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Bacterial Production and Growth Rate Estimation from [^{3}H]Thymidine Incorporation for Attached and Free-Living Bacteria in Aquatic Systems

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Production and specific growth rates of attached and free-living bacteria were estimated in an oligotrophic marine system, La Salvaje Beach, Vizcaya, Spain, and in a freshwater system having a higher nutrient concentration, Butron River, Vizcaya, Spain. Production was calculated from [*methyl*-^{3}H]thymidine incorporation by estimating specific conversion factors (cells or micrograms of C produced per mole of thymidine incorporated) for attached and free-living bacteria, respectively, in each system. Conversion factors were not statistically different between attached and free-living bacteria: 6.812×10^{11} and 8.678×10^{11} μg of C mol^{-1} for free-living and attached bacteria in the freshwater system, and 1.276×10^{11} and 1.354×10^{11} μg of C mol^{-1} for free-living and attached bacteria in the marine system. Therefore, use of a unique conversion factor for the mixed bacterial population is well founded. However, conversion factors were higher in the freshwater system than in the marine system. This could be due to the different trophic conditions of the two systems. Free-living bacteria contributed the most to production in the two systems (85% in the marine system and 67% in the freshwater system) because of their greater contribution to total biomass. Specific growth rates calculated from production data and biomass data were similar for attached and free-living bacteria.

Estimations of bacterial growth rate and production are essential for determining the role of bacteria in the trophic dynamics of aquatic systems. According to recent investigations, bacteria can metabolize from 10 to 60% of photosynthetically produced organic carbon (15, 27, 35), and the production of bacterial biomass is predominantly transferred to protozooplankton (1, 36, 42).

In the last few years, several techniques have been developed to estimate bacterial production. The [^{3}H]thymidine incorporation technique has been widely used, although it does pose problems that must be resolved. One of the problems is the need to calculate a conversion factor to convert thymidine incorporation into bacterial production (15, 16, 24).

Bacteria in aquatic systems occur as both free-living and particle-associated cells. The abundance and percentage of attached bacteria fluctuate among different aquatic systems. Free-living bacteria predominate in surface seawater (2, 13, 21, 48), while in freshwater and estuarine systems the proportion of attached bacteria can become more significant (4, 6, 17). Attached bacteria have been reported to be more active than free-living bacteria in most cases (19, 25, 34, 43), although exceptions have been noted (11, 45). To date, the majority of investigations have been based on the uptake of radiolabeled substrates such as glucose, acetate, or amino acids, but there are only a few studies concerning growth rates and production of the two bacterial types, attached and free-living, from thymidine incorporation (11, 23, 33). These studies have assumed a similar conversion factor for converting thymidine incorporation into production for both bacterial types. Because of activity differences observed between attached and free-living bacteria, we consider that this assumption must be tested.

The object of this work is to estimate production and specific growth rates of free-living and particle-associated aquatic bacteria by determining specific conversion factors from thymidine incorporation to bacterial production for each bacterial type. This study is carried out in two different aquatic systems: an oligotrophic marine system and a freshwater system having higher concentrations of nutrients. Trophic characteristics of these systems were determined in previous studies (3, 21, 22, 31).

MATERIALS AND METHODS

Sampling. Twelve water samples were taken from two locations during 1988 to 1989. Six samples were collected at low tide, 500 m offshore from the coast near Bilbao at La Salvaje Beach, Vizcaya, Spain, and six samples at Butron River, Vizcaya, Spain. All samples were collected from a depth of approximately 0.5 m in polypropylene bottles precleaned with diluted HCl and processed in the laboratory within 2 h of sampling. Subsamples of water were preserved in Formalin (final concentration, 2%) for microscopic observations.

Bacterial abundance and cell volume. Bacterial abundance was measured by acridine orange direct counts (18). Formalin-fixed subsamples were stained with acridine orange (final concentration, 0.01%) for 2 to 3 min and then filtered through prestained (Irgalan black, 0.2% [wt/vol] in 2% acetic acid for 24 h) filters (0.2-μm pore size, 25-mm diameter; Nuclepore Corp., Pleasanton, Calif.) and analyzed by epifluorescence microscopy. Approximately 200 attached and 500 free-living bacteria were counted in each sample.

