Estimating Bacterial Production in Marine Waters from the Simultaneous Incorporation of Thymidine and Leucine

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We examined the simultaneous incorporation of $[^3]H$thymidine and $[^14]C$leucine to obtain two independent indices of bacterial production (DNA and protein syntheses) in a single incubation. Incorporation rates of leucine estimated by the dual-label method were generally higher than those obtained by the single-label method, but the differences were small (dual/single = 1.1 ± 0.2 [mean ± standard deviation]) and were probably due to the presence of labeled leucyl-tRNA in the cold trichloroacetic acid-insoluble fraction. There were no significant differences in thymidine incorporation between dual- and single-label incubations (dual/single = 1.03 ± 0.13). Addition of the two substrates in relatively large amounts (25 nM) did not apparently increase bacterial activity during short incubations (<5 h). With the dual-label method we found that thymidine and leucine incorporation rates covaried over depth profiles of the Chesapeake Bay. Estimates of bacterial production based on thymidine and leucine differed by less than 25%. Although the need for appropriate conversion factors has not been eliminated, the dual-label approach can be used to examine the variation in bacterial production while ensuring that the observed variation in incorporation rates is due to real changes in bacterial production rather than changes in conversion factors or introduction of other artifacts.

Estimates of the rate of biomass production have been instrumental in determinations of the relative importance of heterotrophic bacteria as biomass producers and as mineralizers of organic matter in aquatic ecosystems. The amount of bacterial biomass potentially available to grazers and thus to higher trophic levels can be determined from rates of bacterial production. Coupled with information about assimilation efficiency, production rates can also be used to estimate the total uptake of organic matter by bacteria. In addition, rates of bacterial production can be used to estimate the average growth rate of bacterial assemblages and as an indicator of the response of bacteria to fluctuations in environmental conditions.

Of the various methods available to measure bacterial production, $[^3]H$thymidine incorporation into cold trichloroacetic acid (TCA)-insoluble material (5-7, 20) has become the most widely used. However, the conversion of thymidine incorporation rates into reliable production estimates can be problematic. To achieve this conversion, a factor is often applied that is derived either theoretically from assumptions of the extent of isotope dilution, the amount of DNA per cell, and the thymine content of bacterial DNA (6) or empirically by comparing incorporation rates with increases in bacterial numbers under controlled conditions (15, 21). Since the empirical factor, as well as rates of thymidine incorporation, may vary (1, 4, 15, 21), it is unclear whether changes in the calculated rate of production are real or are due to variations in the conversion factor. Although variations in the conversion factor can be determined empirically, the required experiments are time-consuming, and it is impractical to perform them concurrently with incorporation measurements. A way to circumvent this limitation is to compare rates of thymidine incorporation with other measurements of bacterial production. Agreement between independent measurements made simultaneously provides confidence that observed variations reflect real changes in rates of bacterial production.

Leucine incorporation into protein (hot-TCA-insoluble material) has been proposed as a measure of protein synthesis and bacterial biomass production (14, 17). Kirchman et al. (17) demonstrated that increases in leucine incorporation agreed with increases in cell numbers and protein content. Proteins account for a large percentage of bacterial biomass (ca. 50%) in pure culture (13) and in natural assemblages (9), and their synthesis consumes a large percentage of the cellular energy. Therefore, the rate of protein synthesis may be a good indicator of total biomass production and energy utilization. Rates of leucine incorporation could provide an independent check of the thymidine method. McDonough et al. (18) found that $[^3]H$leucine incorporation covaried with $[^3]H$thymidine incorporation into protein but not with $[^3]H$thymidine incorporation into DNA in depth profiles of a lake with an anoxic hypolimnion. More comparisons of the two methods are needed.

In this study we used a dual-label radioactive method to measure leucine and thymidine incorporation in a single incubation. This approach simplifies the simultaneous measurement of these incorporation rates, reduces the cost in labor and materials, and minimizes errors associated with repeated subsampling. We found that rates of bacterial production as estimated by thymidine and leucine incorporation covaried and differed by less than 25%.

