

Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary

Matthew T. Cottrell and David L. Kirchman¹

University of Delaware, College of Marine Studies, 700 Pilottown Road, Lewes, Delaware 19958

Abstract

Assimilation of ³H-thymidine and ³H-leucine was examined at the single-cell level using a combination of microautoradiography and fluorescent in situ hybridization (Micro-FISH) to determine the contribution of various bacterial groups to bacterial production in aquatic systems. All of the major phylogenetic groups of bacteria examined along the salinity gradient of the Delaware estuary, including alpha-, beta-, and gamma-proteobacteria and *Cytophaga*-like bacteria, assimilated ³H-thymidine and ³H-leucine. However, groups differed substantially in their contribution to the assimilation of these compounds. Alpha-proteobacteria were the dominant substrate-active bacteria at salinities of >9 PSU, whereas beta-proteobacteria were more important in freshwater. At all salinities, *Cytophaga*-like bacteria comprised the second most important group, and gamma-proteobacteria were overall the least important. Bacterial abundance explained about half of the variation in ³H-thymidine and ³H-leucine assimilation by the major bacterial groups. The sizes of silver grains of active bacteria indicate no difference in single-cell activity for the bacterial groups, suggesting that the average growth rates of the groups we examined were similar. However, activity per cell was distributed differently in the phylogenetic groups. Our study suggests that estimates of bacterial production measured using ³H-thymidine and ³H-leucine include bacteria in all of the major phylogenetic groups found in aquatic systems and that growth rates within bacterial groups vary substantially.

Measurements of bacterial biomass production are central to inferring the role of heterotrophic bacteria in marine food webs and their effect in biogeochemical cycles. The production rate of new bacterial biomass gives a measure of the amount of food available to grazers if viral removal of bacterial biomass is taken into account. Along with estimates of bacterial growth efficiency, the rate of dissolved organic matter (DOM) consumption can be calculated from rates of bacterial production to infer the geochemical effect of bacteria on one of the largest labile pools of carbon in the biosphere. Finally, average growth rates of bacterial communities can be calculated from production rates and estimates of bacterial biomass or abundance (Ducklow 2000).

Bacterial production is usually estimated from the incorporation of ³H-thymidine or ³H-leucine (Fuhrman and Azam 1982; Kirchman et al. 1985; Simon and Azam 1989) by the whole bacterial community. However, the application of culture-independent approaches has made clear that bacterial communities in aquatic systems are phylogenetically diverse. Representatives of at least 18 bacterial divisions have been found in aquatic bacterial assemblages, including five subclasses of the Proteobacteria (Giovannoni and Rappé 2000). The ³H-thymidine and ³H-leucine methods provide no information on the production rates of different bacteria comprising aquatic assemblages, which probably vary considerably among environments and over time. For example, bacterial communities in freshwater and marine environments differ greatly (Glöckner et al. 1999). Marine systems are typically dominated by alpha-proteobacteria and *Cytophaga*-like bacteria (Glöckner et al. 1999; Cottrell and

Kirchman 2000b; Kirchman 2002), whereas beta-proteobacteria appear to be the dominant group in freshwater systems (Pernthaler et al. 1998; Methe and Zehr 1999).

Studies examining the metabolism of individual bacterial cells typically reveal differences in activity among heterotrophic bacteria. Consumption of organic matter compounds, including ³H-thymidine and ³H-leucine, varies at the single-cell level (Fuhrman and Azam 1982; Tabor and Neihof 1982; Karner and Fuhrman 1997; Ouverney and Fuhrman 1999). Similarly, phylogenetic groups of bacteria appear to differ in the uptake of high- and low-molecular weight compounds (Cottrell and Kirchman 2000a) and in their contribution to the microbially driven fluxes of dimethylsulphoniopropionate (Zubkov et al. 2001a). Variation in the assimilation of ³H-methionine (Zubkov et al. 2001b) in cells sorted by flow cytometry suggests that protein synthesis varies among individual bacteria, reflecting differences in growth. Respiratory activity, membrane characteristics, and nucleic acid content of estuarine bacteria also vary at the single-cell level (del Giorgio and Bouvier 2002).

The goal of this study was to determine which bacteria in aquatic systems assimilate ³H-thymidine and ³H-leucine and contribute to bacterial production and growth. We hypothesized that some groups of bacteria do not assimilate ³H-thymidine and ³H-leucine and are not included in estimates of bacterial production. This question was examined in an estuary where community composition was expected to vary along a salinity gradient (Bouvier and del Giorgio 2002). We assessed the importance of various phylogenetic groups to bacterial production using microautoradiography to query their assimilation of ³H-thymidine and ³H-leucine in combination with fluorescent in situ hybridization (Micro-FISH) to phylogenetically characterize both active and inactive cells (Cottrell and Kirchman 2000a). Contrary to our original hypothesis, this study revealed that all of the major phy-

¹ Corresponding author.

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logenetetic groups of bacteria in aquatic systems assimilate ^3H -thymidine and ^3H -leucine.

Materials and methods

Sample collection and incubation—Seawater was collected from the Delaware Bay estuary from a depth of 1 m in March 2001. Bacterial production was measured by the incorporation of ^3H -thymidine and ^3H -leucine into cold trichloroacetic acid (TCA)-insoluble material. Triplicate 1.5-ml samples were incubated for 0.5–1 h with 20 nM ^3H -thymidine (83.5 Ci mmol $^{-1}$) and ^3H -leucine (150 Ci mmol $^{-1}$) at in situ temperatures. Killed controls were poisoned with 5% TCA. Macromolecules were extracted by ice-cold TCA extraction, collected using centrifugation, and radioassayed using liquid scintillation counting (Smith and Azam 1992).

Samples for microautoradiography were incubated with 20 nM ^3H -thymidine and ^3H -leucine at the in situ temperature for 4 h. Killed controls were poisoned with 2% paraformaldehyde. Incubations were terminated by adding 20% paraformaldehyde to a final concentration of 2%. Samples were fixed at 4°C overnight and then filtered and rinsed with deionized water on 0.2- μm pore size polycarbonate filters.

Microautoradiography and fluorescent in situ hybridization—Twenty-milliliter aliquots of fixed samples were collected on 0.2- μm pore size polycarbonate filters supported with 0.45- μm nitrocellulose membranes. Samples were rinsed twice with deionized (MilliQ) water and immediately placed on glass coverslips treated within 3 d with a 2% solution of 3-aminopropyltriethoxysilane (AES) (Sigma) (Barer and Entwistle 1991). The nitrocellulose and polycarbonate filters were placed together on the coverslip, and the cells on the surface of the polycarbonate filter were pressed against the coverslip overnight using two glass slides held together with a large paper clip. The polycarbonate filter and coverslip were separated after 18–24 h and stored at -20°C .

