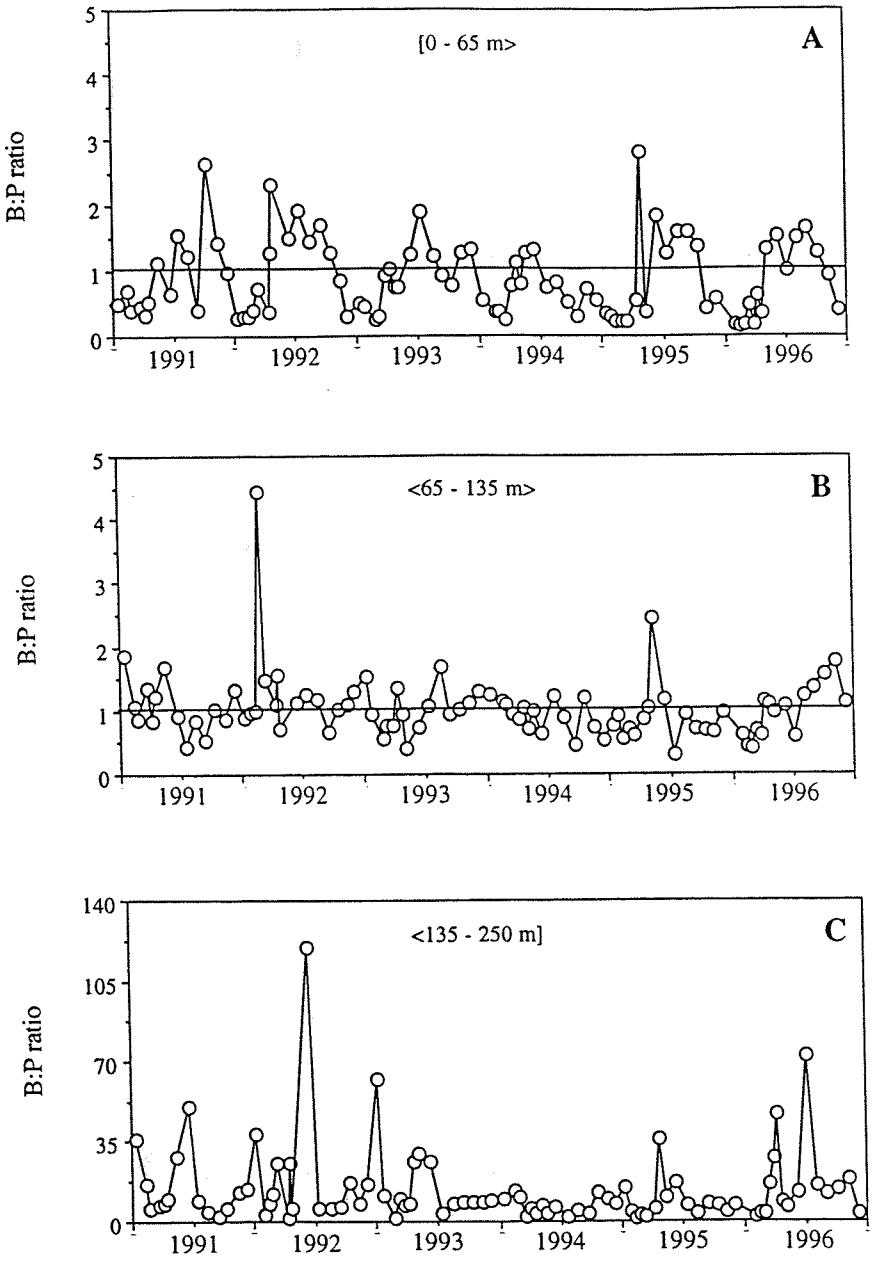

Figure 4-4
The ratio of the integrated biomass of BC and PC (B:P-ratio), integrated from the upper 0-65 m (A), 65-135 m (B) and 135-250 m (C) of the water column at BATS in 1991-1996. Horizontal bar is BC = PC.

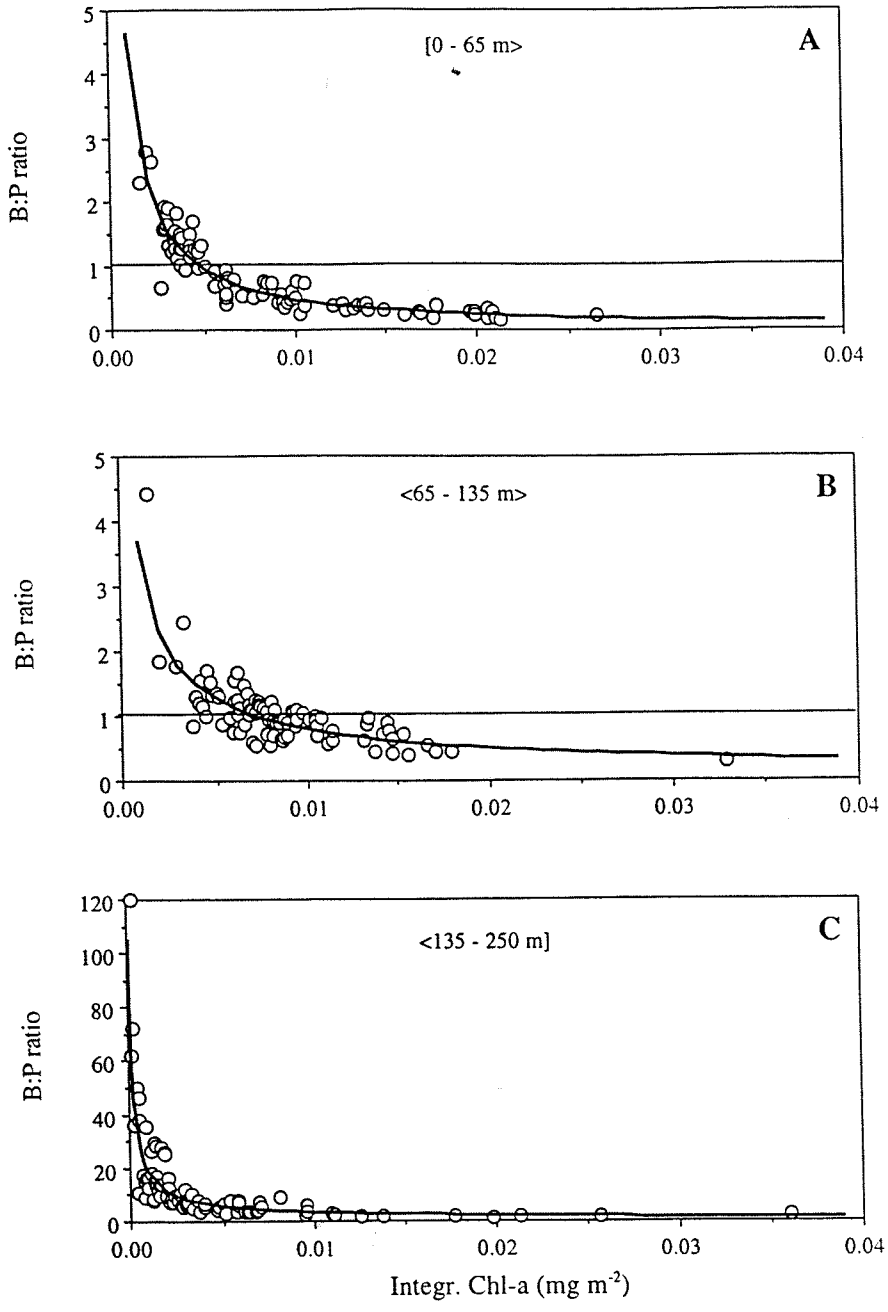

Figure 4-5
The B:P-ratio plotted as a function of Chl-*a* (mg m^{-2}), integrated from the upper 0-65 m (A), 65-135 m (B) and 135-250 m (C) of the water column. The log shaped curve was fitted as a negative power function of the integrated Chl-*a* (0-65 m : $y = 0.005 \cdot x^{-0.995}$, 65-135 m: $y = 0.037 \cdot x^{-0.669}$, 135-250 m: $y = 0.100 \cdot x^{-0.755}$).

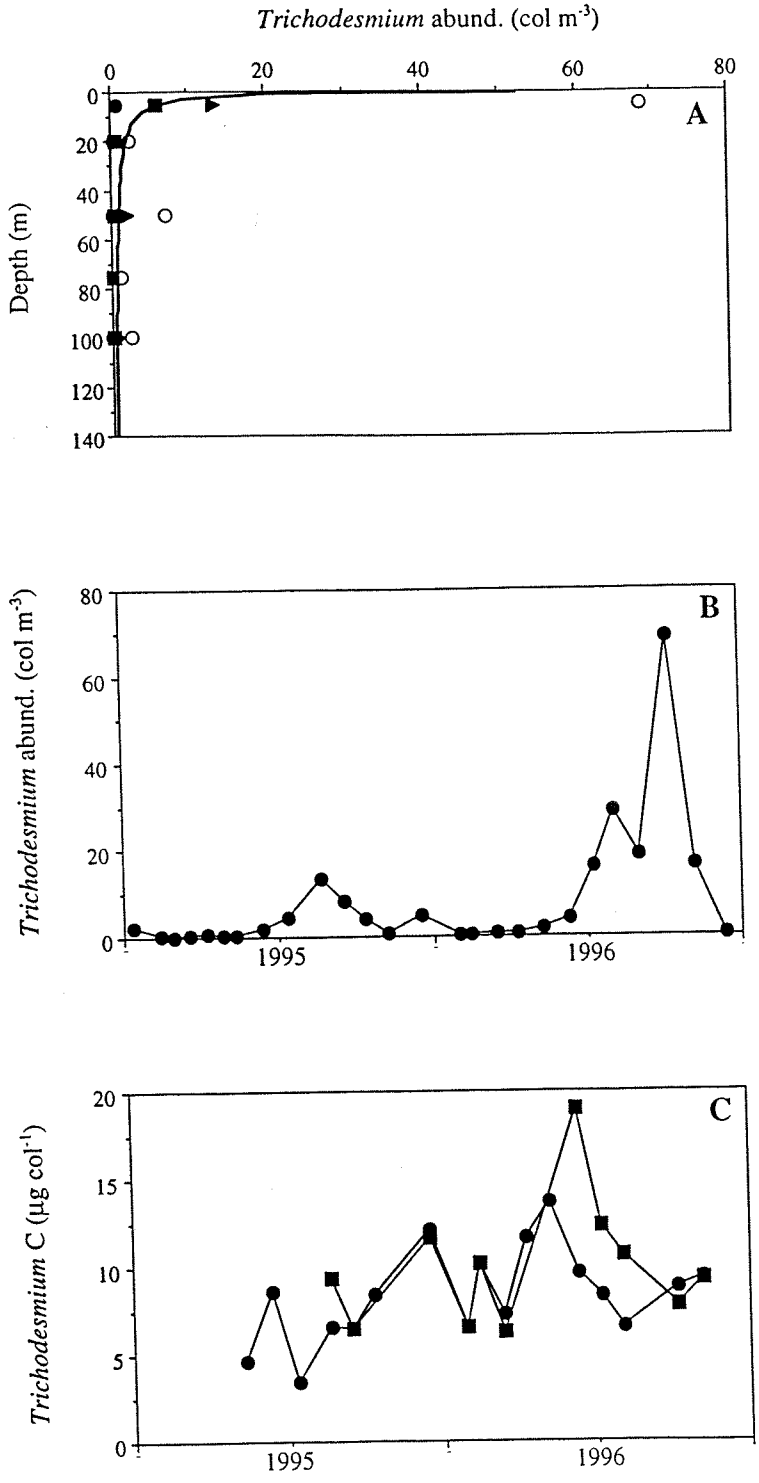

Figure 4-6
A: Composite plot of *Trichodesmium* colonies as a function of the depth at the BATS site in May 1995 (closed circles), August 1995 (closed triangles), October 1995 (closed squares) and October 1996 (open circles). The fitted line is Equation 4-1 (see text). B: Daily average abundance of *Trichodesmium* colonies collected at surface at the BATS site in 1995 and 1996. C: Average *Trichodesmium* C of puffs (closed circles) and tufts (closed squares) collected at surface at the BATS site in 1995 and 1996.

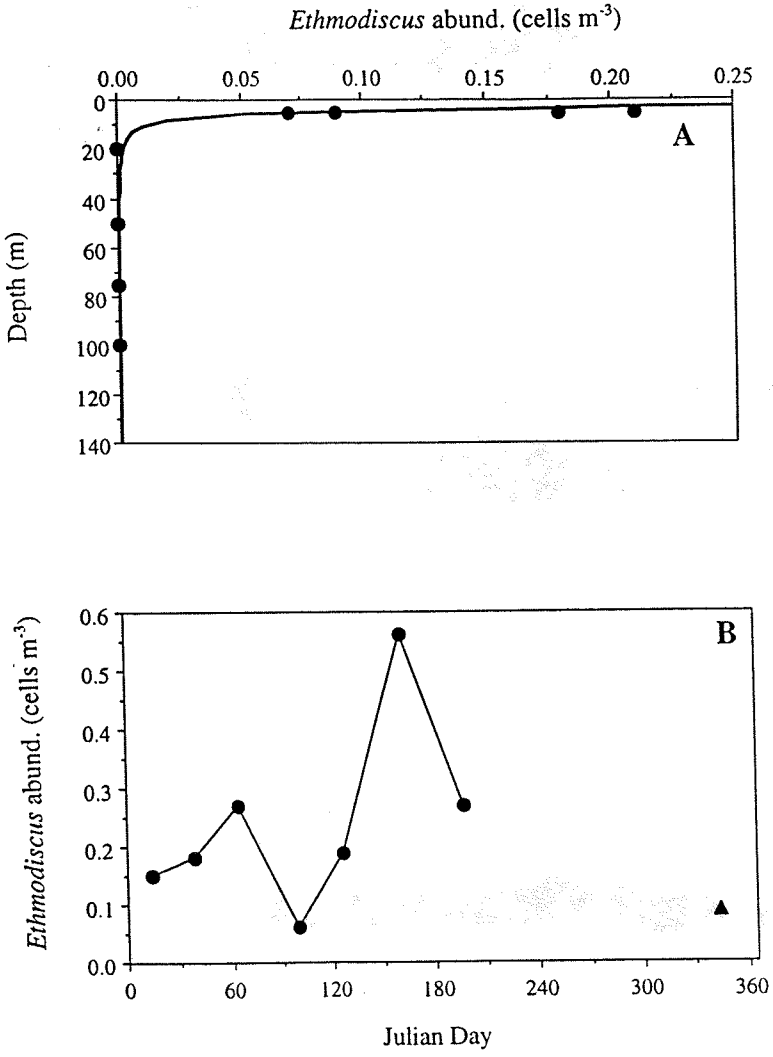

Figure 4-7
A: Distribution of the diatom *Ethmodiscus* as a function of the depth, at BATS in October 1996. The fitted line is Equation 4-2 (see text). B: Composite annual plot of the average distribution of the diatom *Ethmodiscus*, collected at surface at the BATS site in 1996 (closed triangles) and 1997 (closed circles).

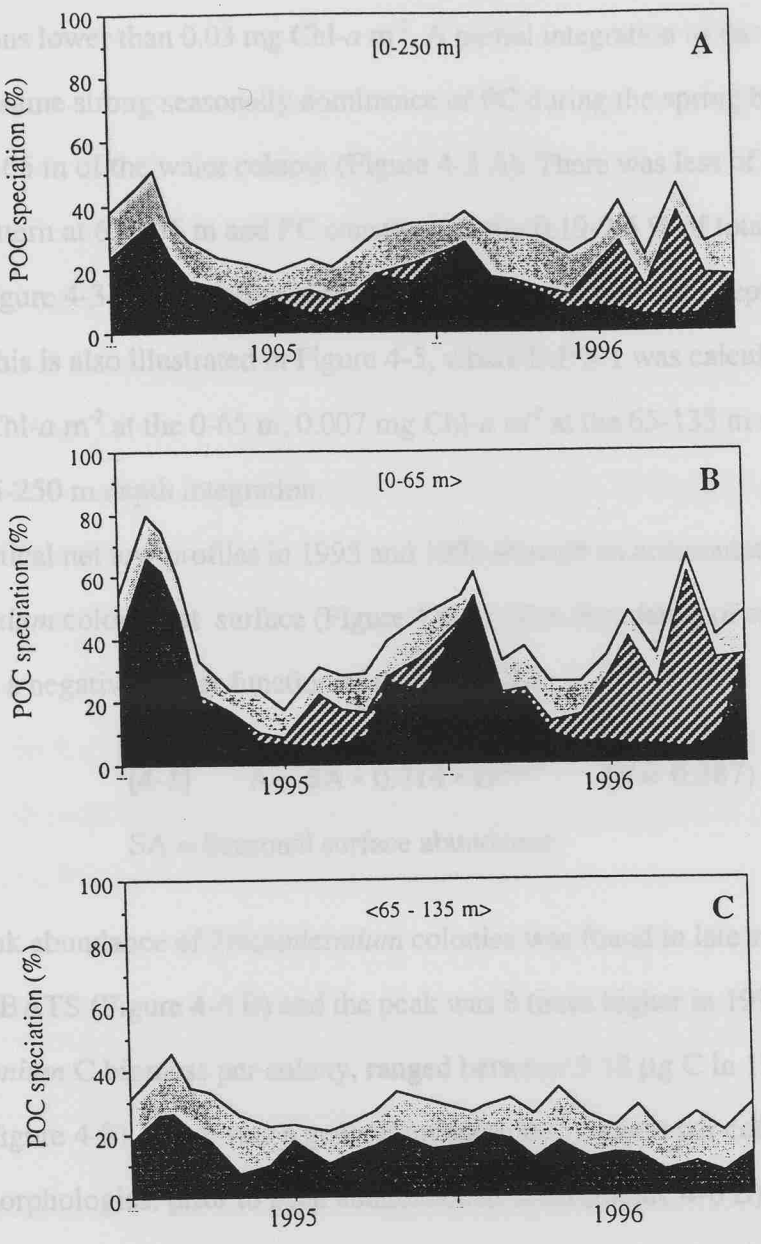

Figure 4-8
Relative distribution of POC (%) integrated from the upper 0-65 m (A), 65-135 m (B) and 135-250 m (C) of the water column at BATS in 1995 and 1996. PC (dark shade) was calculated using the least-squares regression equation of C:Chl-*a* in August, from Malone et al. (1993). BC (grey shade) was calculated using 10 fg C cell^{-1}. *Trichodesmium* C (hatched area) was calculated using Equation 4-1 and 9 μg C col^{-1}. Total POC was corrected for particles sinking below the spigot of a Niskin bottle. OC (white area) was calculated as total POC minus PC, BC and *Trichodesmium* C.

bacterial and phytoplankton biomass (the B:P ratio) could be written as a power function relationship of Chl-*a* (Figure 4-2 C), suggesting a predominance of bacterial C at pigment concentrations lower than 0.03 mg Chl-*a* m^{-2}. A partial integration of the water column showed the same strong seasonally dominance of PC during the spring bloom period in the upper 0-65 m of the water column (Figure 4-3 A). There was less of a marked seasonal pattern at 65-135 m and PC constituted only 0.19-9.6 % of total POC at 135-250 m depth (Figure 4-3). The relative importance of BC increased with depth (Figures 4-3 and 4-4). This is also illustrated in Figure 4-5, where B:P > 1 was calculated to be at 0.005 mg Chl-*a* m^{-2} at the 0-65 m, 0.007 mg Chl-*a* m^{-2} at the 65-135 m and 0.05 mg Chl-*a* m^{-2} at 135-250 m depth integration.

Vertical net tow profiles in 1995 and 1996 showed an accumulation of *Trichodesmium* colonies at surface (Figure 4-6 A). The abundance of colonies (A) declined as a negative power function of the depth (D);

$$[4\text{-}1] \qquad A = SA \bullet 0.714 \bullet D^{-0.890} \qquad (r^2 = 0.387)$$

SA = Seasonal surface abundance

Peak abundance of *Trichodesmium* colonies was found in late summer and autumn at BATS (Figure 4-6 B) and the peak was 3 times higher in 1996 than in 1995. *Trichodesmium* C biomass per colony, ranged between 3-12 µg C in 1995 and 6-18 µg C in 1996 (Figure 4-8). There was a general increase in C content per colony, in both puff and tuft morphologies, prior to peak abundance in 1996 (Figure 4-6 B). Abundance of the single diatom *Ethmodiscus* was only investigated in late 1996 and early 1997. No diatom cells were detected beyond surface depths during mid-day net tows in October 1996 (Figure 4-7 A). The abundance (A) could be written as negative power function of the depth (D);

$$[4\text{-}2] \qquad A = 6.053 \bullet D^{-2.676} \qquad (r^2 = 0.875)$$

There was on average twice as many cells during the spring bloom period of 1997 relative to the previous winter (Figure 4-7 B). *Ethmodiscus* biomass ranged between 2-8 μg C $cell^{-1}$ (Table 4-4).

Equation 4-1 and an average of the measured *Trichodesmium* C (9 μg C col^{-1}), were used to calculate the integrated biomass at 0-250 m, 0-65 m and 65-135 m depths. *Ethmodiscus* C was calculated in the same manner assuming a grand mean biomass content of 3.8 μg C $cell^{-1}$ (Table 4-4) and that the diatom cells accumulate at surface in daytime (Figure 4-7 A and Equation 4-2). *Ethmodiscus* biomass accounted on average for only 0.5 % (range 0.3-0.9 %) at 0-250 m and 1.3 % (range 0.7-2.5 %) at the 0-65 m depth integration and was never found beyond 50 m depth. *Trichodesmium* C constituted only 7 % of total POC at peak abundance in late summer and fall of 1995, but accounted for 33 % of total POC in 1996 at 0-250 m depth (Figure 4-8 A). This trend was even more pronounced at the 0-65 m integration, where *Trichodesmium* C accounted for 17 % (1995) and 56 % (1996) of total POC at peak abundance (Figure 4-8 B). The 65-135 m integration showed that *Trichodesmium* C only accounted for 0.02 % (1995) and 0.1 % (1996) of total POC in this depth range (Figure 4-8 C).

Table 4-4
Average carbon biomass (μg C $cell^{-1}$), C:N ratio [brackets] and the abundance ratio of double (D) versus single (S) diatom cells of *Ethmodiscus*. n = total number of cells used in the CHN analysis. ND = No data or samples available.

Date	Single cell		Double		D:S ratio	n
1996:						
October	ND		5.4	[18]	1.0	3
November	2.5	[25]	5.3	[24]	ND	5
December	2.7	[32]	1.7	[15]	0.2	7
1997:						
January	2.5	[22]	1.6	[11]	0.3	7
February	4.1	[111]	2.8	[33]	0.4	8
March	8.1	[87]	5.8	[37]	1.0	8
April	ND		ND		0.6	ND
May	3.0	[15]	ND		0.1	3
June	4.7	[65]	2.9	[14]	2.3	8
July	2.2	[31]	5.6	[36]	0.2	5

4.4 DISCUSSION

Considering the wide use of Niskin bottles, very few studies have investigated the problem associated with fast settling particles sinking below the spigot before and during sampling. Particle settling in Niskin bottles was already noted in the nineteen seventies; Gardner (1977) found that particles as small as 4 μm in diameter sank below the spigot in Northeast Atlantic seawater samples from surface down to 2600 m. Microscopy showed an overconcentration below the spigot of large diatoms, *Acantharia*, foraminiferans, coccolithophorids, dinoflagellates, faecal pellets, organic aggregates and zooplankton moults and carapaces (Gardner 1977). Two years later, Calvert & McCartney (1979) reported that large sinking particles enriched with Si and Ca, accumulated below the spigot in samples from surface down to 4300 m off the western coast of Portugal. Gardner (1977) concluded that the percentage loss of particles in Niskin bottles was greater in areas with low particle concentrations throughout the water column and could be underestimated with as much as 10-70 % in open ocean waters. An average loss below the spigot of 26 % of total POC in a Niskin bottle was calculated in this study. Since the loss showed little trend with time and depth (Table 4-1), the average of 26 % was applied to correct the measured POC.

Numerous studies have attempted to calculate phytoplankton C by investigating the C:Chl-*a* ratio (Eppley et al. 1977, Cullen & Eppley 1981, Redalje & Laws 1981, Cullen 1982, Cullen et al. 1982, Redalje 1983, Malone et al. 1993). The C:Chl-*a* ratio in these studies ranged from 20 (Malone et al. 1993) to 270 (Redalje & Laws 1981). Although some of the variation may reflect true geographical differences, some of it was probably also due to inaccurate experimental methods. However, a few general trends could be observed; a lack of linearity in the C:Chl-*a* ratio between samples was already observed by Mullin et al. (1966) and Strathmann (1967) found that different phytoplankton species had different the C:Chl-*a* ratios. This was later confirmed in several other studies (Cullen & Eppley 1981, Cullen 1982, Cullen et al. 1982). Eppley et

al. (1977) found low C:Chl-*a* ratios in winter and high ratios in summer off the coast of southern California and suggested that it was due to seasonal changes in the algal speciation and nutrient conditions. Redalje & Laws (1981) introduced the use of ^{14}C-labelled Chl-*a* and measured C:Chl-*a* ratios in the range of 242-272 in coastal waters off Hawaii. They also noted a negative correlation between C:Chl-*a* and phytoplankton growth rate under nutrient limiting conditions (Redalje & Laws 1981). Li et al. (1992) using a Coulter counter and published conversion factors, reported that the C:Chl-*a* ratio changed with depth in the northern Sargasso Sea. Malone et al. (1993) in their study on two cruises at the BATS site in 1990, found that the C:Chl-*a* ratio changed as a power function of the depth. Their least-squares regression equations of the C:Chl-*a* ratio from the upper 150 m of the water column, changed as a function of the depth and varied between 20-100 in August and 20-50 in March-April (Malone et al. 1993). The C:Chl-*a* calculations were based on ^{14}C-labelled Chl-*a* (August) and microscope volume estimates (March-April). The difference between the two cruises reported by Malone et al. (1993), may be due to differences in the surface phytoplankton community, as described in earlier studies (Strathmann 1967, Cullen & Eppley 1981, Cullen 1982, Cullen et al. 1982). However, Redalje (1983) found that microscopic volume estimates of phytoplankton underestimated the C:Chl-*a* ratio by as much as a factor of 2 compared to ^{14}C-labelled Chl-*a* estimates. The negative correlation between the C:Chl-*a* ratio and the growth rate (Redalje 1983) and the reported pigment changes in the plankton community at BATS (Michaels et al. 1994) may all indicate that the C:Chl-*a* ratios calculated in March-April (high primary production) and August (low primary production) were real seasonal changes. Since the high primary production rates indicative of blooms at the BATS site are only found on 1-2 cruises each year (Figure 1-10), the August equation (Malone et al. 1993) was applied to estimate the integrated PC in this study.

A nominal pore size of 0.5 μm (Whatman GF/F) is equivalent to a spherical cell volume of 0.59 μm^3 and the majority of the bacterial cells at the BATS site were smaller than this (Chapter 3, Carlson et al. 1995). Most of the bacteria measured in this study were rod and spiral shapes which are arguably more likely than spherical particles to be

retained in the glassfiber matrix of a GF/F filter. Hence, the finding that an average of 47% of the bacterial cells did pass a GF/F filter is not unreasonable (Figure 4-1). This confirms the Lee et al. (1995) study, who found that 49 % of coastal bacterial cells passed a GF/F filter. Bacterial colonisation of detrital particles, such as materials derived from phytoplankton (Hoppe 1981, Linley & Newell 1984, Fukami et al. 1985), zooplankton (Fukami et al. 1985), faecal pellets (Pomeroy & Diebel 1980, Jacobsen & Azam 1984) and abandoned larvacean houses (Davoll & Silver 1986) and *Trichodesmium* (this study), are other factors that may underestimate total bacterial biomass. However, Alldredge & Youngbluth (1985) found that bacteria attached to marine snow in the Bahamas were 10^4 less abundant than free living bacteria. A similar conclusion was drawn by Simon et al. (1990) off the coast of Southern California where bacteria attached to diatoms, larvacean houses and faecal pellets were 10^3 less abundant than free living bacteria. This study also showed that the number of bacteria attached to *Trichodesmium* colonies were 10^3-10^4 less abundant than the surrounding water (Table 4-3). Alldredge et al. (1986) found slightly higher abundances in marine snow off California, in the NW Atlantic and in Gulf Stream waters, but the number of bacteria were still one to two orders of magnitude less than free living cells. Although bacteria attached to sinking particles may contribute significantly to the C pool in the deeper oceans (Lochte & Turley 1988), this study on buoyant particles and the historical literature suggest that they constitute only a minor part of the bacterial biomass in the euphotic zone of subtropical oligotrophic waters.

Although several studies have compared PC and BC (Caron et al. 1995, Roman et al. 1995), very few have reported the relationship between non-living other C (OC) and living POC (including phytoplankton, *Trichodesmium* and bacteria). This study shows a predominance of non-living C at BATS for the entire period investigated (Figures 4-1 and 4-10). Only the 0-65 m integration showed a dominance of PC and BC in the spring of 1992 to 1996 (Figure 4-2 A). By including *Trichodesmium* C, this conclusion could also be extended to late summer and fall in 1996 (Figure 4-10 B). While PC was higher than BC in the spring (Figure 4-1 B) the rest of the year was dominated by BC when the Chl-*a* concentration decreased below 0.03 mg Chl-*a* m^{-2} (0-250 m), 0.005 mg Chl-*a* m^{-2} (0-65

m), 0.007 mg Chl-*a* m^{-2} (65-135 m) and 0.05 mg Chl-*a* m^{-2}(135-250 m). The latter depth range was always dominated by BC since integrated Chl-*a* at this depth never exceeded 0.04 mg Chl-*a* m^{-2}. A similar seasonal trend was also observed by the U.S.JGOFS cruises at the BATS site in March-April and August 1990 (Caron et al. 1995, Roman et al. 1995). A six year average of the percentage distribution of PC:BC:OC in this study was 15:14:71 (0-250 m), 23:13:64 (0-65 m), 16:15:69 (65-135 m) and 3:17:80 (135-250 m). Roman et al. (1995) did not correct their POC numbers for sinking particles, but reported that bacteria only comprised 56% of the microheterotrophic carbon in a POC sample. Since the remaining 44% was not included in this study the micro-heterotrophic biomass (HC) was most likely underestimated by only accounting for bacteria. By correcting BC to include the missing 44 % of other microheterotrophic C, the PC:HC:OC relationship was 15:21:64 (0-250 m), 23:18:59 (0-65 m), 16:22:62 (65-135 m) and 2:24:74 (135-250 m). By including *Trichodesmium* C in the PC pool, the PC:HC:OC ratios were 20:18:62 (0-250 m), 32:15:54 (0-65 m) and 16:19:65 (65-135 m), while the 135-250 m depth integration remained unchanged due to the lack of *Trichodesmium* beyond 100 m depth (Figure 4-6 B). The seasonal importance of PC versus BC (Figures 4-2, 4-3 and 4-4) demonstrated for the last six years at BATS in this study was also shown by Ducklow et al. (1993) for a much more limited period of time at 47 °N 20 °W, at the North Atlantic Bloom Experiment in April-May 1989.