MATERIALS AND METHODS

Sampling sites. Water samples were collected from the Chesapeake Bay, Delaware Bay, Roosevelt Inlet (Lewes, Del.), and the Mid Atlantic Bight during the spring and summer of 1986 and 1987. Exact locations are given with the results of each experiment. The Chesapeake Bay, Delaware Bay, and Mid Atlantic Bight samples were obtained from various depths with 10 liter Niskin bottles by using a Neil-Brown automated rosette sampler aboard the R.V. Cape Henlopen or the R.V. Cape Hatteras. Roosevelt Inlet samples were obtained from surface waters off the College of Marine Studies dock. Experiments were performed immedi-

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ately after collection, and samples were kept in the dark at the in situ temperature.

Incorporation of [3H]thymidine and [3H]leucine: single-label incubations. We examined the incorporation rate of [3H]thymidine into cold-TCA-insoluble material (6) and of [3H]leucine into hot-TCA-insoluble material (14). Duplicate samples (10 ml) were incubated with either 5 nM (final concentration) [3H]thymidine (specific activity, 84.1 Ci mmol⁻¹) or 11 nM (final concentration) leucine (1 nM [3H]leucine [specific activity, 60 Ci mmol⁻¹]). Incubation times ranged from 30 to 60 min. Abiotic adsorption or radioactivity was measured with TCA-killed controls. Incubations were ended by cooling samples in an ice-cold water bath for 1 min and then adding 1 ml of 50% TCA to reach a final concentration of 5%. [3H]thymidine samples were cooled for an additional 5 min to extract the macromolecular fraction. [3H]leucine samples were heated for 15 min at 85°C to hydrolyze all macromolecules except protein. Following extraction, samples were filtered through Gelman filters (pore size, 0.45 μm). The filters were rinsed twice with 1 ml of ice-cold 5% TCA and once with distilled water and then radioassayed. All radioactive substrates were from New England Nuclear Corp. Nonradioactive leucine was from Sigma Chemical Co.

Incorporation of [3H]thymidine and [14C]leucine: dual-label incubations. To measure simultaneously the incorporation of thymidine and leucine, we added [3H]thymidine and [14C]leucine to a single sample and collected the cold-TCA-insoluble material. [14C]leucine was chosen because [2-14C]thymidine can undergo catabolism and still label DNA owing to the retention of the label in uracil (8). Duplicate water samples (10 ml) were incubated with 5 nM (final concentration) [3H]thymidine (specific activity, 84.1 Ci mmol⁻¹) and 20 nM (final concentration) leucine (10 nM [14C]leucine [specific activity, 328.5 mCi mmol⁻¹]), 10 nM nonradioactive leucine. Because of the low specific activity of [14C]leucine, a higher final concentration (20 nM) was used initially than that of [3H]leucine (10 nM). However, we have been able to measure [14C]leucine incorporation with a 10 nM addition even in oligotrophic waters (D. L. Kirchman, unpublished data). Incubation times ranged from 30 to 60 min. Termination of incubation, cold-TCA extraction of macromolecules, subsequent filtration, and preparation of filters for radioassay proceeded as described above.

Conversion factors. Conversion factors for thymidine and leucine incorporation in dual- and single-label procedures were calculated by comparing increases in cell numbers with the total amount of substrate incorporated (15). Water samples (200 ml) were collected from various depths in the upper Chesapeake Bay during May and July 1987. To minimize grazing of bacteria by protozoa (22), we diluted samples in a ratio of 1:9 with filtered (pore size, 0.22 μm) seawater from the collection site. Samples (final volume, 2 liters) were then kept in darkened polycarbonate bottles (Nalgene) and incubated at the surface seawater temperature. The bottles were subsampled every 6 h for a total of 48 h to measure changes in bacterial abundance and the incorporation of thymidine and leucine during the dual- and single-label procedures. Bacterial abundance was measured by using acridine orange and epifluorescence microscopy (12).

Changes in bacterial abundance and total substrate incorporation were calculated for each sampling interval. The conversion factor was defined as the ratio of total increases in bacterial abundance to the total integrated incorporation. Errors for bacterial abundance and substrate incorporation were propagated to estimate the uncertainty of the conversion factor (2). Estimates of bacterial production derived from these conversion factors were compared with estimates obtained by Fuhrman and Azam (6) with conversion factors for thymidine.