Bacterial cell volumes were calculated after measurement of the shortest and longest axes of the bacteria from photomicrographs. Bacteria were classified as spheres (for cocci) or cylinders (for rods). From 30 to 200 cells were measured from each sample. Cell volume was converted to cell carbon by assuming a conversion factor of 2.2×10^{-7} μg of C μm^{-3} (7).

[^{3}H]thymidine incorporation. Water samples were incubated at in situ temperature in the dark, on a rotatory shaker

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TABLE 1. Bacterial parameters for attached and free-living bacteria in the two systems studied

System	Mean (range; n = 6)			
	Abundance (10^6 cells ml^{-1})	Biomass (ng of C ml^{-1})	Production (ng of C ml^{-1} h^{-1})	Specific growth rate (h^{-1})
Marine				
Free-living	1.51 (0.52–3.45)	17.14 (4.89–38.85)	1.55 (0.15–5.62)	0.060 (0.029–0.145)
Attached	0.08 (0.05–0.12)	2.11 (0.67–4.36)	0.10 (0.02–0.24)	0.066 (0.009–0.182)
Freshwater				
Free-living	1.42 (0.70–2.22)	80.22 (25.41–201.65)	5.80 (3.16–9.23)	0.122 (0.030–0.356)
Attached	0.39 (0.17–0.63)	34.77 (7.94–70.59)	2.97 (0.55–5.68)	0.106 (0.046–0.247)

for 40 min, with 10 nM [*methyl*-^{3}H]thymidine (70 to 85 Ci $mmol^{-1}$; Radiochemical Centre, Amersham, England). The thymidine concentration and incubation time were determined previously through experiments which combined different concentrations with different incubation times. Uptake was linear for the first hour and saturation was reached at 10 nM. After incubation, 5-ml triplicate subsamples were chilled on ice for 1 min and the rest of the water was filtered onto 3-μm-pore-size (25-mm-diameter) Nuclepore membrane filters, to separate attached bacteria. During the filtration procedure, some attached bacteria may have dislodged from particles and may have been able to pass through the filter. To minimize this problem, we used no vacuum pressure for the size fractionation procedure. The filtration was carried out in an ice bath, and all equipment was precooled. Triplicate subsamples, 5 ml, were taken from the filtrate, and an equal volume of ice-cold 10% (wt/vol) trichloroacetic acid was added to each subsample. After 5 min on ice, the mixtures were filtered through 0.2-μm-pore-size membrane filters (pressure differential, 150 mm of mercury) and rinsed five times with 5 ml of ice-cold 5% (wt/vol) trichloroacetic acid. It is necessary to take into account the hypothetic existence of microbacteria in the marine oligotrophic system which could be lost by filtration. The filters were dried at 40 to 50°C for 15 min, chilled, placed in scintillation vials, and radioassayed by liquid scintillation counting on a Tri-Carb 2000CA liquid scintillation spectrometer (Packard Instrument Co., Inc., Rockville, Md.). Quench curves were computed by the external standard method. Controls for abiotic adsorption were processed in a similar manner, except that Formalin (final concentration, 2%) was added.

Conversion factors from [^{3}H]thymidine incorporation to bacterial production. Conversion factors which relate rates of thymidine incorporation to rates of bacterial secondary production were obtained by the empirical dilution technique of Kirchman et al. (24). Water samples were diluted with an equal volume of filtered (0.2-μm pore size) sterilized water, and the mixtures were incubated at in situ temperature, in darkness, on a rotatory shaker. Subsamples for direct counts and for tritiated thymidine uptake measurements were withdrawn during a period of exponential growth (approximately 24 h). Thymidine uptake rate and number and biomass of bacteria were measured by the procedures explained above.

If [^{3}H]thymidine incorporation rate is closely coupled to bacterial growth rate (increase in cell number and biomass), the slope of the regression log of cell biomass versus time (μ_b) will be equal to the slope of the regression log of thymidine incorporation rate versus time (μ_i). The conversion factor (C) can be calculated from the following expression: $C = \mu_i \cdot B_0 \cdot V_0^{-1}$, where μ_i is the slope of the semilogarithmic plot of thymidine incorporation rates versus time, B_0 is the biomass of cells incorporating the radiolabeled compound at the beginning of the experiment (time zero), and V_0 is the thymidine incorporation rate at time zero. However, μ_i larger than μ_b has been found in some cases (24, 33; this study). The possible reason for this is the presence of nondividing cells that do not incorporate [^{3}H]thymidine and dilute the real growth rate of dividing cells. The biomass of cells incorporating the radiolabeled compound at the beginning of the experiment (B_0) can be calculated from the following expression (24): $B_t = B_0 \cdot e^{\mu_i \cdot t} + D$, where B_t is the biomass of bacteria at time t, μ_i is the slope of the semilogarithmic plot of thymidine incorporation rate versus time, and D is the biomass of nondividing or dormant cells. The value of B_0 obtained in this way is used in the first expression given.