Table 4-5
Integrated six year average of the percentage distribution of POC at BATS. PC = phytoplankton C corrected to also include colonial *Trichodesmium* C, HC = heterotrophic C corrected to also include microheterotrophs other than bacteria, OC = other C and LC = living C (PC + HC).

Depth integr.	PC : HC : OC	LC : OC
0-250 m	20 : 18 : 62	38 : 62
0-63 m	31 : 15 : 54	47 : 54
65-135 m	16 : 19 : 65	35 : 65
135-250 m	2 : 14 : 74	16 : 74

A six year average of the biomass estimates in this study, corrected for the presence of *Trichodesmium* in PC and nanoflagellates and microzooplankton (44%) in the heterotrophic carbon pool (Table 4-5), shows a clear dominance of PC in the living POC at the 0-65 m depth range that also contained the maximum primary production rates (Figure 1-10). At the depth range of particle maximum (65-135 m) (Figures 1-11 and 1-12), PC:HC was approximately equal. Beyond particle maximum (135-250 m depth), HC dominated the living POC pool and was 7 times higher than PC (Table 4-5). The ratio between living POC (LC) and OC was almost equal (47:54) at production maximum, but OC was 4.5 times higher below the particle maximum deeper than 135 m (Table 4-5). This study shows that more than half the carbon measured as POC at BATS cannot be accounted for as living C. Non-living C was the single most dominating component of POC and only the euphotic zone (0-65 m) had equal amounts of living versus non-living C (Table 4-5). The non-living C fraction may be dominated by non-pigmented detrital matter such as faecal pellets, organic aggregates, zooplankton moults and carapaces, very similar to the rapidly sinking particles collected in the bottom of Niskin bottles.

Chapter 5: Growth and turnover-time in bacteria and the primary producers

5.1 INTRODUCTION

When Sorokin (1971) first proposed the importance of bacterioplankton in open oceans, estimates of bacterial growth and biomass in biogeochemical cycling were severely underestimated. New methodology for biomass estimates, developed in the late seventies (Hobbie et al. 1977) confirmed Sorokin's hypothesis. During the last two decades, bacterial incorporation assays using methyl-[^{3}H] thymidine [TdR] (Fuhrman & Azam 1980, 1982) and L-[4, 5-^{3}H] leucine [Leu] (Kirchman et al. 1985, 1986), have become the two most commonly used radiotracer techniques for measuring bacterial growth in seawater. Although, Leu has been used in specific studies of bacterial protein synthesis (Kirchman et al. 1985, Simon & Azam 1989), both TdR and Leu is more commonly applied as a radiotracer in studies of bacterial growth and C synthesis. The latter approach involves a two step calibrated conversion from the incorporation rate of TdR or Leu, to a cell multiplication factor and a final calculation using a specific C per cell conversion. Carlson et al. (1995) found that Leu underestimated their bacterial growth estimates at BATS 5 out of 8 times relative to the TdR assay and suggested that unbalanced growth (Kirchman et al. 1985, Chin-Leo & Kirchman 1990) caused the remaining discrepancies. In their review on bacterial growth, Ducklow & Carlson (1992) found that more than half of all publications on bacterial growth had applied the TdR incorporation technique. Although popular, the method has remained controversial. Factors such as bacterial DNA content, TdR isotope dilution, repeatability and non-specific incorporation of TdR should all be taken into consideration when applying the

TdR technique (Fuhrman & Azam 1982, Moriarty 1985, Robarts et al. 1986, Hollibaugh 1988, Chrzanowski 1988, Jeffrey & Paul 1988). This can all be avoided, Kirchman et al. (1982) argued, by using empirically derived conversion factors for TdR incorporation and subsequent cell or carbon based growth rates. Bell (1990) was able to reduce the variability in the conversion factor (cell mol^{-1} TdR) by increasing the final concentration (> 5 nM) of TdR. A number of studies have investigated TdR conversion factors (Ducklow & Hill 1985, Riemann et al. 1987, Rivkin et al. 1989). Ducklow & Carlson (1992) reported a wide range of TdR conversion factors from the literature (0.2-60 • 10^{18} cells mol^{-1}) with a median value of 2 • 10^{18} cells mol^{-1}.

The issue of active versus inactive bacterial cells, has been raised intermittently through the last three decades (Jannasch 1969, Stevenson 1978, Moyer & Morita 1989). More recently, Zweifel & Hagstrøm (1994) questioned again whether all bacteria enumerated by epifluorescence (DAPI or acridine orange) could be living cells. They concluded the following year that only 2-30 % of the total number of bacteria contained nucleoids (Zweifel & Hagstrøm 1995). Their finding was widely discussed due to the propanol extraction procedure used in order to distinguish the nucleoid containing cells. Gazol et al. (1995) found a correlation between bacterial cell size and growth activity by using the redox dye 5-cyano-2, 3-ditolyl tetrazolium chloride (CTC) and they concluded that smaller cells (< 0.06 μm^3) were dormant and that larger bacteria were more likely to be active (Gazol et al. 1995). Recently, CTC was shown to be toxic to bacteria (Ullrich et al. 1996) and the growth activity pattern observed by Gazol et al. (1995) may have been due to the fact that larger bacteria survived the CTC treatment more effectively than smaller cells. In their evaluation of active marine bacterioplankton, Karner & Fuhrman (1997) recently showed that an average of 60 % (range 22-78 %) of the cells enumerated by DAPI staining were also detected by the 16S rRNA universal-probe hybridisation technique. Karner & Fuhrman (1997) also applied the propanol extraction procedure used by Zweifel & Hagstrøm (1995) and found that an average of 30 % (range 4-52 %) of the bacteria enumerated by DAPI, were nucleoid-containing cells. Choi et al. (1996) were

also able to show that the proportion of active bacterioplankton cells increased as a function of the growth rate.

An average TdR conversion factor of $1.6 \bullet 10^{18}$ cells mol^{-1} (Carlson et al. 1995) and a conversion factor of 10 fg C $cell^{-1}$ (Chapter 3), was used in order to assess the bacterial growth rate at the BATS site. Samples from surface down to 140 m depth were assayed by the TdR incorporation technique on monthly cruises in 1991-1996. Primary production is the main source of DOC for bacterial growth and two significant groups of primary producers (single cell phytoplankton and *Trichodesmium*) were identified in surface waters at BATS (Chapter 4). Bacterial and primary production turnover times were calculated using the biomass and growth rate integrations from the upper 140 m of the water column.

5.2 MATERIALS AND METHODS

Bacterial growth was measured as the incorporation rate of ^3H labelled TdR in seawater from the early morning Kevlar cast at the BATS site (Chapter 1), using the method of Fuhrman and Azam (1980, 1982). Samples for the TdR assay were collected from GoFlo bottles from 8 discrete depths between surface and 140 m and powder free polyethylene gloves were worn during all stages of the assay. TdR was added to the samples to a final concentration of 10 nM [specific activity varied from 80-90 Ci $mmol^{-1}$ TdR] (Carlson 1994). At the end of the incubation, the bacterial samples were collected on 0.2 μm cellulose nitrate filters and extraceted in ice cold 5 % TCA followed by an 80 % ethanol. Samples from all depths in a BATS core TdR assay, are routinely incubated in the dark on the ship at the current seawater temperature at 100 m depth. Potentially, this may influence the bacterial incorporation rate of TdR at depths with seawater temperatures that differ from the 100 m depth temperature. In July 1997, a TdR method comparison was made between two sets of triplicate samples of seawater (from 0-140 m depth)

incubated at a fixed temperature (BATS core) and the TdR assay run at simulated depth specific *in-situ* temperatures in an incubator.

No single factor exists for the conversion from pmole TdR taken up by the bacteria as a function of time, to numbers of bacterial cells and amount of carbon biomass produced. However, the TdR technique has been extensively studied at the BATS site by Carlson (1994). Bacterial growth in this study is initially reported as pmol TdR $l^{-1}h^{-1}$. The bacterial cell growth, when applicable, was calculated from the incorporation of TdR using a conversion factor of $1.6 \bullet 10^{18}$ cells per mol TdR (Carlson et al. 1995) and a factor of 10 fg C $cell^{-1}$ for the calculation of bacterial biomass (Chapter 3).

Integrated phytoplankton growth rate and biomass (Chapter 1) and bacterial growth rate and biomass (Chapter 4 and this chapter), was used to calculate the generation time (GT). The average specific growth rate (μ) for each cruise in the time-series, was calculated from trapezoid integrations of growth (G) and biomass (B) as a function of the depth;

$$\mu = G / B$$

$$GT \text{ (days)} = \ln 2 / \mu$$

The primary production rate of surface *Trichodesmium* colonies was measured in 1995 and 1996. Colonies were picked from subsamples of a net tow (Chapter 4) and placed in surface seawater containing a ^{14}C-bicarbonate solution as for the phytoplankton primary production assay (Chapter 1). The *Trichodesmium* primary production assay was run for the entire day (10-12 h) in a deckboard incubator, with a flow-through surface seawater system and covered with tinted glass mimicking the light conditions at 10 m depth. If it was not possible to run the assay from dawn to dusk, the incorporation rate was extrapolated to a full day, assuming a 10-12 h daylight period and a linear uptake of ^{14}C-bicarbonate throughout the day. At the end of the incubation, each colony was placed in 250 µl of 5 % HCl in a scintillation vial. The vials were left open overnight, in order to evaporate the inorganic ^{14}C. The colonies were dissolved in 750 µl of Solouene-350 (Packard) tissue homogeniser and left overnight. Finally, 9 ml of Hionic-Fluor (Packard)

was added and the samples were vortex mixed and counted on a Packard scintillation counter (external channel ratio method) the following day. A study of the primary production versus irradiance (PI) curve was done in October 1996 and in July 1997 for both puff and tuft morphologies of *Trichodesmium* using incubation bottles covered with screens reducing surface incident irradiance to 15, 26, 47 and 81 % of the surface light level in the incubator.

5.3 RESULTS

Bacterial incorporation of TdR was measured in the upper 140 m of the water column at BATS between 1991-1996 (Figure 5-1). The TdR incorporation rate showed seasonal maxima similar to the primary production rate (Figure 1-10). A summer maximum, equivalent or larger in size to the spring bloom, was evident in all six years (Figure 5-1). Figure 5-2 shows a comparison of the TdR incorporation rate at a fixed temperature (21.5 °C) and the TdR incorporation rate at simulated depth specific *in-situ* temperatures. The horizontal error bars in Figure 5-2 B, shows the variance in the simulated temperature during the 3 h incubation period. There was no significant difference between the rates measured at any of the depths investigated (Table 5-1).

The bacterial growth rate integrated from the upper 140 m of the water column, was calculated from the TdR incorporation rate using a conversion factor of $1.6 \cdot 10^{18}$ cells mol^{-1} and 10 fg C per bacterial cell. Distinct peaks in the integrated (0-140 m) bacterial growth were evident in the spring and summer/fall of each of the six years (Figure 5-3 A), following the spring primary production maximum each year (Figure 5-7 A). The annual average of the integrated bacterial growth rate was higher by a factor of nearly two in 1993-1996 (0.038-0.067 g C $m^{-2}d^{-1}$) relative to 1991-1992 (Table 5-2), while the integrated bacterial biomass remained unchanged (0.64-0.78 g C m^{-2}). Consequently, the generation time decreased by a factor of two in 1993-1996 relative to the previous two years (Figure 5-3 C and Table 5-2). The annual fluctuation of the

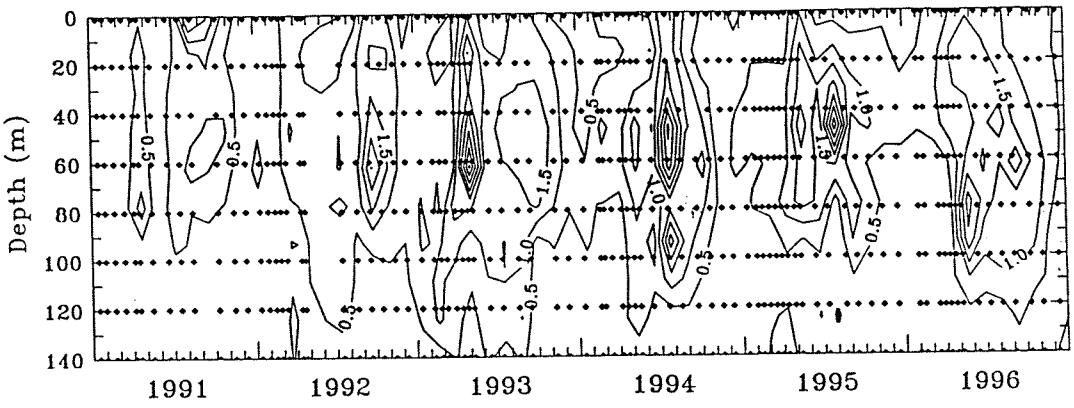

Figure 5-1
Contour plot of the bacterial growth rate measured as the incorporation of TdR (pmol $l^{-1}h^{-1}$) at BATS in 1991-1996. Contour interval is 0.5 pmol $l^{-1}h^{-1}$. Black diamonds shows sampling depths and sampling frequency.

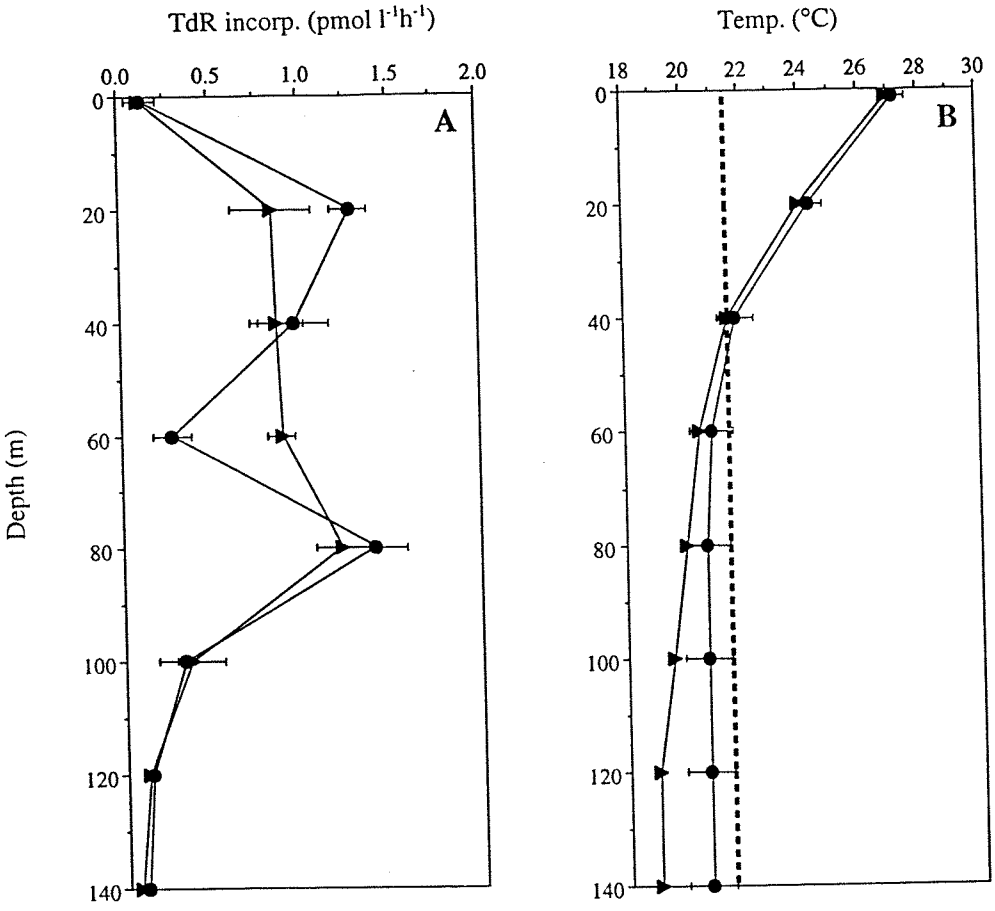

Figure 5-2
A: Depth profile of the TdR incorporation rate in natural seawater samples collected at BATS in July 1997. The seawater samples were incubated at a fixed temperature (closed triangles) and at simulated *in-situ* temperatures (closed circles). Horizontal bars are the variances between 3 replicates at each depth.
B: Temperature profile at BATS in July 1997 (closed triangles) and the temperature profile simulated on deck (closed circles). Vertical broken line is the fixed temperature used in the comparison.

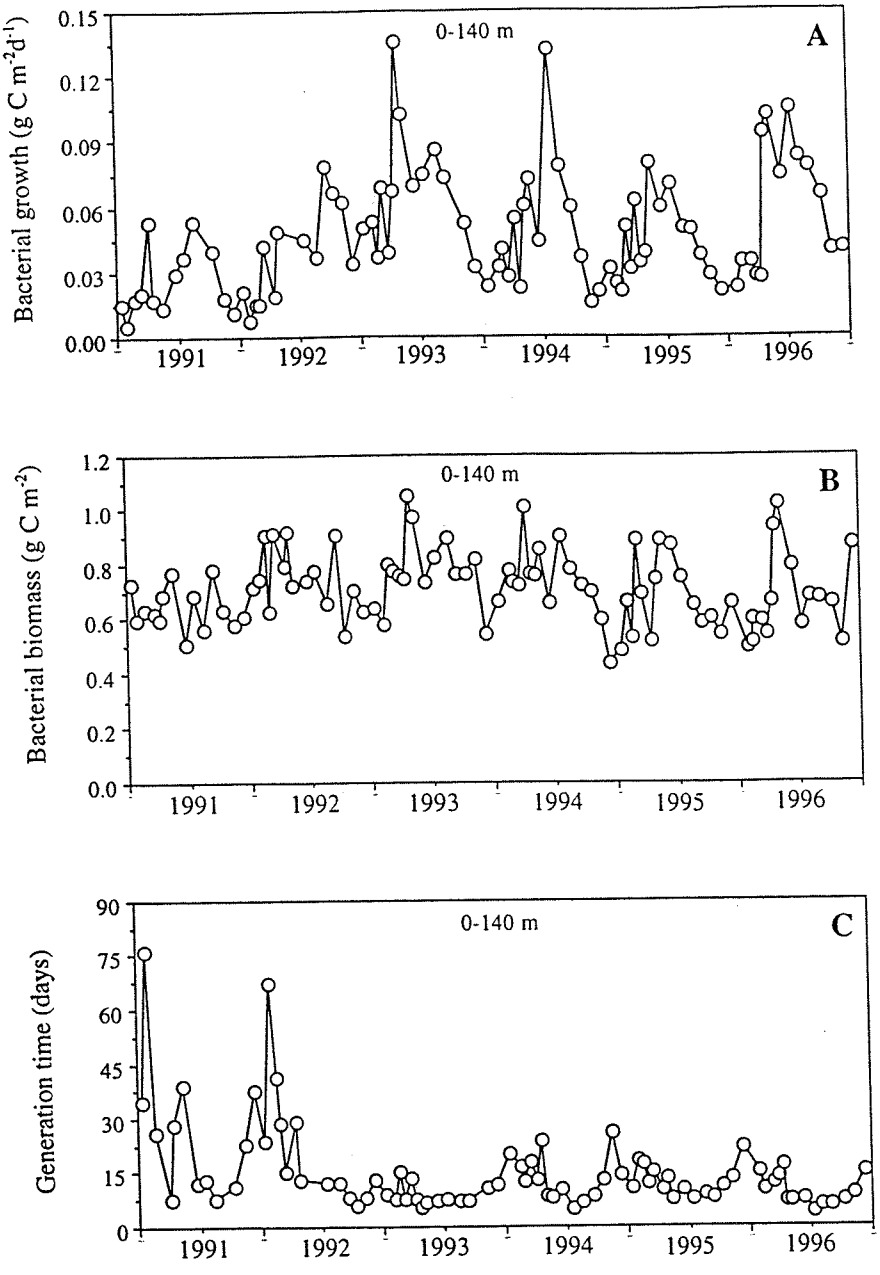

Figure 5-3
Calculated bacterial growth rate (A), bacterial C biomass (B) and the generation time (C), integrated from the upper 0-140 m of the water column.

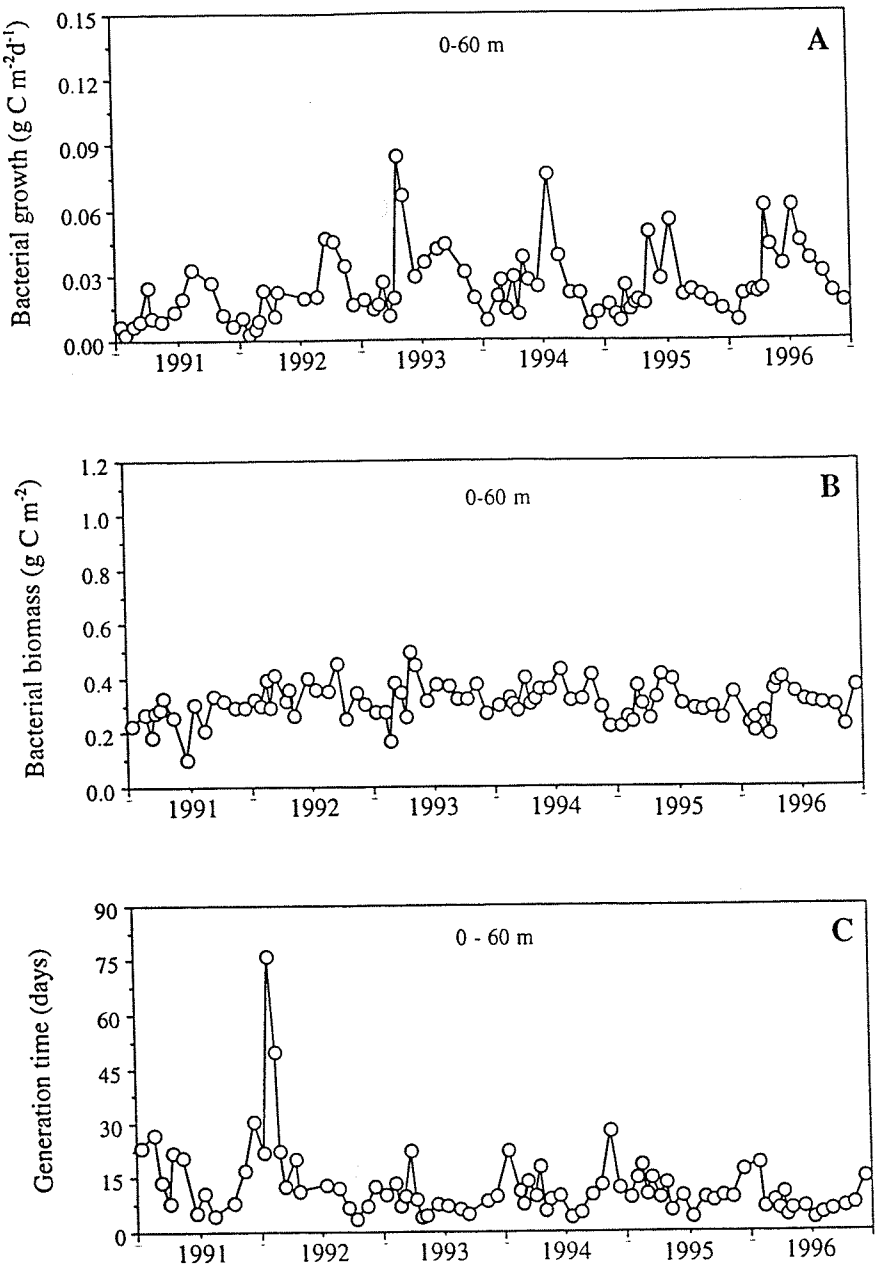

Figure 5-4
Calculated bacterial growth rate (A), bacterial C biomass (B) and the generation time (C), integrated from the upper 0-60 m of the water column.

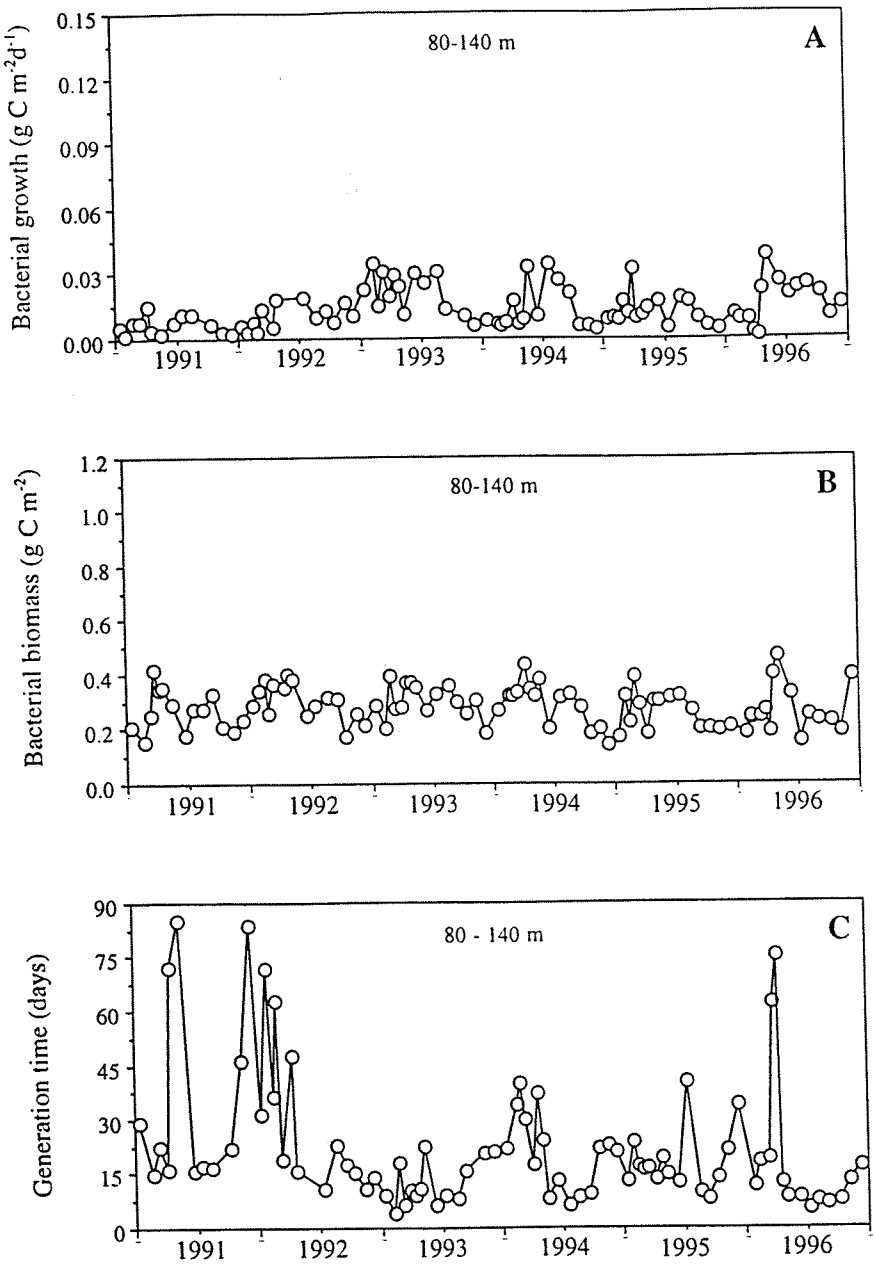

Figure 5-5
Calculated bacterial growth rate (A), bacterial C biomass (B) and the generation time (C), integrated from the upper 80-140 m of the water column.

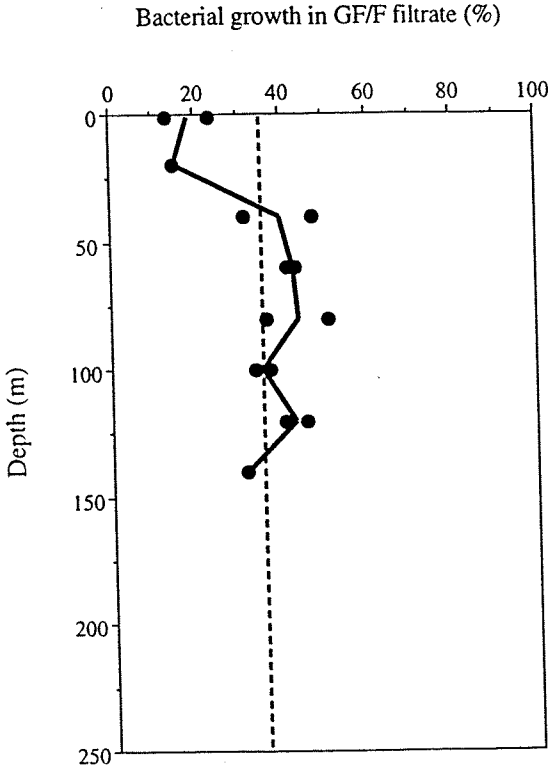

Figure 5-6
Bacterial TdR incorporation in GF/F filtered seawater from 0-140 m depth, in August and September 1993. The GF/F size separation was done at the termination of the assay and the TdR incorporation rate was calculated as the percentage of the rate measured in natural seawater. Vertical broken line is the grand average of 35 %, calculated from all depths.

