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Activity of marine bacteria under incubated and *in situ* conditions

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ABSTRACT: We evaluated the extent to which the average metabolic activity of open ocean bacterioplankton changed during 2 to 3 d incubations of 1 µm filtered seawater at *in situ* temperature. Indices of bacterial activity during incubation experiments were compared to those of *in situ* bacterioplankton, which were repeatedly sampled at each oceanic site. Indices included: total and cell-specific incorporation rates of tritiated leucine ([^{3}H]-Leu) and thymidine ([^{3}H]-TdR), ratio of Leu to TdR incorporation, and percentage of highly active cells as determined by detectable reduction of the fluorogenic dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC). Patterns of change in metabolic activity were similar for experiments with water collected in 3 regions: midshelf, slope, and offshore, off the Oregon coast, USA, and incubated at *in situ* temperatures of 9.5 to 18°C. At the beginning of the experiments, cell-specific incorporation rates of [^{3}H]-Leu and [^{3}H]-TdR were lower compared to *in situ* rates, but after 19 to 28 h, incorporation rates of [^{3}H]-Leu and [^{3}H]-TdR increased dramatically, by more than an order of magnitude, compared to *in situ* activity. When scaled to the abundance of CTC positive (CTC+) cells, rates of Leu and TdR incorporation were 2.8- to 8.1-fold higher during the incubations compared to activity of *in situ* CTC+ cells. In this study, marine bacteria in 1 µm, incubated filtrate exhibited a much larger variation in metabolic activity than did *in situ* bacterioplankton. In addition, the proportion of CTC+ cells was closely related to cell-specific rates of Leu and TdR incorporation.

KEY WORDS: Marine bacteria · Incubation · Metabolism · Cell-specific activity · CTC

INTRODUCTION

From results of a variety of assays, it appears that a large fraction (>50 to 90%) of *in situ* bacterioplankton cells enumerated via epifluorescence microscopy have minimal, or no, metabolic activity (del Giorgio & Scarborough 1995, Zweifel & Hagström 1995, Heissenberger et al. 1996, Choi et al. 1999, Sherr et al. 1999). Inactive bacteria may be dead, dormant, or slowly growing (Morita 1997, Schut et al. 1997, Sherr et al. 1999). However, Choi et al. (1999) demonstrated that substantial fractions (30 to 85%) of marine bacterial cells with relatively inactive electron transport systems (ETS), based on lack of detectable reduction of the fluorogenic dye, 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), can become CTC positive (CTC+) within 1 to 2 d after addition of organic substrate and/or increase in water temperature. This result suggests that a significant portion of less active bacteria have the capacity to change their physiological state to become highly metabolically active under conditions favorable for growth.

Investigations of growth rate and metabolic capabilities of bacterioplankton are often based on incubation of bacteria in size-fractionated or diluted seawater over periods of one to several days (e.g. Kirchman et al. 1990, Pomeroy et al. 1994, Carlson & Ducklow 1996, Cherrier et al. 1996, Sieracki et al. 1999). Understanding how the cell-specific metabolic activity of incubated bacteria compares to that of *in situ* bacteria in the same system is crucial for interpretation of data resulting from such experiments. Ferguson et al. (1984) reviewed earlier criticisms of the use of container experiments in this regard, and showed that incubated marine bacteria increased in cell number, in average cell volume, in turnover rate of amino acids, and in the percentage of culturable cells with time.

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In this study, we extended our previous work (Choi et al. 1996, 1999) to evaluate the time course of change of average metabolic activity of open ocean bacterioplankton during incubation, via variation in rates of incorporation of tritiated leucine ([^{3}H]-Leu) and of tritiated thymidine ([^{3}H]-TdR), as proxies for rates of bacterial protein and DNA synthesis, respectively, and in proportion of cells with highly active ETS, using the CTC assay. We also calculated ratios of Leu:TdR incorporation, a parameter which has been suggested as an index of growth status of bacteria *in situ* (Simon & Azam 1989, Chin-Leo & Kirchman 1990, Tibbles 1996, Shiah & Ducklow 1997). The metabolic indices of bacteria in the incubations were compared to the same indices for *in situ* bacterioplankton, which were repeatedly sampled at each site over the same 2 to 3 d period that each incubation experiment took place.

The objectives of this study were (1) to demonstrate that oceanic bacterioplankton subjected to experimental manipulation, in this case size fractionation to remove bacterial grazers, can exhibit much higher cell-specific metabolic activities in comparison to those found for *in situ* bacterioplankton, and (2) to evaluate the relationship of the abundance of CTC+ cells to metabolic activity of marine bacteria. Criticisms of the use of CTC reduction as an index of relative activity of bacteria cells (Ullrich et al. 1996, Karner and Fuhrman 1997) have been addressed by Sherr et al. (1999). Sherr et al. (1999) concluded that the CTC method is a useful approach for detecting the most highly active cells in bacterial assemblages. The results of the present study support that conclusion.

METHODS

Sampling. Water samples were collected during a 3 wk research cruise (August 31 to September 18, 1998) off the Oregon coast, USA, at 3 locations: midshelf (44° 38.3' N, 124° 18.5' W, bottom depth 80 m), slope (44° 38.3' N, 124° 48.0' W, bottom depth 350 m), and offshore (44° 38.65' N, 127° 10' W, bottom depth >2000 m). The ship remained at each location for 3 to 5 d. While at each station, abundance and metabolic activity of *in situ* bacterioplankton were sampled 2 to 3 times d^{-1}, including both light and dark periods, at 2 to 3 depths in the upper 20 to 40 m of the mixed layer using a CTD rosette with 10 l Niskin bottles.