Samples for in situ hybridization were obtained by cutting coverslips in quarters and placing the cell-adherent side in contact with a 30- μl drop of hybridization solution containing 2 ng probe μl^{-1} on a glass slide covered with parafilm. The relative abundance of major phylogenetic groups was determined using CY3-labeled (Operon Technologies) probe Eub338 for eubacteria (Amann et al. 1990), Alf968 for alpha-proteobacteria (Glöckner et al. 1999), Bet42a for beta-proteobacteria (Manz et al. 1992), Gam42a for gamma-proteobacteria, CF319a for the *Cytophaga*-like bacterial group (Manz et al. 1996), and a negative control probe for non-specific binding (Karner and Fuhrman 1997). Samples were incubated for 18–20 h at 42°C. The hybridization solution contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.4), 0.01% sodium dodecyl sulfate, and the concentration of formamide determined to achieve specificity for the bacterial groups targeted by the different probes (Zarda et al. 1997; Eilers et al. 2000b). After hybridization, the sample was transferred to a wash solution containing 20 mM Tris-HCl (pH 7.4), 5 mM edetic acid (EDTA), 0.01% sodium dodecyl sulfate, and a concentration of NaCl appropriate for the probe (Zarda et al. 1997; Eilers et al. 2000b). The sample was then rinsed with

water, air dried, and mounted with oil on a glass slide with the cells facing away from the slide.

Polycarbonate filters, which retained about half of the cell after being separated from the AES-treated glass coverslips, were prepared for autoradiography using the approach introduced by Tabor and Neihof (1982; see also Carman 1993). A series of exposure times, typically 1, 3, 8, 24, and 48 h, was tested to determine two exposure times that would yield approximately 10 and 30% of the total community with silver grains. These exposure times were then used in autoradiography of the glass coverslip samples. Short thymidine and leucine exposure times ranged from 8 to 24 h and 1 to 3 h, respectively. Long thymidine and leucine exposure times ranged from 28 to 48 h and 3 to 7 h, respectively.

Samples on glass coverslips were prepared for microautoradiography by dipping the glass slide, with coverslip attached, into a molten (43°C) solution of NTB-2 emulsion (Kodak) diluted to two parts emulsion and one part deionized water. The glass slides were placed on an ice-cold aluminum block for 10 min to solidify the emulsion before being transferred to light-tight boxes for autoradiographic exposure times that ranged from 1 to 48 h. The exposures for 8 h or less were done at room temperature, whereas longer exposures were done at 4°C. Emulsion was developed using Dektol developer (Kodak), a deionized water stop bath, and fixer (Kodak) following the manufacturer's instructions. The slides were then rinsed in water for 10 min, dried overnight in a vacuum chamber with desiccant, then stained in a 2- $\mu\text{g ml}^{-1}$ solution of 4',6-diamidino-2-phenylindole (DAPI) for 2 min. After a final rinse in deionized water, the emulsion was once again dried overnight in the vacuum desiccator, and the coverslip was transferred to a clean glass slide and mounted with the cells facing toward the slide using a 4:1 mixture of the antifade mountants Citifluor (Ted Pella) and Vectashield (Vector Labs).

Semiautomated microscopy and image analysis—Samples were assayed using semiautomated microscopy and image analysis. The microscope setup consisted of a SPOT-RT monochrome charge-coupled device (CCD) camera (Diagnostics Instruments) mounted on an Olympus Provis AX70 microscope equipped with a U-MCB multicontroller used to control a shutter in front of the 100-W mercury lamp and a turret holding filter sets consisting of excitation, emission, and beam-splitting filters for epifluorescence microscopy. The multicontroller also controlled the tungsten lamp used for transmitted light microscopy. The microscope was controlled using ScopePro (Media Cybernetics), whereas image acquisition and processing were accomplished using ImagePro (Media Cybernetics). Numerical calculations and data storage were done in Microsoft Excel. Microscope control, image acquisition, image processing, and numerical calculations were coordinated through a graphical user interface designed with Visual Basic 6 (Microsoft).

Three images were acquired for each field of view using a $\times 100$ UPlanApo oil immersion objective. Pixel size in the eight-bit images was 0.0737 μm . DAPI images were acquired using automatic exposure with a gain limit of four using the band-pass emission filter set 31000 (Chroma), whereas Cy3 images were collected using manual exposure

with filter set 41007 (Chroma). Manual exposure times for Cy3 images were optimized using samples with the negative control probe to restrict background counts to <5% of the DAPI count. Images of silver grains were collected using transmitted light illumination with automatic exposure adjusted to overexpose the image, thus eliminating objects with low optical density and capturing only objects with high optical density against a uniform white background.

Bacteria were detected in images captured with epifluorescent illumination using three image processing steps. In the first step, a modified Marr–Hildreth kernel (Ramsing et al. 1996) was applied to the DAPI and Cy3 images to detect the gradient in pixel brightness at the cell edges (Viles and Sieracki 1992). After applying the Marr–Hildreth kernel, the images were smoothed using three passes of a median filter with dimensions of 3×3 pixels. The gray scale value of each pixel was subsequently increased by 128, and binary images representing DAPI-stained and Cy3-positive objects were produced by segmenting the DAPI and Cy3 images at gray scale values of 0 and 65, respectively.

Silver grains were detected in transmitted light images without the edge detection step because the raw images had only two features with different pixel intensities that were readily distinguished. The silver grains appeared as black objects on a uniform white background without any other objects. Raw images were segmented at a gray scale value of 250 to produce binary images of the silver grains. The size of silver grains of active alpha-, beta-, and gamma-proteobacteria and *Cytophaga*-like bacteria was determined from their projected area. We sampled only a small number of active beta-proteobacteria, which limited the analysis of activity in this group.

Probe-positive cells in Cy3 images were distinguished from other nonbacterial objects visible with the Cy3 filter set using image analysis to compare the DAPI and Cy3 images. We used restricted dilation to accomplish a boolean AND of objects in different images (Russ 1999) to distinguish the probe-positive cells that appeared in both the DAPI and Cy3 images from other objects that appeared in only the Cy3 image. Objects appearing in both images were defined as those that touched when the binary DAPI and binary Cy3 images were overlaid. After the images were overlaid, pixels forming objects in the Cy3 image were grown up to the boundaries of the objects they touched in the DAPI image. The result was a third image containing the objects that appeared in both the DAPI and Cy3 images (i.e., the probe-positive bacteria). Objects in the third image had boundaries corresponding to their appearance in the DAPI image. Silver grains associated with cells and cells that generated silver grains were identified using the restricted dilation as well with the assumption that images of silver grains touch the images of cells that produced them.