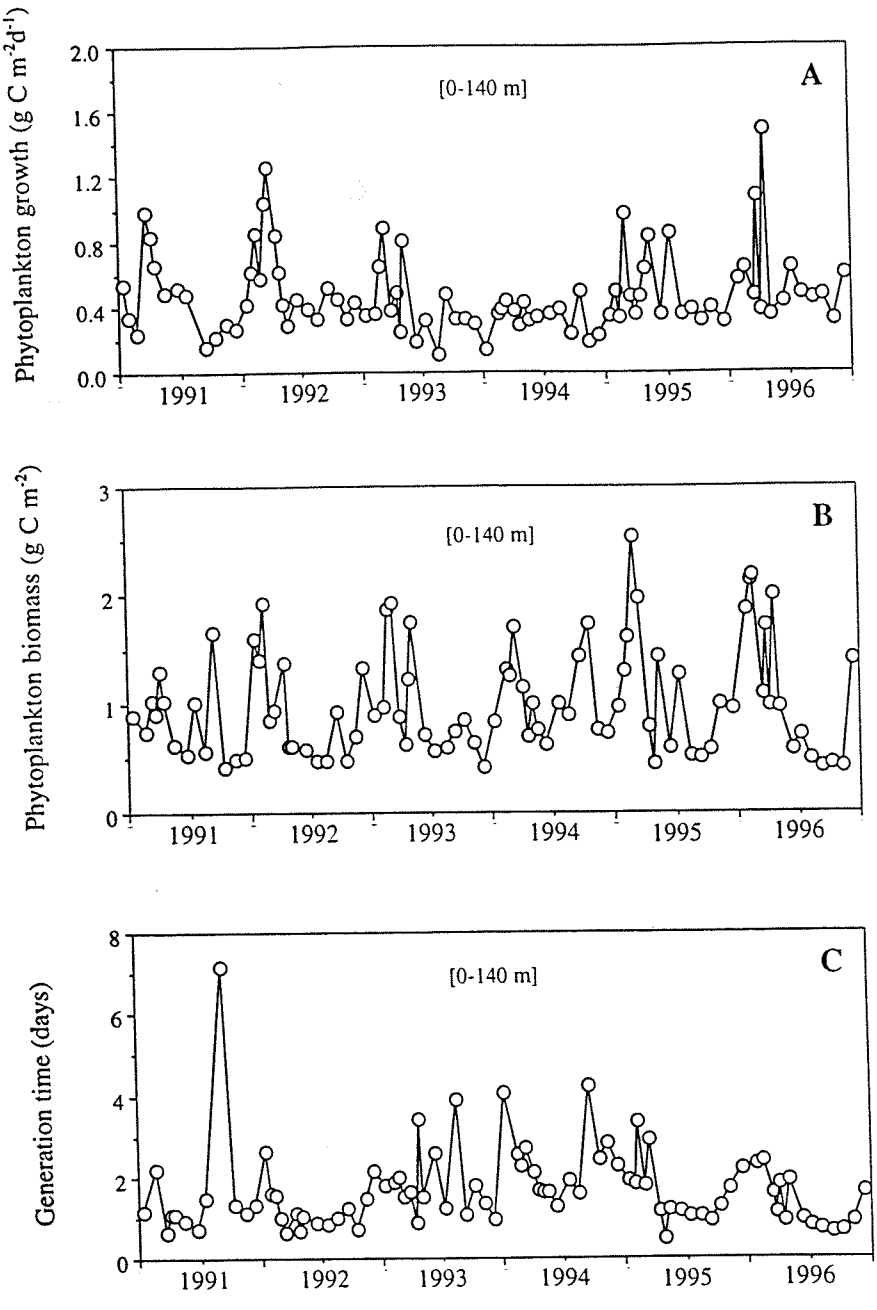

Figure 5-7
Calculated phytoplankton primary production (A), phytoplankton C biomass (B) and the generation time (C), integrated from the upper 0-140 m of the water column.

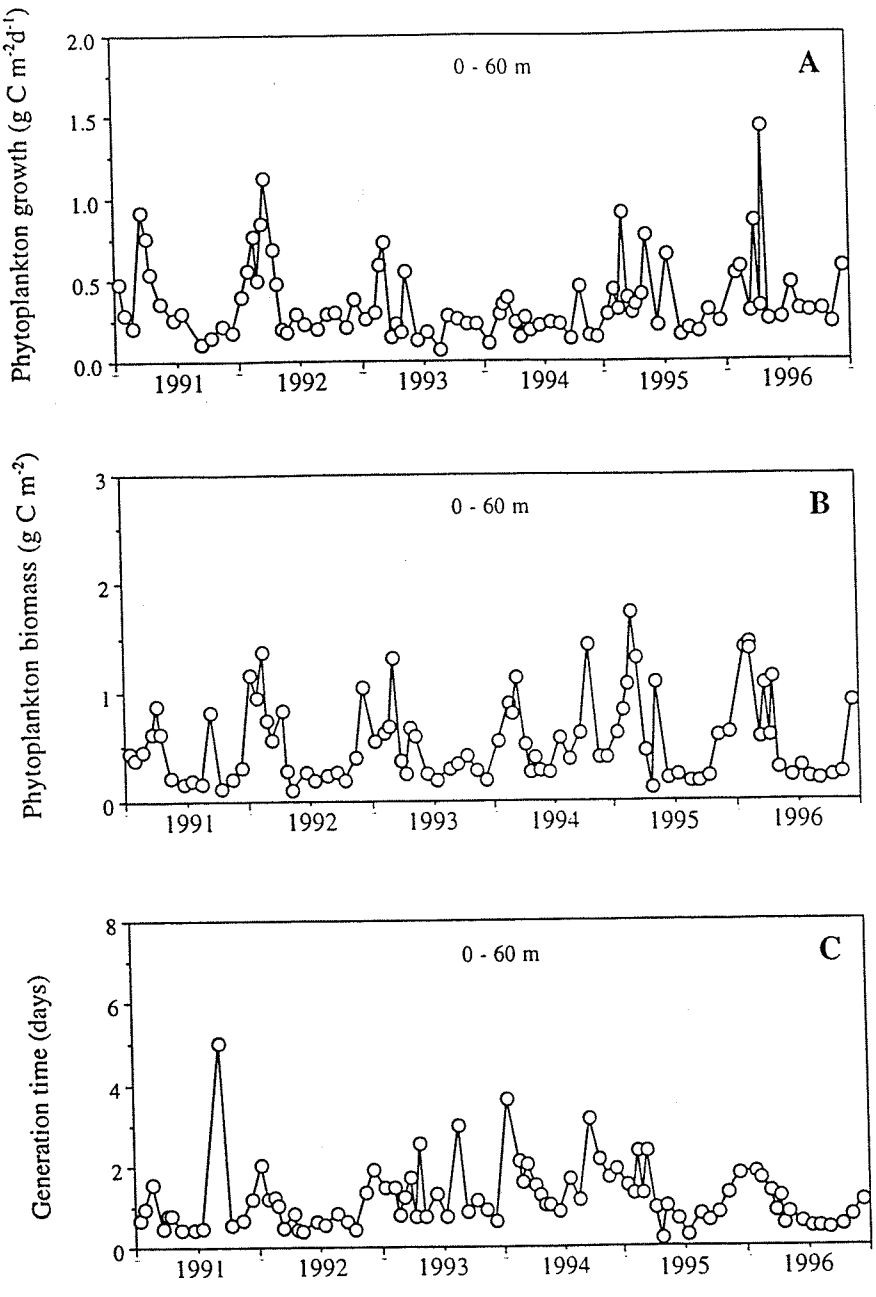

Figure 5-8
Calculated phytoplankton primary production (A), phytoplankton C biomass (B) and the generation time (C), integrated from the upper 0-60 m of the water column.

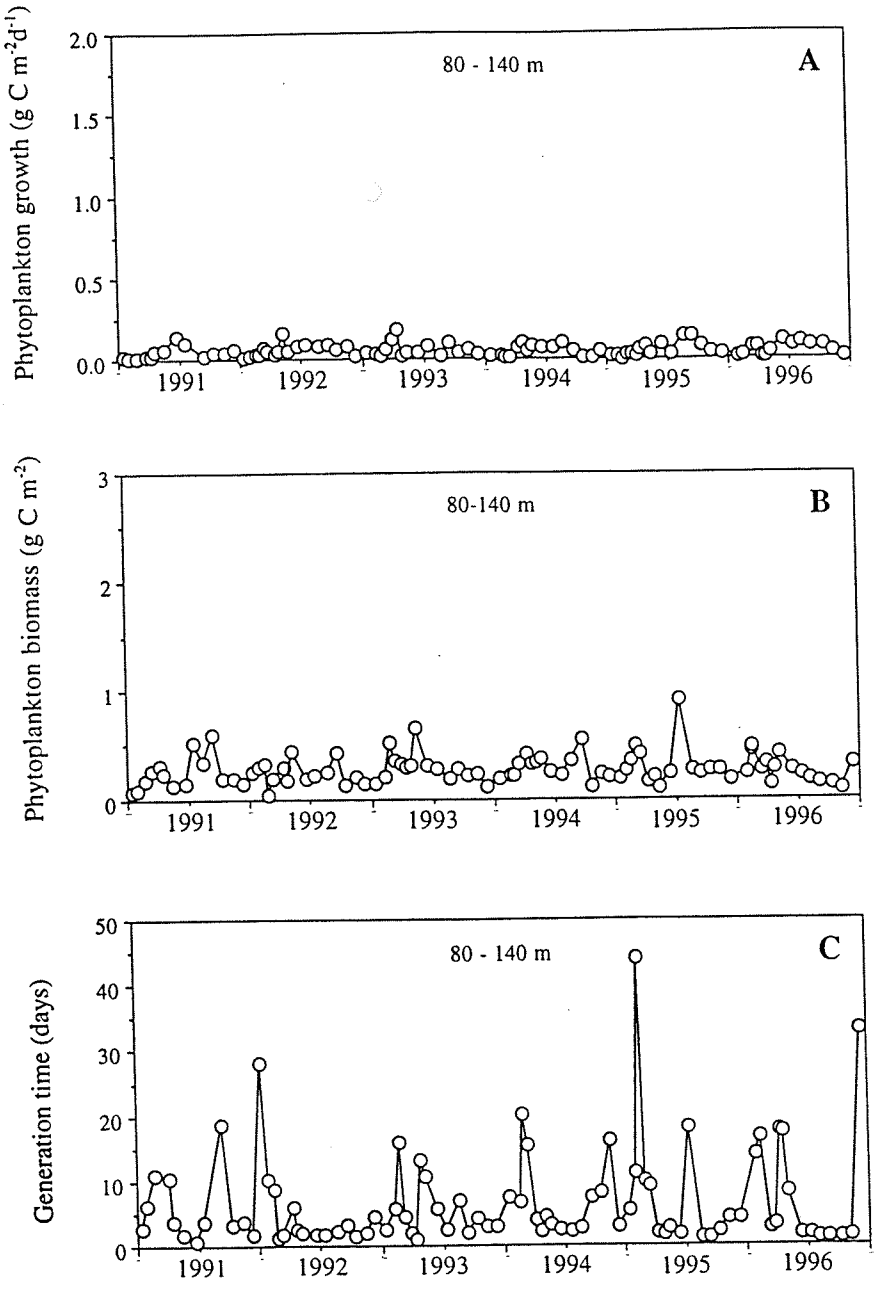

Figure 5-9
Calculated phytoplankton primary production (A), phytoplankton C biomass (B) and the generation time (C), integrated from the upper 80-140 m of the water column.

Table 5-1
Average bacterial incorporation rate of TdR (pmol $l^{-1}h^{-1}$) at BATS in July 1997. The assay was run at 21.5 °C (BATS core) and at simulated *in-situ* temperatures (Incubator) as shown in Figure 5-1. Degrees of freedom was 4 at each depth and the variances (brackets) were not significantly different in the two sets of data. The two assay incubations were tested for equalnes (null hypothesis $\mu_1 - \mu_2 = 0$) using a Students t-test.

Depth	BATS core	Incubator	Significance
1	0.12 (0.18)	0.13 (0.29)	$t_s << t_{0.9[4]}$
20	0.84 (0.48)	1.28 (0.32)	$t_s << t_{0.9[4]}$
40	0.86 (0.39)	0.96 (0.45)	$t_s << t_{0.9[4]}$
60	0.89 (0.28)	0.27 (0.32)	$t_s < t_{0.5[4]}$
80	1.22 (0.39)	1.40 (0.43)	$t_s << t_{0.9[4]}$
100	0.36 (0.42)	0.33 (0.21)	$t_s < t_{0.9[4]}$
120	0.12 (0.13)	0.14 (0.14)	$t_s << t_{0.9[4]}$

integrated bacterial growth rate was also evident in the 0-60 m (Figure 5-4) and 80-140 m (Figure 5-5) depth integrations. While the generation time (GT) at 0-60 m was similar to the 0-140 m depth integration, GT at 80-140 m was 1.3-1.9 times longer than 0-140 m and 1.3-2.4 times longer than the GT calculated from the surface 0-60 m integration (Table 5-2). The bacterial TdR incorporation rate of particles that passed a GF/F filter was 35 % of natural seawater (both collected on 0.2 μm cellulose nitrate filters) from the equivalent depths between surface and 140 m (Figure 5-6).

The annual averages of the phytoplankton primary production rates as well as the calculated phytoplankton C biomass at 0-140 m showed strong seasonal fluctuations, but remained very much the same for all years except for 1994, in which no distinct primary production peak was detected (Figure 5-7, Table 5-3). Phytoplankton generation time (GT) was 8-11 times faster than the bacterial GT (Tables 5-2 and 5-3). Phytoplankton primary production GT at 0-60 m was slightly faster (1.3-1.5 times) than the 0-140 m integration, while GT at 80-140 m was 3-7 times slower than 0-140 m and 5-10 times slower than the surface 0-60 m integration (Figures 5-7, 5-8, 5-9 and Table 5-3).

The lowest integrated bacterial abundance recorded in any year investigated, was assumed to be dominated by dormant and slow growing cells. This minimum was

Table 5-2
Annual averages of bacterial growth (BG; g C $m^{-2}d^{-1}$), biomass (BC; g C m^{-2}) and generation time (GT; days), integrated from 0-140 m, 0-60 m and 80-140 m depth.

	1991	1992	1993	1994	1995	1996
0-140 m:						
BG	0.025	0.038	0.067	0.048	0.043	0.059
BC	0.64	0.75	0.78	0.74	0.67	0.68
GT	26	21	9	14	12	10
0-60 m:						
BG	0.014	0.021	0.033	0.025	0.022	0.032
BC	0.26	0.34	0.33	0.33	0.30	0.30
GT	16	21	9	12	11	8
80-140m:						
BG	0.0064	0.010	0.022	0.014	0.013	0.017
BC	0.26	0.30	0.30	0.29	0.26	0.2
GT	40	28	12	20	18	19

Table 5-3
Annual averages of primary production (PP; g C $m^{-2}d^{-1}$), biomass (PC; g C m^{-2}) and generation time (GT; days), integrated from 0-140 m, 0-60 m and 80-140 m depth.

	1991	1992	1993	1994	1995	1996
0-140 m:						
PP	0.46	0.58	0.41	0.33	0.49	0.59
PC	0.84	0.95	0.98	1.06	1.10	1.16
GT	1.7	1.2	1.8	2.3	1.6	1.3
0-60 m:						
PP	0.37	0.45	0.29	0.24	0.38	0.48
PC	0.40	0.58	0.47	0.60	0.63	0.68
GT	1.1	0.9	1.3	1.8	1.2	0.9
80-140m:						
PP	0.045	0.058	0.058	0.048	0.054	0.051
PC	0.24	0.24	0.30	0.29	0.31	0.26
GT	5.6	5.2	5.5	7.1	9.7	8.7

Table 5-4
Average bacterial growth (g C m^{-2} d^{-1}), biomass (g C m^{-2}) and generation time (days) integrations from 1991-1996, using total number of bacteria and abundance corrected for non-living cells.

Integration	0-140 m	0-60 m	80-140 m
Growth	0.047	0.025	0.014
Biomass	0.71	0.31	0.28
Generation time	15	13	22
Living cells (%)	39	68	50
Biomass	0.28	0.21	0.14
Generation time	5	8	10

Table 5-5
Integrated (0-140 m) average bacterial growth and loss by protistan grazing and viral lysis (g C m^{-2} d^{-1}) during the spring bloom period in 1991-1996. The impact of grazing pressure, and viral lysis (% Loss) was calculated as the fraction of bacterial growth measured by TdR, not accounted for in the growth estimate (BG) using the change of bacterial abundance over time (linear integration over a minimum of 3 cruise dates).

Integration	BG	Viral lysis & Grazing	% Loss
1991	0.002	0.019	92
1992	0.006	0.022	80
1993	0.006	0.058	91
1994	0.004	0.033	89
1995	0.008	0.024	74
1996	0.009	0.040	81
Grand average	0.006	0.032	85

subtracted from the total integrated abundance and used in order to create a measure of the actively growing bacterial biomass. The corrected integrated bacterial abundance in any other month, outside the month with the minimum (30-60 % of total bacterial biomass), was used to calculate a new BC and GT for all three depth integrations (Table 5-4). This approach resulted in an average bacterial GT that was 2-3 times faster, but still 1.4-6.7 times slower than the primary production (Tables 5-3 and 5-4).

The bacterial growth rate, measured by the TdR incorporation technique, does not take into account the loss of bacterial biomass by protistan grazing, viral lysis and UV damage. Therefore, the difference in bacterial growth measured from the change in bacterial abundance as a function of time (linear regression of a minimumof 3 cruise dates) and the TdR incorporation technique, may show the potential loss of bacterial biomass relative to the bacterial growth rate (% Loss). The results shown in Table 5-5 suggest that an average of 85 % of the bacterial growth, was lost due to protistan grazing, viral lysis and UV damage (range 74-92 %) during the spring bloom season.

Primary production of *Trichodesmium* puffs showed a strong seasonal peak in summer, both in 1995 and 1996 (Figure 5-10 A). *Trichodesmium* GT varied between 4-12 days most of the year, but was as fast as 0.5-2 days during the period of peak primary production (Figure 5-10 B). Integrated *Trichodesmium* primary production was calculated, using the colony depth distribution given in Chapter 4 (Equation 4-1 and Figure 4-6) and the primary production rates shown in Figure 5-10 A. The light saturation level was on average 40 % for both puffs and tufts, with the exception of tufts in October 1996 (Table 5-6) which were not light saturated at any light level (> 100 %). *Trichodesmium* primary production was measured on puff colonies, since they are present all year around and a surface light saturation level of 40 % was used for further calculations of the integrated growth rate. Primary production of *Trichodesmium* was only measured in surface light incubations (equivalent to 10 m depth) and the integrated primary production rate at depth was corrected in the following way:

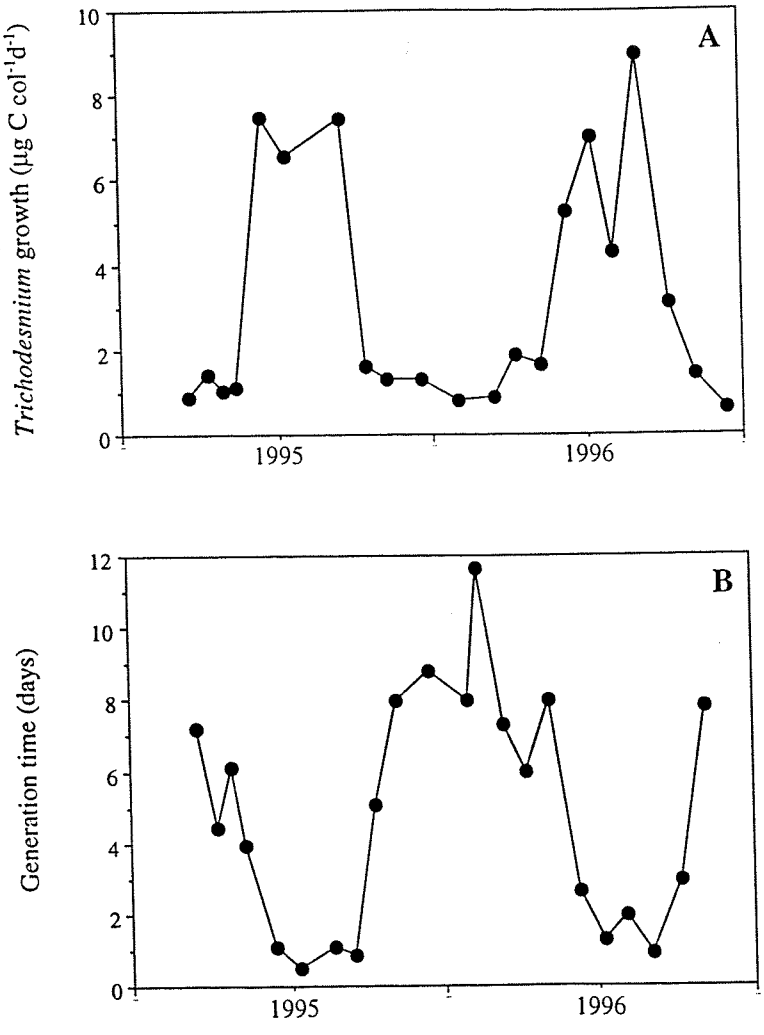

Figure 5-10
Trichodesmium primary production (A) of puffs and the calculated generation time (B) at surface at BATS in 1995 and 1996.

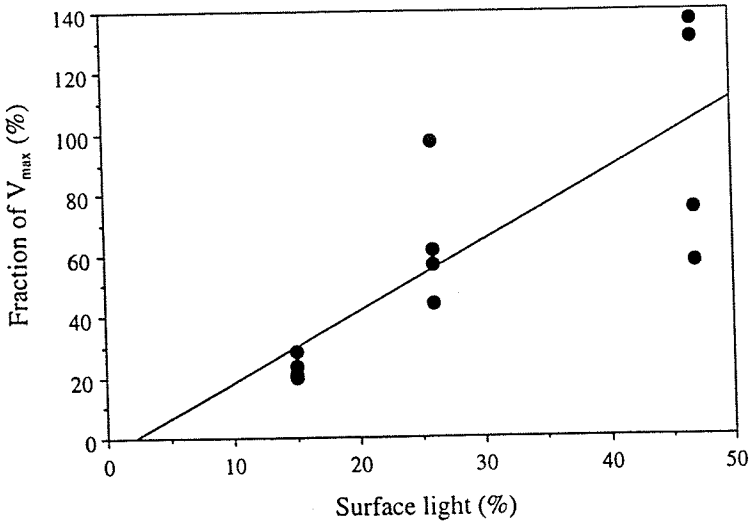

Figure 5-11
The rate of primary production by *Trichodesmium*, calculated as the percentage of P_{max} and plotted as a function of the light level. The correction factor (CF_z) used to correct *Trichodesmium* PP at a given depth with light level I_z (Equation 5-1) is listed in Table 5-7.

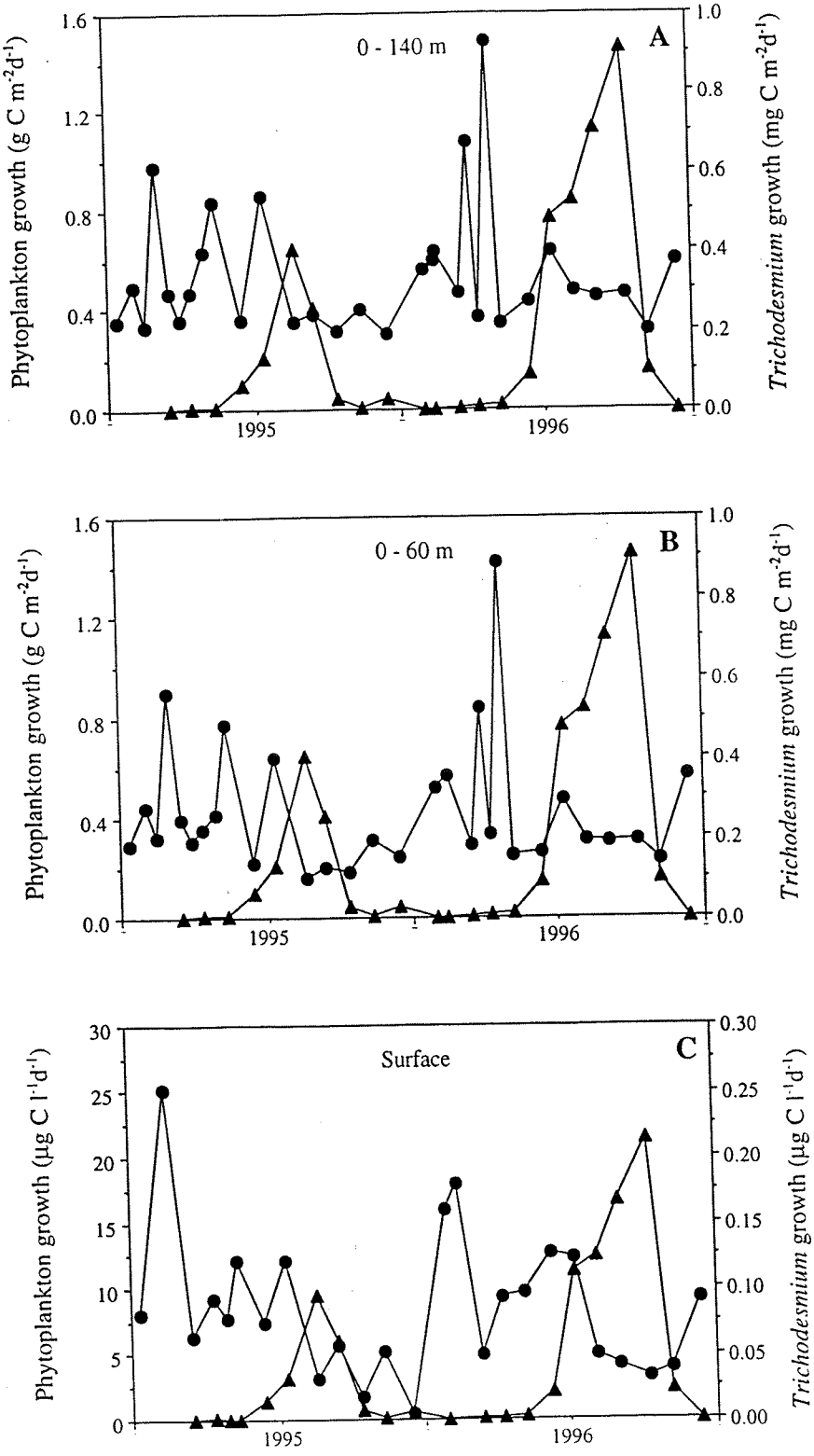

Figure 5-12
Calculated phytoplankton primary production and *Trichodesmium* primary production (*Trichodesmium* PP) integrated at 0-140 m (A) and 0-60 m depth (B). Phytoplankton primary production was compared to *Trichodesmium* PP at surface (C). Note the different scales on the Y-axis between phytoplankton primary production and *Trichodesmium* PP (1:1000 in A, B and 1:100 in C).

Table 5-6
Calculated half light saturation (I_k) expressed as % of surface light level at 15 m depth and maximum (P_{max}) rate of primary production ($\mu g\ C\ col^{-1}d^{-1}$) for puff and tuft morphologies of *Trichodesmium* on two cruises at BATS in 1996 and 1997.

	Puff			Tuft		
	P_{max}	I_k	n	P_{max}	I_k	n
October 1996	3.7	18	3	7.4	70	4
July 1997	2.1	23	4	2.2	19	4

A 1992-1996 average of the 1% light level, was found to be at 94 m depth (CV% = 12) at BATS (D. Siegel, pers. com.). Since the average 1% light level is known and by using Beers equation (Equation 5-1), the only unknown is the average extinction coefficient (k) which can be estimated for the BATS site (k = 0.049). This is only an approximation, since k also depends on the particle concentration in the water column which does not always change as a power function of the depth. The particle concentration at BATS, however, does in general increase as a positive power function of the depth down to the 1 % light level associated with the particle and Chl-*a* maximum. Hence, the assumption made about k and the use of Beers Law in this study, is valid;

[5-1] $$I_z = I_0 \cdot e^{-kz}$$

z = depth
I_z = light level at depth z
I_0 = light level at surface
k = light extinction coefficient in water

According to Beers Law, the average 40 % light level at BATS would then be at 19 m depth. Therefore, the surface primary production rate at depths below 19 m were corrected using a correction factor (CF_z) for a given depth z, by assuming a linear relationship between the primary production rate and the light level at depth z (Figure 5-11).

$$CF_z = I_z \cdot 2.31 \qquad (r^2 = 0.631,\ n = 12)$$

A list of the calculated correction factors applied, as a function of depth (z) and the light level (I_z), is given in Table 5-7.

The integrated primary production rates of phytoplankton and *Trichodesmium* was calculated for the 0-140 m and the 0-60 m depth interval (Figure 5-12 A, B). Peak rates of *Trichodesmium* primary production were measured in late summer and fall, but these were a factor of 10^3 lower than the spring phytoplankton primary production peak (Figure 5-12 A, B). The calculated *Trichodesmium* primary production rate was on average 0.008 % (range 0.00004-0.04 %) of the total primary production rate in the 0-140 m depth integration and 0.06 % (range 0.0001-0.3 %) in the 0-60 m depth integration. At the surface, the *Trichodesmium* primary production rates were on average 1% of the total primary production rate (range 0.002-6%) (Figure 5-12 C).

Table 5-7
Depth (z), light irradiance (I_z) and correction factor (CF_z) applied to primary production rates calculated from surface *Trichodesmium* primary production measurements.

Depth (z)	I_z	CF_z
1	0.95	1.00
5	0.78	1.00
10	0.61	1.00
15	0.48	1.00
20	0.38	0.87
25	0.29	0.68
30	0.23	0.53
35	0.18	0.42
40	0.14	0.33
45	0.11	0.25
50	0.09	0.20
55	0.07	0.16
60	0.05	0.12
65	0.04	0.10
70	0.03	0.07
75	0.03	0.06
80	0.02	0.04
85	0.02	0.03
90	0.01	0.03
95	0.01	0.02

5.4 DISCUSSION

This study is the first to report a continuous time series of the seasonal changes of bacterial growth rates in the Sargasso Sea (Figure 5-1) and the numbers are in the same order of magnitude as the TdR incorporation rates reported by Carlson et al. (1995), from 8 cruises in the vicinity of Hydrostation 'S' and BATS between 1991 and 1993. The TdR incorporation rates assayed at 100 m depth seawater temperature were not significantly different from the assay run at simulated *in-situ* temperatures in July 1997 (Figure 5-2, Table 5-1), suggesting that the BATS core TdR assay data (using one set temperature for all depths) was not significantly influenced by potential temperature changes during the 2-3 h incubation period.