Time-course experiments. During the first sampling day at each site, 2 l of water taken from the upper 20 m were gently filtered (vacuum <5 in Hg) through 1 µm pore size membrane filters. The filtrate was used to pre-rinse and then fill 2 clean, autoclaved 500 ml polycarbonate bottles, which were placed in the dark in a water bath set at the *in situ* temperature of the mixed layer at each site. Subsampling from the bottles was begun at approximately 1 h after the bottles were placed in the incubator, and then at 6 to 15 h intervals for 2 to 3 d. At each time point, duplicate samples were taken from each bottle for bacterial abundance and percentage of CTC+ cells, and separate triplicate subsamples for rate of incorporation of tritiated [^{3}H]-Leu and [^{3}H]-TdR. At the end of each experiment, 25 ml samples were preserved, stained with DAPI, and filtered onto 0.8 µm black membrane filters to check for growth of bacterivorous flagellates (Sherr et al. 1993).

Bacterial abundance. Duplicate 2 ml subsamples were fixed with 5% final concentration of borate-buffered formalin, stained with 25 µg ml^{-1} final concentration of DAPI for 7 min (Suzuki et al. 1993) and filtered onto 25 mm, 0.2 µm black-stained membrane filters. The filters were mounted onto slides and stored frozen. Upon return to the laboratory, slides were thawed and bacterial cells enumerated at 1250× with a Zeiss Universal microscope outfitted for epifluorescence microscopy, using Zeiss filter set 4877-02 (excitation 365 nm/barrier 420 nm).

Percentage of highly active (CTC+) cells. Duplicate 1 ml subsamples were pipetted into 1.5 ml cryovials. 50 mM CTC was added to yield a final concentration of 5 mM, and the samples were incubated in the dark at *in situ* temperature for 2 to 3 h (Choi et al. 1996, 1999). After fixation with 5% final concentration formalin, the samples were frozen at –20°C until later analysis. On return to the laboratory, each CTC sample was thawed, counterstained with DAPI (25 µg ml^{-1}), settled onto a 0.2 µm black membrane filter, mounted onto a slide, and the number of CTC+ cells immediately enumerated (Choi et al. 1996, 1999). Bacterial cells were visualized at 1250× by DAPI fluorescence, then a blue excitation filter set (Zeiss 4877-09, excitation 365 nm/barrier 420 nm) was used to check each cell for red-orange fluorescence. The percentage of CTC+ cells was calculated by dividing the number of CTC+ cells by the number of separately enumerated total bacterial cells for each sample.

Rate of incorporation of [^{3}H]-Leu and of [^{3}H]-TdR. We used the centrifugation method of Smith & Azam (1992). For each sample, triplicate 1.7 ml aliquots were pipetted into 2 ml microcentrifuge tubes for each incorporation assay. A fourth aliquot in each set was amended with 5% final concentration TCA as a killed control for abiotic uptake into the macromolecular fraction. Final concentrations of 20 nM [^{3}H]-Leu (NEN, NET460, 159 Ci mmol^{-1}) and 10 nM [methyl-^{3}H]-TdR (NEN, NET512, 102.8 Ci mmol^{-1}) were added to each live sample and killed control. These were saturating concentrations as determined by concentration-dependent incorporation experiments carried out at the 3 sites. The microcentrifuge tubes were incubated in a

dark water bath at *in situ* temperature for 1 h. Preliminary experiments showed linear uptake of both [^{3}H]-TdR and [^{3}H]-Leu during 1 to 2 h incubations of seawater samples. Live samples were killed by addition of 100% TCA (5% final volume) and processed on board within 24 h via high-speed centrifugation, with a 5% TCA and an 80% ethanol treatment, as described by Smith & Azam (1992). When processing the [^{3}H]-TdR incorporation samples, killed samples and the TCA and ethanol reagents were kept ice-cold prior to and during the procedure. The refrigerated centrifuge was kept at 2°C for [^{3}H]-TdR samples. After addition of 0.5 ml of Ultima-Gold scintillation cocktail, each microcentrifuge tube was placed in a 7 ml plastic scintillation vial. On return to the laboratory, activity in the samples was determined via 30 min counts on a Wallac 1411 LSC. Killed control DPM was subtracted from the average of triplicate live DPM, and molar incorporation rates of [^{3}H]-Leu and [^{3}H]-TdR calculated.

Growth rates and [^{3}H]-TdR and [^{3}H]-Leu conversion factors. Growth rates were calculated via regression analysis of increase in the natural log of cell abundance over time during the growth phase of bacterioplankton in the experiments. Conversion factors (CFs) for [^{3}H]-TdR and for [^{3}H]-Leu incorporation (cells produced per mole of TdR or Leu incorporated) were calculated over the growth phase by the modified derivative method (Simon & Azam 1989, Kirchman & Ducklow 1993). CFs were separately calculated for each of the replicate incubation bottles. Assuming on average 20 fg C per bacterial cell (Lee & Fuhrman 1987, Fukuda et al. 1998), a carbon-based conversion factor was also determined for Leu incorporation (kg bacterial C produced per mole of Leu incorporated).

RESULTS

Environmental conditions

The study was carried out during the end of the upwelling season off the Oregon coast. The midshelf station was characterized by low sea surface temperature, 8.5 to 10°C, and mixed layer chlorophyll *a* concentrations of 3.0 ± 0.35 µg l^{-1}. The slope station had intermediate upper water temperatures, 14.5 to 16°C, and lower chlorophyll concentrations, 0.52 ± 0.02 µg l^{-1}. The offshore station had the highest mixed layer temperatures, 18 to 19°C, and a mean chlorophyll concentration of 0.76 ± 0.03 µg l^{-1}. The number of discrete samples used for analysis of *in situ* bacterial parameters during the incubations was 10 for the midshelf, 6 for the slope, and 15 for the offshore sites; these included samples taken during both day and night.