Two conversion factors were calculated for each sample, one for free-living bacteria (removing attached bacteria after incubation by filtration through 3.0-μm-pore-size filters) and another for bacteria attached to particles (subtracting the free-living bacteria incorporation from the incorporation value of total population). The production associated with free-living bacteria and that associated with attached bacteria can be calculated, in this way, by multiplying the thymidine incorporation rate by the corresponding conversion factor for each sample. The specific growth rates of the two types of bacteria were estimated from production rates normalized for bacterial biomass.

RESULTS

Bacterial abundance and biomass. In La Salvaje Beach, attached bacteria represented a low percentage of the total population density, with an average value of 6.79%. In comparison, Butron River showed an average value of 23.03% (Table 1). The number of bacteria per particle was low and similar for the two systems, with an average value of 0.9 cell per particle. Because of their larger size, attached bacteria contributed proportionally more to total biomass than to total numbers. In the marine system, attached bacteria represented an average value of 16.74% of total biomass, the mean for the freshwater system being 33.11%.

Conversion factors from [^{3}H]thymidine incorporation to bacterial production. Average conversion factors were 1.276×10^{11} and 1.354×10^{11} μg of C mol of incorporated $thymidine^{-1}$ for free-living and attached bacteria in the marine system and 6.812×10^{11} and 8.678×10^{11} μg of C mol^{-1} for free-living and attached bacteria in the freshwater system. We noted that conversion factors were higher in the freshwater than in the marine system ($P < 0.05$). Statistically significant differences (Student's t test) were not observed between the conversion factors of attached and free-living bacteria in each system (Table 2).

TABLE 2. Conversion factors from [^{3}H]thymidine incorporation to bacterial production expressed as number and biomass

System	Mean conversion factor (range; n = 6) expressed as:			
	Number (10^{18} cells mol^{-1})		Biomass (10^{11} μg of C mol^{-1})	
	Free-living	Attached	Free-living	Attached
Marine	2.514 (0.657–4.743)	2.331 (0.077–8.855)	1.276 (0.384–2.679)	1.354 (0.429–3.366)
Freshwater	8.671 (1.543–23.454)	5.762 (1.305–11.346)	6.812 (1.223–18.110)	8.678 (0.722–23.748)

The means of thymidine incorporation rates in the marine system were 9.0×10^{-15} and 0.9×10^{-15} mol ml^{-1} h^{-1} for free-living and attached bacteria; in the freshwater system, values were 21.4×10^{-15} and 12.3×10^{-15} mol ml^{-1} h^{-1}, respectively. Bacterial production rates were lower in the marine system, with average values of 1.55 and 0.1 ng of C ml^{-1} h^{-1} for free-living and attached bacteria. In the freshwater system, the average production rates were 5.8 and 2.97 ng of C ml^{-1} h^{-1} for free-living and attached bacteria. Attached bacteria represented 14.9% of total production in the marine system and 33.4% in the freshwater system (Table 1).

Specific growth rates were not statistically different between the two systems or between attached and free-living bacteria, although somewhat higher values were found in the river: 0.122 h^{-1} for free-living bacteria and 0.106 h^{-1} for attached bacteria in the freshwater system, and 0.060 h^{-1} for free-living bacteria and 0.066 h^{-1} for attached bacteria in the marine system (Table 1).

DISCUSSION

The conversion factor necessary for transforming thymidine incorporation into bacterial production can be calculated on the basis of two approaches: (i) the theoretical approach used by Fuhrman and Azam (15, 16), which was developed from considerations of several biochemical parameters; and (ii) the empirical approach, which involves the incubation of diluted samples in the laboratory (24). According to the first approach, factors obtained have ranged from 0.2×10^{18} to 2.4×10^{18} cells mol of thymidine incorporated^{-1} (8, 15, 16, 30, 38). Factors obtained by the second approach have ranged from 1.9×10^{18} to 68×10^{18} cells mol^{-1} (5, 9, 10, 24, 28, 32, 33, 41, 47). Some authors (29, 39, 41) have observed that grazing estimations agreed better with production estimations based on an empirical factor than with those based on the theoretical factor. In this study, the empirical approach was used and the conversion factors derived for each bacterial type, attached and free-living (Table 2), were in the range of factors for the total mixed bacterial population derived by other authors who also used the empirical approach.