Bacterial growth in spring was closely associated with the increase in primary production (Figure 1-10 and 5-7 B). A secondary increase in bacterial growth of the same magnitude as the bacterial spring bloom occurred each year (Figure 5-1) but this event appeared not to be a reflection of a further increase in the phytoplankton primary production (Figure 1-10). However, the oxygen anomaly data (Figure 1-9) shows a pattern similar to bacterial growth, suggesting the presence of autotrophic production that the BATS core ^{14}C-assay apparently did not measure. Integrated bacterial C biomass (using 10 fg C cell^{-1}), bacterial growth rate (using $1.7 \cdot 10^{18}$ cells mol TdR^{-1}) and GT (days) all showed the same trend, although not in the same order of magnitude, at all depth integrations (Figure 5-3, 5-4 and 5-5): Bacterial biomass did not show any direct seasonal trends outside the spring bloom, but the bacterial growth rate showed distinct peaks above the annual average after the spring bloom and into the summer/fall (Figures 5-3, 5-4 and 5-5). Annual averages of bacterial growth were approximately the same in 1991 and 1992, but almost doubled in 1993-1996 (Table 5-2). This increase was also observed by Carlson et al. (1995) in their single TdR incorporation profiles at BATS in the summers of 1992 and 1993. As the biomass did not change much with the season (Figures 5-3, 5-4, 5-5 and Table 5-2) bacterial GT was a mirror image of the growth rate (Figures 5-3 C, 5-4 C and 5-5 C). There is no obvious explanation for the much higher

growth rates in 1993-1996 relative to the years 1991-1992 (Table 5-2). The winter mixing event took place in all three years at BATS (Figure 1-3) and extended to approximately the same depth each year. In fact, primary production, the main source of DOC for bacterial consumption, was higher the year prior to 1993 (Figure 5-7) and in the same order of magnitude as in 1995 and 1996 (Table 5-3). The primary production bloom event was followed by minor peaks during summer-fall and did not decline as steeply in 1993-1996 as in 1991-1992 (Figure 1-10). This was also evident in the oxygen anomaly data in the upper 80 m of the water column, which had a subsurface maximum (+15 μmol kg^{-1}) as late as September-October in 1993-1996 (Figure 1-7). Sources of primary production that were not included in the BATS core primary production assay may have been the cause of elevated O_2-levels and bacterial growth rates (Figures 5-3, 5-4 and 5-5). Michaels et al. (1994) and Bates et al. (1996) reported a decrease in TCO_2 at BATS in spring-summer and concluded that this draw-down was mainly caused by biological factors. Due to their low total biomass relative to other phytoplankton, rare and large diatoms (Villareal 1988) were ruled out as a significant source of primary production (Chapter 4). Mass occurrences of cyanobacterial colonies such as *Trichodesmium*, may at times be of significance in the upper 0-60 m of the water column (Chapter 4). However, due to their negative exponentially shaped depth distribution (Figure 4-6) and low surface primary production rate relative to phytoplankton (Figure 5-12 C), the calculated integrations of the *Trichodesmium* primary production rates were in the order of 10^3 times less active than phytoplankton primary production (Figure 5-12 A and B). Hence, *Trichodesmium* colonies alone could not account for the TCO_2 drawdown reported by Michaels et al. (1994) and Bates et al. (1996) at BATS in summer and fall. However, Orcutt (1998) reported that *Trichodesmium* biomass was underestimated by 9-49 times in June-August of 1996, if only the colonies were accounted for, since the majority of the diazotrophs exists as single trichomes. In her enumeration of *Trichodesmium* trichomes, Orcutt (1998) filtered 2-4 l of seawater. Hulburt et al. (1960) did a similar study but filtered only 0.25 l per sample from the upper 100 m of the water column, twice a month for a whole year at Hydrostation 'S' and detected trichomes only once in June at 10 m depth. Since

the BATS primary production measurements typically inoculate 0.25 l per sample depth and only 0.05 l of this volume is filtered, *Trichodesmium* trichomes were most likely not included in the core assay. Michaels et al. (1994) estimated the TCO_2 drawdown to be 2.6 mol C m^{-2} over a 275 day period in the upper 150 m of the water column, between April and December at BATS. This is equivalent to 0.017 mol C m^{-2} d^{-1} from the first of June to the end of August, or 204 mg C m^{-2} d^{-1}. By assuming that the trichomes have the same rate of primary production as the colonies, the potential rate was 11-38 mg C m^{-2} d^{-1} over a 3 month period. This rate of primary production by *Trichodesmium* is within the same order of magnitude and 6-19 % of the measured TCO_2 drawdown reported by Michaels et al. (1994) at BATS. Hence, the reported TCO_2 drawdown (Michaels et al. 1994) and the positive oxygen anomaly (Figure 1-9) in summer and fall at BATS, may to some extent be caused by *Trichodesmium* trichome and colony primary production.

It has been argued that smaller bacteria in natural seawater are in a suboptimal medium and mostly consists of inactive cells in a resting mode (Jannash 1969). More recently, Stevenson et al. (1981) and Moyer & Morita (1989) came to a similar conclusion that small bacterial cells were not viable. This study shows that bacteria passing a GF/F filter constituted an average of 35 % of the TdR incorporation rate (Figure 5-6), suggesting that smaller cells are as viable as the ones retained on the GF/F filter. This finding was also supported by Fuhrman (1981), who found an active smaller fraction (< 0.6 μm diameter) of bacteria off the coast of southern California. More recent findings however, show that there is a fraction of the bacterial population enumerated by the DAPI epifluorescence technique that consists of dead or dormant cells (Zweifel & Hagstrøm 1995, Karner & Fuhrman 1997). This phenomenon seems not to be related to any particular size group of bacteria and the fraction of inactive cells may change depending on the stage of growth activity in the bacterial population (Choi et al. 1996). Karner & Fuhrman (1997) estimated that only half the bacteria enumerated by DAPI epifluorescence were actually active cells. The estimated average amount of active bacterial cells in this study, based on seasonal changes in the bacterial biomass (Table 5-4), is in the same order of magnitude as the Karner & Fuhrman (1997) study. Interestingly the amount of

active cells calculated from the 0-140 and 80-140 m depth integration (40 and 50 % respectively) was less than the 0-60 m depth integration (70 % active cells). The 0-60 m depth integration interval had the highest primary production rates (Figure 1-10) and was also associated with a higher level of bacterial growth than deeper waters (Figure 5-1). This lends support to the Choi et al. (1996) study that higher growth rates are associated with a higher frequency of active bacterial cells. By excluding the number of inactive bacterial cells, the recalculated bacterial GT was only 7 times slower at the 0-60 m depth interval and almost equal to (1.4 times slower) the phytoplankton GT at the 80-140 m depth integration (Table 5-4).

Several processes can reduce the number of actively growing bacterial cells; 1) UV-light may inactivate or permanently damage bacteria (Guerro & Jones 1996a, b); 2) viral (phage) infections may inactivate and eventually kill their host (Bergh et al. 1989) and; 3) protistan grazing and egestion may produce non-living "ghost cells" (Cole & Wynne 1974) . Guerro & Jones (1996 a) recently showed that nitrifying bacteria are UV-light sensitive. Ammonium and nitrite oxidising bacteria had different levels of sensitivity to UV-light and the nitrite oxidisers failed to recover their activity after being exposed 2-4 h of sunlight (Guerro & Jones 1996 b). Jeffrey et al. (1996) were able to demonstrate that the damage effects of UV-B irradiation on natural bacterioplankton in the Gulf of Mexico extended no deeper than 10 m in calm weather. Slightly turbulent conditions saw no net accumulation of UV damaged cells (Jeffrey et al. 1996). UV-light has been shown to induce infections and viral lysis of bacteria cells (Jiang & Paul 1996). However, viral infectivity has itself been shown to be light sensitive to both UV-B and UV-A light (Suttle & Chan 1992) suggesting that the virus (or phage) will only be able to utilise UV-damaged bacteria during night time hours. However, UV-light damage of bacterial cells is likely to be of minor importance on a depth integrated scale of 140 m, due to the fact that the majority of bacteria at BATS are associated with the particle and pigment maximum situated at 50-110 m depth (Figures 1-11, 1-12 and 2-6).

Until a decade ago, viral infections of bacteria, were considered to be of minor importance (Wiggins & Alexander 1985). However, Bergh et al. (1989), using a novel

harvesting technique with an ultracentrifuge, were able to show that earlier abundance estimates of aquatic virus were significantly underestimated. High viral abundances in the marine environment, exceeding bacterial abundance by two orders of magnitude (Børsheim et al. 1990, Bratbak et al. 1990) and rapid viral decay rates (Heldal & Bratbak 1991) have demonstrated that viruses play an important role in controlling the bacterial growth cycle. Spatial distribution of viruses is closely associated with bacterial abundance (Cochlan et al. 1993, Boehme et al. 1993) and a threshold abundance of bacteria, ranging between 10^5 and 10^8 bacterial cells l^{-1}, has been suggested as one of the pre-requisites for a viral infection (Suttle & Chan 1994, Weinbauer & Peduzzi 1994). Fuhrman & Noble (1995) did not investigate the diel periodicity of viral infections, but their mesocosm study on coastal seawater suggested an approximate equal contribution to the disappearance of bacteria due to protistan grazing and viral infections. In this study, only spring bloom bacterial abundances showed a positive unidirectional increase past more than two sampling time points, making it possible to generate an estimate of the net biomass increase in terms of bacterial cells. Due to the sudden input of nutrients and elevated primary production rate, this is potentially the only time of the year when there is a distinct uncoupling between bacterial growth and the loss rate caused by grazing and viral lysis. Table 5-5 shows that as much as 85 % of the bacterial growth may have been lost due to grazing and viral lysis in spring. Assuming that protistan grazing and viral lysis each caused half of the losses of bacterial biomass (Karner & Fuhrman 1997), these processes may have reduced the bacterial growth rate by 43 % each.

Historically, grazing on bacteria was evaluated by reducing or eliminating the protozoan predators (Newell et al. 1983, Fuhrman & McManus 1984, Landry et al. 1984, Wright & Coffin 1984, Sanders & Porter 1986, Sherr et al. 1987). Subsequently, more sophisticated methods, requiring less manipulation and shorter incubation time, were developed. These methods include the minicell technique introduced by Wikner et al. (1986), the TdR radiotracer technique of Lessard & Swift (1985) and the protozoan uptake of bacteria-sized fluorescent microspheres (Børsheim 1984, Cynar & Sieburth 1986, McManus & Fuhrman 1986) and fluorescently labelled bacteria [FLB] (Sherr et al.

1987). In a comparative study, Sherr et al. (1987) were able to show that the FLB technique gave much higher clearance rates than previously established methods. The use of different labelling techniques may explain the wide range of clearance and ingestion rates reported in the early studies. Grazing studies using FLB additions to natural seawater, have only been applied for the last ten years (Wikner et al. 1990, Weisse & Scheffel-Möser 1991, Marrasé et al. 1992, Gonzalez & Suttle 1993, Laurion et al. 1995, Leaky et al. 1996). Of these studies, only Weisse & Scheffel-Möser (1991) have reported heterotrophic nanoflagellate (HNF) clearance and ingestion rates from the North Atlantic. Weisse & Scheffel-Möser (1991) found an ingestion rate ranging from 9-36 bacteria flagellate^{-1} h^{-1} and counted an average of 0.5-1 • 10^{6} HNF l^{-1} in the upper 100 m of the water column. By assuming 10 fg C bacterial cell, this is equivalent to a loss of 4.5-36 • 10^{6} bacterial cells h^{-1} or 1.1-8.6 mg bacterial C m^{-3}d^{-1}. This study showed a six year spring time average loss of 32 mg bacterial C m^{-2}d^{-1} (range 19-58 mg C m^{-2}d^{-1}) in the upper 0-140 m of the water column (Table 5-5) which is equivalent to 0.23 mg C m^{-3}d^{-1} (range 0.14-0.41 mg C m^{-3}d^{-1}). By assuming that half the loss was caused by viral infections (Karner & Fuhrman 1997), bacterial grazing constituted 0.11 mg C m^{-3}d^{-1} (range 0.070-0.21 mg C m^{-3}d^{-1}) of the loss rate. The latter estimate is an order of magnitude less than the lower range calculated by Weisse & Scheffel-Möser (1991) for the North Atlantic in the spring of 1989.

Bacterial C biomass either remained unchanged or decreased during summer in the 0-140 m depth integration from 1991 to 1996 in this study (Figure 5-3 B). The calculated loss rate of bacterial C in this study (Table 5-5) was balanced in spring by the measured bacterial growth rate (TdR incorporation). During summer however, the calculated spring loss rate (Table 5-5) was a factor of 2 lower than the rate of bacterial growth. By assuming the same HNF ingestion rate in summer and a balance between bacterial growth (by TdR) and the loss rate of bacterial C caused by grazing and viral lysis (due to a close coupling between growth and the loss rate), then the loss rate should be a factor of 2 higher than the one calculated for the spring bloom. Hence, two different scenarios can be distinguished regarding bacterial growth and the loss of bacterial C caused by grazing and

viral lysis: 1) The spring phytoplankton bloom and the associated DOC excretion initiated an increase in bacterial growth. The rate of removal of bacterial C by grazing and viral lysis was low, allowing a build up bacterial biomass. 2) Eventually, the loss rate of bacterial C caused by grazing and viral lysis, exceeded bacterial growth and effectively controlled bacterial abundance and biomass until next spring bloom event.

Chapter 6: DOC excretion, exoenzymatic peptidase activity and nutrient limitations for bacterial growth

6.1 INTRODUCTION

The significance of DOC excretion by phytoplankton was debated as early as the nineteen thirties (Krogh & Keys 1934). However, renewed interest occurred at the time of fluorochrome staining of bacteria and the discovery of the importance of microbial biomass (Hobbie et al. 1977). The main bulk of dissolved organic matter (DOM) in open oceans originates from phytoplankton primary production and the main portion of DOM is low molecular weight < 1000 Da (Jensen 1983, Lancelot 1984). Past studies have demonstrated that only a quarter of oceanic DOM is high molecular weight (Benner et al. 1992, Carlson et al. 1995). Eppley & Sloan (1965) pointed out the importance of including DOC in order to balance dissolved inorganic carbon (DIC) uptake with organic carbon synthesis and ever since then, a number of studies have investigated the rate of DOC excretion by the primary producers. The first reports on DOC excretion using the ^{14}C-bicarbonate label approach, was published simultaneously by Sharp (1977) and Wiebe & Smith (1977). Williams (1990) described three different stages in a time course of DOC excretion, using ^{14}C-bicarbonate: 1) The early phase, is the time-lag period prior to equilibrium between external and internal cell pool of inorganic carbon. 2) The second stage is when the internal cell pool is saturated with ^{14}C (inorganic and organic) and the exudation of DO^{14}C is entering the surrounding seawater medium. 3) The last stage occurs when the external DO^{14}C pool is saturated and the time course is in steady-state. At the second stage the linear DO^{14}C excretion as a function of time, equals the exudation rate of DOC from the primary production. In order to calculate the DOC accumulation rate, we have to assume an equilibrium between ^{14}C and naturally occurring carbon in

both the internal and external inorganic carbon, as well as a constant $DO^{14}C$/DOC-ratio. The presence of heterotrophs in the surrounding seawater is likely to consume and alter the primary excretion of DOC: bacteria are consuming DOC as it is excreted and there is also a release of DOC caused by sloppy-feeding bacterivores and herbivores grazing on the actively growing bacteria and phytoplankton. The measured net DOC excretion rate is the end product of all these processes and also constitutes the potential daily build-up of the DOC pool.

The use of fluorogenic tracers in exoenzyme measurements have been known for more than two decades (Perry 1972). Exoenzymes hydrolyse complex molecular compounds into substances that could be incorporated and utilised as building blocks in biomass synthesis, were first thought to be solely a nutrient acquisition pathway for bacterial growth. However, later studies have shown that exoenzymes such as Leucine aminopeptidases (LAP), is produced by both auto and heterotrophic micro-organisms, released through grazing by sloppy-feeding as well as by excretion through the cell membrane. Berges & Falkowski (1996) found that extracts of cell-associated phytoplankton proteases such as LAP, was surprisingly stable outside the cell. They concluded that once released by grazing or viral lysis, the LAP activity level would persist and function in the environment for some time (Berges & Falkowski 1996). Two simultaneous studies (Davies et al. 1994, Karner et al. 1994) suggested that LAP was not only associated with bacteria. However, this was already known by Palenik et al. (1989) who reported that the exoenzyme activity associated with phytoplankton, transformed external DON and incorporated only the end product (NH_4^+) of the process. Martinez & Azam (1993) reported elevated exoenzyme activities associated with 2 strains of the blue-green algae *Synechococcus* and Berges & Falkowski (1996) were able to show that a significant amount of the LAP activity was associated with 5 species of phytoplankton, including one species of cyanobacteria. Karner & Herndl (1992) and Smith et al. (1992) simultaneously reported elevated exoenzyme activities associated with marine snow and aggregates. The first reports on exoenzyme activity in marine waters appeared two decades ago (Petterson & Jansson 1978, Hoppe 1983, Somville & Billen 1983). A

seasonal pattern was observed early in studies, suggesting that certain precursors are regulating the exoenzyme activity (Chrost et al. 1986, Rath et al. 1993). Proteins and peptides have been shown to initiate LAP activity (McDonald & Chambers 1966, Litchfield & Prescott 1970, Fontigny et al. 1987) while specific aminoacids will have the adverse effect (Long et al. 1981, Fontigny et al. 1987). Several studies have found a good correlation between the LAP activity and bacterial biomass (Fontigny et al. 1987, Rosso & Azam 1987). The bacterial incorporation rate of aminoacids have also been shown to be closely associated with the LAP activity rate (Somville & Billen 1983, Rosso & Azam 1987). These findings suggest that the exoenzyme activity at times may play an instrumental role in microplankton nutrient acquisition in the marine environment. Seasonal accumulation of DOM at the end of phytoplankton blooms have been demonstrated (Barlow 1980, Carlson et al. 1994) and in some areas the dissolved pool has been found to exceed that of particulate organic matter during blooms (Williams 1995). Open ocean C, N and P cycles are closely linked to each other through production and remineralisation processes (Redfield et al. 1963) and the availability of these essential elements have important consequences for growth synthesis as well as the fate of organic matter in the sea (Dugdale & Goering 1967).

Since studies in the past have shown that low $DO^{14}C$ concentrations are easily compromised (Sharp 1977, Williams 1990), a series of method checks were conducted in order to avoid inorganic ^{14}C contamination. Natural surface seawater from BATS was incubated with nutrient supplements in order to study the DOC excretion dynamics in a culture. Rates of DOC excretion in natural surface seawater and seawater samples with added *Trichodesmium* colonies, were investigated at BATS in 1996 and 1997 using the ^{14}C-radiolabel technique. The seasonality of LAP and ^{3}H-Leu incorporation rates was measured in the upper 250 m of the water column at BATS in 1995. LAP enzyme kinetics in natural surface seawater and seawater with *Trichodesmium* colonies were investigated in 1993 and 1994. Potential nutrient limitations, the seasonality of bacterial growth and the bacterial growth efficiency at the BATS site was evaluated.

6.2 MATERIALS AND METHODS

$DO^{14}C$ excretion experiments

During the primary production assay ^{14}C -bicarbonate is taken up by the primary producers, which in the course of the photosynthesis incorporate the isotope into the carbon biomass ($PO^{14}C$) or internal pool of $DO^{14}C$. Depending on the growth stage and condition of the primary producer, various amounts of the ^{14}C-label is excreted as $DO^{14}C$ and expired as $^{14}CO_2$ during the course of photosynthesis. A number of caveats to be avoided when using the $DO^{14}C$ excretion technique, were summarised by Williams (1990): 1) Inorganic ^{14}C can easily contaminate the $DO^{14}C$ sample, particularly in waters with low DOC excretion rates. 2) Seawater may quench the scintillation cocktail and consequently underestimate the amount of $DO^{14}C$. 3) It is important to avoid leakage of $DO^{14}C$ from phytoplankton cells, e.g. during filtration and handling of the samples. 4) Since the DOC excretion time course has a sigmoid shape and only the linear log phase of the curve is used to measure the actual DOC excretion rate, it is imperative that the rate of primary production (i.e. the $PO^{14}C$ synthesis) is linear during the course of an entire day.

Method checks of the $DO^{14}C$ C excretion assay

In order to measure the excreted $DO^{14}C$, it was necessary to purge all the remaining inorganic ^{14}C from the seawater sample. This was done by releasing all bicarbonate as CO_2 from the seawater sample by acidifying the seawater sample (pH = 0.6). It was essential to purge the seawater sample with a gas as soluble as CO_2 for a period of time in order to release the isotope-labelled gas from the water phase. In order to check for remaining contamination of inorganic ^{14}C in acidified radioactive seawater samples, a time course study of sparging the samples with air was done from 5-60 min.

Scintillation cocktails commonly used for ß-emitting isotopes, are able to absorb only a limited amount of water before quenching in the sample starts interfering with the

counting efficiency. Due to the low primary production activity at the BATS site (Lohrenz et al. 1992, Michaels et al. 1994) it was necessary to maximise the amount of seawater added to the scintillation cocktail in order to get detectable measurements of $DO^{14}C$ above background radiation. A series of samples with a known amount of ^{14}C-label, but with a series of different amounts of seawater added to the Aquasol scintillation cocktail (Packard), was made in order to find the maximum amount of seawater that would not interfere with the counting efficiency.

In order to measure the amount of $DO^{14}C$ in a sample, it was necessary to remove particulate ^{14}C from the sample by gentle filtration. A sample volume of 10 ml was collected in 50 ml polypropylene syringe and the solution was filtered through a 0.2 µm Acrodisc (evacuated for air) by gently pushing the piston by hand. Cell damage and potential loss of intracellular $DO^{14}C$ was investigated during a DOC excretion time course in February 1997, by comparing unfiltered seawater samples with the filtered ones from the same assay.

In May 1996, a test of linear ^{14}C uptake was done on surface seawater at BATS. The incubation was run like an ordinary primary production assay (see Methods in Chapter 1), with the exception that a 1 l polycarbonate bottle was used and seven time point samples were filtered during the course of the day. Three replicates were taken at the start and at the end of the assay.

The $DO^{14}C$ excretion assay in a phytoplankton culture experiment

In order to study phytoplankton bloom dynamics and the rate of DOC excretion, a natural seawater culture with nutrient supplements was set up *in vitro*. Natural Sargasso surface seawater from BATS in May 1996 was supplemented with nutrients (10 µM NO_3^- and 1 µM PO_4^{2-}) and incubated in natural daylight mimicking 15 m depth at the BATS site (see next paragraph for more detail) using the running seawater system on the dock at BBSR. Over a time course of 10 days DIN, DIP, Chl-*a*, DOC content and the rate of primary production was monitored daily. $DO^{14}C$ excretion experiments were conducted in

natural seawater without nutrient supplements at start of the experiment, on day 5 during pre-bloom conditions, on day 6 during the exponential growth phase, on day 8 during maximum growth and on day 10 during the senescence of the phytoplankton culture. The primary production assay and the DIN, DIP and Chl-*a* analysis were described in Chapter 1. Total amount of DOC was analysed by the High Temperature Combustion (HTC) method as described by Sharp et al. (1994).

DO^{14}C excretion in natural surface seawater samples at BATS

In order to quantify the DOC excretion rate, time course measurements of the ^{14}C-labelled DOC excretion (DO^{14}C) were made concurrently with the primary production assay (Chapter 1) using 20 m seawater collected in a GoFlo bottle at BATS in 1996 and 1997. The assay was run in a deckboard incubator, using the ships surface seawater flow-through system. The incubator was covered with blue-tinted Plexiglas (50 % total sunlight penetration and 80 % light penetration at 488 nm) to mimic subsurface light conditions at 15 m depth and prevent UV damage and photoinhibition of the phytoplankton. During the course of the day 6-8 time points were sampled (1-3 h intervals); 10 ml seawater samples were siphoned out using a 50 ml polyethylene syringe with a tubing. The sample was filtered through a 0.2 μm Acrodisc filter and an aliquot of 7 ml was transferred to a scintillation vial containing 1 ml of 6 N HCl. The sample was sparged with air for a minimum of 30 min using a Pasteur pipette connected to an aquarium pump. This sparging was repeated for another 30 min after the sample had been vigorously shaken. Then, the lid on the scintillation vial was cleaned off with a Kimwipe tissue paper and to the sample was added 10 ml of Aquasol scintillation cocktail. Finally, the vial was capped and homogenised on a Vortex mixer. The samples were left in the dark for 5 - 7 days before being counted on a Packard Tricarb 2000 CA Liquid Scintillation Analyzer, using the external channel ratio method. The primary production assay was run concurrently in the same incubator and time points were filtered at the start of the assay and 30 min past sunset (Chapter 1). The primary production assay in the

incubator usually commenced after sunrise. Daily rate calculations were therefore corrected for the actual length of the day (10-12 h).

The Leucine aminopeptidase (LAP) activity assay

LAP was assayed in natural seawater samples in discrete depth profiles between surface and 250 m depth at the BATS site in 1994 and 1995. The LAP activity was also measured in surface waters, comparing natural seawater and the enzyme activity associated with *Trichodesmium*, at the BATS site in 1994 and 1996. Natural unfiltered seawater was collected in Niskin bottles attached to a CTD (Chapter 1) for all the assays. LAP was assayed according to Chrost & Velimirov (1991) in polypropylene centrifuge tubes containing 10 ml of unfiltered seawater. The LAP activity was measured in natural surface seawater with *Trichodesmium* colonies (2 colonies per 10 ml of unfiltered seawater). The *Trichodesmium* colonies were collected as described in Chapter 4. The LAP activity associated with the *Trichodesmium* colonies was calculated by subtracting the activity in the adjacent unfiltered seawater incubation. A substrate saturated concentration of 308 μM of L-leucine 4-methyl-7-coumarinylamide hydrochloride (Leu-MCA) was used for the LAP depth profiles and the comparison of enzyme activity between natural seawater and *Trichodesmium*. The fluorescent end product, 7-amino-4-methylcoumarin (AMC) was measured on a Sequoia-Turner Fluorometer using a 360 nm narrow band filter excitation and a 430 nm sharp cut emission filter. All the LAP assays were done in real time at sea during the BATS cruises.

The LAP activity at BATS

Several method checks of the assay were performed in order to get a better understanding of factors controlling the LAP activity. The impact of L-leucine (L-2-Amino-4-methylpentatoic acid), the non-fluorescing end product and a potential inhibitor of the LAP activity, was investigated in March 1997. The substrate saturation concentration was investigated five times in natural surface seawater, four times for *Trichodesmium* puffs and once for *Trichodesmium* tufts, between December 1993 and July 1994. The LAP activity fitted a Michaelis-Menten curve as a function of the substrate concentration (range 40-600 μM) and K_m and V_{max} were extracted using a Lineweaver-Burk plot of the same set of data.

Nutrient limitations for bacterial growth

From the time of vertical mixing of the water column at BATS in the last months of the winter, the organisms living in the euphotic zone are subjected to a dramatic change of events in their environment starting with the spring bloom mixing followed by the stabilisation and strengthening of the surface layers of the ocean in early summer. In order to investigate the order and possible causes of increased bacterial growth in surface waters during the spring bloom period, LAP activity (this chapter), DIN (Chapter 1), the bacterial C:N and C:P ratios (Chapter 3), phytoplankton primary production (Chapter 1), net daily DOC accumulation (this chapter) and the bacterial incorporation rate of both Leu and TdR (Chapter 5) were evaluated for the years of 1995, 1996 and 1997.

Measurements of the bacterial incorporation rate of L-[4, 5-^{3}H] leucine (Leu) was done according to Kirchman et al. (1985, 1986) and is identical in conduct to the TdR assay (Chapter 5). The Leu incorporation by bacteria at the BATS site has been found to be saturated at concentrations of 21 nM or higher (Carlson 1994) and this concentration was used in the Leu incorporation assay.

6.3 RESULTS

Method checks of the DO^{14}C excretion assay

In order to avoid contamination during the DO^{14}C excretion assay, it was essential to purge the remaining inorganic ^{14}C at each time-point. A time course check of acidified ^{14}C-bicarbonate labelled seawater (pH = 0.6) purged with air, showed that there was no detectable inorganic isotope label left above background radiation after 10 minutes of purging (Figure 6-1 A). Background was defined as the radiation in a clean scintillation vial filled only with scintillation cocktail.

Aquasol scintillation cocktail absorbed as much as 40 % seawater (percentage of seawater mixed into total volume of Aquasol) without quenching interfering with the counting efficiency (Figure 6-1 B). The scintillation cocktail turned into a thick gel, when mixed with 40 % of seawater. Seawater concentrations above and below 40 %, either left a precipitate at the bottom of the scintillation vial (< 40 %) or eventually separated into two distinct phases(> 40 %).

Figure 6-2 A shows a time course from a DOC excretion experiment in February 1997, where filtered and unfiltered samples were compared 9 h into the time-course. Unfiltered samples had 3 times as much DPM and a pairwise Students t-test comparison showed that they were significantly higher ($P < 0.001$) than the filtered ones (Figure 6-2 A). Autotrophic incorporation of ^{14}C-bicarbonate into the particulate fraction retained on a GF/F filter, was checked for linearity in May 1996. The amount of ^{14}C-bicarbonate taken up per unit was linear during the course of the day (Figure 6-2 B). An example of a DOC excretion time course is shown in Figure 6-3 A, using ^{14}C-bicarbonate as the initial tracer in a 5 day old natural seawater culture supplemented with nutrients. The initial time lag (0-7.5 h) was followed by a linear phase (8-11 h) of DOC excretion (Figure 6-3 A). Subsequently, the external DOC-pool became saturated with DO^{14}C (> 11 h) and a

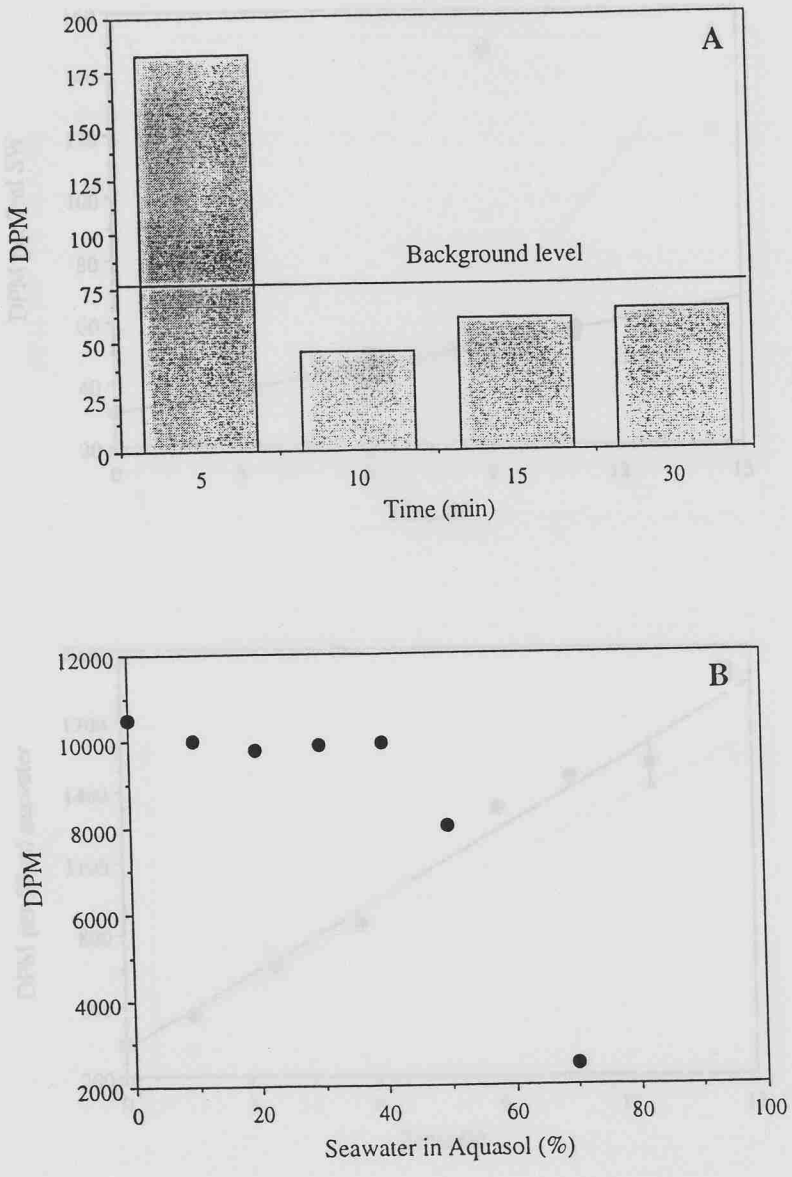

Figure 6-1
A: Measured radioactivity of ^{14}C-bicarbonate (DPM) in acidified seawater samples (7 ml) added 10 ml of Aquasol scintillation cocktail. The acidified seawater samples were purged with air for 5, 10, 15 and 30 min prior to the addition of Aquasol.
B: Impact of quenching by seawater in Aquasol added ^{14}C-bicarbonate.