Results

Environmental setting—The contribution of Proteobacteria and *Cytophaga*-like bacteria to the assimilation of ^3H -thymidine and ^3H -leucine was examined at four stations along a 200-km transect of the Delaware Bay estuary, span-

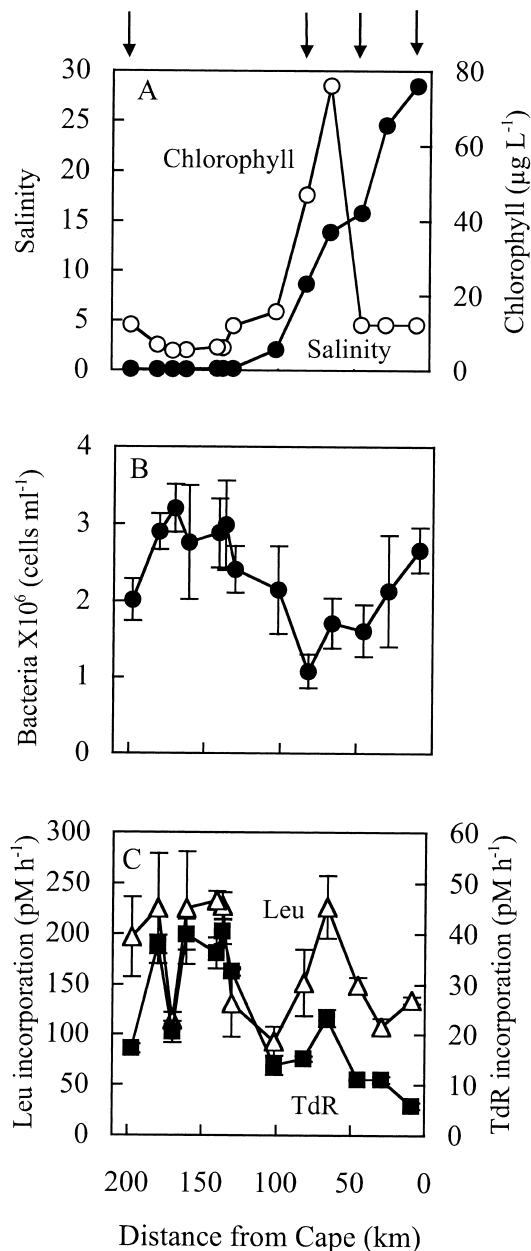


Fig. 1. Distribution of (A) salinity and chlorophyll, (B) bacterial abundance, and (C) ^3H -thymidine and ^3H -leucine incorporation in the Delaware Bay. Double-headed arrows indicate the location of stations where the assimilation of ^3H -thymidine and ^3H -leucine by bacterial groups was measured using autoradiography and FISH. Cape refers to Cape Henlopen at the mouth of the Delaware Bay. Error bars indicate standard deviations.

ning a range of salinities from 0.1 to 29 PSU (Fig. 1A). The concentration of chlorophyll along the transect varied almost 10-fold, from 5.3 to 46.9 $\mu\text{g L}^{-1}$. There was a peak in chlorophyll 66 km from Cape Henlopen at a salinity of 13.9 PSU (Fig. 1A). The chlorophyll concentration was the same (12.2 $\mu\text{g L}^{-1}$) at three stations sampled for ^3H -thymidine and ^3H -leucine Micro-FISH, whereas it was fourfold higher at the fourth station (46.9 $\mu\text{g L}^{-1}$). Bacterial abundance varied threefold (Fig. 1B), whereas rates of thymidine and leucine

incorporation varied by factors of 6 and 1.5, respectively, along the 200-km transect (Fig. 1C). Thymidine and leucine incorporation rates varied from 6 to 17 pM h⁻¹ and 134 to 197 pM h⁻¹, respectively, at the four stations sampled for Micro-FISH.

Bacterial community composition—Alpha, beta-, and gamma-proteobacteria and *Cytophaga*-like bacteria were present at all four stations (Fig. 2A). Bacterial communities at the two stations with the lowest salinity were dominated by the *Cytophaga*-like bacterial group and comprised 40 and 20% of the communities at the 0.1 and 9 PSU stations, respectively. In contrast, no single group dominated the communities at the 16 and 29 PSU stations. The *Cytophaga*-like and alpha-proteobacteria groups were equally abundant at the high-salinity stations, with each group comprising 20% of the community. The alpha-proteobacteria group was less abundant at the lower salinity stations, making up 4 and 14% of the communities at the 0.1 and 9 PSU stations, respectively.

The beta-proteobacterial and gamma-proteobacterial groups were typically minor members of the communities at our four main stations. Gamma-proteobacteria accounted for 4–15% of the communities at the four stations (Fig. 2A), whereas the relative abundance of beta-proteobacteria was generally low—<10% of the communities except at the 0.1 PSU station. At that station, beta-proteobacterial abundance was exceptionally high (42%), second only to the *Cytophaga*-like bacteria (Fig. 2A). At other freshwater stations not sampled by Micro-FISH, beta-proteobacteria were most abundant (data not shown). On average, 59 ± 13% of total bacteria were detected by FISH with probes for alpha-, beta-, and gamma-proteobacteria and *Cytophaga*-like bacteria, whereas 57 ± 18% of cells were detected with the general probe for bacteria (Eub338).

Bacterial groups assimilating ³H-thymidine and ³H-leucine—Alpha-proteobacteria were the most important bacteria assimilating ³H-thymidine and ³H-leucine at all but the freshwater station. Thirty-four percent to 49% of the bacteria assimilating ³H-thymidine and ³H-leucine, respectively, at the 9, 16, and 29 PSU stations were alpha-proteobacteria (Fig. 2B,C). In contrast, alpha-proteobacteria made up only a small fraction (6 ± 3%) of the bacteria assimilating ³H-thymidine and ³H-leucine at the 0.1 PSU station. At this freshwater station, beta-proteobacteria dominated the assimilation of ³H-thymidine and ³H-leucine, accounting for 65 ± 9% of the ³H-thymidine- and ³H-leucine-active bacteria. Beta-proteobacteria made up 0–14% of the ³H-thymidine- and ³H-leucine-assimilating bacteria at the other three stations. At all four stations, <10% of the ³H-thymidine- and ³H-leucine-assimilating bacteria were gamma-proteobacteria. Forty-nine percent (±5%) of the ³H-thymidine- and ³H-leucine-assimilating bacteria were *Cytophaga*-like bacteria at the 0.1 PSU station, whereas 14–17% of the ³H-thymidine- and ³H-leucine-active bacteria at the 9, 16, and 29 PSU stations belonged to this group. Eighty-five percent (±26%) and 80% (±31%) of the bacteria assimilating ³H-thymidine and ³H-leucine, respectively, were detected with probes for the phylogenetic groups of bacteria we examined.