Time course of bacterial abundance and Leu incorporation

A similar pattern of change in abundance and rates of [^{3}H]-Leu incorporation was found for all 3 experiments (Fig. 1). Bacterial abundances began to increase after 43 h in the midshelf experiment, and after 19 h in the slope and offshore experiments. Bacterial numbers approximately doubled during each incubation, from 0.68 to 1.14 × 10^9 cells l^{-1} at time 0 to 1.65 to 2.21 × 10^9 cells l^{-1} by 53 to 67.5 h (Fig. 1). Rates of [^{3}H]-Leu incorporation began to increase prior to increase in abundance, and then accelerated to rates 50- to 130-fold greater than were observed at the start of incubation (Fig. 1). In the slope and offshore experiments, rates of [^{3}H]-Leu incorporation decreased sharply by the end of the incubations. Samples were checked for presence of

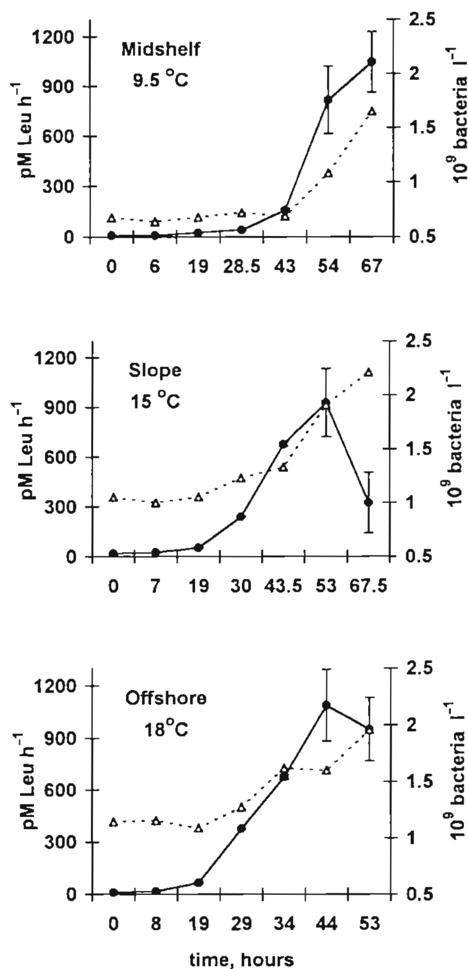

Fig. 1. Change in rate of [3H]-Leu incorporation (●, pM Leu h^{-1}) and in abundance of bacteria (△, 10^9 cells l^{-1}) over time during the 3 incubation experiments at the midshelf, slope, and offshore sites. Average of values for duplicate bottles ±1 standard deviation for [3H]-Leu incorporation rates

bacterivorous flagellates at the end of each experiment; protists were found only in the slope site experiment at concentrations of 10 to 25 cells ml^{-1}, too few to have any significant impact on bacterial abundance or activity. Natural abundances of heterotrophic flagellates in this region are in the range of 120 to 1300 cells ml^{-1} (Gonzalez et al. 1993).

Time course of Leu:TdR ratios

Leu:TdR ratios were minimal (8.6 to 15.5) at the beginning of the experiments, and increased to 33–36.2 by the end (Fig. 2). In the offshore experiment, the Leu:TdR ratio was high initially, but dropped to 14.6 by 8 h and then increased steadily thereafter. However, midshelf Leu:TdR ratios did not begin to increase until after 19 h, and slope ratios until after 43 h (Fig. 2).

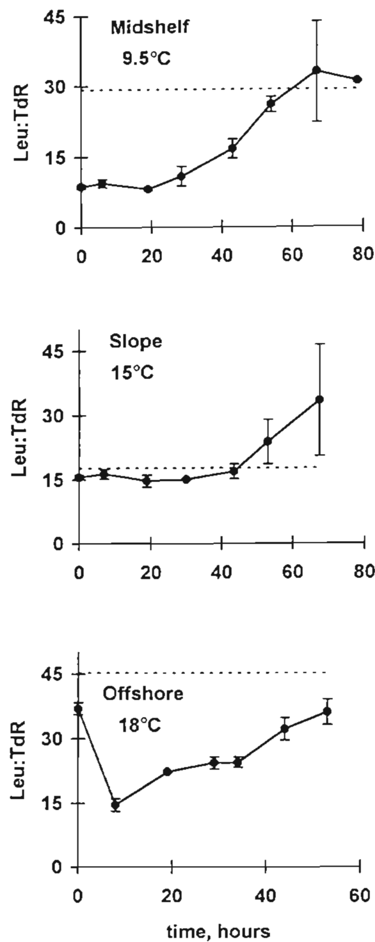

Fig. 2. Change in ratio of Leu incorporation: TdR incorporation over time during the 3 incubation experiments, compared to the average Leu:TdR ratio found for *in situ* bacterioplankton at each site (dotted line). Average of values for duplicate bottles, ±1 standard deviation

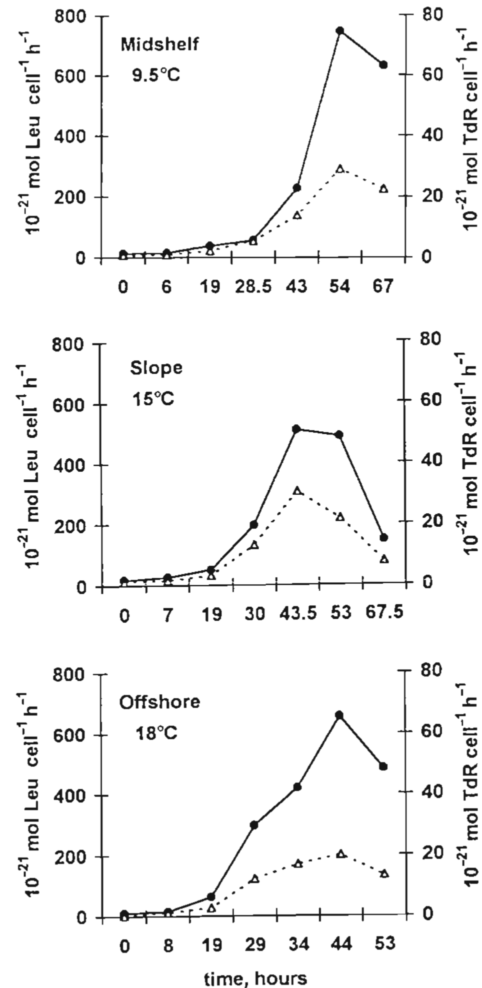

Fig. 3. Change in cell-specific rates of [^{3}H]-Leu incorporation (●, 10^{-21} mol Leu $cell^{-1}$ h^{-1}) and in cell-specific rates of [^{3}H]-TdR incorporation (▲, 10^{-21} mol TdR $cell^{-1}$ h^{-1}) over time during the 3 incubation experiments

Time course of cell-specific rates of incorporation of Leu and TdR

Cell-specific protein and DNA synthesis, assessed by rates of incorporation of [^{3}H]-Leu and [^{3}H]-TdR, respectively, began to increase after about 8 to 19 h in the 3 incubations (Fig. 3). Cell-specific incorporation rates were maximal at 43.5 to 54 h, and then decreased.