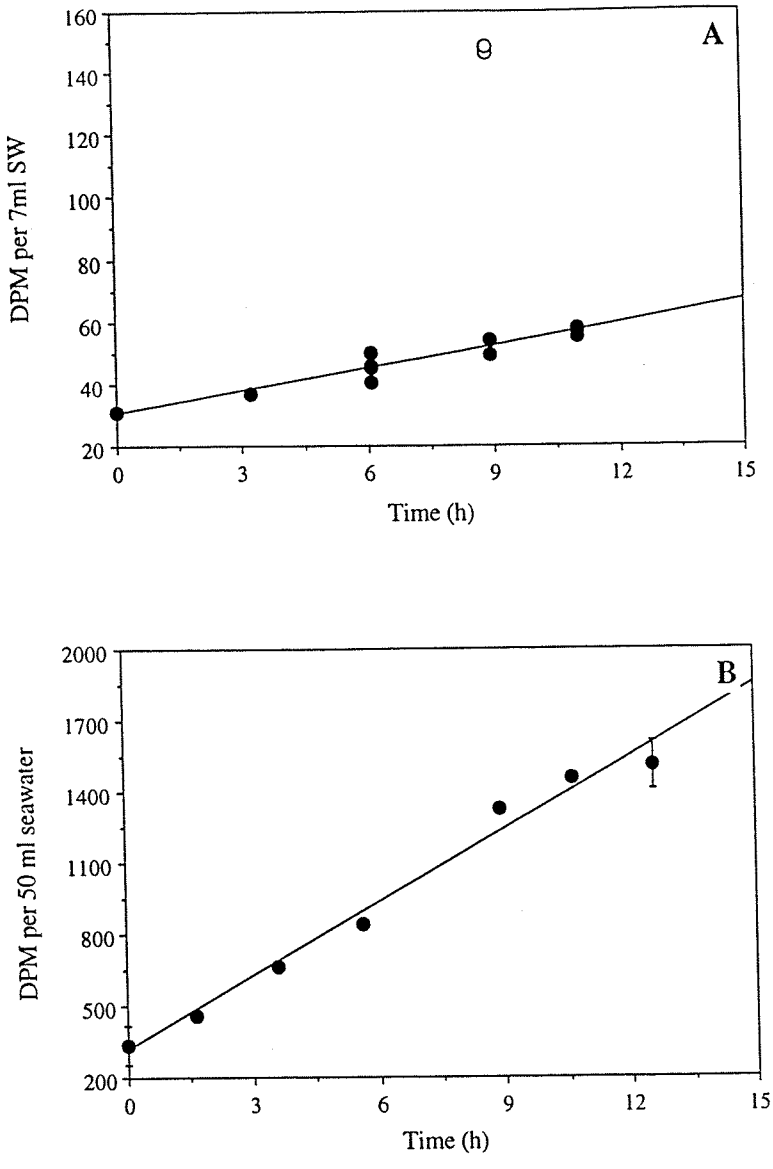

Figure 6-2
A: Time course of DO^{14}C excretion (DPM) in surface seawater at BATS in February 1997 (closed circles). After 9 h, a set of two unfiltered subsamples were taken for comparison (open circles). $y = 30.5 + 2.4\ x$, $r^2 = 0.921$, $n = 12$, $F_{[1,10]} = 118.49$, $p << 0.001$.
B: Phytoplankton incorporation of ^{14}C-bicarbonate (DPM) over the course of a day in surface seawater at BATS in May 1996. $y = 313.9 + 102.3\ x$, $r^2 = 0.971$, $n = 11$, $F_{[1,9]} = 297.84$, $p << 0.001$.

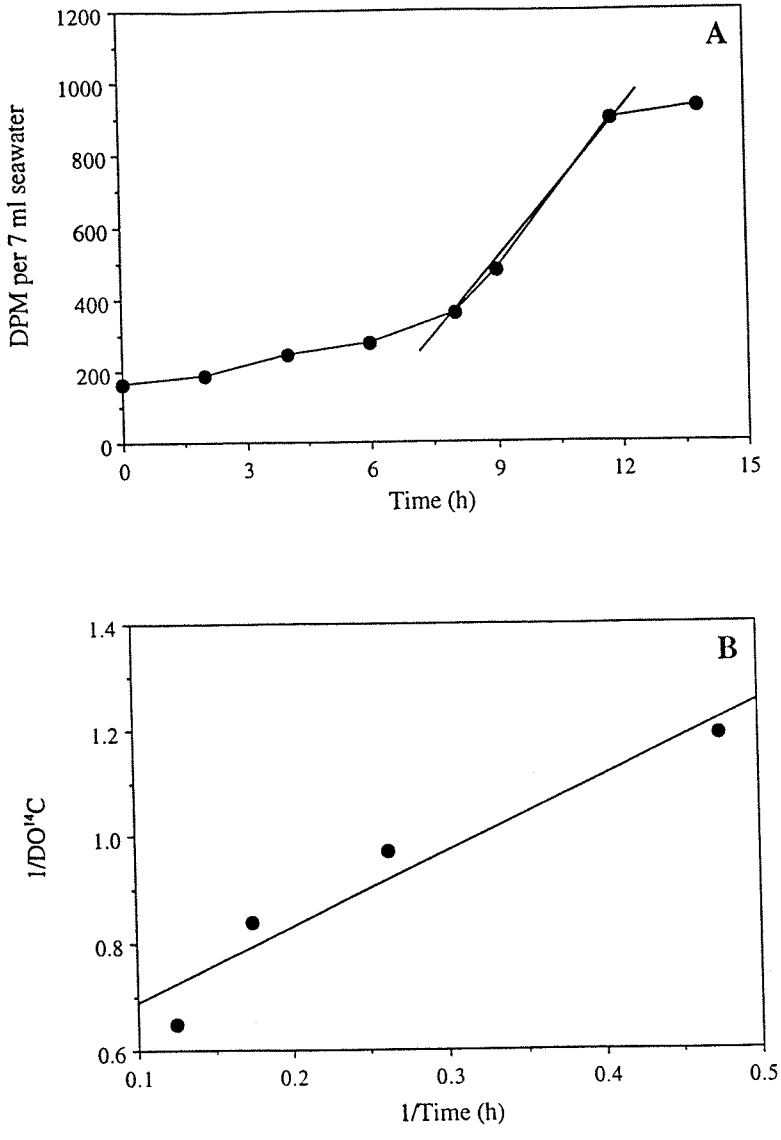

Figure 6-3
A: Time course of $DO^{14}C$ excretion in a 5 day old surface seawater culture with nutrient supplements, taken from BATS in March 1996. The bar fitted by eye shows the area of balanced $DO^{14}C$ excretion.
B: Lineweaver-Burk plot of the log-phase and steady-state of the $DO^{14}C$ excretion time course. Data past 7 h of the time course are included. $y = 0.55 + 1.41$, $r^2 = 0.929$.

steady-state was reached beyond this point in time (Figure 6-3 A). Since the primary production rate was linear over a day (Figure 6-2 B), the DOC excretion rate (CER) could be calculated from the linear (8-11 h) midsection of the curve (Figure 6-3 A).

The steady state section of Figure 6-3 A (> 11 h) depicts the total amount of accumulated net $DO^{14}C$ excreted at a given time (Williams 1990). Recently excreted DOC is only a smaller part of total pool of DOC measured by the high temperature combustion (HTC) technique. The linear phase and the steady-state section of the $DO^{14}C$ excretion time course (Figure 6-3 A) was fitted to a Michaelis-Menten curve. The data were graphed as a Lineweaver-Burk plot in order to get a more accurate estimate of the total amount of net $DO^{14}C$ present (Figure 6-3 B). As a continuation at the saturation level at steady-state of the Michaelis-Menten curve, when T approaches infinity, T^{-1} approaches zero and the intercept (DOC^{-1}) which is the reciprocal of the daily net $DO^{14}C$ (DOC_{Net}) accumulation, can be calculated;

$$DOC_{Net}^{-1} = a \bullet T^{-1} + b$$

when $T^{-1} \rightarrow 0$; $\quad DOC_{Net}^{-1} = b$

[6-1] $\quad DOC_{Net} = b^{-1}$

The $DO^{14}C$ excretion assay in a phytoplankton culture experiment

Natural Sargasso surface seawater from BATS was supplemented with nutrients and incubated in natural daylight mimicking 15 m depth at the BATS site. The daily rate of primary production during the entire experiment (10 days) and the Chl-*a* concentration, is shown in Figure 6-4 A. The decline in DIN and DIP (Figure 6-4 B) was followed by a primary production peak on day 8 and a Chl-*a* peak one day later (Figure 6-4 A). On day 8 the DIN pool was exhausted and both primary production and Chl-*a* were in a steady decline by the end of the experiment at day 10 (Figure 6-4). The total DOC concentration and the assayed CER was measured 5 times during the time course experiment (Figure 6-5 A). CER followed a similar pattern as the rate of primary production (Figure 6-4 A),

while total DOC continued to accumulate from day 8 to 10 (Figure 6-5 A). The dissolved inorganic N:P ratio (mw) remained in the range of 8.5-14.5 until day 7 (Figure 6-5 B). The nutrient concentrations and the N:P ratio declined rapidly after day 7 as the rate of bacterial growth increased by a factor of 5 (Figures 6-4 B and 6-5 B).

The $DO^{14}C$ excretion time course was fitted to a Michaelis-Menten curve and the net $DO^{14}C$ pool was calculated from a Lineweaver-Burk plot using equation 6-1. The concentration of DOC_{Net} and the calculated percentage $DO^{14}C$ of DOC_{Net} increased until day 6 (Table 6-1). At this point in time, bacterial growth increased rapidly (Figure 6-5 B) and DOC_{Net} started declining towards day 10 (Table 6-1). There was a linear relationship between the rate of primary production and the CER ($r^2 = 0.729$, $p < 0.25$) at all stages of the culture experiment except for the senescence on day 10 (Figure 6-6 A). CER and bacterial growth correlated well ($r^2 = 0.959$, $p < 0.025$) over the 4 stages of the growth cycle investigated (Figure 6-6 B).

Table 6-1
The phytoplankton DOC excretion rate (CER) was calculated assuming a 24 h excretion period, total DOC concentration (DOC_{Total}) was measured by the HTC technique and net maximum DOC excretion (DOC_{Net}) was derived from equation 6-1. DOC_{Net} was also calculated as a percentage of DOC_{Total} (% Net).

Day	CER $\mu M\ d^{-1}$	DOC_{Total} μM	DOC_{Net} μM	% Net
0	0.06	63.2	0.7	1.1
5	0.26	65.9	1.8	2.8
6	1.36	67.8	16.1	23.8
8	10.10	76.7	14.5	18.9
10	6.12	79.4	13.4	16.9

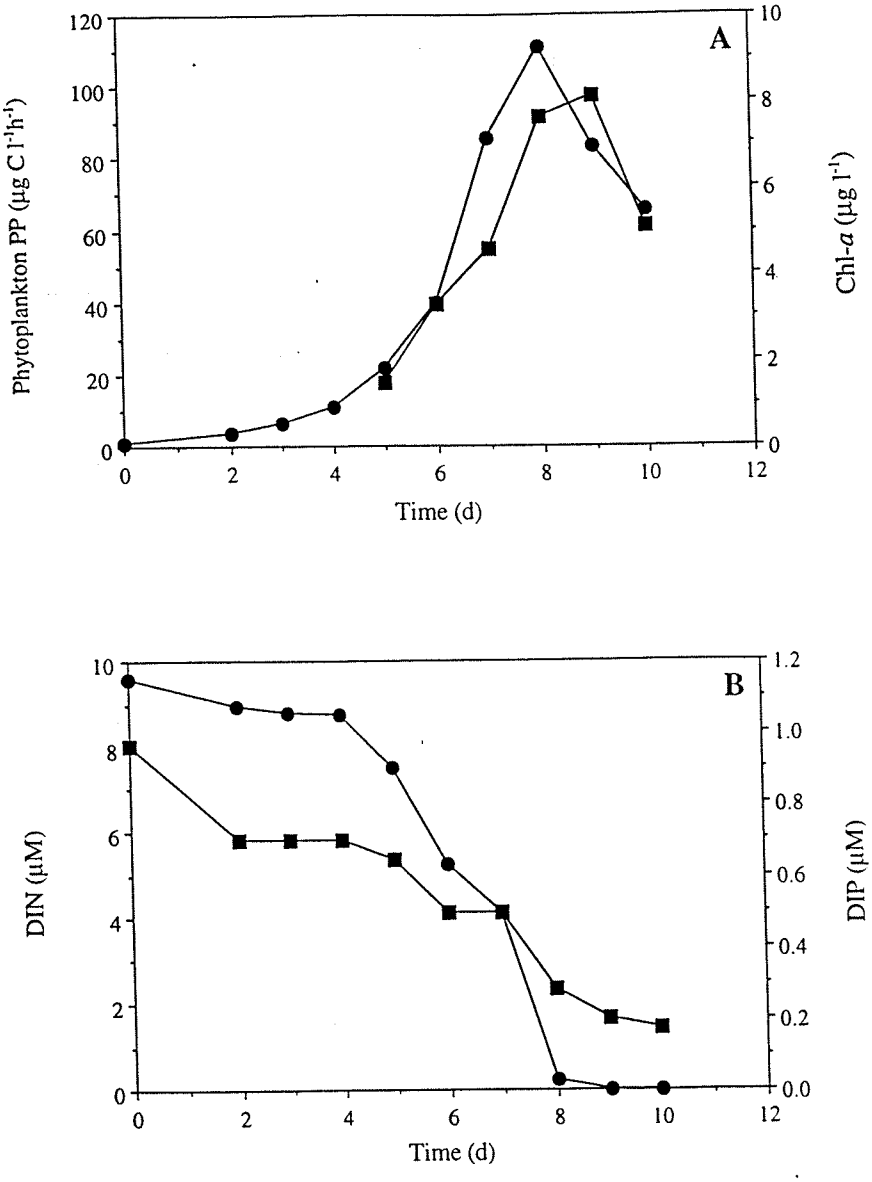

Figure 6-4
Time course experiment with surface seawater from BATS in March 1996. A: The phytoplankton primary production (PP) rate (closed circles) and the Chl-*a* concentration (closed squares) was measured daily during the 10 d experiment. B: The seawater was added DIN (closed circles) and DIP (closed squares) at the beginning of the experiment.

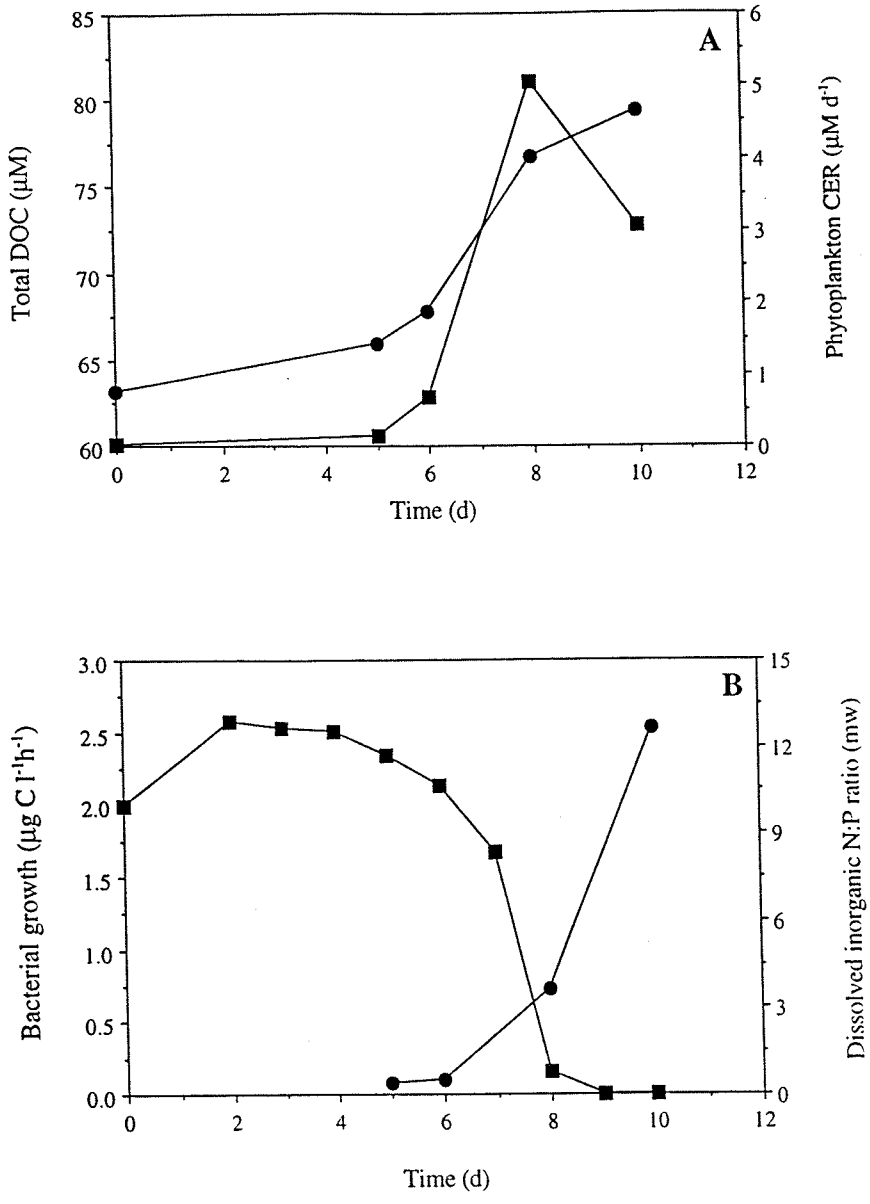

Figure 6-5
Time course experiment with surface seawater from BATS in March 1996. A: The total DOC concentration (closed circles) and the DO^{14}C excretion rate (closed squares) was measured 5 times during the time course. B: Bacterial growth was measured 4 times as the incorporation rate of TdR (closed circles) and the dissolved inorganic N:P ratio (closed squares) was calculated for the entire 10 d period.

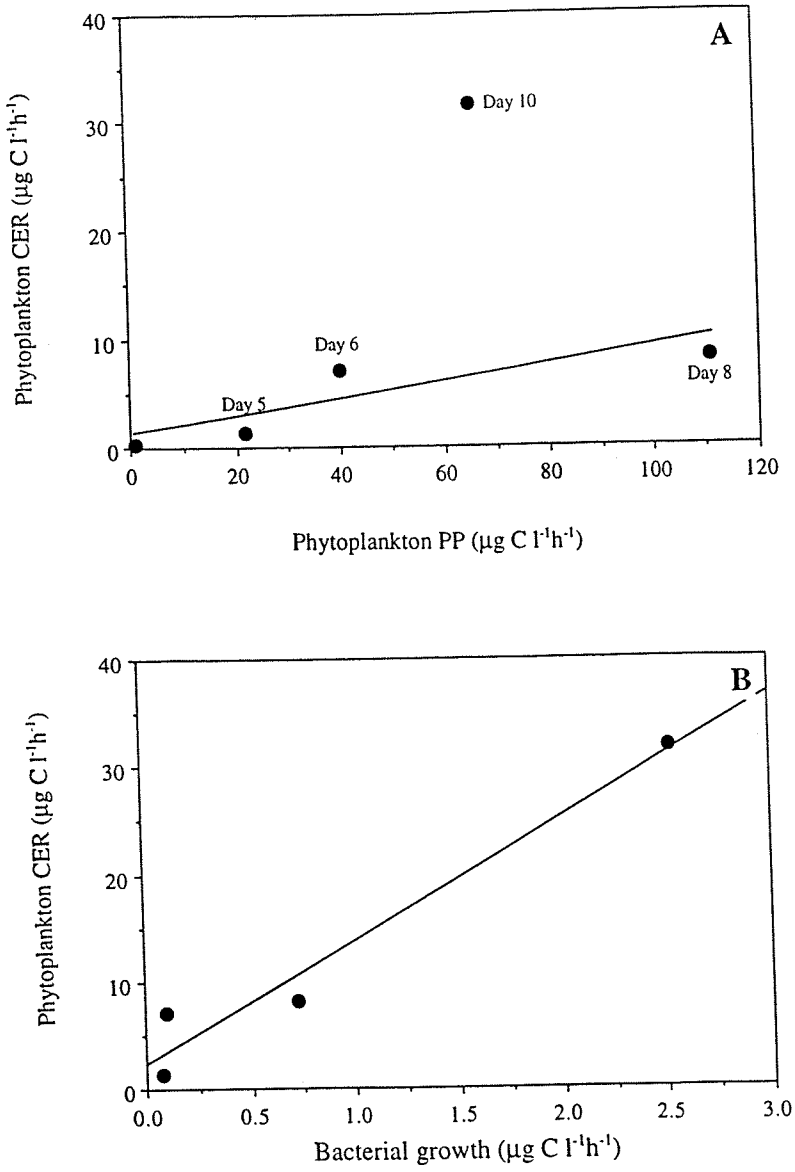

Figure 6-6
A: The measured net DO^{14}C excretion rate as a function of the phytoplankton primary production rate (PP) in aliquots of surface seawater from BATS in March 1996, measured at 0, 5, 6, 8 and 10 days of the culture. Day 10 was treated as an outlier and was not included in the regression. $y = 1.12 + 0.07\ x$, $r^2 = 0.729$, $n = 4$, $F_{[1,2]} = 5.39$, $p < 0.25$.
B: The phytoplankton DO^{14}C excretion rate was also plotted as a function of bacterial growth (BG) at day 5, 6, 8 and 10 of the culture experiment. $y = 2.27 + 11.38\ x$, $r^2 = 0.959$, $n = 4$, $F_{[1,2]} = 46.25$, $p < 0.025$.

DO^{14}C excretion in natural surface seawater samples at BATS

The DOC excretion rate was measured in surface seawater samples at BATS on 15 cruises between January 1996 and May 1997 (Table 6-2). The carbon excretion rate (CER) had a geometric average of 42 % of the primary production rate (range 6-74 %).There was a linear relationship ($r^2 = 0.806$) between the measured CER and the primary production rates (Figure 6-7 A), but no apparent trend was observed between CER and bacterial growth (Figure 6-7 B). Total CER as a percentage of PP in surface waters at BATS , did not change linearly as a power function of PP (Figure 6-8 A). DOC excretion due to *Trichodesmium* primary production (*Trichodesmium* PP) was measured on 8 cruises between June 1996 and February 1997 (Table 6-3).

Table 6-2
Phytoplankton primary production (PP) and the net carbon excretion rate (CER) in natural seawater from 20 m depth at BATS in 1996 and 1997. PP was calculated using 10-11 h daily light cycle (depending on the time of the year) and CER was calculated assuming that the process of DOC excretion takes place 24 h a day (Mague et al. 1980). CER was also calculated as the percentage of PP (CER % of PP).

	PP $\mu g\ C\ l^{-1}\ d^{-1}$	CER $\mu g\ C\ l^{-1}\ d^{-1}$	CER % of PP
1996:			
February	17.88	8.98	50.2
February$_{\text{Bloom cruise}}$	12.59	4.18	33.2
March	4.98	3.66	73.4
April	9.29	3.96	42.6
May	9.68	5.36	55.4
June	12.63	8.58	68.0
July	12.28	5.54	45.2
October	3.95	0.22	5.6
November	3.93	1.36	34.6
1997:			
January	8.97	2.62	29.2
January $_{\text{Bloom cruise}}$	17.76	6.92	39.0
February	5.49	2.26	41.2
March	2.69	0.70	26.0
March	4.51	1.84	40.8
May	4.78	2.22	46.4

Table 6-3
Primary production (PP) and the net carbon excretion rate (CER) associated with the two *Trichodesmium* morphologies, Puff and Tuft. PP was calculated by using a 10 - 11 h daily light cycle (depending on the time of the year) and CER was calculated assuming that the process of DOC excretion takes place 24 h a day (Mague et al. 1979). CER was also calculated as the percentage of *Trichodesmium* PP (CER % of PP).

	PP μg C col^{-1} d^{-1}	CER μg C col^{-1} d^{-1}	CER % of PP
Puff:			
June'96	5.24	0.40	7.6
October'96	3.52	0.25	7.1
November'96	1.71	0.33	19.3
January'97	1.08	0.06	5.5
February'97	1.31	0.24	18.3
Tuft:			
October'96	4.26	0.57	13.4
November'96	2.50	0.42	16.8
February'97	1.98	0.79	39.9

CER was on average 12 % for the puff (range 6-19 %) and 23 % for the tuft (range 7-20 %) primary production rate. A linear regression of LOG CER (puffs and tufts) plotted as a function of LOG *Trichodesmium* PP (Figure 6-8 B) was weak, but showed that CER could be written as a power function of PP;

[6-2] *Trichodesmium* $CER = 0.0798 \bullet PP^{0.784}$ $r^2 = 0.327$, n = 7,

$F_{[1,5]} = 7.95$, $p < 0.005$

The net DOC excretion rate was calculated for surface waters from all BATS cruises in 1995 and 1996, assuming a phytoplankton CER of 42 % of the phytoplankton primary production rate and *Trichodesmium* CER calculated as a power function of *Trichodesmium* PP (Equation 6-2). The daily net $DO^{14}C$ accumulation (DOC_{Net}) was calculated from the $DO^{14}C$ excretion assay (Table 6-2) as described in the culture experiment (Equation 6-1) assuming that the excretion data fitted a Michaelis-Menten curve. The estimated daily accumulation of DOC_{Net} ranged between 0.03-0.73 μM and was on average 0.3 % of DOC_{Total} (range 0.04-1.1 %) (Table 6-4).

Table 6-4
Calculated daily pool of net excreted $DO^{14}C$ (DOC_{Net}) and total DOC measured by the high temperature combustion (HTC) technique of samples from 20 m depth at BATS (DOC_{Total}). DOC_{Net} was also calculated as the percentage of DOC_{Total} (% Net). The DOC_{Total} data are courtesy of D. Hansell and C. Carlson.

Date	$[DOC]_{Net}$ µM	$[DOC]_{Total}$ µM	% Net
1996:			
February	0.35	63.1	0.6
$February_{Bloom\ cruise}$	0.06	63.0	0.1
March	0.10	64.0	0.2
April	0.19	65.8	0.3
May	0.73	68.0	1.1
June	0.25	67.4	0.4
July	0.18	66.5	0.3
October	0.03	67.8	0.04
November	0.04	66.7	0.1
1997:			
January	0.32	62.5	0.5
$January_{Bloom\ cruise}$	0.33	64.4	0.5
February	0.11	64.0	0.2
March	0.04	66.7	0.1
$March_{Bloom\ cruise}$	0.14	65.6	0.2
May	0.07	65.2	0.1

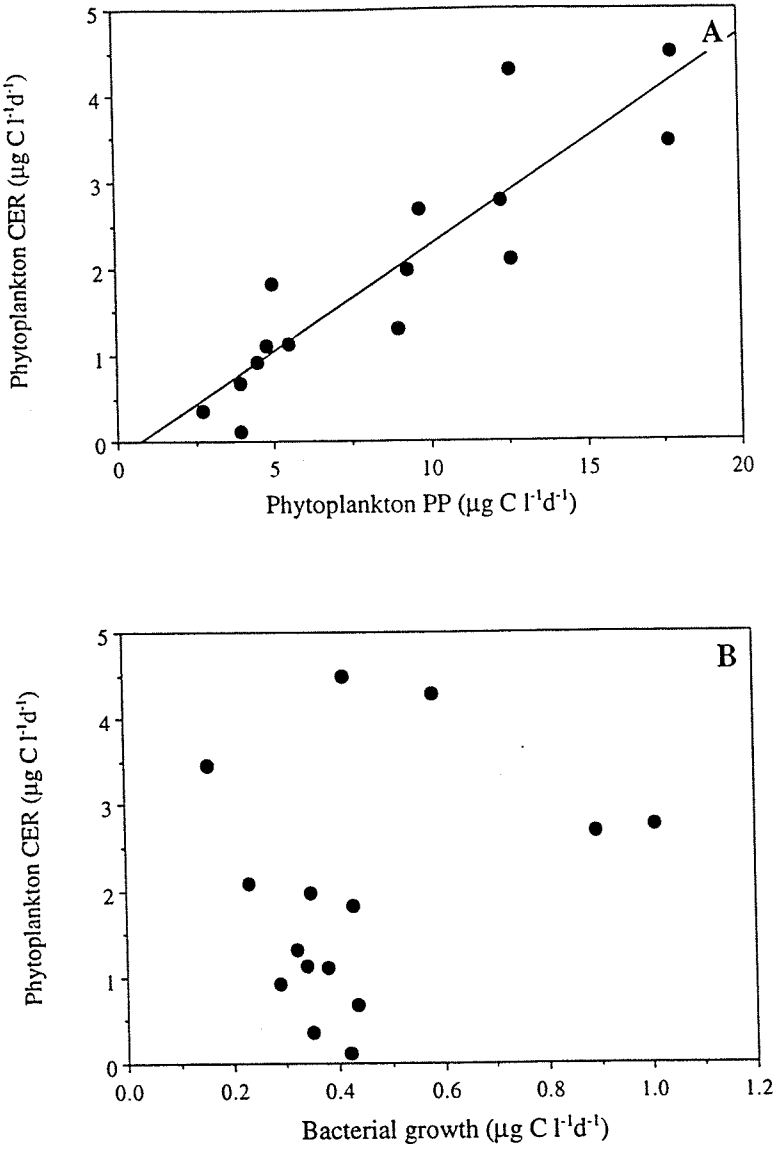

Figure 6-7
A: Net DO^{14}C excretion rate (CER) as a function of the phytoplankton primary production rate (PP) in surface seawater from BATS on cruises in 1996 and 1997.
$y = -0.20 + 0.24\ x$, $r^2 = 0.806$, $n = 15$, $F_{[1,13]} = 53.93$, $p << 0.001$.
B: Net phytoplankton DO^{14}C excretion rate (CER) plotted as a function of bacterial growth (BG) measured on BATS cruises in 1996 and 1997.