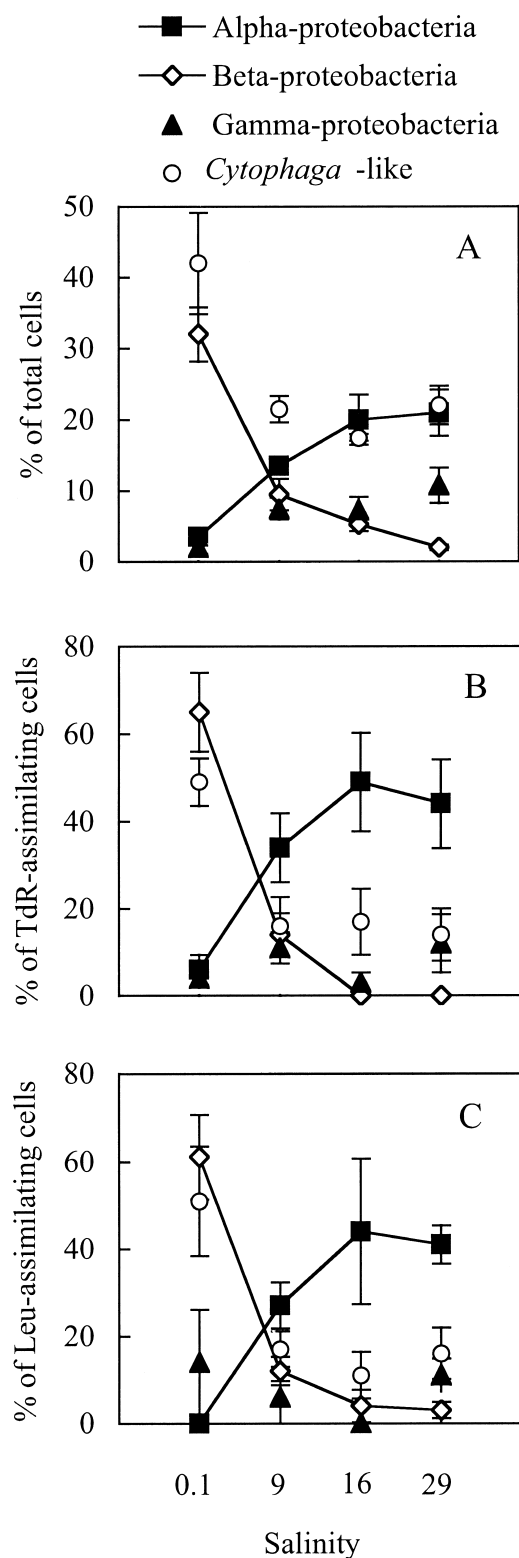


Fig. 2. Relative abundance of bacterial groups in (A) the total bacterial community, (B) the ³H-thymidine-assimilating population, and (C) the ³H-leucine-assimilating population. Alpha-, beta-, and gamma-proteobacterial subclasses and the *Cytophaga*-like bacterial group were detected with oligonucleotide probes Alf968, Bet42a, Gam42a, and CF319a, respectively. Error bars indicate standard errors.

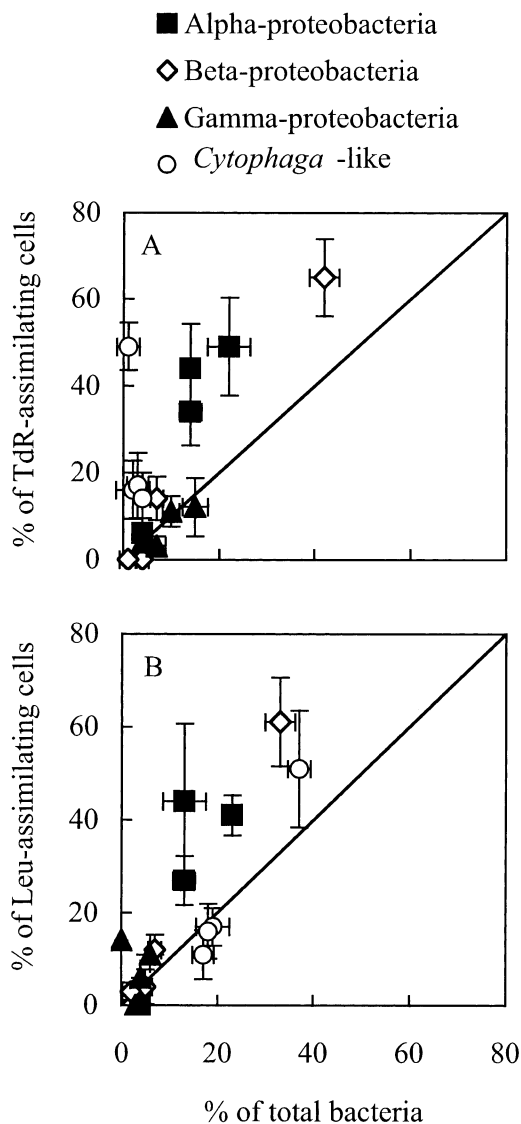


Fig. 3. Relative abundance of bacterial groups in (A) the ^3H -thymidine-assimilating and (B) the ^3H -leucine-assimilating populations plotted against their abundance in the total community. Samples were given the shorter autoradiographic exposure. Alpha-, beta-, and gamma-proteobacterial subclasses and the *Cytophaga*-like bacterial group were detected with oligonucleotide probes Alf968, Bet42a, Gam42a, and CF319a, respectively. Error bars indicate standard errors. The diagonal line indicates a 1:1 relationship.

Community structure explained about half of the variation in the assimilation of ^3H -thymidine and ^3H -leucine by different bacteria. Regression analysis indicated that, at the community level, 65 and 56% of the variation in ^3H -thymidine and ^3H -leucine assimilation, respectively, was explained by the relative abundance of bacterial groups (Fig. 3A,B). As expected, minor bacterial groups contributed less to ^3H -thymidine and ^3H -leucine assimilation than abundant groups. For example, at the 9, 16, and 29 PSU stations, beta-proteobacteria and gamma-proteobacteria comprised <10% of the total community and the populations assimilating ^3H -

thymidine and ^3H -leucine (Fig. 3A,B). The 0.1 PSU station was the only exception; at this station, gamma-proteobacteria comprised <1% of the total community, but 14% of the ^3H -leucine-assimilating bacteria.

In contrast, abundance was not an adequate predictor of a bacterial group's contribution to bacterial production when that group was a large fraction of the total community. For example, ^3H -thymidine assimilation by alpha-proteobacteria was greater than would be predicted solely from their abundance (Fig. 3A). Alpha-proteobacteria comprised 14–22% of the total community (Fig. 2A), but 44–49% of the ^3H -thymidine-assimilating bacteria at the 16 and 29 PSU stations (Fig. 2B). Alpha-proteobacteria were also disproportionately important in the assimilation of ^3H -leucine where these bacteria were abundant (9, 16, and 29 PSU stations). Alpha-proteobacteria comprised 27–44% of the populations assimilating ^3H -leucine (Fig. 2C), which was two- to threefold higher than their contribution to total bacterial abundance (Fig. 2A,C).