Time course of percentage of CTC+ cells, incorporation rates scaled to CTC+ cells, and relation of % CTC+ cells to cell-specific Leu and TdR incorporation

The percentage of CTC+ cells increased by 3.5- to 6-fold, from 3.2–4.9 % at the beginning of the incuba-

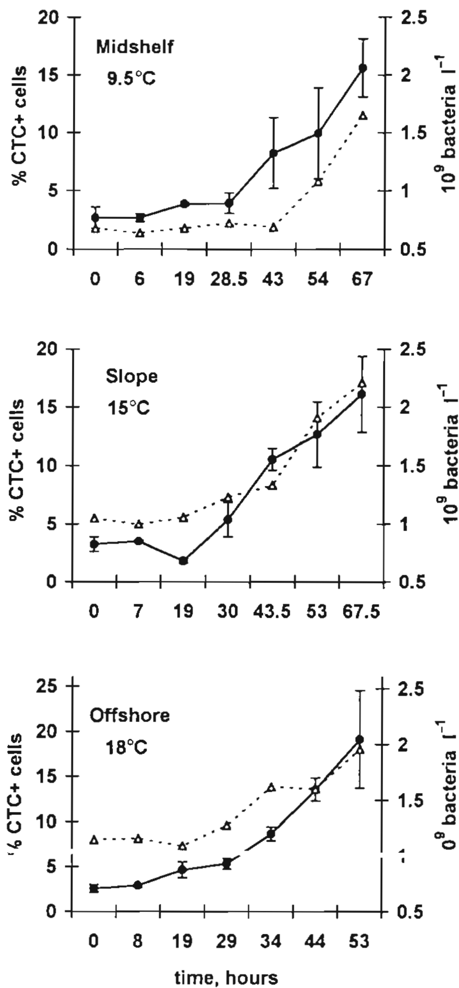

Fig. 4. Change in percentage of CTC+ cells (●) and in abundance of bacteria (▵, 10^9 cells l^{-1}) over time during the 3 incubation experiments at the midshelf, slope, and deepwater sites. Average of values for duplicate bottles ±1 standard deviation for % CTC+ cells

tions to 16.1–19.1% at the end (Fig. 4). Increase in % CTC+ began just prior to increase in cell number. Scaling incorporations rates of [^{3}H]-Leu to only the CTC+ cells (Fig. 5) resulted in cell-specific activities 20- to 32-fold higher than Leu incorporation rates scaled to total bacterial abundance. The percentage of CTC+ cells during lag and growth phases was highly correlated with cell-specific incorporation rates of both [^{3}H]-Leu and [^{3}H]-TdR (Fig. 6).

Comparison of incubated bacteria with *in situ* bacteria

Abundances and metabolic indices for *in situ* bacterioplankton compared to those of bacteria at the start of, and maximum values during, the incubations are shown in Table 1. At time 0, bacterial abundances in the 1 µm filtered seawater were 0.64 to 0.92×, and by the end of the experiments were 1.09 to 2.23×, the average cell abundance of *in situ* bacteria at the 3 sites. At the beginning of all 3 experiments, [^{3}H]-Leu incorporation, in terms of both volumetric and cell-specific rates, was substantially lower compared to *in situ* rates (Table 1). In the midshelf experiment, all metabolic indices were lower at the start of the experiment: 10-fold lower for [^{3}H]-Leu incorporation, 6.6-fold lower for [^{3}H]-TdR incorporation, 3.4-fold lower for Leu:TdR ratio, and 1.8-fold lower for % CTC+ cells. In this experiment, the indices did not begin to approach those found for *in situ* bacterioplankton until after about a day.

By the end of the incubations, [^{3}H]-Leu incorporation rates had increased dramatically, by 14- to 28-fold

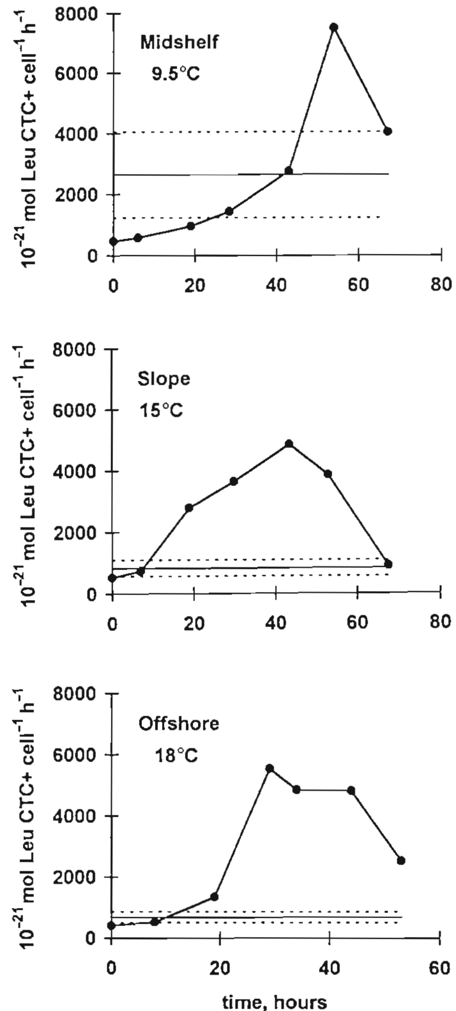

Fig. 5. Change in CTC+ cell-specific rates of [3H]-Leu incorporation (10^{-21} mol Leu cell^{-1} h^{-1}) over time during the 3 incubation experiments, compared to the average (solid line) and standard deviation (dotted lines) of CTC+ cell-specific rates of [^{3}H]-Leu incorporation found for the *in situ* samples at each site