To date, reported investigations of bacterial production have used the same conversion factor for the two bacterial types (11, 23, 33); we do not know of any work in which different conversion factors for each type have been estimated. Our results indicate that in a given system there are no differences between conversion factors of attached and free-living bacteria (Table 2). Therefore, we can deduce that the use of the same factor for both bacterial types is correct, since in a specific aquatic system attached and free-living bacteria seem to use the incorporated thymidine with equal efficiency in the production of new bacterial biomass. However, it must be taken into account that particles might act as selective barriers, thereby preventing the specific activity of the radiolabeled compound from being the same in the microenvironment surrounding attached and free-living bacteria (23). If the specific activity were lower around particle-attached bacteria, then conversion factors of attached and free-living bacteria would not be comparable. However, in any case, those conversion factors estimated by the empirical approach for the two bacterial types could be useful in obtaining realistic estimations of production of attached and free-living bacteria.

We did find differences in the conversion factors between the two systems studied, with higher values in the freshwater system than in the oligotrophic marine system. Some authors have analyzed possible causes of variability in conversion factors, but to date the results on this subject are limited and contradictory. Riemann et al. (37) found a uniform factor in seawater (1.1×10^{18} cells mol^{-1}) which did not change significantly with media (aged, normal, or enriched seawater), temperature, or generation time, although other studies (46) carried out in freshwater showed higher conversion factors with increased temperature and decreased generation time. These authors suggested that the [*methyl*-^{3}H]thymidine uptake limits the incorporation in fast-growing cells, favoring de novo synthesis. Scavia and Laird (40), working on samples from Lake Michigan, obtained factors that varied seasonally, being lowest in midsummer. We have obtained lower factors in oligotrophic marine waters (Table 2). This may be due to the fact that bacteria in nutrient-limited systems more frequently metabolize nucleic acids and proteins to obtain their maintenance energy (20, 26). Thus, the incorporated [^{3}H]thymidine is used as an energy source rather than for the production of new biomass. According to the results obtained in this study and the wide range of values reported by other authors in different systems, the conversion factor to transform thymidine incorporation into bacterial production is quite variable. For that reason, it is necessary to calculate conversion factors for each system under accurate conditions, so that bacterial production estimates from the thymidine incorporation method might prove more reliable and comparable.

With respect to bacterial production in the aquatic systems we studied, most of it was attributed to free-living bacteria, especially in the marine system. This is mainly due to a greater contribution of the free-living bacteria to the density and biomass of the whole community (Table 1). From papers on this subject, in those systems in which production associated with free-living and attached bacteria has been measured, greater production has usually been attributed to free-living bacteria (11, 12, 23, 33, 44).

The specific growth rate, determined by dividing the production between the biomass, is an indicator of the capacity of the bacterial population to replace its biomass. In this respect, we did not find any differences in specific growth rates between the attached and free-living communities. However, attached bacteria have been reported to be

more active than free-living bacteria when glucose and glutamate (21, 25), phosphate (34), and amino acids (43) are used as radiolabeled substrates. A possible explanation of these apparently contradictory results could be that both attached and free-living bacteria grow at the same rate but attached bacteria need more of these substrates to synthesize the extracellular material with which they attach to the particles (14, 23). To date, there are not many studies on the growth rates of attached and free-living bacteria and the results are contradictory. Thus, in the Hudson River Plume, Ducklow and Kirchman (11) found similar growth rates for the two bacterial types from thymidine incorporation, and in Lake Constance, Simon (45) showed that the difference between biomass turnover times of free-living and attached bacteria was not as pronounced as the difference between specific uptake rates of amino acids by both fractions; on the contrary, in blackwater rivers, Edwards and Meyer (12) reported shorter turnover times in attached than in free-living bacteria by using thymidine incorporation. Therefore, further work is obviously necessary to determine accurately the growth rates of attached bacteria. We think that it is important to take into account the nature of the radiolabeled substrate to determine the differences between attached and free-living bacterial activity.

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