Beta-proteobacteria appeared to account for more ^3H -thymidine and ^3H -leucine assimilation than expected based on abundance. When beta-proteobacteria were abundant (0.1 PSU station), these bacteria comprised a large fraction of the total community and a disproportionately large fraction of the ^3H -thymidine- and ^3H -leucine-assimilating bacteria. At this freshwater station, beta-proteobacteria made up 32% of the community and were twofold more abundant among the ^3H -thymidine- and ^3H -leucine-active bacteria than in the total community (Fig. 2B,C).

The *Cytophaga*-like bacterial group, which was a major component of the bacteria at all stations, exhibited a different relationship between abundance and ^3H -thymidine and ^3H -leucine assimilation. In contrast to alpha- and beta-proteobacteria, the contribution of *Cytophaga*-like bacteria to ^3H -thymidine and ^3H -leucine assimilation reflected the abundance of this bacterial group (Fig. 3A,B). The *Cytophaga*-like bacterial group, which comprised 17–53% of the total community at all four stations (Fig. 2A), accounted for 14–49% and 11–51% of the ^3H -thymidine- and ^3H -leucine-assimilating bacteria, respectively (Fig. 2B,C).

The percentage of active cells in different bacterial groups varied substantially. The fraction of ^3H -thymidine- and ^3H -leucine-active *Cytophaga*-like bacteria in freshwater decreased from 40 to 10% in seawater with a salinity of 29 PSU. In contrast, the fraction of ^3H -leucine-active alpha-proteobacteria increased from 10 to 50% over the same salinity range. The distribution of ^3H -thymidine-active alpha-proteobacteria was different. Forty percent of alpha-proteobacteria were active at 9 PSU, whereas 10 and 20% were active at 0.1 and 29 PSU, respectively. Active beta- and gamma-proteobacteria were distributed differently as well. At all salinities, the fraction of ^3H -leucine-active beta-proteobacteria was >30%, with a maximum of 60% in 29 PSU seawater, whereas the fraction of ^3H -thymidine-active beta-proteobacteria varied from 65% to <10% in the estuary. The percentage of active gamma-proteobacteria varied from nil to 50% for ^3H -thymidine-active bacteria in freshwater and ^3H -leucine-active bacteria at 29 PSU, respectively (Cottrell and Kirchman unpubl. data).

Table 1. Percentage of cells with silver grains (\pm SD) in ^3H -thymidine and ^3H -leucine microautoradiography at four locations in the Delaware Bay estuary. Long exposures were two- to threefold longer than the short exposures.

Salinity	% Cells with silver grains			
	^3H -thymidine		^3H -leucine	
	Short exposure	Long exposure	Short exposure	Long exposure
0.1	27(3.9)	43(4.5)	9(5.9)	27(5.2)
6	15(0.6)	18(3.7)	20(3.1)	30(4.9)
16	6(0.4)	21(1.0)	7(0.5)	31(0.5)
29	8(1.5)	13(2.0)	22(4.4)	34(6.1)

Populations detected with different autoradiographic exposure times—Varying the autoradiographic exposure time gave different perspectives on the populations of bacteria assimilating ^3H -thymidine and ^3H -leucine. The results presented up to this point are from microautoradiographic samples exposed for a short time. On average, 14% of cells were apparently active with the short exposure; these bacteria probably had the highest levels of ^3H -thymidine and ^3H -leucine assimilation. The long exposure produced autoradiographs with a larger fraction (27%) of apparently active cells (Table 1). The short exposure revealed no difference in the community composition of ^3H -thymidine- and ^3H -leucine-active bacteria. Alpha-, beta-, and gamma-proteobacteria and the *Cytophaga*-like bacterial groups comprised equal fractions of the populations assimilating ^3H -thymidine and ^3H -leucine (Fig. 4A).

In contrast, long-exposure samples revealed differences in the importance of bacterial groups assimilating ^3H -thymidine compared to ^3H -leucine. In assemblages at the 16 and 29 PSU stations, alpha-proteobacteria were 1.5-fold more abundant among bacteria assimilating ^3H -leucine than ^3H -thymidine, whereas the opposite was true at the 9 PSU station; alpha-proteobacteria were 1.5-fold more abundant in the ^3H -thymidine-assimilating populations (Fig. 4B). *Cytophaga*-like bacteria in the 16 and 29 PSU assemblages were 2- to 2.5-fold more abundant in the populations assimilating ^3H -thymidine. Beta-proteobacteria in all but the 29 PSU assemblage were 2- to 2.5-fold more important in the populations assimilating ^3H -thymidine than in the ^3H -leucine-assimilating populations. Gamma-proteobacteria were seven times more abundant in the ^3H -leucine-assimilating population at the 9 PSU station than in ^3H -thymidine-assimilating populations. Differences in ^3H -thymidine and ^3H -leucine assimilation revealed by the long exposure can be attributed to slower growing bacteria or cells with different capacities to take up or incorporate the label with lower ^3H -thymidine and ^3H -leucine activity.

Single-cell activity—The results presented up to this point have considered the importance of bacterial groups in terms of the number of bacteria assimilating ^3H -thymidine or ^3H -leucine. Because the level of activity per cell can vary, we also used the size of silver grain clusters to examine assimilation by various bacterial groups. This analysis assumes