compared to *in situ* rates (Table 1). Maximum cell-specific rates of [^{3}H]-Leu and [^{3}H]-TdR incorporation were higher by 6- to 30-fold compared to rates of *in situ* bacteria. During the incubations, CTC+ cell-specific Leu incorporation rates increased to values 2.8-fold (midshelf), 5.8-fold (slope) and 8.1-fold (offshore) higher compared to *in situ* CTC+ cells (Table 1). However, the Leu:TdR ratios found by the end of the incubations were similar to average *in situ* ratios for the midshelf and offshore sites, and only twice as high as *in situ* ratios at the slope site (Table 1). The maximum percentages of CTC+ cells in the experiments were up to 6-fold higher than *in situ* % CTC+ values.

Growth rates and [^{3}H]-TdR and [^{3}H]-Leu conversion factors

Growth rates estimated for bacteria during the incubations were highest (0.36 h^{-1}: doubling time [T_d] of 19.1 h) for the midshelf experiment, even though surface water temperatures were lowest at this site. Growth rates were lower (0.018 to 0.016 h^{-1}, T_d of 38.3 to 43.3 h) in the slope and offshore experiments (Table 2). CFs for [^{3}H]-Leu and [^{3}H]-TdR incorporation rates showed a pattern of highest CFs for midshelf bacteria, and lowest CFs for offshore bacteria (Table 2).

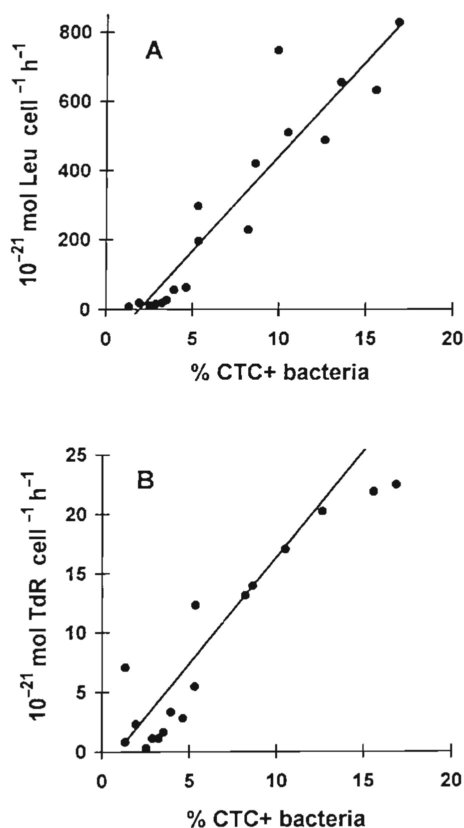

Fig. 6. Relation between percentage of CTC+ cells and: (A) cell-specific rates of [^{3}H]-Leu incorporation (regression line: $y = -10.5 + 5.6x$, $r^2 = 0.89$; (B) cell-specific rates of [^{3}H]-TdR incorporation (regression line: $y = -0.16 + 0.18x$, $r^2 = 0.77$)

DISCUSSION

Variability in bacterial metabolic activity during incubation

We found that freshly collected oceanic bacterioplankton exhibited a large range in metabolic activity during 2 to 3 d incubations. The patterns of metabolic activities showed 2 shifts compared to *in situ* bacterial activity. First, at the beginning of the experiments, protein and DNA synthesis rates, assayed via Leu and TdR incorporation, were somewhat lower compared to average rates of synthesis by *in situ* bacterioplankton at the same sites (Table 1). This decline was apparent for incorporation rates scaled both to total bacteria and to only CTC+ bacteria. Bacteria at the midshelf site showed the most dramatic decrease in metabolic activity. It may be that a fraction of the most active cells, which tend to be larger and associated with particles (Sherr et al. 1999), was removed by the 1 µm filtration. It is also possible that bacterial physiology was affected by the manipulations involved in setting up the experiment. Using flow cytometry to quantify cell-specific amounts of CTC reduced by marine bacteria in dilution culture, Sieracki et al. (1999) noted a decline in cell-specific fluorescence during the first 30 h of incubation. These authors speculated that this effect could be an artifact of sampling and containment.

Second, after 19 to 28.5 h the bacteria showed a 'shift-up' in metabolic activity coincident with, or just prior to, increase in cell number. While cell abundances only doubled, rates of [^{3}H]-Leu and [^{3}H]-TdR incorporation, and percentage of CTC+ cells, showed increases in the range of 5- to 30-fold greater compared to the metabolic indices of *in situ* bacterioplankton at the 3 sites. The greatest proportional increases in cell-specific indices were observed in the offshore experiment. These increases in metabolic activity demonstrated that bacterioplankton at all 3 sampling sites were capable of responding, on the time scale of a day, to change in environmental conditions. Pomeroy et al. (1994) also observed an initial decline, followed by an order-of-magnitude increase, in [^{3}H]-Leu incorporation rate for marine bacterioplankton incubated in bottles.