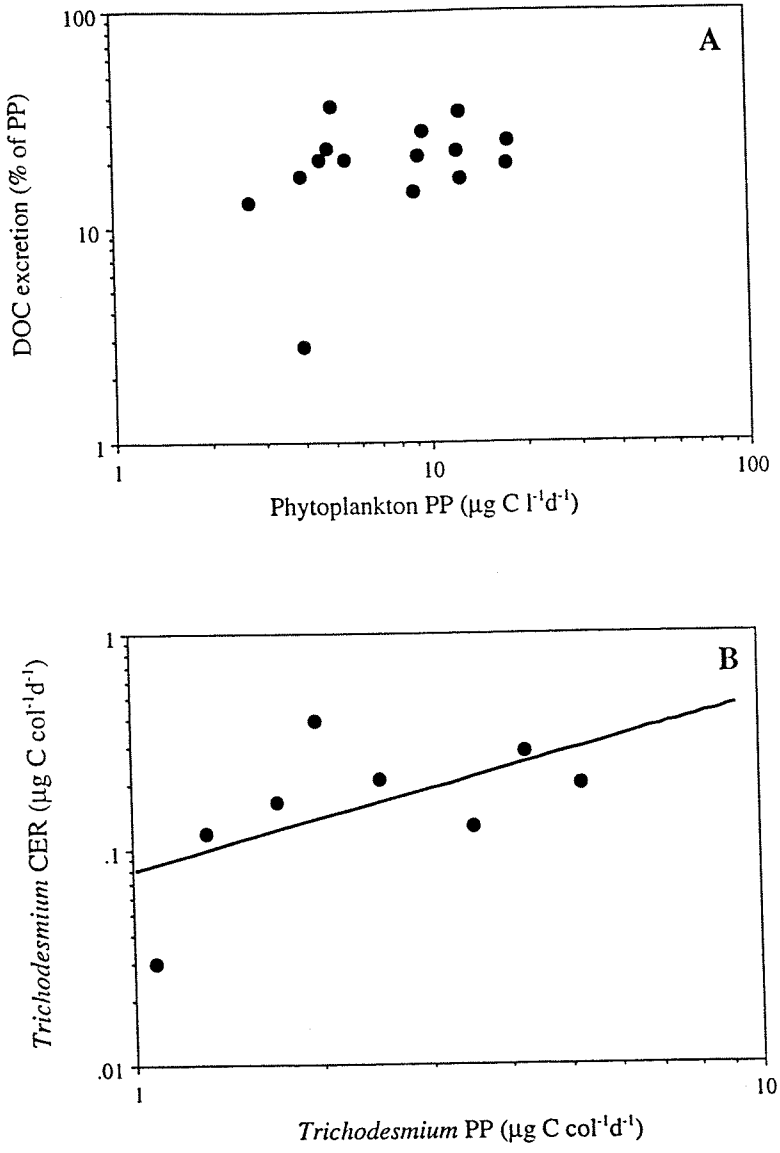

Figure 6-8
A: Phytoplankton DOC excretion rate (CER) calculated as the percentage of the rate of primary production, as a function of phytoplankton primary production in 1996 and 1997.
B: The net $DO^{14}C$ excretion rate of *Trichodesmium* (*Trichodesmium* CER) as a function of *Trichodesmium* primary production (*Trichodesmium* PP). Note the LOG scale.

The LAP activity at BATS

LAP was closely associated with the rate of primary production at BATS: A low primary production rate was recorded in the spring of 1994 with a minor peak in the fall (Figure 1-10) and this was also reflected in the LAP rate the same year (Figure 6-9). The rate of primary production was more than twice as high in 1995 and extended into the summer (Figure 1-10). The same pattern was observed for the LAP rate in 1995 which doubled during the spring bloom and summer (Figure 6-9).

Table 6-5
Leucine aminopeptidase activity (LAP) in surface seawater at BATS in March 1997, manipulated with leucine supplements (Leu). LAP was calculated as the percentage of the activity in natural seawater (% Nat. SW).

[Leu] nM	LAP (nM h^{-1})	% Nat. SW
0	3.3	100
1	3.4	101
3	1.0	31
5	1.7	51
10	2.1	62
21	1.6	50

The LAP activity in natural seawater was lowered by 31-62 % when compared to samples containing L-Leucine concentrations ranging from 3-21 nM, but appeared to have the same level of activity at Leu concentrations less than 3 nM (Table 6-5).

The Leu-MCA half saturation constant (K_m) ranged from 85.1-454.8 μM for bacteria and 37.8-516.0 μM for the puffs and the tufts (Table 6-6). The K_m in natural seawater was five times as high in April and July 1994 while the half saturation constant increased by a factor of fourteen in natural seawater with puffs in July 1994 (Table 6-6). Maximum LAP activity associated with bacteria (V_{max}) ranged from 1.4-25.7 μM h^{-1} while the enzyme activity associated with the *Trichodesmium* puff colonies in January-April 1994 increased by a factor of 6-8 (Table 6-6). LAP assays with a substrate concentration of 308 μM Leu-MCA showed the same range of activity for bacteria in natural surface

seawater (1.5-17.4 nM h^{-1}), while the activity associated with *Trichodesmium* colonies, normalised to volume seawater, was 10^3 times lower in spring but approached the same order of magnitude as natural seawater in the fall (Table 6-7).

Table 6-6
LAP in natural surface seawater (nM h^{-1}) and associated with *Trichodesmium* colonies (nmol $col^{-1}h^{-1}$). The half saturation constant K_m (μM) and V_{max} was estimated from a Lineweaver-Burk plot and n is the number of substrate concentrations used in the assay.

	K_m	V_{max}	n	r^2	F_s	p
Natural seawater:						
December 1993	85.1	25.7	4	0.992	261.8	<0.005
April 1994	454.8	17.4	5	0.997	861.0	<<0.001
July 1994	436.5	1.4	7	0.986	341.0	<<0.001
Puff:						
December 1993	98.3	33.2	4	0.610	3.1	<0.25
January 1994*	68.5	265.5	4	0.538	1.1	<0.5
April 1994	37.8	197.8	4	0.361	2.3	<0.5
July 1994	516.0	50.7	6	0.793	11.5	<0.05
Tuft:						
January 1994*	107.5	343.4	3	0.992	120.3	<0.005

* = LAP assay run in surface seawater from Hydrostation 'S'

Table 6-7
A comparison of LAP in natural surface seawater and associated with *Trichodesmium* colonies at BATS in 1994 and 1996. LAP activity was calculated per colony (nmol col^{-1} h^{-1}) and per volume (pM h^{-1}), using the surface *Trichodesmium* colony abundance data from Chapter 4. ND = No data available.

	Seawater (nM h^{-1})	Puff (nmol col^{-1} h^{-1})	Abundance (col m^{-3})	Puff (pM h^{-1})	Tuft (nmol col^{-1} h^{-1})	Abundance (col m^{-3})	Tuft (pM h^{-1})
1994:							
January	4.5	206.9	0.02	4.1	240.3	0.01	2.4
April	7.9	210.0	0.67	140.7	274.0	0.21	57.5
July	1.5	26.2	2.08	54.5			
1996:							
June	3.7	178.4	3.17	565.5	ND	ND	ND
November 6	5.0	155.6	6.28	977.2	ND	ND	ND
November 13	17.4	155.6	6.28	977.2	ND	ND	ND

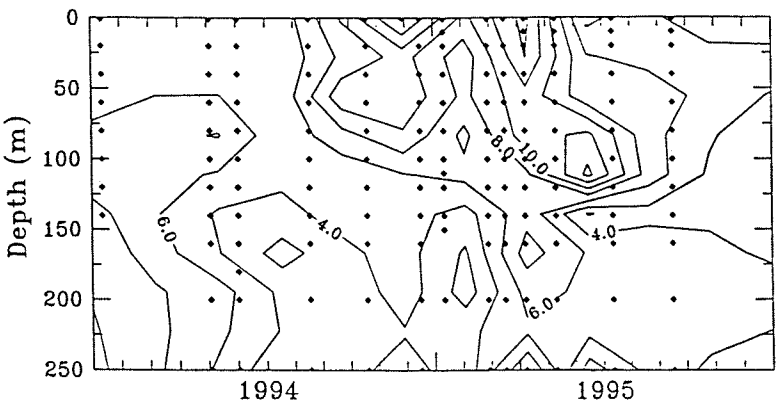

Figure 6-9
Contour plot of the leucine aminopeptidase activity (LAP) at the BATS site in 1994 and 1995. Contour interval is 2 nM h^{-1}. The black diamonds shows sampling depths and sampling frequency of the assay.

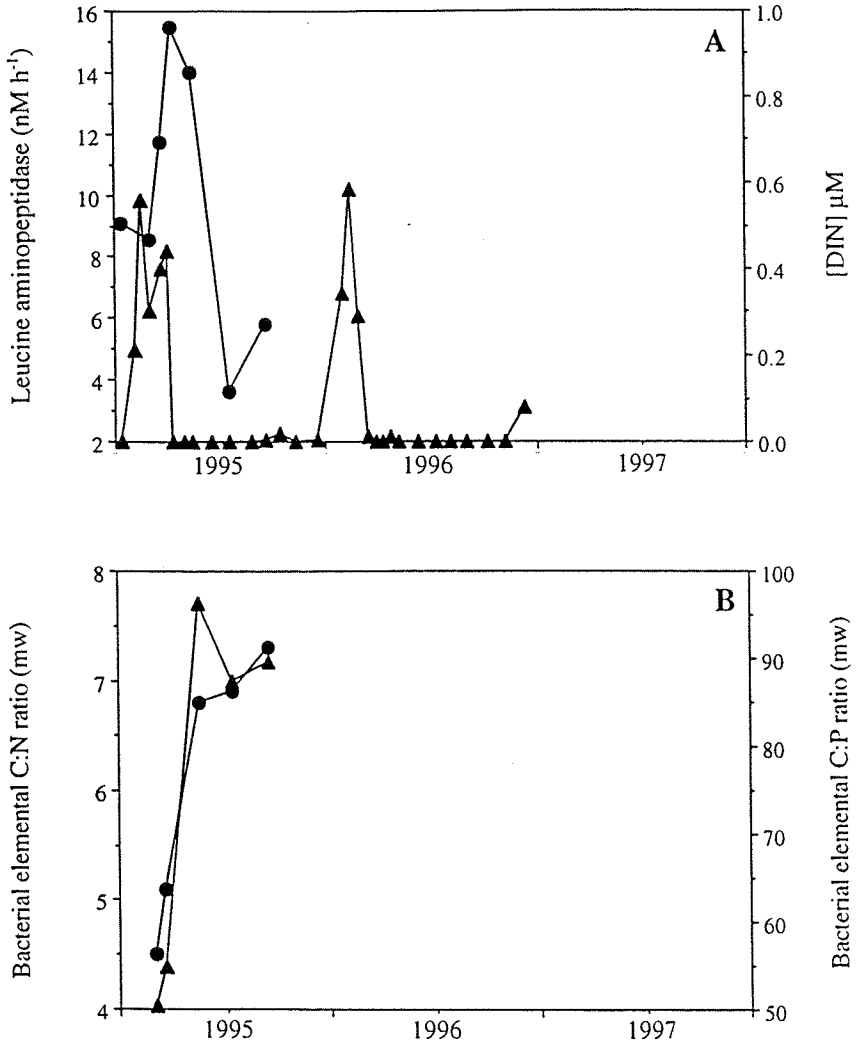

Figure 6-10
A: Surface leucine aminopeptidase (LAP) activity (closed circles) and the concentration of dissolved inorganic nitrogen (DIN) at the BATS site (closed triangles). The LAP activity rates and DIN concentrations were averaged from 1, 10 and 20 m depth.
B: The elemental C:N (closed circles) and C:P (mw) ratio (closed triangles) in bacteria from 10 m depth at the BATS site.

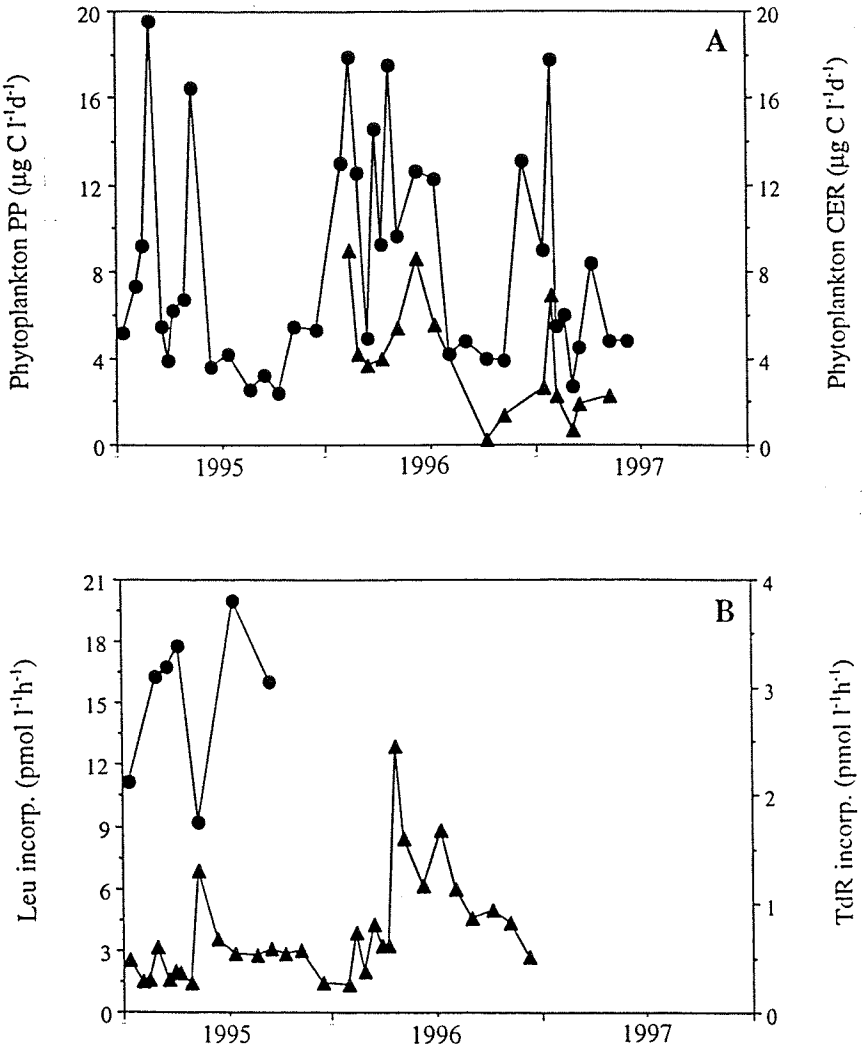

Figure 6-11
A: The average rate of phytoplankton primary production (PP) from 1 and 20 m depth (closed circles) and the net daily DOC concentration (DOC_{Net}) calculated from the $DO^{14}C$ excretion assay from 20 m depth at BATS (closed triangles).
B: The leucine (Leu) incorporation rate (closed circles) and thymidine (TdR) incorporation rate (closed triangles) of bacteria in surface waters at BATS. Leu was calculated as the average of samples assayed from 1, 10 and 20 m depth and TdR was calculated as the average from samples assayed from 1 and 20 m depth.

Nutrient limitations for bacterial growth

Surface seawater concentrations of dissolved inorganic nitrogen (DIN) appeared as a near surface peak each spring in 1995 and 1996, following the winter mixing of the water column (Figure 6-10 A). Approximately a month after the DIN peak the LAP activity rate was at a maximum (Figure 6-10 A). In the same period of time the average single cell elemental C:N and C:P (mw) ratios of bacteria doubled (Figure 6-10 B). Even though the primary production and the LAP assay was done on natural seawater sampled 2-3 days apart at the BATS site, the peak period of primary production in 1995 (Figure 6-11 A) very often appeared to co-occur with peak LAP activity (Figure 6-10 A). The measured CER activity in surface waters at the BATS site in 1996 and 1997, was on average 2.5 times less active than the rate of primary production and occurred during or at the end of peaks in phytoplankton carbon assimilation (Figure 6-11 A). Bacterial carbon assimilation of leucine (Leu) co-occurred with the primary production maximum and peak LAP in 1995 (Figures 6-10 A and 6-11 B). The increase and build-up of bacterial biomass (Leu incorporation) was followed by a peak in cell synthesis and TdR incorporation in 1995 (Figure 6-11 B). Peak TdR incorporation occurred as Leu incorporation was at a minimum and the two rates appeared to be inversely related (Figure 6-11 B).

6.4 DISCUSSION

DOC excretion at BATS

In order to calculate the DOC excretion rate by using the ^{14}C -label approach, a few assumptions needed to be made: 1) The isotope tracer (^{14}C) should be identical and non-preferentially taken up chemically to the inorganic carbon (^{12}C and ^{13}C) present in seawater. If isotope discrimination occurs, the calculated rate should be corrected for this.

2) There should be no imbalance between the external and internal ^{14}C-labelled DIC pool of the autotrophic algal cell, when measuring the DOC excretion rate. 3) Since there is an initial time-lag due to the isotope dilution inside the cell and only the mid part of the sigmoid excretion curve can be used to calculate the DOC excretion rate, the diel rate of photosynthesis must be linear. 4) With the presence of microheterotrophs (i.e. bacteria) and bacterivore and herbivore grazers, the measured CER is only the net and not the gross rate of DOC accumulation.

Most isotope tracer studies, including ^{14}C-bicarbonate, have excess amounts of the label added to the medium in order to avoid or disseminate the uptake of unlabelled substrate. The ^{14}C isotope has been reported however, to be taken up at a slower rate than the natural ^{12}C and ^{13}C isotopes. This is usually compensated for by a correction factor ($f = 1.05$) applied to the primary production rate calculation (Strickland & Parsons 1972). The imbalance between external and internally labelled pools of $DI^{14}C$ was evident from the first 7 h of the culture experiment (Figure 6-3 A) and hence, only the time course after label saturation (and prior to steady-state) was used for the CER calculation (Williams 1990). In order to do this rate calculation, the rate of primary production (PP) had to be linear over the course of a day. Linear autotrophic carbon assimilation as measured by the ^{14}C technique (Figure 6-2 B), has also been reported in several other studies (Dring & Jewson 1982, Li et al. 1980, Li & Harrison 1982, Goldman et al. 1981 and Marra et al. 1988). In an ecosystem characterised by a tight coupling between DOC excretion and heterotrophic growth, the primary DOC used by the bacteria may not only appear as a part of the bulk DOC present in seawater. A major source of the DOC excretion from primary production is also likely to be immediately incorporated by the bacterial cells (Chapter 5). CER may also be a product of "secondary DOC" that is not immediately used by the bacteria and the by-products from sloppy feeding performed by the bacterial grazers and other herbivore and carnivorous zooplankton.

Method checks of the $DO^{14}C$ excretion assay

Due to the apparent rapid response of bacteria to the increase in primary production at BATS (Figure 5-1), CER and DOC accumulation was expected to be low compared to areas with higher primary production rates and relatively slower rates of heterotrophic C incorporation. Consequently, it was of crucial importance to eliminate any possible contamination of inorganic ^{14}C and quenching by seawater, in samples analysed for $DO^{14}C$. Typical background radiation for natural seawater is 50-100 DPM on a Packard Tricarb 2000 CA Liquid Scintillation Analyzer. In order to remove all inorganic ^{14}C from the $DO^{14}C$ samples, acidified seawater samples were always purged with air (containing more than 80 % N_2) for more than an hour. Sharp (1977), Wiebe & Smith (1977) and Mague et al. (1980), applied the same method check and purged the acidified samples with N_2, air and CO_2 respectively. Coincident with this experiment (Figure 6-1 A) inorganic ^{14}C was successfully purged within 5-10 min below background level in all three studies (Sharp 1977, Wiebe & Smith 1977, Mague et al. 1980). The amount of seawater exceeding 40 % of the total volume in a scintillation cocktail sample, may interfere (quench) with the counting efficiency of $DO^{14}C$ (Figure 6-1 B). Therefore, the amount of seawater and scintillation cocktail was mixed in a ratio of 7 to 10 respectively.

The $DO^{14}C$ excretion assay in a phytoplankton culture experiment

In order to simulate four different scenarios of a phytoplankton primary production growth cycle, an experiment with nutrient enriched Sargasso seawater was conducted in May 1996. As the primary production responded to the initial nutrient input (9.8 μM DIN and 1 μM DIP), the level of inorganic nutrients decreased (Figure 6-4). Depletion of DIN at day 8 (Figure 6-4 B) eventually caused a decline in the primary production (Figure 6-4 A). Four different phases of the primary production (pre-bloom, log-phase growth, steady state and decline of the bloom) during different levels of nutrient stress, were chosen during the time course, in order to investigate the relation between phytoplankton primary production and CER. A linear relationship ($p < 0.25$)

was found between the rate of primary production and CER (Figure 6-6), with the only exception on day 10 during bloom senescence when CER was more than 4 times higher than the primary production rate (Figure 6-6 A).

DO^{14}C excretion in natural surface seawater samples at BATS

An average CER of 42 % (SE= 17) of the rate of primary production, was calculated from the field DOC excretion measurements in this study (Table 6-2). Sharp (1977) gave an overview of net DOC excretion rates up until 1975 and found CER in the range of 1-67 % of the rate of primary production. More recently, net DOC excretion rates were reported from Antarctic waters (40-50 %; Karl et al. 1991), Arctic waters (50-70 %; Passow et al. 1994, Vernet et al. 1994) and coastal waters such as the English Channel (20-60 %; Lancelot 1979) and Chesapeake Bay (15 %; Malone et al. 1991). Malinsky-Rushansky & Legrand (1996) reported that larger cells excreted less DOC (4-5 % of primary production) than pico-eukaryotes (29 %). They also found that the excretion rates were more related to algal speciation than to cell volumes (Malinsky-Rushansky & Legrand 1996). Søndergaard & Middelboe (1995) recently reviewed the literature on DOC excretion rates and concluded that eutrophic as opposed to oligotrophic systems, experienced higher concentrations of labile DOC. Lancelot (1979) concluded as Sharp did two years earlier, that the wide range of reported excretion rates may to some degree be caused by method problems associated with eliminating superfluous inorganic ^{14}C. Contamination can be avoided by maximising the sample volume and by purging the seawater sample in order to eliminate contaminating ^{14}C-bicarbonate (Thomas 1971, Williams & Yentsch 1976, Sharp 1977, Wiebe & Smith 1977 and Mague et al. 1980). The samples in this study were purged for a minimum of 30 min, re-shaken and purged again for another 30 min. Great care was taken to clean the inside of the cap of each scintillation vial with a tissue paper before scintillation cocktail was added to the sample. Finally, the sample was capped and homogenised on a vortex mixer.

When a tight coupling exist between the source of DOC production and heterotrophic growth, bacteria can effectively modify the composition of the excretory

products (Smith 1974, Nalewajko et al. 1976). Under these circumstances, CER is the net DOC excretion rate that is being measured. Lancelot (1979) found an average excretion rate of 26 % in the North Atlantic and Johnson et al. (1981) reported a net excretion rate of 23-43 % from Caribbean waters, which is within the same range as the estimated average of 42% of the daily primary production rate found in this study (Table 6-2). DOC is produced in a series of processes, such as phytoplankton exudation (Lancelot 1979), exoenzyme hydrolysis of particulates and high molecular compounds (Smith et al. 1992), viral lysis of cells (Bratbak et al. 1990, Suttle et al. 1990) and is also released during zooplankton grazing (Lampert 1978, Jumars et al. 1989, Nagata & Kirchman 1992, Peduzzi & Herndl 1992). Although a DOM release pathway through zooplankton feeding behaviour and excretion has been known for some time (Lampert 1978), the concept received renewed interest in the late eighties when it was reintroduced by Jumars et al. (1989). Jumars et al. (1989) proposed a second source/pathway for DOC production; sloppy-feeding and DOC dissolution from faecal pellets. Peduzzi & Herndl (1992) later showed that cultures of phytoplankton and zooplankton had elevated concentrations of monomeric carbohydrates and higher bacterial abundance. They identified three pathways of zooplankton DOC excretion; dissolution from faecal matter, excretory release and breakage/leaking through sloppy-feeding. Due to the small size of the incubation bottles used in this study (250-1000 ml), macro-zooplankters were not adequately represented in the DOC excretion measurements. Fortunately, macro-zooplankton biomass at BATS is negligible (Roman et al. 1995) and hence, their impact on the DOC excretion rate was considered of minor importance. Due to their size, meso, nano and micro-plankton would all be included in the seawater sample volumes used for the DOC excretion time course experiment. Apart from bacteria, these groups also constituted the most dominant heterotrophic biomass at BATS by 14, 34 and 7 % respectively of the total biomass (Roman et al. 1995).

Net DOC only accumulates in the mixed layer at BATS after the spring primary production peak (Carlson et al. 1994 and Table 6-4). The majority of the accumulated DOC has been suggested to be of semi-labile quality (Carlson & Ducklow 1996) and is

not as readily consumed as "freshly produced" DOC. Three different pathways can be identified for inorganic carbon (^{14}C) entering the primary production synthesis: Particle assimilation of ^{14}C (PP), net $DO^{14}C$ excretion (CER) and microbial consumption of $DO^{14}C$. Since the term "labile DOC" has already been designated to the secondary DOC not immediately taken up by the bacteria (Carlson et al. 1995), a new set of terms is proposed in this study in order to describe the different fractions of DOC (Figure 6-12 A): *Primary DOC* is taken up directly by bacteria from the source of primary production. This source of DOC is not present as a part of the stock dissolved in seawater as long as bacteria are C-limited and can only be released when there is a decoupling between bacterial growth and the rate of primary production. Consequently, elevated concentrations of bulk DOC may appear more frequent in eutrophic than in oligotrophic ecosystems. *Secondary DOC* (labile DOC) is not taken up by bacteria directly, but is utilised at a later stage when primary production and the access to primary DOC is low. Secondary DOC, measured as CER (rate) and DOC_{Net} (bulk) in this study, may also to some extent be a by-product of viral lysis of bacterial cells and sloppy-feeding caused by meso, nano and micro-heterotrophic plankton grazing on bacteria. Secondary DOC is utilised after the springbloom period in the Sargasso Sea (Carlson et al. 1994). Recent studies have also shown that during situations such as a decoupling between the producers and the heterotrophs, excreted primary DOC that is not utilised immediately is "ageing" and may turn into secondary DOC that is not as readily obtainable after some time (Tranvik & Kokalj 1998). Therefore, bacterial growth efficiency (BGE) calculations made on bulk DOC material present *in situ*, may not reflect the actual direct uptake and growth efficiency that exist between the primary producers and the heterotrophs. *Tertiary DOC* (non-labile DOC) is a major part of bulk DOC present in the euphotic zone and this source of DOC is not readily available as a carbon source for bacterial growth. Tertiary DOC has been found to be utilised by bacteria when introduced to waters below the seasonal mixed layer at BATS during the winter mixing (Carlson et al. 1994). It is still uncertain however, whether this draw-down of the DOC concentration is caused by different strains of bacteria below the euphotic zone (Carlson et al. 1994) or by the mere

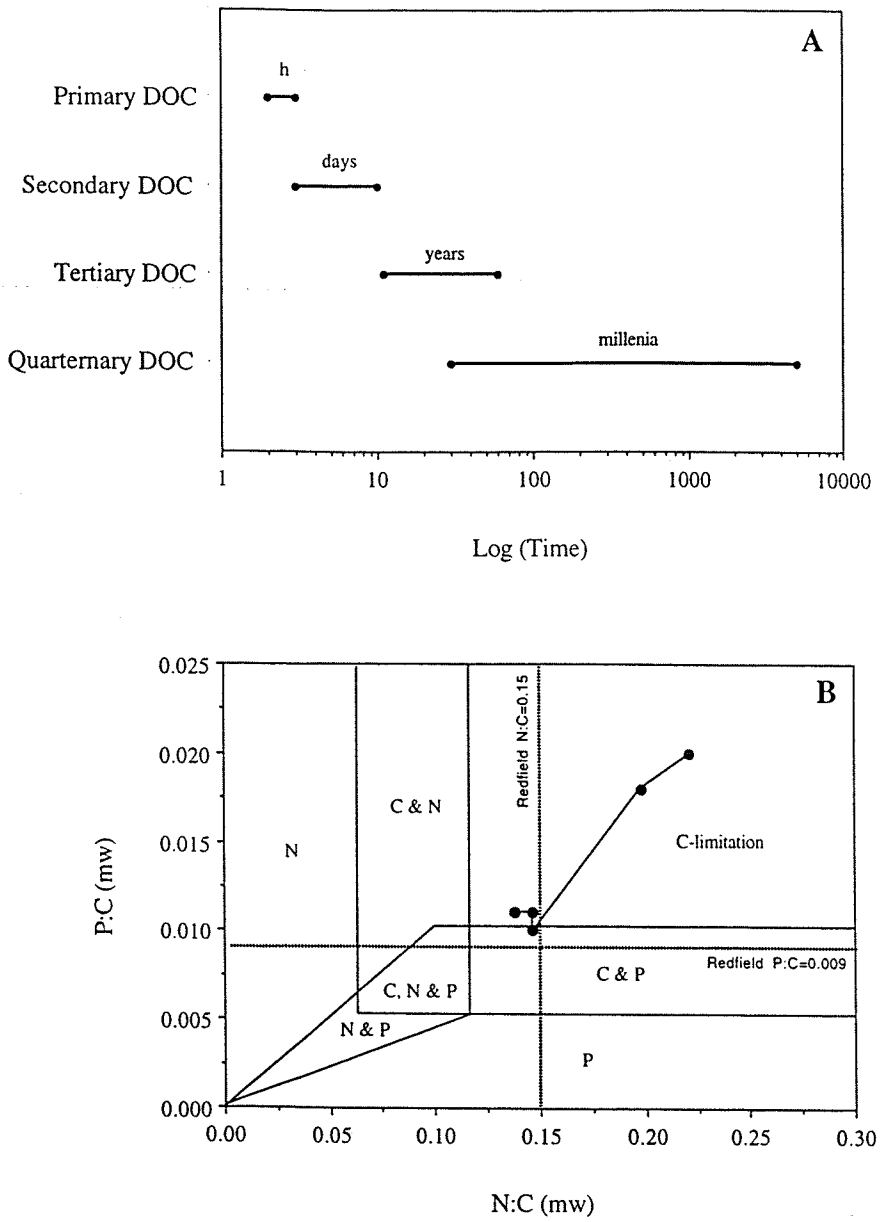

Figure 6-12
A: Schematic presentation of the turnover time (hours, days, years and millennia) in different fractions of DOC. The graph is a compilation based on information from Barber (1968), Menzel (1970) and Carlson et al. (1994).
B: Elemental N:C versus P:C ratio (mw) in bacteria. Border limitations were derived by Martinussen & Thingstad (1987) from continuos cultures of bacteria subjected to various levels of C, N and P stress. Dotted lines are the inverted Redfield ratios of C:N (6.7) and C:P (106). Filled circles are data from Table 3-7 in this study.

Table 6-8
Overview of bacterial growth efficiencies (BGE) measured in natural seawater at ambient DOC concentrations.