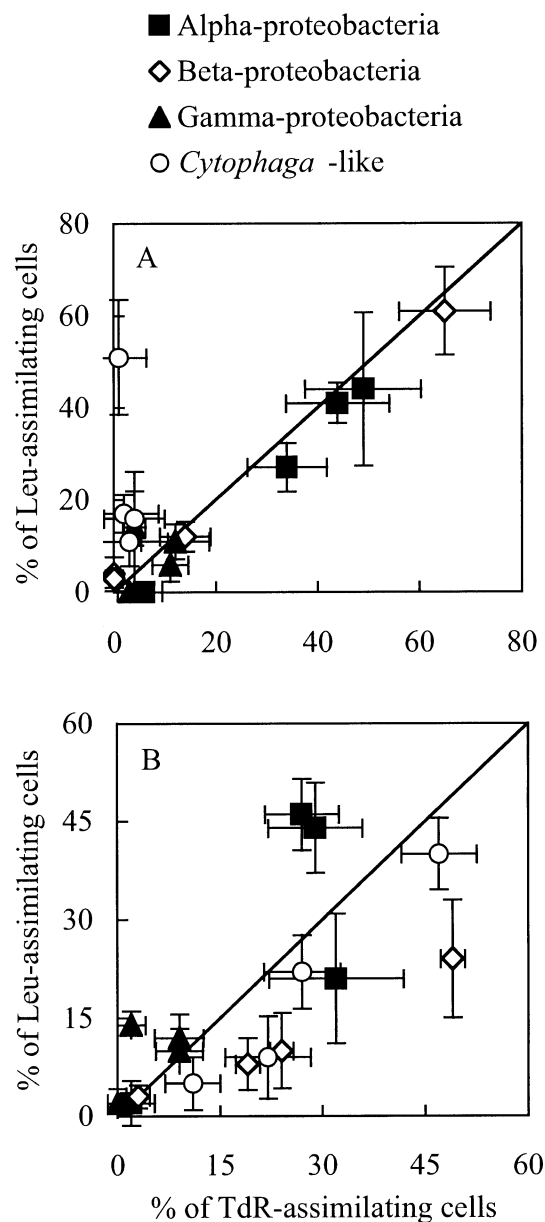


Fig. 4. Relative abundance of bacterial groups in the ^3H -leucine-assimilating population plotted versus their relative abundance in the ^3H -thymidine-assimilating population in samples exposed for (A) shorter and (B) longer times. Alpha-, beta-, and gamma-proteobacterial subclasses and the *Cytophaga*-like bacterial group were detected with oligonucleotide probes Alf968, Bet42a, Gam42a, and CF319a, respectively. Error bars indicate standard errors. The diagonal line indicates a 1:1 relationship.

that a larger silver grain cluster area indicates higher single-cell activity (Rogers 1979).

Silver grain clusters were larger and more variable in size in samples given a longer autoradiographic exposure time. The average size of silver grain clusters increased 2.5-fold from $1.04 \mu\text{m}^2$ to $2.70 \mu\text{m}^2$ with a 2.3-fold longer exposure time (Fig. 5A,B). The size of silver grain clusters varied considerably with coefficients of variation equal to 139 and 191% for the short and long exposure times, respectively.

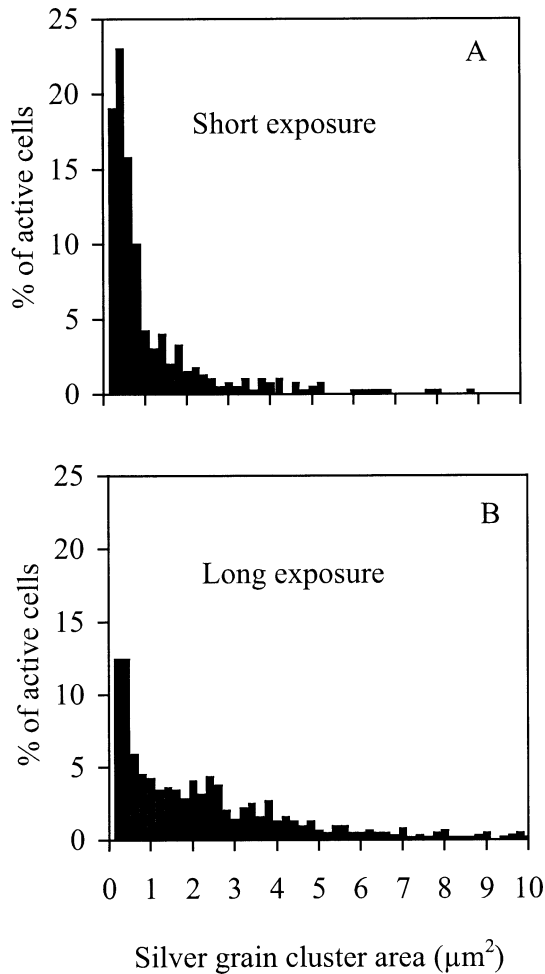


Fig. 5. Frequency distribution of silver grain cluster area in ^3H -thymidine and ^3H -leucine microautoradiographs exposed for a (A) short time and (B) long time. The y-axis value (% active cells) for each size class was calculated as the percentage of total silver grain area in silver grain clusters of that size class.

These data indicate substantial differences in the assimilation of ^3H -thymidine and ^3H -leucine per cell and, by implication, growth rates. The standard deviation of silver grain cluster size was 3.6-fold larger in the long exposure than the short exposure, suggesting that bacteria with a broader range of activity per cell (growth rate) were detected with the long exposure time. In the ^3H -leucine samples with the long exposure, average silver grain area varied from 2.14 to 3.30 μm^2 for gamma-proteobacteria and *Cytophaga*-like bacteria, respectively (Table 2). Here we present results from the analysis of silver grain cluster size in the long-exposure samples.

Alpha-proteobacteria accounted for the largest fraction of the ^3H -thymidine and ^3H -leucine silver grain area at the 9, 16, and 29 PSU stations. Of the ^3H -thymidine and ^3H -leucine silver grain area, 36–46% and 22–50%, respectively, were associated with alpha-proteobacteria where they were abundant (9, 16, and 29 PSU stations) (Fig. 6A,B). In contrast, only 3% of the ^3H -thymidine and ^3H -leucine silver grain area was associated with alpha-proteobacteria at the 0.1 PSU station. The beta-proteobacteria group was the most important

Table 2. Size of silver grain clusters associated with the major phylogenetic groups of bacteria assimilating ^3H -leucine at four stations in the Delaware Bay estuary. *N*, number of bacteria examined for each group.

	Protobacteria			<i>Cytophaga</i> -like
	Alpha	Beta	Gamma	
Mean (μm^2)	2.69	3.04	2.14	3.30
Median (μm^2)	0.84	2.50	1.73	1.94
SD	8.76	2.81	1.63	6.13
CV (%)	325	92	76	186
N	104	19	35	127