In contrast to the large range of metabolic activity shown by bacteria in the incubation experiments, *in situ* bacterioplankton, repeatedly sampled at the same

Table 1. Comparison of values for cell abundance and for metabolic parameters found for *in situ* bacterioplankton assemblages and for bacteria in the incubation experiments at time 0 and at the time at which maximum values of the parameters occurred. Mean ± 1 standard deviation. M = midshelf, S = slope, O = offshore

Parameter	Site	*In situ*	Incubation, time 0	Incubation, maximum
Bacterial abundance (10^9 cells l^{-1})	M	0.74 ± 0.12	0.68 ± 0.05	1.65 ± 0.5
	S	1.41 ± 0.51	1.05 ± 0.0	2.21 ± 0.04
	O	1.79 ± 0.38	1.14 ± 0.02	1.96 ± 0.2
[^{3}H]-Leu incorporation (pM h^{-1})	M	76 ± 40	8.2 ± 0.05	1050 ± 290
	S	41 ± 18	18 ± 1.2	930 ± 50
	O	38 ± 11	12 ± 0.7	1090 ± 180
Leu:TdR ratio	M	29 ± 10	8.6 ± 0.4	33 ± 11
	S	18 ± 6	16 ± 0.4	34 ± 13
	O	45 ± 29	37 ± 1.4	36 ± 3
[^{3}H]-Leu specific activity scaled to total bacteria (10^{-21} mol cell^{-1} h^{-1})	M	132 ± 43	13 ± 0.6	750 ± 110
	S	29 ± 8	18 ± 1.0	490 ± 20
	O	21 ± 3.8	10 ± 0.3	650 ± 130
[^{3}H]-Leu specific activity scaled to CTC+ bacteria (10^{-21} mol cell^{-1} h^{-1})	M	2640 ± 1420	470 ± 160	7510 ± 1500
	S	840 ± 27	540 ± 130	4840 ± 1190
	O	690 ± 18	407 ± 75	5550 ± 2680
[^{3}H]-TdR specific activity scaled to total bacteria (10^{-21} mol cell^{-1} h^{-1})	M	4.6 ± 0.8	1.4 ± 0.7	29 ± 5.6
	S	1.7 ± 0.1	1.1 ± 0.05	31 ± 0.7
	O	0.6 ± 0.4	0.3 ± 0.03	20 ± 2.4
[^{3}H]-TdR specific activity scaled to CTC+ bacteria (10^{-21} mol cell^{-1} h^{-1})	M	39 ± 12	26 ± 21	290 ± 70
	S	51 ± 19	34 ± 8	290 ± 66
	O	17 ± 15	11 ± 3	230 ± 93
% CTC+	M	4.9 ± 1.1	2.7 ± 0.8	17 ± 0.9
	S	3.6 ± 1.4	3.2 ± 0.6	16 ± 3.3
	O	3.2 ± 0.6	2.6 ± 0.4	19 ± 5.4

sites during each of the incubations, showed a narrower range of values for each of the parameters. *In situ* bacteria also had lower values for cell-specific leucine and thymidine incorporation rates, and for % CTC+ cells, compared to those attained by the incubated bacteria (Table 1).

The observed difference in metabolic activity between incubated and *in situ* bacterioplankton could be due to filtration/manipulation of samples providing additional available substrate for the bacteria compared to *in situ* bacterioplankton. In each experiment, about 1×10^9 bacteria l^{-1} were produced. At 12 to 30 fg C cell^{-1} (Lee & Fuhrman 1987, Fukuda et al. 1998), the bacteria would have required 48 to 120 µg C l^{-1} of substrate to yield that number of cells, assuming their carbon conversion efficiencies were about 25% (Biddanda & Benner 1997, del Giorgio et al. 1997). Chlorophyll concentrations during the sampling period were in the range of 0.5 to 3 µg l^{-1}. Assuming a C:chl *a* ratio of 50, the phytoplankton standing stock would represent 25 to 150 µg C l^{-1}. Concentrations of dissolved organic carbon (DOC) in surface seawater off the Oregon coast during summer are in the general range of 1200 to 1700 µg C l^{-1}, although most of this material is likely to be fairly refractory (P. A. Wheeler & J. Hill unpubl. data). It is possible that sample handling made some fraction of the organic matter in plankton and in DOC more available for incubated bacteria.

Growing bacteria may also have accumulated in the bacterioplankton assemblage due to exclusion of bacterivores. Previous studies have demonstrated that when relieved from grazing pressure, bacterioplankton cells become on average larger in biovolume, have a higher frequency of dividing cells and percentage of CTC+ cells, and show higher growth rates compared to bacteria in the presence of grazers (Gonzalez et al. 1990, Sherr et al. 1992, del Giorgio et al. 1996). Mortality due to viral lysis (Fuhrman & Noble 1995) should not be significantly affected by filtration. The fact that there were fairly long lag times, 19 to 43 h, before bacterial abundance increased above initial abundance in the experiments suggests that bacteria were undergoing a 'shift-up' in average metabolic activity. If bacterivores selectively graze larger, actively growing cells, then accumulation of highly active cells would be expected in bacterial assemblages separated from grazers (Sherr et al. 1992, del Giorgio et al. 1996, Choi et al. 1999), resulting in a lag time as observed here.

Filtration and bottle incubation may also select for bacterial genotypes which are rare in natural assemblages but have a competitive advantage in 1 µm filtrate. If 1 or 10% of the initial bacterial assemblage was responsible for the observed growth, then doubling times for just these bacteria would have been about 7 to 14 h rather than 19 to 43 h calculated for the whole assemblage (Table 2). These doubling times do not seem unreasonable. Selective growth of specific strains of bacteria might explain the observation of Ferguson et al. (1984) that the percentage of culturable bacterial cells increased during incubation. Using LH-PCR, Suzuki (1999) found that filtration through 0.8 µm did not dramatically change the community structure of bacterioplankton present in Oregon coastal waters. However, after about 24 h of incubation, the pheno-

typic composition of bacteria in 0.8 µm filtered seawater showed major shifts compared to that of bacterioplankton in the presence of bacterivorous protists. Suzuki (1999) speculated that removal of bacterivores allowed rarer phenotypic groups to grow up in the incubations. Hain & Hofle (1998) and Jurgens et al. (1999) have also demonstrated compositional shifts among bacteria due to protist grazing. On the other hand, Choi et al. (1999) found that with substrate addition and/or increase in temperature, up to 85% of bacterial cells initially present in coastal seawater samples became highly metabolically active (CTC+). Their result suggests that a large fraction of bacteria in seawater may have the capacity to become highly active, not just initially rare opportunists.