Location	% BGE (range)	Technique	Reference
Freshwater, brackish water and estuaries:			
Lake Erken	25 (20-27)	ΔO_2, TdR	Bell & Kuparinen 1984
Roskilde Estuary	20 (11-27)	ΔDOC, ΔPOC	Bjørnsen 1986
Roskilde Estuary	42 (ND)	ΔO_2, TdR	Sand-Jensen et al. 1990
Mississippi River plume	19 (10-22)	ΔO_2, TdR	Chin-Leo & Benner 1992
Mississippi River plume	29 (9-42)	ΔO_2, Leu	–
Freshwater	31 (22-46)	ΔDIC, ΔPOC	Kristiansen et al. 1992
NW Florida Estuaries	43 (28-58)	ΔO_2, ΔPOC	Coffin et al. 1993
Florida, inshore waters	47 (32-61)	ΔDOC, ΔPOC	Kroer 1993
Baltic Sea	27 (11-54)	ΔDOC, ΔPOC	Zweifel et al. 1993
Coastal areas:			
Off Georgia	6 (2-11)	ΔO_2, TdR	Griffith et al. 1990
Santa Rosa Sound, Florida	16 (0.4-35)	ΔO_2, ΔPOC	Coffin et al. 1993
Santa Rosa Sound, Florida	30 (26-33)	ΔDOC, ΔPOC	Kroer 1993
Gulf of Mexico	61 (ND)	ΔDOC, ΔPOC	–
Gulf of Mexico, shelf	45 (38-55)	ΔO_2, Leu	Biddanda et al. 1994
Open ocean:			
North Atlantic	27 (25-28)	$\Delta DO^{14}C$, $\Delta PO^{14}C$	Lancelot 1979
Southern Ocean	39 (38-40)	ΔDIC, TdR	Bjørnsen & Kuparinen 1991
North Atlantic Ocean	5 (2-8)	ΔDOC, ΔPOC	Kirchman et al. 1991
Gulf of Mexico, slope	23 (19-26)	ΔO_2, Leu	Biddanda et al. 1994
Sargasso Sea	7 (4-9)	ΔCO_2, TdR	Hansell et al. 1995
Gulf of Mexico, off shore	6 (2-16)	ΔO_2, Leu	Pomeroy et al. 1995
NW Sargasso Sea	14 (7-19)	ΔDOC, ΔPOC	Carlson & Ducklow 1996

presence of inorganic nutrients (N and P) at these depths. *Quaternary DOC* (refractory DOC) is the largest part of bulk DOC in the ocean. This fraction of the DOC is highly refractive and has an estimated turnovertime of several 1000 years (Barber 1968, Menzel 1970).

Very little is still known about the exact composition of DOM excreted from phytoplankton. However, it has been shown that DOM is rich in carbohydrates, nitrogen compounds such as aminoacids, proteins and polypeptides, fatty acids and organic acids (Hellebust 1974, Lancelot 1984). A summary of the BGE from the literature (Table 6-8),

Table 6-9

Overview of bacterial growth efficiencies (BGE) in detrital organic matter and pure substrate additions to natural seawater.

Substrates	% BGE (range)	Technique	Reference
Detrital organic matter:			
Phytoplankton detritus	10 (8-13)	ΔDOC, ΔBiovolume	Newell et al. 1981
Plant detritus	13 (9-24)	ΔPOC, ΔBiomass	Linley & Newell 1984
Phytoplankton	ND (13-24)	ΔPOC, ΔBiomass	Linley & Newell 1984
Dolioid faeces	ND (10-20)	ΔPOC, TdR	Pomeroy et al. 1984
Phytoplankton detritus	23 (17-27)	ΔO_2, ΔBiovolume	Bauerfeind 1985
Pure substrates and exudates:			
Amino acids (short term)	78 (66-87)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Williams 1970
Glucose (short term)	67 (57-77)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	–
Phytoplankton hydrolysate	18 (14-26)	ΔO_2, ΔBiovolume	Sorokin 1971
Amino acids (short term)	69 (40-97)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Crawford et al. 1974
Exudates (short term)	77 (69-82)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Iturriaga & Hoppe 1977
Exudates (short term)	65 (57-70)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Bell & Sakshaug 1980
Amino acids (short term)	32 (16-50)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Billen et al. 1980
Glucose (short and long term)	35 (20-50)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	–
Glucose	ND (40-69)[1]	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Iturriaga & Zsolnay 1981
Glycine	ND (24-76)[1]	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	–
Glycolic acid	ND (10-25)[1]	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	–
Amino acids (short term)	ND (50-95)	$\Delta^{14}CO_2$, $\Delta PO^{14}C$	Williams 1981
Amino acids (monosaccharide)	ND (10-30)	TdR	Billen & Fontigny 1987
Glucose and NH_4^+	ND (40-94)	ΔPOC (fraction of C_{max})	Goldman et al. 1987
Amino acids (short term)	ND (53-65)	Δ^3H_20, ΔPOC	Fuhrman 1990
Amino acids (short term)	34 (ND)	ΔDFAA, TdR	Kirchman 1990

[1)] BGE depending on the duration of the incubation time

shows that a higher BGE can be found in coastal areas (average 32 %), freshwater, brackish water and estuaries (average 31 %), while offshore regions (average 17 %) are generally lower. The average BGE from coastal marine and limnetic areas, as shown in Table 6-8, is closer to the growth efficiencies measured from pure substrates (average 55 %, Table 6-9), while the offshore BGE closely resembles the one derived from detrital organic matter (average 15 %). Tables 6-8 and 6-9 indicate that the calculated BGE is source dependant; large amounts of pure or easily assimilated substrates will result in a high growth efficiency. The immediate response in the bacterial growth rate to the primary production spring bloom at BATS, may also suggest that the phytoplankton excretory products are immediately utilised by the bacteria at this stage and that the DOC excreted is

closer in resemblance to pure substrates than detrital organic matter. Due to the initial C-limitation in bacteria and the apparent tight coupling between the rate of DOC excretion and the bacterial growth rate, photosynthetic products excreted from the primary producers as primary DOC may never appear in the quantifiable bulk fraction at BATS during the spring bloom. Hence, if primary DOC rarely exists in the dissolved organic phase, BGE measurements done on bulk DOC will not reflect the true growth efficiency in this environment. The higher rates of BGE measured from ambient DOC concentrations in inshore waters also indicates that these waters are experiencing a decoupling between the primary producers (DOC excretion) and bacteria (DOC consumption) during bloom conditions and hence, primary DOC is more likely to be present as a part of bulk DOC due to the mere size and longevity of the bloom, resulting in a higher growth efficiency than in offshore waters. During the spring bloom period at BATS, characterised by high excretion rates of primary DOC, BGE may approach the efficiencies obtained from pure substrates in Table 6-9 (average 50 %). However, in summer and fall, when the build-up of secondary DOC from the spring bloom period is utilised to a greater extent in addition to the continuing but lesser flow of primary DOC from the primary producers, the actual BGE at the BATS site may resemble the growth efficiencies found in coastal waters (average 30 %) in Table 6-9. This is possible since the grazing rate on the bacterial population is approximately equal to the growth rate at the time and hence, the presence of recyclable DON and DOP from sloppy feeding, digestion and egestion will be at a maximum. Therefore, a true BGE for the BATS site may be between 30-50 % depending on the time of year, which is 2-4 times higher than the BGE derived from bulk DOC measurements in the same area (Carlson & Ducklow 1996).

The LAP activity at BATS

The similar time-series patterns observed for LAP and phytoplankton PP at BATS (Figure 6-9, Figure 1-10) shows that elevated rates of phytoplankton photosynthesis and hence, increasing rates of DOM excretion, was reflected in the rate of LAP activity. This

time-series pattern of LAP was expected, since primary production excretory products are known to be rich in complex nitrogen compounds such as proteins and polypeptides (Hellebust 1965, Lancelot 1984). As DIN and DIP is depleted from the euphotic zone, excretory products and exoenzyme activities are increasingly important in order to create incorporable N and P products to maintain cell growth.

A negative feedback inhibition of the LAP rate, was observed in natural seawater with added leucine concentrations equal to or higher than 3 nM (Table 6-5). During the infancy of aminoacid-analysis in oligotrophic oceans such as the Sargasso Sea, Lee & Bada (1977) found a concentration of dissolved free amino acids (DFAA) of approximately 5 nM. However, their blank values were 4 times higher than this and they also reported no detectable concentrations in the surface waters (Lee & Bada 1977). Later studies have concluded that *in-situ* leucine concentration are in the order of 1 nM (Kirchman et al. 1986). Leucine is one of the primary building blocks in cell synthesis and constitutes one of the major components of DFAA in seawater. In their measurements of DFAA in the Sargasso Sea, Suttle et al. (1991) found that leucine concentrations ranged from 0.1-1.7 nM in surface waters. Therefore, the LAP activity only appears to be substrate limited (depending on excretory nitrogen compounds from photosynthesis) and not end-product inhibited since the presence of leucine concentrations rarely exceeds the 2 nM level in the Sargasso Sea.

The LAP activity associated with *Trichodesmium* colonies, normalised to volume of seawater, approached the same order of magnitude as natural seawater in the fall of 1996 (Table 6-7), suggesting that the cyanobacterial colonies at times are an important source and mediator of nutrients in the nutrient depleted surface waters at BATS. Elevated enzyme activities have also been detected in marine snow aggregates (Karner & Herndl 1992, Smith et al. 1992). The K_m values in this study (Table 6-6) shows that the LAP activity associated with natural seawater at BATS was adjusted to relatively high concentrations of DON on all cruises with the exception of December 1993 (Table 6-6). The highest K_m for both natural seawater and *Trichodesmium* puffs (Table 6-6) was recorded in July 1994. July coincides with the time of the year when the increase in

Trichodesmium PP was observed in 1995 and 1996 (Figure 5-10 A). By assuming that *Trichodesmium* PP in July 1994 was equally high to the rates that appeared in both 1995 and 1996, the high K_m in July may have been a reflection of the increase in biomass turnover time (Figure 5-10 B) and the associated increase in the rates of DON release to the seawater surrounding the cyanobacterial colonies. The high K_m for natural seawater in April 1994 (Table 6-6) occurred prior to the annual increase in *Trichodesmium* PP, but coincided with elevated phytoplankton PP at the surface in April 1994 (Figure 1-10).

The rate of DOC release from phytoplankton is not only resulting as an overflow of photosynthetic products (Mague et al. 1980) and we can assume that the products of grazing and viral decay, caused by sloppy feeding and cell senescence of both auto and hetero-microtrophs, also plays an important and integral part in the release of DOM from the particulate phase. This study shows a tight coupling between the phytoplankton PP and the measured DOC excretion rates (Figure 6-7) and only minor amounts of DOC appears to accumulate in the euphotic zone at BATS (Carlson et al. 1994). No major LAP activity could be observed outside the phytoplankton PP peaks, suggesting that there was not an accumulation of DON in the euphotic zone. However, the K_m values for LAP reported in this study (Table 6-6), are high compared to other studies in the Caribbean (Rath et al. 1993) and in culture strains of *Synechococcus* (Martinez & Azam 1993). In fact, Rath et al. (1993) considered K_m values in the order of 50 μM to be indicative of eutrophic waters off the coast of Belize. The K_m values found in natural seawater at BATS were 1-10 times higher than the highest values reported from the Rath et al. (1993) study. Since the Sargasso Sea is considered a highly oligotrophic ocean and we can assume that only minor amounts of DON accumulates in the euphotic zone, the high K_m values at BATS may suggest a high and fairly constant flow rate of DON caused by cell excretion, sloppy feeding from cell grazing and viral cell lysis. Due to a constant and tight coupling between the autotrophs and the heterotrophs outside the spring bloom period at BATS, we can assume that the released DON is rapidly re-assimilated for cell synthesis.

Nutrient limitations for bacterial growth

Bacterial growth and abundance, is regulated by temperature, substrate supply and mortality caused by predation and viral lysis (Caron 1991, Ducklow & Carlson 1992, Shiah & Ducklow 1994, Fuhrman & Noble 1995). Predation and viral lysis of bacterial cells may reduce the stock of bacteria by an average of 85 % in spring and be equal to the growth rate during the summer months at BATS (Chapter 5). Although there seems to be a general consensus about the importance and relative magnitude of predation and viral lysis of bacteria, rather contradictive reports have emerged on what is the limiting nutrient source for bacterial growth. Recently, Carlson & Ducklow (1996) concluded that bacteria in the Sargasso Sea are carbon limited. However, this conclusion was argued by Rivkin & Andersson (1997) and Cotner et al. (1997) who claimed that DIP (PO_4^{2-}) was the single most important factor contributing to bacterial growth in the Sargasso Sea.

Unlike other investigations, this study of the elemental composition of bacteria at the BATS site did not include *in-vitro* incubations and nutrient manipulations of natural seawater. Until recently, we were not able to measure the actual elemental composition of bacteria and hence, left with 'cause and effect' studies including nutrient amendments, as the only means of studying nutrient limitations of bacterial growth. Only one available method, the TEM x-ray microanalysis, can actually evaluate the limiting element or elements in bacteria by accurately measuring the elemental composition of individual cells. Martinussen & Thingstad (1987) studied continuous cultures of bacteria subjected to C, N and P depletion. Due to the fact that the experiments were done on continuous bacteria cultures, the C, N and P elements could be easily measured by conventional methods without detection problems (Martinussen & Thingstad 1987). Their N:C versus P:C plot is shown in Figure 6-12 B together with the inverted elemental ratios from Chapter 3 (Table 3-6). Bacteria in surface waters at BATS appeared to be C-limited prior to the spring bloom period in 1995 (Figure 6-12 B). Bacteria in both freshwater and marine habitats, have been known to be able to take up both DIN and DIP (Currie et al. 1986, Currie 1990, Kirchman 1990). Mainly due to their high surface to volume ratio, bacteria

can effectively compete with phytoplankton for inorganic nutrients (Faust & Correll 1976, Mann 1982, Currie & Kalff 1984, Bratbak & Thingstad 1985, Suttle et al. 1990, Hoch & Kirchman 1995). Prior to the spring bloom period, bacterivory and sloppy feeding from the herbivores are at a low since primary production is low. This may be the only way the primary producers, the only primary source of DOC excretion, can compete with bacteria for inorganic nutrients during the pre-bloom winter mixing. As long as bacteria are C-limited, they will also be true remineralisers of N and P (Martinussen & Thingstad 1987, Jürgens & Güde 1990).

Due to the increase in primary production at the onset of the spring bloom and the proportionately higher rate of C-excretion (Figure 6-7, Figure 6-11 A), bacteria are being supplied with increasing amounts of DOC and will again be competing with phytoplankton for inorganic nutrients. Bacteria are at this stage going from being extremely C-limited towards C, N and possible P limitation (Figure 6-12 B). Obernoster & Herndl (1995) found that DOC was not taken up effectively by bacteria during P-limitation in the Adriatic Sea. P and N-limitation, combined with the fact that bacterial generation time was slower than phytoplankton, may therefore be a likely cause for the DOC accumulation that Carlson et al. (1994) observed at BATS at the peak or subsequently to the spring bloom. This pool of secondary DOC, seems not to be as easily degradeable by the bacteria in the euphotic zone (Carlson et al. 1994). Tranvik & Kokalj (1998) found that accumulated DOC "aged" and changed character with time. This is consistent with Heissenberger & Herndl (1994), who observed a shift of LMW ^{14}C-labelled leucine transforming into HMW-DOM in surface waters of the Adriatic Sea. They concluded that flagellates only had a minor influence on the formation of HMW-DOM. However, the consumption of the HMW-DOM by the flagellates represented a novel non-predatory transfer of carbon from the bacteria to their predators (Heissenberger & Herndl 1994). The decoupling between bacterial growth and the bacterivore grazers is evident as a build-up of bacterial biomass at the peak of the spring bloom. Since all measureable pools of DIN and DIP are exhausted after the spring bloom, all growth from then on will be generated from neat DON, DOP and the LMW organic and inorganic N

and P compounds derived from the organic pool by exoenzymes. Assuming a Redfield ratio of the elemental C:N and C:P, the bacterial cells appeared to be closer to N than P-limitation in May, July and September 1995 (Figure 6-12 B). After the initial abundance peak associated with the spring bloom, no further increase was observed in the bacterial biomass and the estimated grazing rate was at its maximum and approximately equal to the growth rate (Chapter 5).

The summer period was characterised by a tight coupling between bacterial growth and bacterivore grazing in a system were primary production to a large extent is depending on regenerated nutrients and the only new source of N and P to the euphotic zone is the cyanobacterial colonies of *Trichodesmium* developing at the nutricline (Orcutt 1998). The appearance of *Trichodesmium* colonies in surface waters was well documented in this study (Chapter 4 and Orcutt 1998). However, the proposed growth cycle of *Trichodesmium* (Orcutt 1998) suggest that the main bulk of the cyanobacterial colonies at surface, originally developed at the nutricline as trichomes and appeared as fully grown colonies one month after the peak in trichome abundance. Assuming that 200 trichomes are equivalent to approximately one colony, the number of trichomes at the nutricline was equivalent to the number of *Trichodesmium* colonies found at the surface one month later (Orcutt 1998). This phenomenon provides a renewed input of nutrients to an otherwise nutrient depleted and strongly stratified euphotic zone.

Proportionately more DIP than DIN is incorporated by the trichomes at the nutricline (Karl et al. 1992, Orcutt 1998). Karl et al. (1992) suggested a migratory model for *Trichodesmium* in the Pacific that may be similar for the Sargasso Sea: The cyanobacterial colonies incorporated DIP at the base of the nutricline and transformed this into positively buoyant polyphosphate storage products, that would enable them to migrate to the surface. At the surface the diazotrophic colonies would be able to photosynthesise and have a balanced growth until the P storage products were exhausted and the excess carbohydrate storage products would make the colonies increasingly negatively buoyant and enable them to migrate down to the nutricline to acquire more DIP. The Karl et al. (1992) model provides a repeated flux of P into the euphotic zone at

the same time as N is provided by the N-fixing activity at the surface. Glibert and Bronk (1994) found that as much as 50 % of the N fixed by *Trichodesmium* colonies, was released as DON in surface waters off the Caribbean Islands. Capone et al. (1994) came to a similar conclusion when they discovered that the release of glutamate alone was at least one quarter of the N-fixation rate. Elevated phosphatase and peptidase exoenzyme activities associated with *Trichodesmium* have been reported from the BATS site (Elardo et al. 1994) suggesting that DOP may be released by the cyanobacterial colonies in a similar order of magnitude to DON. This study suggests that *Trichodesmium* colonies in surface waters at BATS may provide the euphotic zone with DON and DOP, subsequently transformed into LMW N and P compounds, in summer and fall when bulk inorganic nutrients are depleted in the euphotic zone. The elemental C:N and C:P ratios of single cell bacteria in surface waters at BATS appears to never reach a true N or P limitation, due to a steady source of new and regenerated nutrients provided to a large extent by the cyanobacterial colonies. Hence, *Trichodesmium* may constitute an important nutrient source for bacterial growth in surface waters at BATS in summer and fall.

Chapter 7: Summary

The seasonality in hydrography at the BATS site, as illustrated in Chapter 1, is similar to the description given by Menzel & Ryther (1960, 1961) of the Sargasso Sea four decades ago. Since then, longitudinal transect studies have shown that the BATS site is situated in a meridional transition zone between two, hydrographically, distinct areas (Siegel et al. 1990). A strong seasonality in winter mixing and the ventilation of nutrient rich mode water into the euphotic zone is typical for the area north of the BATS site (Talley & Raymer 1982, Ebbesmeyer & Lindstrøm 1986), while permanent stratification and low nutrient levels can be found south of BATS (Siegel et al. 1990). The complex mesoscale physical structures observed by Lai & Richardson (1977), Cornillion et al. (1986) and Ebbesmeyer & Lindstrøm (1986) in the Sargasso Sea, are still not fully understood and a continued effort by means of trans and cross-sectional cruises in the area surrounding the BATS site is still being implemented. The seasonal pattern in stratification, production and biomass, already noted by Menzel & Ryther (1960, 1961) and the extreme nutrient dynamics later confirmed by Michaels et al. (1994), makes the BATS site a unique environment to study the biogeochemical factors controlling bacterial growth and biomass in open oceans.

The last two decades have seen a rapid development and improvements of methods in microbial research and recent advances have enabled us to measure elemental properties at the level of a single microbial cell. Results from this study have shown that several assumptions based on previous investigations of microbial communities in coastal waters did not fully apply to open ocean oligotrophic waters. Hence, it was evident that a reassessment of the role of bacterial growth and biomass in the Sargasso Sea was necessary.

The issue of storing preserved bacteria samples was already questioned by Nishino (1986) and Spinrad et al. (1989), when they noted a loss of cells in samples stored for weeks and months. However, the magnitude of this problem was not fully understood until after the first year of the North Atlantic Bloom Experiment (NABE) when Turley & Hughes (1992) reported that an average of 39 % of the bacterial cells preserved in glutaraldehyde, disappeared over a 40 day period. Seawater samples routinely preserved in glutaraldehyde and stored at +4 °C, are susceptible to a number of processes that may cause the loss of bacterial cells and no single study has been able to rectify this problem. This study found a loss of glutaraldehyde preserved bacteria from the Sargasso Sea, stored for no more than 1 week at +4 °C, to be in the order of 24-50 % of the original concentration (Chapter 2). The loss was mainly attributed to lysis activity dissolving the cells from the inside, as the use of a known proteolytic inhibitor (PMSF) showed a reduced loss of 17-18 % over a 30 day period. A recommendation therefore, from this study, is that bacterial samples preserved in glutaraldehyde and stored at +4 °C should be processed for enumeration within 2-3 days of sampling and the microscope slides stored at -20 °C prior to enumeration.

Until recently, very few studies had managed to accurately measure the elemental content of single individual bacterial cells. This was mainly due to the problems associated with separating bacteria from other living cells and detrital matter prior to conventional elemental analysis. Several studies over the last four years have reported accurate measurements of the elemental composition of C, N and P in natural bacteria (Gundersen et al. 1994, Norland et al. 1995, Tuomi et al. 1995, Fagerbakke et al. 1996), using the novel TEM x-ray microanalysis technique on single bacterial cells. The TEM x-ray microanalysis technique, first reported by Heldal et al. (1985), was used in this study in order to accurately measure the elemental C, N and P content of single cell bacteria from the BATS site (Chapter 3). A wide range of variation was found between single cells in all the elements investigated and this could be expressed as a function of the cell size. By applying an average cell volume from the BATS site (Carlson et al. 1995) an

average elemental content of 10 fg C, 1.9 fg N and 0.28 fg P could be calculated per bacterial cell.

High concentrations of bacteria associated with detrital particles, aggregates and living colonial organisms such as the cyanobacterium *Trichodesmium* may, if not accounted for, underestimate the total C biomass. Although important on a micro-scale level, these aggregations of bacteria are less abundant by three to four orders of magnitude, when compared per volume to free living bacteria in open ocean oligotrophic waters. Another potential underestimation of bacterial C contributing to the total POC, may be caused by the filtration of POC samples, since Lee et al. (1995) found that an average of half the bacterial cells were lost during filtration on a GF/F filter. This was practically the same average (47 %) calculated from filtered and natural seawater samples from the upper 250 m of the water column in this study (Chapter 4). The calculated relative proportion of bacterial C biomass retained on a GF/F filter in this study, was corrected by a factor of two in order to compensate for the loss of cells during the filtration of total POC.

Finally, the issue of rapidly sinking particles not accounted for in seawater samples, collected through the spigot of a Niskin watersample bottle, has been poorly addressed since it was first noted by Gardner (1977) more than two decades ago. Gardner (1977) concluded that the percentage loss of particles sinking out prior to sampling, was of great importance in areas with low particle concentrations, such as the Sargasso Sea. This study of the upper 250 m of the water column, showed that an average of 26 % of total POC would not be accounted for if the dregs sinking below the spigot were not included in the sample analysis (Chapter 4).

Based on these findings, recalculated bacterial biomass estimates were compared to phytoplankton (including *Trichodesmium* colonies) and detrital non-living matter. Total microheterotrophic biomass, integrated over the upper 250 m of the water column and averaged over a year, constituted 18 % of total C. Phytoplankton C made up 20 % of the remaining total C and the remainder consisted of non-living detrital matter. In the upper 65 m of the surface waters at BATS, phytoplankton C biomass was always higher than

bacteria during the spring bloom season. However, this was reversed for the remainder of the year, unless late summer and fall blooms of the colonial cyanobacterium *Trichodesmium*, occurred. The annual average ratio between living C (including all phytoplankton and microheterotrophs) and non-living detrital matter, was equal to one in the euphotic zone at BATS. Deeper than 135 m however, non-living detrital C was 4.5 times higher than living C biomass.

This study is the first to present a continuous time series of depth profiles of bacterial growth rates at BATS (Chapter 5). Bacterial growth in spring was closely associated with the phytoplankton primary production peak. However, a secondary increase in bacterial growth in summer and fall, coinciding with a TCO_2 drawdown reported at BATS (Michaels et al. 1994) was not reflected in the phytoplankton primary production measurements, but could be attributed to *Trichodesmium* primary production. Bacterial generation time was estimated to be 15 days for the upper 140 m of the water column. If non-living bacteria were excluded from this estimate, generation time was reduced to 7 days which is still four times longer than the phytoplankton generation time. Grazing and viral lysis removed 85 % of the bacterial growth rate in spring, with each constituting a loss of 0.07-0.21 mg C $m^{-3}d^{-1}$. This loss rate was estimated to be twice as high for the remainder of the year, when grazing and viral lysis totally controlled bacterial abundance,

Access to DOC excretory products derived from the phytoplankton primary production, may play a key role in regulating bacterial growth in surface waters. Recently, it has been debated whether bacterial incorporation of DOC was slowed down due to depletion of P in the euphotic zone in the Sargasso Sea, making it the primary cause for DOC build-up in the water column in spring (Rivkin & Andersson 1997). The rates of net DOC excretion measured at BATS in this study (Chapter 6) could be expressed as a linear function of primary production and constituted an average of 42 % of the rate of carbon fixation. Results from this study has shown that bacteria are strongly

C-limited during the winter mixing and the surface intrusion of nutrient rich mode water prior to the spring bloom. Due to the rapid increase in the bacterial growth rate concomitant to the increase in phytoplankton primary production, we can assume that DOC is directly and effectively incorporated by the bacteria. By assuming a conservative estimate of the bacterial growth efficiency of 14 % (Carlson & Ducklow 1996), the actual rate of DOC excretion was equal to the rate of primary production. Hence, by not accounting for the DOC excretion, the rate of primary production as measured in the BATS program, was underestimated by a factor of two.

The presence of readily available DOC and the measured increase in bacterial growth during the initial stages of the spring bloom formation, suggests that the actual bacterial growth efficiency is closer to 50 %, which is the average for pure substrates reported in the literature. This study showed that excess amounts of DOC were excreted during the senescence in a natural seawater culture and such an event, following the peak in phytoplankton primary production at BATS, may cause a DOC build-up in the water column. Bacteria during this period were not severely N, P or C-limited. However, due to the slower generation time relative to phytoplankton, the cells may not have been able to incorporate the excess DOC excreted over such a short period of time. Tranvik & Kokalj (1989) found that excess DOC changes character over time and this may explain why the DOC build-up at BATS in spring is not readily incorporated over the course of the summer and fall (Carlson & Ducklow 1996).

Bacterial growth through the summer and fall may be sustained by regenerated nutrients from grazing and viral decay and new production generated by the *Trichodesmium*. The cyanobacterium *Trichodesmium* appears to be a key mediator of new N and P to the euphotic zone at BATS in summer. Organic N is made available by new production through the process of N-fixation and orthophosphate is incorporated at the nutricline and brought to the surface by vertically migrating colonies of *Trichodesmium* (Karl et al. 1992, Orcutt 1998). The high LAP rates measured in this study and the alkaline phosphatase activity associated with *Trichodesmium* (Elardo et al. 1994), suggests that the diazotrophs represents a significant source of regenerated

nutrients in summer and fall. Bacteria at BATS appear to be strongly C-limited in spring, indicative of a bottom-up controlled trophic system. This is also the only time of the year a decoupling appeared between the bacterial growth rate and the removal rate of bacterial cells, due to grazing and viral lysis. During the remainder of the year, grazing and viral lysis appeared to be closely coupled to the bacteria growth rate, suggesting a top-down controlled trophic system.

APPENDIX 1

Data for the contour plots are available on the Bermuda Biological Station for Research Home Page (http://www.bbsr.edu/bats) at;

http://www.bbsr.edu/users/ctd/nisklist.html
- for Niskin bottle wet data, and

http://www.bbsr.edu/users/ctd/goflolist.html
- for primary production and bacterial incorporation of ^{3}H -thymidine data

Chapter 1

Figure 1-3
Seawater temperature at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: Temperature (C) (IPTS-68)

Figure 1-4
Salinity at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: CTD Salinity-1 (PSS-78)

Figure 1-5
Dissolved Inorganic Nitrogen (DIN) at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: Nitrate+Nitrite-1 (µmol/kg)

Figure 1-6
Dissolved Inorganic Phosphorous (DIP) at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: Phosphate-1 (µmol/kg)

Figure 1-7
Oxygen anomaly at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: Oxygen anomaly-1 (µmol/kg)

Figure 1-8
Seawater temperature on BATS Val. 10, 12 and 14:
BATS Validation data (0-500 m): BATS Val.10 = 1-4 February 1994
BATS Val.12 = 26 September - 2 October 1995
BATS Val.14 = 25-29 July 1995
Click on: Temperature (C) (IPTS-68)

Figure 1-9
Oxygen anomaly on BATS Val. 10, 12 and 14:
BATS Validation data (0-500 m): BATS Val.10 = 1-4 February 1994
BATS Val.12 = 26 September - 2 October 1995
BATS Val.14 = 25-29 July 1995
Click on: Oxygen anomaly-1 (µmol/kg)

APPENDIX 1 (CONT.)

Figure 1-10
Primary production (PP) at BATS:
BATS core and BATS Bloom data (0-140 m) from 1 January 1991 to 31 December 1996
Click on: 14C Primary Production mean light values - dark values (mgC/m^3/d)

Figure 1-11
Particulate Organic Carbon (POC) at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: POC (µg/kg)

Figure 1-12
Chlorophyll-*a* (Chl-*a*) at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
Click on: Turner Chlorophyll (µg/kg)

Chapter 2

Figure 2-6
Bacterial abundance at BATS:
BATS core and BATS Bloom data (0-1000 m) from 1 January 1991 to 31 December 1996
*Click on: Bacteria (cell*10^8/kg)*

Chapter 5

Figure 5-1
Bacterial incorporation of ^{3}H-thymidine (TdR) at BATS:
BATS core and BATS Bloom data (0-140 m) from 1 January 1991 to 31 December 1996
Click on: Bacterial Growth rate by 3H-thymidine (pmol/l/h)

APPENDIX 2

Chapter 6: Data for contour plot of leucine aminopeptidase (LAP) in Figure 6-9.