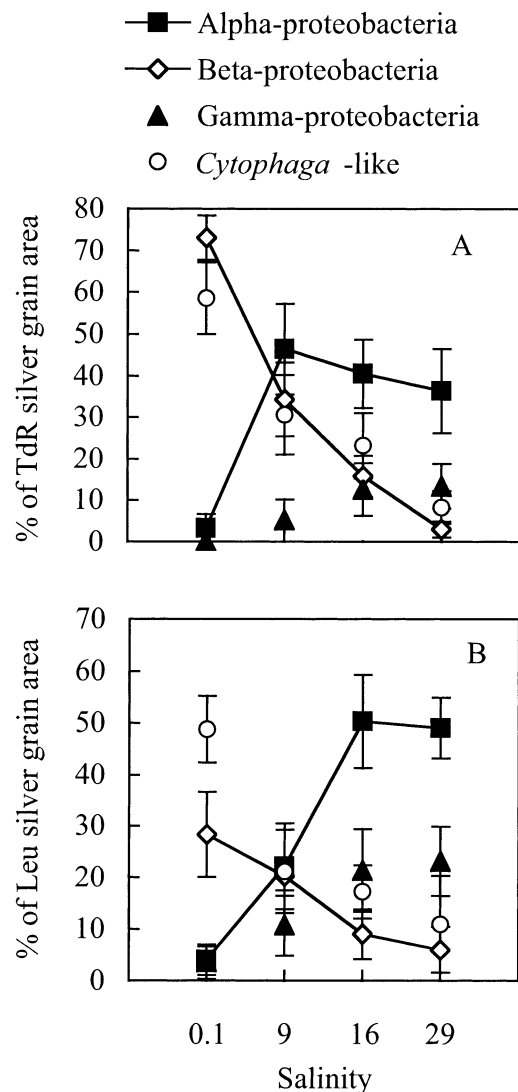


Fig. 6. Percentage of (A) ^3H -thymidine and (B) ^3H -leucine silver grain cluster area associated with different bacterial groups at four stations in the Delaware Bay. Samples were treated with the long autoradiographic exposure time (see Materials and methods). Alpha-, beta-, and gamma-proteobacterial subclasses and the *Cytophaga*-like bacterial group were detected with oligonucleotide probes Alf968, Bet42a, Gam42a, and CF319a, respectively. Error bars indicate standard errors.

group of ^3H -thymidine- and ^3H -leucine-assimilating bacteria at the 0.1 PSU station (73 and 28% of the ^3H -thymidine and ^3H -leucine silver grain area, respectively). Beta-proteobacteria were less important in the assimilation of ^3H -thymidine and ^3H -leucine at higher salinities. In contrast, gamma-proteobacteria were more important at higher salinity. Gamma-proteobacteria accounted for none of the ^3H -thymidine silver grain area and only 4% of the ^3H -leucine silver grain area at the 0.1 PSU station, but 13 and 23% of the ^3H -thymidine and ^3H -leucine silver grain area, respectively, was associated with gamma-proteobacteria at the 29 PSU station. The *Cytophaga*-like bacterial group was also less important in ^3H -thymidine and ^3H -leucine assimilation at higher salinity than in lower salinity waters. At the 0.1 PSU station, 59 and 49% of the ^3H -thymidine and ^3H -leucine silver grain area, respectively, was associated with the *Cytophaga*-like bacterial group, whereas 8 and 11% of the ^3H -thymidine and ^3H -leucine silver grain area, respectively, was associated with this group at the 29 PSU station.

The size distributions of silver grains associated with ^3H -thymidine- and ^3H -leucine-active bacteria were different (Fig. 7). The silver grains of the alpha-proteobacteria and *Cytophaga*-like bacterial groups were the most variable, with coefficients of variation of 325 and 186%, respectively (Table 2; Fig. 7). The silver grain area of beta-proteobacteria and gamma-proteobacteria varied less (C.V. = 92 and 76%, respectively). Differences in variation in the size of silver grains probably reflect variation in growth rates of bacteria composing these phylogenetic groups.

Analysis of silver grain area and the abundance of substrate-active bacteria lead to different conclusions about the importance of various groups in assimilating ^3H -thymidine and ^3H -leucine. For the short exposure time, conclusions from the two analyses were not different, although for the long exposure, the percentage of abundance and the percentage of silver grain area were statistically different (paired *t*-test, $p < 0.01$). In every case but four, the implied contribution of a group to bacterial production based on abundance was not substantially different (less than a factor of 2) than that based on area. The only substantial differences were for ^3H -leucine. Beta-proteobacteria, gamma-proteobacteria, and *Cytophaga*-like bacteria at the 9 PSU station and *Cytophaga*-like bacteria at the 29 PSU station were two-fold more important in bacterial production when silver grain area was considered compared to abundance.

Discussion

The results of this study indicate that all of the major groups of bacteria we assayed assimilate ^3H -thymidine and ^3H -leucine. This result is surprising because the fraction of ^3H -thymidine- and ^3H -leucine-active cells is typically $\leq 50\%$ of total prokaryotic abundance (Fuhrman and Azam 1982; Kirchman et al. 1985; Douglas et al. 1987). The fraction of bacteria assimilating ^3H -glucose and ^3H -amino acid mixtures is usually considerably higher (Fuhrman and Azam 1982; Douglas et al. 1987), and there is a high correlation between the number of bacteria counted by FISH and amino acid microautoradiography (Karner and Fuhrman 1997). We ex-

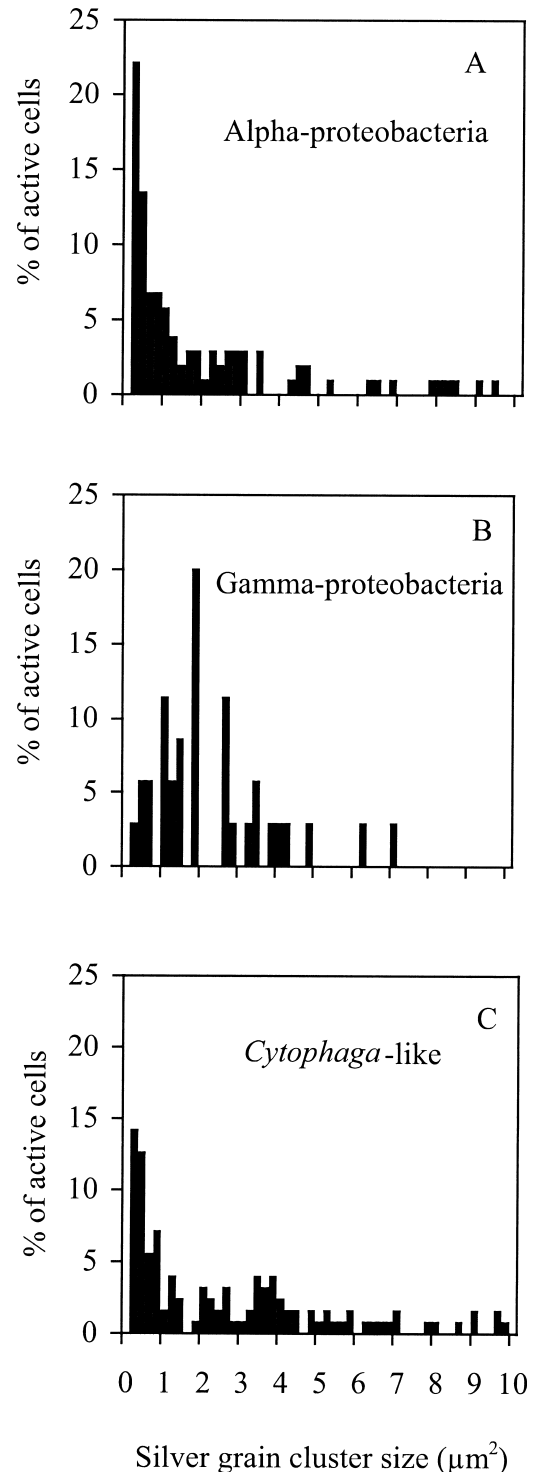


Fig. 7. Frequency distribution of silver grain cluster area in ^3H -thymidine and ^3H -leucine microautoradiographs of (A) alpha-proteobacteria, (B) gamma-proteobacteria, and (C) *Cytophaga*-like bacteria. Silver grain areas were binned at 0.2- μm intervals. The y-axis value (% active cells) for each size class was calculated as the percentage of total silver grain area in silver grain clusters of that size class.