Leu:TdR ratio

Among the 3 experiments, there was a consistent pattern in Leu:TdR ratio change over time. At the beginning of the experiments, Leu:TdR was low (8.6 to 15), and the ratio increased as the cells began to grow, to final ratios of 33 to 36 (Fig. 2). Chin-Leo & Kirchman (1990) observed a similar increase of Leu:TdR ratio with bacterial growth in seawater cultures.

The average *in situ* Leu:TdR ratios in the mixed layer at the 3 sites varied from 17.7 to 45.3. These values are in the range found by other investigators for mesotrophic marine systems. In seawater culture experiments, Chin-Leo & Kirchman (1990) found that the Leu:TdR ratio varied from 5 to 80, and that changes in the ratio occurred during shifts in bacterial growth rate. In a later study, Shiah & Ducklow (1997) reported that during seasonal sampling of estuarine bacterioplankton assemblages, the molar ratio of [^{3}H]-Leu to [^{3}H]-TdR uptake varied about 5-fold, from 6 to 30, and that the Leu:Tdr ratio decreased with increasing temperature. In contrast, Tibbles (1996) found a positive relation between Leu:TdR incorporation ratio and temperature in marine ecosystems. Average Leu:TdR ratio was 26 ± 10 in Subantarctic Surface Water (ca 10°C) and 47 ± 19 in Subtropical Surface Water (ca 20°C) (Tibbles 1996).

In this study, Leu:TdR varied over about the same range (15 to 40) in the 3 experiments, despite a 9°C range in experimental temperature. Gasol et al. (1998) reported a large range in Leu:TdR ratio in the oligotrophic Mediterranean Sea, with ratios generally <100 above the deep chlorophyll maximum at 50 m, and very high ratios, >100 to 400, below this depth. We found the highest ratios at the offshore station (maximum ratio of 99 at 5 m depth), although bacterial abundances ($1.79 \pm 0.38 \times 10^9$ l^{-1}) and chlorophyll concentrations (0.76 ± 0.03 µg l^{-1}) suggested that surface waters at the offshore site were not particularly oligotrophic.

Cell-specific metabolic indices

We assessed 3 separate cell-specific indices: cell-specific rates of protein synthesis (rate of [^{3}H]-Leu incorporation), cell-specific rates of DNA synthesis (rate of [^{3}H]-TdR incorporation) and percentage of cells with highly active ETS (%CTC+). In our previous work, we have found that the percentage of CTC+ cells is a sensitive index of metabolic state, and is highly correlated with cell growth (Choi et al. 1996, 1999). In this study, percent CTC+ values were significantly correlated with cell-specific [^{3}H]-Leu and [^{3}H]-TdR incorporation rates (Fig. 6). Others have shown a positive relationship of abundance of CTC+ cells with rate of [^{3}H]-TdR or [^{3}H]-Leu incorporation (Lovejoy et al. 1996, Sherr et al. 1999), with bacterial cell size (Gasol et al. 1995), and with rate of respiration in the microbial (<3 µm) size fraction of seawater (Smith 1998).

In our experiments, %CTC+ values increased just prior to increase in cell abundance and then rose in tandem with cell numbers (Fig. 4). The maximum numbers of CTC+ cells were equivalent to 26.5, 35.8 and 55.7% of the increase in bacterial abundance in the 3 experiments. This may suggest that some growing cells were not detectable as metabolically active with our microscope-based CTC method. Flow cytometric analysis detects a higher number of CTC+ cells compared to microscopy (Sherr et al. 1999, Sieracki et al. 1999). The results of Choi et al. (1996, 1999) indicated

Table 2. Summary of growth rates, doubling times (T_d), [^{3}H]-TdR conversion factor (CF, 10^{18} cells mol^{-1} TdR), and [^{3}H]-Leu conversion factors (CF, 10^{17} cells mol^{-1} Leu and kg bacterial C mol^{-1} Leu). CFs are averages of values calculated for duplicate bottles in each incubation

Site	Temp (°C)	Growth rate (h^{-1})	T_d (h)	TdR CF (10^{18} cells mol^{-1})	Leu CF (10^{17} cells mol^{-1})	Leu CF (kg C mol^{-1})
Midshelf	9.5	0.036	19.1	2.2 ± 0.4	1.2 ± 0.2	2.5 ± 0.3
Slope	15	0.018	38.3	1.4 ± 0.5	0.9 ± 0.2	2.3 ± 0.5
Offshore	18	0.016	43.3	1.3 ± 0.2	0.5 ± 0.1	1.6 ± 0.2

that bacterial cells may cycle in and out of high metabolic activity during growth. Sieracki et al. (1999) also found that abundance of CTC-negative cells increased during the growth phase of incubated bacterioplankton, and speculated that a portion of the progeny of active cells becomes inactive.

The cell-specific [^{3}H]-Leu and [^{3}H]-TdR incorporation rates we found for *in situ* bacteria and for the early time points in the time course experiments (Fig. 3, Table 1) are within the range of values reported for other open ocean systems. Gasol et al. (1998) measured [^{3}H]-Leu specific activities of 3.4 to 10×10^{-21} mol cell^{-1} h^{-1}, and [^{3}H]-TdR specific activities of 3.3 to 11.8×10^{-21} mol cell^{-1} h^{-1} for bacteria in the western Mediterranean Sea. In surface seawater in the Subtropical Convergence, bacterial [^{3}H]-TdR specific activities were generally less than 10×10^{-21} mol cell^{-1} h^{-1}, and [^{3}H]-Leu specific activities less than 200×10^{-21} mol cell^{-1} h^{-1} (Tibbles 1996). By the end of our incubations, however, cell-specific incorporation rates had increased substantially (Fig. 3, Table 1), and were in the range of specific activities reported for bacteria in eutrophic coastal waters. Shiah & Ducklow (1997) found [^{3}H]-TdR specific activities in the range of 5 to 50×10^{-21} mol cell^{-1} h^{-1}, with values >10 for water temperature above 20°C, for bacterioplankton sampled in a temperate salt marsh tidal creek. In a coastal marine lagoon, [^{3}H]-TdR specific activities were 20 to 120×10^{-21} mol cell^{-1} h^{-1}, and [^{3}H]-Leu specific activities were 100 to 1500×10^{-21} mol cell^{-1} h^{-1}, with higher values at higher temperature (Tibbles 1996).