Decal Year	Depth (m)	LAP (nM/h)	Decal Year	Depth (m)	LAP (nM/h)	Decal Year	Depth (m)	LAP (nM/h)	Decal Year	Depth (m)	LAP (nM/h)
1994,03	1	5,26	1994,80	1	10,34	1995,21	1	12,19	1995,71	1	5,46
1994,03	20	5,02	1994,80	20	9,73	1995,21	20	12,41	1995,71	10	6,61
1994,03	40	5,62	1994,80	40	10,09	1995,21	40	10,61	1995,71	20	5,31
1994,03	60	4,66	1994,80	60	12,03	1995,21	60	9,82	1995,71	40	5,74
1994,03	80	6,95	1994,80	80	7,76	1995,21	80	8,63	1995,71	60	6,81
1994,03	100	6,33	1994,80	100	5,87	1995,21	100	9,60	1995,71	80	6,24
1994,03	120	7,48	1994,80	120	4,67	1995,21	120	6,22	1995,71	100	3,70
1994,03	140	8,59	1994,80	140	4,83	1995,21	140	7,37	1995,71	120	7,71
1994,34	1	5,21	1994,80	160	4,50	1995,21	160	10,86	1995,71	140	3,41
1994,34	20	4,61	1994,80	200	2,90	1995,21	200	4,93	1995,71	160	4,97
1994,34	40	6,69	1994,80	250	3,35	1995,21	250	16,11	1995,71	200	4,62
1994,34	60	9,08	1994,80	300	3,04	1995,21	300	11,29	1995,71	250	4,68
1994,34	80	7,86	1994,96	1	16,29	1995,27	1	16,04	1995,71	140	3,41
1994,34	100	4,26	1994,96	20	10,46	1995,27	10	15,48	1995,71	160	4,97
1994,34	120	9,99	1994,96	40	10,56	1995,27	20	14,92	1995,71	200	4,62
1994,34	140	3,43	1994,96	60	10,89	1995,27	40	13,94	1995,71	250	4,68
1994,34	160	3,61	1994,96	80	9,50	1995,27	60	11,64			
1994,34	200	5,43	1994,96	100	7,25	1995,27	80	12,77			
1994,34	250	5,39	1994,96	120	5,50	1995,27	100	13,61			
1994,34	300	3,95	1994,96	140	4,64	1995,27	120	11,97			
1994,42	1	3,93	1994,96	160	4,34	1995,27	140	12,25			
1994,42	20	3,20	1994,96	200	4,64	1995,27	160	11,78			
1994,42	40	3,48	1994,96	250	4,73	1995,27	200	11,04			
1994,42	60	3,44	1994,96	300	4,73	1995,27	250	11,93			
1994,42	80	8,01	1995,03	1	8,82	1995,36	1	15,76			
1994,42	100	4,95	1995,03	10	9,76	1995,36	10	12,02			
1994,42	120	3,86	1995,03	20	8,68	1995,36	20	14,17			
1994,42	140	4,00	1995,03	40	5,90	1995,36	40	12,28			
1994,42	160	3,55	1995,03	60	8,96	1995,36	60	11,07			
1994,42	180	4,21	1995,03	80	4,51	1995,36	80	11,96			
1994,42	200	7,97	1995,03	100	4,97	1995,36	100	4,18			
1994,42	250	5,86	1995,03	110	6,98	1995,36	120	1,52			
1994,63	1	4,77	1995,03	120	5,59	1995,36	140	1,93			
1994,63	20	6,42	1995,03	140	0,56	1995,36	160	2,72			
1994,63	40	4,95	1995,03	150	1,32	1995,36	200	2,85			
1994,63	60	5,20	1995,03	200	0,00	1995,36	250	1,90			
1994,63	80	5,04	1995,16	1	10,08	1995,53	1	1,45			
1994,63	100	4,65	1995,16	20	7,40	1995,53	10	5,34			
1994,63	120	3,94	1995,16	40	8,17	1995,53	20	4,17			
1994,63	140	3,61	1995,16	60	6,81	1995,53	40	6,21			
1994,63	160	1,74	1995,16	80	7,36	1995,53	60	6,86			
1994,63	200	2,90	1995,16	100	7,70	1995,53	80	11,98			
1994,63	250	2,17	1995,16	120	6,76	1995,53	100	24,78			
1994,63	300	3,39	1995,16	140	6,93	1995,53	120	2,56			
			1995,16	160	2,77	1995,53	140	1,69			
			1995,16	200	3,49	1995,53	160	4,77			
			1995,16	250	2,89	1995,53	200	5,10			
			1995,16	300	1,79	1995,53	250	0,91			

APPENDIX 3

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APPENDIX 4

Overview of BATS core data sample analysis from 1991 to 1996. Common share (CS) responsibilities were rotated on a regular basis within a smaller group of 3 people in the program. My personal responsibilities are highlighted with a shade of grey.

Rachael Dow	RD	Nick Bates Lab	NB	Shannon Stone	SS
Rodney Johnson	RJ	Alice Doyle	AD	Karen Orcutt	KO
Kjell Gundersen	KG	Fred Bahr	FB	Cathy Rathbun	CR
Jens Sorensen	JS	Susan Becker	SB	Marta Sanderson	MS
Ann Close	AC	Peter Countway	PC	Mark Brzezinski Lab	MB
Tye Waterhouse	TW	Becky Little	BL	Common share	CS

1991

	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ
Salinity (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Oxygen (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Nutients	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	KG	KG	KG	KG	KG	JS	JS	JS	JS	JS	JS	JS
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Sediment traps	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG

1992

	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ
Salinity (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Oxygen (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Nutients	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS	JS
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Sediment traps	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG

APPENDIX 4 (CONT.)

Overview of BATS core data sample analysis from 1991 to 1996. Common share (CS) responsibilities were rotated on a regular basis within a smaller group of 3 people in the program. My personal responsibilities are highlighted with a shade of grey.

Rachael Dow	RD	Nick Bates Lab	NB	Claire Michaels	CM
Rodney Johnson	RJ	Alice Doyle	AD	Shannon Stone	SS
Kjell Gundersen	KG	Fred Bahr	FB	Karen Orcutt	KO
Jens Sorensen	JS	Susan Becker	SB	Cathy Rathbun	CR
Ann Close	AC	Peter Countway	PC	Marta Sanderson	MS
Tye Waterhouse	TW	Becky Little	BL	Mark Brzezinski Lab	MB

1993

	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	RJ	RJ	RJ	RJ	RJ	RJ	RJ	RJ	FB	FB	FB	FB
Salinity (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Oxygen (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS
Nutients	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	JS	JS	JS	JS	JS	JS	TW	TW	TW	TW	TW	TW
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Sediment traps	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG

1994

	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	FB	FB	FB	FB	FB	FB	FB	FB	FB	FB	FB/SS	FB/SS
Salinity (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	BL
Oxygen (wet samples)	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	RD/CS	BL
Nutients	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	TW	TW	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Sediment traps	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	KG	CM/KG	CM/KG	CM/KG	CM/KG	CM/KG	KO/KG

APPENDIX 4 (CONT.)

Overview of BATS core data sample analysis from 1991 to 1996. Common share (CS) responsibilities were rotated on a regular basis within a smaller group of 3 people in the program. My personal responsibilit are highlighted with a shade of grey.

Rachael Dow	RD	Nick Bates Lab	NB	Shannon Stone	SS
Rodney Johnson	RJ	Alice Doyle	AD	Karen Orcutt	KO
Kjell Gundersen	KG	Fred Bahr	FB	Cathy Rathbun	CR
Jens Sorensen	JS	Susan Becker	SB	Marta Sanderson	MS
Ann Close	AC	Peter Countway	PC	Mark Brzezinski Lab	MB
Tye Waterhouse	TW	Becky Little	BL		

1995	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS
Salinity (wet samples)	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL
Oxygen (wet samples)	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL
Nutients	SB	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC	PC
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC	AC
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD	AD
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KG	KG	KO	KO	KO	KO	KO	KO	KO	KO	KO	KO
Sediment traps	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG

1996	January	February	March	April	May	June	July	August	September	October	November	December
CTD (O, T, S)	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS	FB/SS
Salinity (wet samples)	BL	BL	BL	BL	BL	SB	SB	SB	SB	SB	SB	SB
Oxygen (wet samples)	BL	BL	BL	BL	BL	SB	SB	SB	SB	SB	SB	SB
Nutients	PC	PC	PC	PC	MS	MS	MS	MS	MS	MS	MS	MS
DIC (wet samples)	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB	NB
Primary Production	AC	AC	AC	CR	CR	CR	CR	CR	CR	CR	CR	CR
Bacterial growth (TdR)	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Chlorophyll-a	AD	AD	AD	CR	CR	CR	CR	CR	CR	CR	CR	CR
POC, PON	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG	KG
Part. Silicate	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB	MB
Bacterial abundance	KO	KO	KO	KO	KO	KO	KO	KO	KO	KO	KO	KO
Sediment traps	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG	KO/KG

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Factors influencing the loss of bacteria in preserved seawater samples

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ABSTRACT: Several time course storage experiments with preserved seawater samples were conducted to study the loss of bacterial cells as a function of storage time. The number of bacteria decreased by 24 to 50% within 7 to 29 d in samples preserved with 2.5% glutaraldehyde (final conc.). A comparison between epifluorescence and electron microscope counts showed that the decrease was not due to filtration artefacts. Only 0.4 to 0.6% of the bacterial cells were found to be attached to the walls of the sample containers after 1 yr of storage. There was no positive correlation between the frequency of virus-infected cells at the start of the storage experiments and the loss of bacteria as a function of storage time. Numbers of bacteria declined by only 5% the first 9 d in samples preserved in glutaraldehyde and stored at −20°C. By adding phenolmethylsulfonylfluoride (PMSF), a protease inhibitor, prior to the addition of glutaraldehyde, the loss of bacterial cells only 17 to 18% over a 30 to 35 d period. Our study shows that protease activity may be a major cause of bacterial loss in glutaraldehyde preserved samples.

KEY WORDS: Bacteria · Preservation · Glutaraldehyde · PMSF

INTRODUCTION

Bacteria are considered to be the main heterotrophic link between particles and dissolved organic matter, in open ocean and coastal ecosystems (Pomeroy 1974, Azam et al. 1983). Although still widely discussed (Li et al. 1992, Caron et al. 1995, Roman et al. 1995), bacterial biomass has also been suggested to be a major part of the particulate organic carbon pool in open ocean and coastal water communities (Dortch & Packard 1989, Fuhrman et al. 1989, Cho & Azam 1990). Therefore, an accurate assessment of bacterial abundance and biomass is important in the study of aquatic biogeochemical cycling of carbon, nitrogen and phosphorus. Direct microscopic counts of bacteria stained with fluorescent dyes (Zimmerman & Meyer-Reil 1974, Hobbie et al. 1977, Porter & Feig 1980) has been a standard procedure the last 2 decades for enumerating bacteria in natural water samples. Due to the relative ease of handling and low cost, epifluoresecence microscopy (EFM) is by far the most widely used technique to enumerate bacteria. Bacterial samples preserved with formaldehyde or glutaraldehyde are routinely stored at 4°C for weeks, months and sometimes years before counting.

Questions have been raised about the preservation of bacteria in stored samples. In a comparative study of bacterial preservatives, Nishino (1986) found Lugol's iodine to be superior to glutaraldehyde preservation of marine bacteria, but the number of bacteria declined in both treatments as a function of storage time. A loss of 45 and 69% of the initial bacteria concentration was found in seawater samples preserved with glutaraldehyde after 1 and 6 mo storage at +4°C (Nishino 1986). Spinrad et al. (1989) noted a loss of bacteria in formaldehyde preserved samples stored for 2 mo and applied a logarithmic function to correct the bacterial cell concentration. Indeed, a loss of bacteria has been observed with most commonly used poisons or preservatives, such as Lugol's solution (Nishino 1986), formaldehyde (Spinrad et al. 1989) and glutaraldehyde (Nishino 1986, Turley & Hughes 1992).

There are several potential processes that may cause a loss of bacteria in preserved samples. These include:

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(1) attachment of cells to the bottle surface and other surfaces (aggregates) in the sample (Turley & Hughes 1992); (2) cell shrinkage, which may cause an increased loss by filtration and preparation of the sample; (3) virus-infected bacteria in the lytic cycle may lyse and disintegrate cells in the preserved sample; (4) continued enzymatic activity and cell dissolution in glutaraldehyde preserved samples.

In one extensive study, Turley & Hughes (1992) investigated and discussed the possible causes of loss of bacteria in preserved seawater samples. They suggested that cells adhering to the wall of the sample bottle could account for 41 to 48% of the original bacterial concentration in samples stored for about 11 mo. However, 27 to 51% of the total loss could not be accounted for. Bacterial DNA and RNA are not preserved by aldehydes (Hayat 1981), and enzymatic breakdown activity may also have a deleterious effect on the nucleotides present in bacteria. Continued enzymatic activity in glutaraldehyde and formaldehyde preserved organic matter has been reported in several studies (Fahimi & Drochmans 1968, Kolb-Bachofen 1977, Synowiecki et al. 1982, Shepard et al. 1983).

The purpose of this study was to investigate the various processes that may cause the apparent bacterial cell loss in preserved samples. Therefore, we have introduced a new preservation technique, a protease inhibitor in a solution with glutaraldehyde, in order to inhibit enzymatic breakdown of bacteria to improve the preservation and storage time of bacterial samples.

MATERIAL AND METHODS

Samples. Open ocean water samples for this investigation were collected in the Sargasso Sea at Stn 'S' (32°10'N, 64°30'W) and at the Bermuda Atlantic Time-series Study (BATS) site (31°50'N, 64°10'W) at 80 and 100 m depth. Coastal and estuarine water samples were collected at the surface in Bergen Harbour and from Raunefjorden 20 km south of Bergen, Norway. We used unbuffered, unfiltered glutaraldehyde (25% v/v Baker Analyzed Reagent Grade in Bermuda and 25% v/v Merck Electron Microscope Grade in Norway) to preserve the seawater samples.

Bacterial cell counts by transmission electron microscopy (TEM) and EFM. Preparations for the TEM analysis were made by centrifuging algae, bacteria and viral particles in the water sample onto electron microscope grids with carbon stabilized formvar film (Bratbak & Heldal 1993). Bacteria were counted using a JEOL 100CX TEM at 20000× magnification. Similar preparations were used for counting of bacteria containing mature virus particles. For every TEM sample, a minimum of 100 bacterial cells were counted, which results in a counting error of less than 10% of the calculated mean.

For the EFM analysis, 20 ml of open ocean seawater samples and 5 ml of coastal and estuarine water samples were filtered and prepared according to the method of Porter & Feig (1980). The filtered bacteria were stained using a concentration of 25 µg 4',6-diamidino-2-phenylindole (DAPI) per ml of Milli-Q water. Since our time course samples were all closely related in time and distance (Kirchman et al. 1983), only 1 filter per sample per time point was enumerated by the EFM method. An earlier replication study from the Sargasso Sea, using a 2-level nested ANOVA (Sokal & Rohlf 1969), did not show any significant difference between replicate sample bottles ($p > 0.1$), and 76 to 82% of the variance component was found between the fields counted on a filter (K. Gundersen unpubl. data).

Loss caused by cell shrinkage and by attachment of cells to the bottle surface. Bacterial samples for the time course experiments were collected at 1 m depth from Bergen Harbour in January 1993. One sample was preserved with 2.5% glutaraldehyde and aliquots were distributed into a series of 20 ml polyethylene scintillation vials (1 to 3 for each time point). In addition, aliquots of 100 and 1000 ml were stored in glass bottles to check for any effects of volume-to-surface ratio of the sample container. All samples were stored at 4°C in the dark. Bacteria were enumerated over a time course of 94 d by the EFM and the TEM methods. On the last day of the experiment, we also counted bacteria in the 100 ml and the 1000 ml samples.

Bacterial adhesion to the inside surface of a sample container, with surface seawater from Raunefjorden, was studied by direct observation using both the EFM and the TEM techniques. The glass sample bottle containing a 1 yr old glutaraldehyde fixed seawater sample was emptied without agitation and the bottle was broken into pieces. Unwashed pieces from the side of the broken bottle were stained with DAPI as described above and mounted in liquid paraffin on a glass slide, with the inside surface facing upwards and with a coverslip on the top. We inspected more than 40 fields of view using EFM, each with an area of 400 µm^2 at 1000× magnification. Replicas for TEM of the inside surface of the bottle were made by coating unwashed pieces (3 to 6 mm^3) of the crushed bottle with platinum and carbon in a Balzers BAF 400 Freezetcher: platinum at 45°C, 1900 V, 80 mA (2 nm) and carbon at 90°C, 2400 V, 120 mA (20 nm). The replicas were transferred to electron microscope grids and inspected in the TEM. More than 200 fields of view, with an area of 50 µm^2 at 20000× magnification, were inspected by the TEM

technique. The inside area of the glass bottle that was covered by the seawater sample was calculated to be 100 cm^2.

Disappearance of cells with mature viral particles. During a mesocosm experiment conducted in May 1993 at the Marine Biological Field Station at Raunefjorden, the fraction of bacteria containing mature viral particles varied from <1% to about 30% (Heimdal et al. 1995). In order to check for a loss of bacterial cells, selected samples from these experiments were preserved with 2.5% glutaraldehyde, stored for 72 d at 4°C and then recounted in the TEM.

Enzyme activity. In a first attempt to inhibit the impact of enzymatic activity on cell lysis and disintegration of bacteria, water was collected from 100 m depth in November 1992 at Stn 'S' in the Sargasso Sea, quick frozen in liquid nitrogen and stored at –20°C. Over a time course of 43 d, quick frozen samples of natural seawater and seawater preserved in 2.5% glutaraldehyde, were compared to samples preserved in 2.5% glutaraldehyde stored at +4°C. The frozen samples were thawed in cold tap water and processed immediately. The number of bacteria was counted by the EFM method.

A protease inhibitor, phenylmethylsulfonylfluoride (PMSF; Turini et al. 1969, James 1978, Moss & Fahrney 1978), was added to bottles containing Sargasso Sea water from 80 m depth at Stn 'S' in September 1993 and from 80 m depth at the BATS site in April 1994. PMSF is not readily soluble in aqueous solutions and we found that final concentrations higher than 1 µM precipitated out of the seawater. Stock solutions of PMSF, stored in pure alcohol, were stable for at least a month (James 1978). A 100 µM stock solution of PMSF was prepared fresh in 95% ethanol a couple of days prior to use. In the September 1993 time course experiment, one series of seawater samples was supplemented with PMSF to a final concentration of 1 µM. After 5 min at room-temperature, glutaraldehyde was added to a final concentration of 2.5%. Another series of seawater samples was preserved in 2.5% glutaraldehyde. All the sample bottles were stored at +4°C. A time zero sample was filtered, stained with DAPI and prepared immediately from both treatments. All stained preparations were stored at –20°C. At the end of the time course, all samples were thawed and enumerated by the EFM method. One sample, designating a single time point, was prepared and counted from each bottle.

In the April 1994 time course experiment, Sargasso Sea water with 2 different concentrations of PMSF (0.5 and 1.0 µM final conc.) and 2.5% glutaraldehyde was compared to seawater samples preserved in 2.5% glutaraldehyde only. The samples were prepared and enumerated by the EFM method.

RESULTS AND DISCUSSION

Loss of bacteria by filtration and cells adhering to the container's inside surface

A comparison of the EFM and the TEM techniques (Fig. 1) shows that, with both techniques, the number of bacteria decreased on average by 43% over the 94 d time course. The basic difference between these 2 methods is that the preparation for EFM is based on filtration and staining of cellular DNA with the fluorescent dye DAPI, while preparation for TEM is based on centrifugation onto a formvar film and contrast staining of the cells with uralyl acetate. This leads us to conclude that the loss of cells cannot be caused by increased filtrational loss due to cell shrinkage as a function of storage time and that the EFM method of enumerating bacteria worked as well as the TEM method.

Less than 0.6% of the original number of bacteria from Bergen Harbour (2×10^9 l^{-1}) enumerated by the TEM replica technique was found to be attached to the inside surface of the sample bottle after 1 yr storage time. Corresponding values obtained with DAPI staining and the EFM method were less than 0.4%. Moreover, there was no significant difference ($p > 0.05$) in bacterial numbers in samples stored in bottles with volumes ranging from 20 ml to 1 l (Fig. 1) although the surface-to-volume ratio of these sample containers differed by a factor of 4.

Turley & Hughes (1992) were able to recover 37 to 70% (percentage of initial number of bacteria) by a

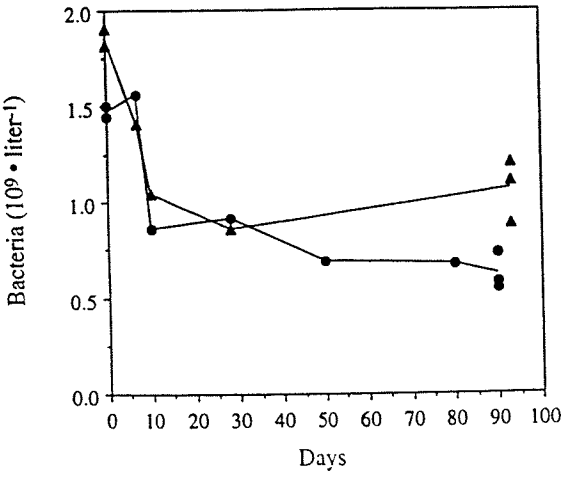

Fig. 1. Bacterial number as a function of storage time in seawater samples preserved with 2.5% glutaraldehyde (final conc.) from Bergen Harbour in January 1993. The samples were stored in 20 ml scintillation vials. Bacteria were enumerated by the EFM (●) and the TEM (▲) methods. The triplicates at the end of the time course are bacteria samples stored in 20, 100 and 1000 ml glass bottles

4 min sonication of samples stored for 338 to 340 d. We sonicated 4 wk old glutaraldehyde fixed seawater samples from the Sargasso Sea in an attempt to recover the missing 30% of the bacteria. We were not able to recover any significant amount of bacteria ($p > 0.9$) after 1 min of sonication prior to counting the samples by epifluorescence microscopy. More than 1 min continuous sonication reduced the number of bacteria (K. Gundersen unpubl. data). In comparison, Turley & Hughes (1992) found an increase of 1.5 to 2.5 times the initial cell number after only 1 min sonication of samples stored for 338 to 340 d.

These results suggest that the loss of bacteria in our samples cannot be explained by attachment to the walls of the sample container. Turley & Hughes (1992) also found that 30 to 90% of bacteria remained unaccounted for and concluded that other factors as well as attachment must play a significant role in cell loss with time. Although there is a considerable difference in storage time between these 2 sonication studies, the reason for the discrepancy between our observations is not readily explained. Different properties of the bacterial communities used in the experiments may be one possibility, since Turley & Hughes (1992) used bacteria from the North Atlantic, whereas our collections were from the Sargasso Sea. Wiik (1984) noted that sonicating cultured bacteria longer than 2 min damaged and disrupted the cells. However, Turley & Hughes (1992) studied the effect of sonication on fresh seawater samples and found that 4 × 1 min sonication with cooling and shaking in between resulted in no decrease in bacterial cell numbers (C. Turley pers. comm.). Different surface properties of the sample containers could be another possible explanation, since Turley & Hughes (1992) used polystyrene tissue culture flasks, whereas we used glass bottles and polyethylene scintillation vials in our study. More recently, however, Turley & Hughes (1994) found no significant difference in bacterial cell numbers between polystyrene tissue culture flasks and nalgene bottles.

Viral lysis

The potential loss of cells in preserved samples due to lysis and disintegration of cells in the late stage of the lytic cycle was not supported by our results (Fig. 2). We observed a slightly negative correlation, suggesting that the cell losses were lower in samples where there were more cells in lysis. We only detected bacteria containing mature viral particles. If it had been possible to include cells at any stage in the lytic cycle (i.e. all cells that were bound for lysis at the time the sample was preserved) the results may have been different.

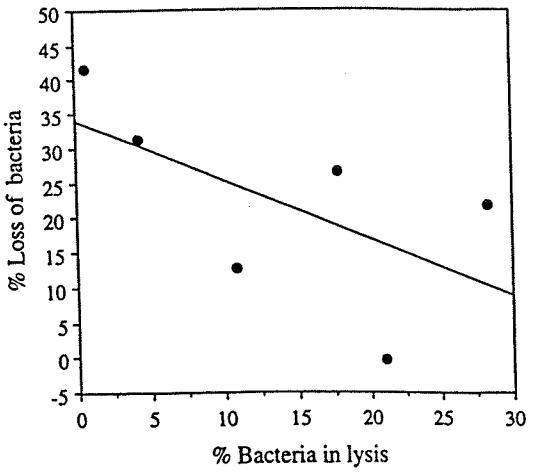

Fig. 2. Loss of bacteria in seawater samples preserved with 2.5% glutaraldehyde (final concentration) and stored for 72 d, as a function of the percentage of cells containing mature viral particles at the time of sampling ($r^2 = 0.361$, $p = 0.207$)

Enzyme activity

Bacterial numbers in samples preserved with glutaraldehyde, quick frozen and stored at −20°C declined by only 5% after 9 d of storage (Fig. 3). Bacteria in the preserved samples stored at 4°C and in the unpreserved frozen samples had at this time point reached 72 and 81% of the initial concentration. After 16 d the bacterial numbers were reduced to 56–62% of the initial concentation in all treatments.

By adding 1.0 µM PMSF to Sargasso Sea water samples prior to the addition of 2.5% glutaraldehyde, we

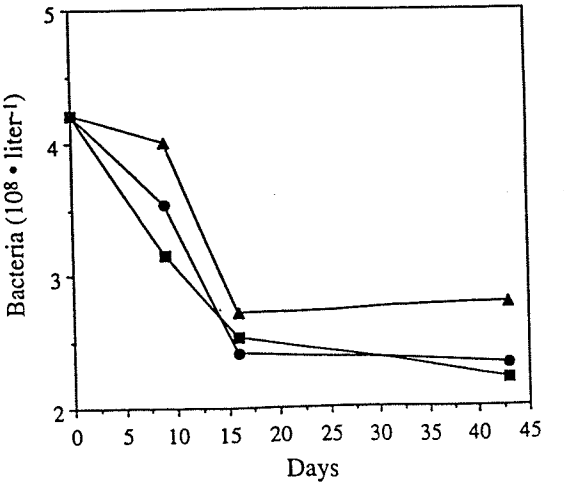

Fig. 3. Bacterial number as a function of storage time in seawater samples taken from 100 m depth at Stn 'S' in the Sargasso Sea in September 1992. (●) Unpreserved seawater, quick-frozen in liquid nitrogen and stored at −20°C; (▲) seawater preserved in 2.5% glutaraldehyde (final conc.), quick-frozen in liquid nitrogen and stored at −20°C; (■) seawater preserved in 2.5% glutaraldehyde and stored at +4°C

lost only 17 and 18% of the bacterial cells after 21 and 36 d of storage time (Fig. 4A, B). During the same time course, bacteria in seawater samples preserved only with glutaraldehyde decreased by 50 and 25% (Fig. 4A, B). By using 1.0 µM PMSF prior to the addition of glutaraldehyde the loss was only 7 and 13% of the initial concentration of bacteria in both experiments after 9 and 8 d (Fig. 4). Although the difference between the treatments was less pronounced in the latter experiment (Fig. 4B), the same trend over time was observed: seawater samples preserved with 1.0 µM PMSF and glutaraldehyde had a higher number of bacteria than seawater preserved only with glutaraldehyde (Fig. 4B). In the second time course experiment we used final concentrations of 0.5 and 1.0 µM PMSF and we found a positive linear correlation between number of bacterial cells preserved and the concentration of PMSF used after 9 and 21 d of storage ($r^2 = 0.626$, n = 4).

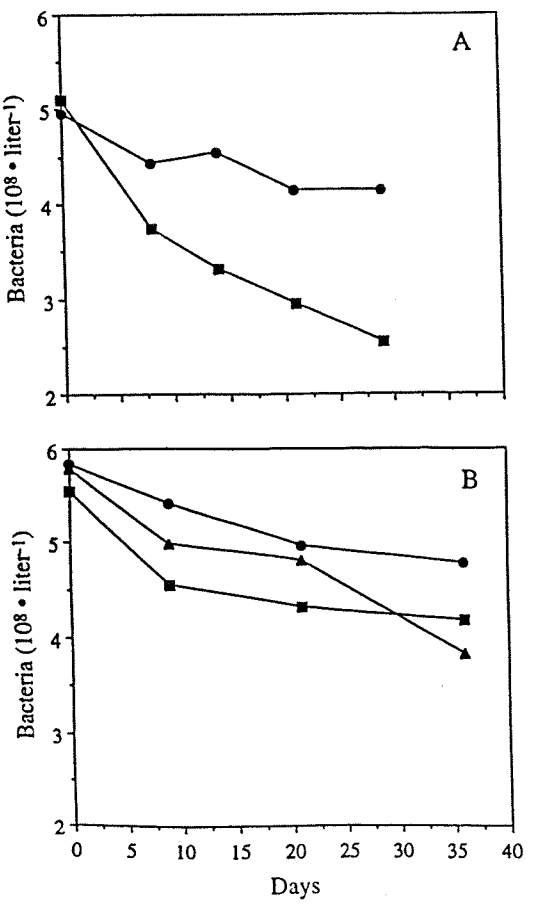

Fig. 4. Bacterial number as a function of storage time in seawater samples taken from 80 m depth (A) at Stn 'S' in September 1993 and (B) at the BATS station in April 1994. (●) Seawater preserved in 1.0 µM PMSF and 2.5% glutaraldehyde (final conc.); (▲) seawater preserved in 0.5 µM PMSF and 2.5% glutaraldehyde; (■) seawater preserved in 2.5% glutaraldehyde. All samples were stored at +4°C during the time course

These results suggest that the loss of bacterial cells can be delayed several days by freezing seawater samples at −20°C and that the addition of a protease inhibitor (PMSF) can slow down the loss significantly. Furthermore, our findings indicate that a considerable part of the loss of bacteria must be related to enzymatic breakdown of cells in the preserved sample. Addition of 2.5% glutaraldehyde may thus not be sufficient to stop enzymatic activity in the water or in the bacterial cells, leading to cell lysis and disintegration.

PMSF is unstable in aqueous solutions and we found that a precipitate was formed in our seawater samples at concentrations higher than 1 µM. Thus, other protease inhibitors may be better suited for inhibiting protease activity in preserved seawater. James (1978) found that PMSF inhibited enzymes are not reactivated when the inhibitor is deactivated. We observed a minor reduction of bacterial cells (3×10^6 l^{-1} d^{-1}) in samples pretreated with 1 µM PMSF (Fig. 4A, B). This result may suggest that PMSF insufficiently inhibited the enzymatic breakdown of bacterial cells or that factors other than proteolytic activity are responsible for the loss of bacteria as a function of storage time.

Summary

Turley & Hughes (1992) concluded that 41 to 48% of the original concentration of bacteria was lost due to cells adhering to the wall of the sample containers. In the same study, 27 to 51% of the observed loss of bacterial cells could not be accounted for (Turley & Hughes 1992). Our study shows that proteolytic breakdown activity of the cells may be a major cause of the documented losses of bacteria in glutaraldehyde preserved samples. We were able to store frozen seawater samples (−20°C) preserved in glutaraldehyde, and seawater samples treated with a protease inhibitor such as PMSF prior to the glutaraldehyde addition, for as long as 1 wk without any major loss of bacterial cells. Nevertheless, we support the recommendation made by Turley & Hughes (1992) that bacteria samples should be preserved, stained, filtered and mounted on slides as soon after sampling as possible and then stored frozen (−20°C) until enumerated.

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