pected that some groups assimilate amino acids, including ^3H -leucine, but not ^3H -thymidine (Ramsay 1974). Our data indicate that some members of all major groups of bacteria are ^3H -thymidine and ^3H -leucine active. Probes for bacteria comprising subgroups (e.g., SAR11 within the alpha-proteobacteria) of the phylogenetically broad groups we examined would be necessary to determine whether some members of these broad groups do not assimilate these compounds. However, it is clear that bacteria assimilating ^3H -thymidine and ^3H -leucine are dominated by the Proteobacteria subclasses and the *Cytophaga*-like bacteria in aquatic systems.

About 50% of the variation in the assimilation of ^3H -thymidine and ^3H -leucine by bacterial groups was explained by community composition. This level of correlation suggests that abundance and activity are at least partially controlled by the same factors. The quantity and quality of DOM can be primary factors limiting bacterial growth and biomass accumulation in aquatic systems (Kirchman et al. 2000). Bacterial mortality is also a factor influencing both growth and abundance. Viral lysis and protozoan grazing are density-dependent agents of bacterial mortality that can recycle limiting nutrients, resulting in higher growth rates at higher levels of bacterial abundance (Fuhrman 1992). However, a substantial fraction of the assimilation of ^3H -thymidine and ^3H -leucine cannot be explained by the abundance of bacterial groups, indicating that additional factors that separately influence production and abundance of bacterial groups could be important as well. Factors affecting the fraction of bacteria in the various groups that are metabolically active (Cottrell and Kirchman unpubl. data) are likely candidates.

One aim of this study was to examine the contribution of the major groups of aquatic bacteria to bacterial production estimated from the incorporation of ^3H -thymidine or ^3H -leucine. The data support the hypothesis that relative abundance of bacterial groups reflects differences in their contribution to bacterial production. For example, the *Cytophaga*-like bacterial group was more abundant than the gamma-proteobacterial group at all four stations, and it was more important in terms of the abundance of ^3H -thymidine- and ^3H -leucine-assimilating bacteria. The link between abundance and importance in bacterial production was also observed for alpha- and beta-proteobacteria whose abundance changed greatly along the salinity gradient. Alpha-proteobacteria dominated the total community and the substrate-active bacteria at the 16 PSU station, but at the 0.1 PSU station, the alpha-proteobacteria group was a minor member of the total community and of the bacteria assimilating ^3H -thymidine and ^3H -leucine. Similarly, beta-proteobacteria were more important in terms of abundance in the whole community at the freshwater station, where this group also dominated the assimilation of ^3H -thymidine and ^3H -leucine. At the 16 PSU station, the roles were reversed, with alpha-proteobacteria dominating both the whole community and the substrate-active bacteria. This analysis suggests that bacterial groups with high abundance determined by FISH are also important contributors to bacterial production.

However, the 16 and 29 PSU stations present an interesting comparison because, at these stations, alpha-proteobacteria and *Cytophaga*-like bacteria were equally abundant but clearly not equally important for bacterial production. The

alpha-proteobacteria group appeared to account for threefold more assimilation of ^3H -thymidine and ^3H -leucine than *Cytophaga*-like bacteria. The greater contribution of alpha-proteobacteria to bacterial production might be a common feature of marine bacteria. Alpha-proteobacteria contributed more to bacterial production (methionine incorporation) than the *Cytophaga*-like bacteria in the Celtic Sea (Zubkov et al. 2001b). The differences in biomass production by bacterial groups might reflect the supply of DOM. A previous study indicated that alpha-proteobacteria and *Cytophaga*-like bacteria differ in the assimilation of high- and low-molecular weight DOM (Cottrell and Kirchman 2000a). The Micro-FISH data suggest that the *Cytophaga*-like bacterial group dominates the consumption of high-molecular weight materials, whereas the alpha-proteobacteria group was more important for low-molecular weight compounds. A greater supply of low-molecular weight DOM compared to high-molecular weight material could explain the greater contribution of alpha-proteobacteria in bacterial biomass production.

Another objective of our study was to determine whether rare bacterial groups contribute disproportionately to bacterial production. Gamma-proteobacteria are typically not abundant members of aquatic bacterial communities, but they have the potential for rapid growth (Eilers et al. 2000b), enabling them to overgrow other bacteria in enrichment cultures. Consistent with the rapid response in dilution cultures, gamma-proteobacteria also have high cellular protein content (Fuchs et al. 2000). One possible explanation for the rapid growth potential in gamma proteobacteria (Pernthaler et al. 2001) is adaptation to patchy distributions of substrates (Azam 1998). However, our data do not support the hypothesis that gamma-proteobacteria grow more rapidly than other bacteria. The average size of silver grain clusters associated with gamma-proteobacteria was not different (ANOVA, $p > 0.05$) from the other bacterial groups (Table 2). Furthermore, the number of cells with different levels of activity does not support this hypothesis (Fig. 7). The median silver grain size of gamma-proteobacteria ($1.7 \mu\text{m}^2$) was within the range of the other groups (0.84 – $2.5 \mu\text{m}^2$, Table 2), suggesting that the gamma-proteobacterial group has no more highly active cells (high growth rate) than any other group.

Understanding which bacteria contribute to bacterial biomass production is an important first step in deciphering the role of microbial diversity in bacterial metabolism in aquatic environments. The results of this study indicate that bacterial abundance generally reflects the importance of bacterial groups, at least as far as dominant and rare groups are concerned. However, half of the variation in the importance of bacterial groups was not explained by their relative abundance, and our analysis of silver grain area suggested that accounting for variation in activity per cell would not improve the correlation. The *Cytophaga*-like bacteria and proteobacterial groups we examined are phylogenetically diverse, so examining more closely related bacteria within these groups might help us understand more fully the relationship between standing stock and biomass production by selected bacterial groups.

The data presented here indicate that proteobacteria and *Cytophaga*-like bacteria are not only abundant but they also

account for most of the heterotrophic bacterial production in this estuarine system. Data on the phylogenetic composition of these bacterial groups is key, but understanding their metabolic variation is equally important. Bulk properties of communities, such as DOM consumption and total biomass production, are the aggregate of the metabolism of several different bacteria. Our data on the variation in growth of different bacteria suggest that information on variation of metabolism at the single-cell level will be essential for understanding the effect of bacterial communities on carbon fluxes in aquatic systems.

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