Cell-specific activities scaled to only CTC+ cells showed a pattern similar to specific activities scaled to total bacteria (Table 1, Fig. 5), although the values were higher by 10-fold or more. The actual values for cell-specific activities must lie between the 2 estimates, since some fraction, and probably a high fraction, of cells not detectably CTC+ may be able to assimilate substrate (Ouverney & Fuhrman 1999, Sherr et al. 1999); while at the same time, a variable fraction of the bacterial assemblage may be completely inactive, or dead (Zweifel & Hagström 1995, Heissenberger et al. 1996, Williams et al. 1998). Scaling activities to CTC+ cell abundance did show that the high rates of metabolic activity found for the incubated bacteria were not simply due to accumulation of CTC+ cells during the experiments, as the CTC+-specific activities were much greater than found for *in situ* CTC+ cells (Table 1, Fig. 5).

[^{3}H]-TdR and [^{3}H]-Leu conversion factors

In order to convert [^{3}H]-Leu and [^{3}H]-TdR incorporation rates into bacterial production rates, as increase in either carbon biomass or cell number, empirical factors are often calculated via experimental incubations of bacterioplankton in size-fractionated or diluted water (Ducklow & Carlson 1992, Kirchman & Ducklow 1993, Robarts & Zohary 1993). Our CFs for TdR incorporation, 1.3 to 2.2×10^{18} cells mol^{-1} TdR, were in the range of TdR CFs reported for mesotrophic to oligotrophic ocean systems. Fuhrman & Azam (1982) calculated CFs of 1.7 and 2.4×10^{18} cells mol^{-1} TdR for 2 stations off the California coast. According to Ducklow & Carlson (1992), the median value for TdR CFs derived from a wide range of marine systems is 2×10^{18} cells mol^{-1} TdR. After reviewing the literature, Robarts & Zohary (1993) concluded that TdR CFs between 1 and 2×10^{18} cells mol^{-1} TdR are most realistic.

Fewer estimates have been made for [^{3}H]-Leu CFs. Using bacterial parameters provided by Simon & Azam (1989), and assuming isotope dilution to be 2-fold, the theoretical relationship between Leu incorporation and bacterial carbon production is 3.1 kg C mol^{-1} Leu (Kirchman 1993). Kirchman (1993) reported that the average empirically derived [^{3}H]-Leu CF for open ocean bacteria was 1.15×10^{17} cells mol^{-1} Leu. Assuming 12 to 30 fg C bacterium^{-1} (Lee & Fuhrman 1987, Fukuda et al. 1998), this factor would be equivalent to 1.4–3.4 kg bacterial C mol^{-1} Leu. Gasol et al. (1998) used empirically derived carbon-based CFs of 0.3, 1.5 and 2.1 kg C mol^{-1} Leu for a offshore, slope, and coastal station in the northwestern Mediterranean Sea. Our calculated [^{3}H]-Leu CFs, assuming a median bacterial carbon content of 20 fg C cell^{-1}, were 0.5 to 1.2×10^{17} cells mol^{-1} Leu, or 1.6 to 2.5 kg C mol^{-1} Leu (Table 2), in the range of these previously reported estimates. In a pattern similar to that found by Gasol et al. (1998), highest CFs were found for the midshelf site, and lowest CFs at the offshore site.

CONCLUSIONS

We found that average metabolic activity of oceanic bacteria in bottle incubations can vary widely compared to average metabolic activity of *in situ* bacterioplankton in the water mass from which the experimental sample was taken. Incubated bacteria increased their average metabolic activity by 10-fold or more compared to the cell-specific activity of *in situ* bacterioplankton. Water temperature did not appear to affect patterns of bacterial activity observed in the incubations. Highest growth rate was found in the midshelf experiment, which was carried out at a lower temperature (9.5°C) compared to the slope (15°C) and offshore (18°C) sites. In these experiments, incubated bacteria also showed a lag, or shift-up phase, of up to a day, before significant increase in cell-specific activity was observed.

The results of this study support the idea that CTC+ cells are the most active cells in bacterioplankton assemblages (Smith 1998, Choi et al. 1999, Sherr et al. 1999). Cell-specific rates of incorporation of [^{3}H]-TdR and of [^{3}H]-Leu, and cell-specific ETS activity based on %CTC+ cells, appear to be indices of cell metabolism that are are positively related to cell growth, and that are highly correlated with each other. Flow cytometry can be used to more sensitively enumerate CTC+ cells, and to quantify cell-specific amounts of the reduced compound (Sherr et al. 1999, Sieracki et al. 1999).

Finally, incubation experiments designed to evaluate performance and metabolism of *in situ* bacteria may be complicated by the fact that incubated bacteria can attain order-of-magnitude greater rates of cell-specific protein and DNA synthesis, may have different ratios of protein:DNA synthesis, and can exhibit much higher proportions of cells with active ETS systems (%CTC+), compared to *in situ* bacteria. Our results reinforce the earlier warning of Ferguson et al. (1984) that one should use caution in carrying out, and interpreting data derived from, container incubation of bacterioplankton, especially for experiments longer than 12 to 24 h. Development of alternative non-incubation, or shorter-incubation, methods for investigating bacterial activity, such as the combined 16S rRNA probe and autoradiography protocol of Ouverney & Fuhrman (1999) is encouraged.

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