Measurement of incorporated radioactivity. Radioactivity incorporated into cellular material was counted with a Beckman LS 3801 liquid scintillation spectrometer. Quenching was corrected with the external standard in the Compton edge shift mode (H#). Spillover of 14C into the 3H spectrum and vice versa were corrected with the Beckman Automated Quench Control program based on H#. Reading windows were adjusted with the Automated Quench Control program as a function of quenching (H#) to minimize 14C spill. The Beckman manual for this instrument warns against attempting to calculate disintegrations per minute in a dual-label experiment in which the ratio of 14C to 3H counts per minute is greater than 15. In our experiments, the 14C/3H ratio of counts per minute incorporated into cellular material was never greater than 1.

RESULTS

The dual-label method consists of adding both [3H]thymidine and [14C]leucine to a single sample. After incubation the cold-TCA insoluble material is collected on filters. Bacterial production is estimated from incorporation rates of the radiolabeled compounds by using measured (see below) conversion factors or factors taken from the literature (6).

Hot versus cold extraction of leucine incorporation. The leucine method involves the use of a hot-TCA extraction to hydrolyze the nonprotein macromolecules. In the dual-label approach, leucine incorporation into leucyl-tRNA which remains in the cold-TCA-insoluble fraction may lead to overestimates of the amount of leucine incorporated into proteins. To determine the magnitude of this overestimate, we compared incorporation rates of [3H]leucine following hot- and cold-TCA extractions over time in samples from Delaware Bay (Roosevelt Inlet) and the Mid Atlantic Bight. The amount of [3H]leucine incorporated into cold-TCA-insoluble material was in close agreement with that incorporated into hot-TCA-insoluble material (Fig. 1). The average difference was <10% of the [3H]leucine recovered in cold-TCA-insoluble material and was within the experimental error of the measurements. This difference was consistent
throughout the experiment (120 min). Similar results were observed in experiments performed at various times and locations (Table 1). The average ratio of \(^3\)H\text{leucine} recovered after a cold-TCA extraction to that recovered after a hot-TCA extraction was 1.0 ± 0.2. In the waters tested, a cold-TCA extraction did not appear to lead to a substantial overestimate of the level of \(^3\)H\text{leucine} incorporation into proteins.

Comparison of single-label and dual-label incubations. The dual-label method requires the addition of two different substrates in high concentrations. This organic enrichment could potentially affect incorporation by natural bacterial assemblages. To test this possibility, we compared rates of incorporation obtained by the dual-label method with those obtained from single-label incubations at various times in samples taken from various locations in Delaware Bay, the Chesapeake Bay, and the Mid Atlantic Bight.

Leucine incorporation was generally higher (85% of all comparisons, \(n = 48\)) in dual-label than in single-label incubations, but this difference was small (average ratio of 1.13 ± 0.2 [Table 2]). A scatter plot of all dual- and single-label leucine incorporation values yielded a linear relation (Fig. 2) with a correlation factor, \(r\), of 0.96 (\(n = 48\)). The slope of the regression line was 1.10 ± 0.07 (standard error), which is not significantly different from the expected slope of 1 (\(P > 0.05\); Student’s \(t\) test).

During the growth experiments designed to calculate conversion factors, however, leucine incorporation rates were significantly higher in dual-label than in single-label incubations. This difference was much greater than that observed in normal samples and occurred during the initial increases in bacterial abundance (Fig. 3).

Measurements of \(^3\)H\text{thymidine} incorporation by the dual-and single-label methods covaried along the axis of Delaware Bay (Fig. 4) and were in close agreement. The correlation coefficient for these values was 0.93 (\(n = 9\)). No obvious trend was observed in the differences between these measurements; the average ratio of thymidine incorporation obtained by the dual-label procedure to that obtained by the single-label approach was 1.0 ± 0.1.

Comparison of rates of thymine and thymidine incorporation obtained by the dual- and single-label procedures indicate that for the environments tested, the presence of both leucine and thymidine in the dual-label method does not significantly affect their individual incorporation.

Conversion factors. Conversion factors for \(^3\)H\text{thymidine} and \(^3\)H\text{leucine} by using the dual-label method and of \(^3\)H\text{leucine} by using the single-label method were calculated for a sample from the Mid Atlantic Bight and for samples obtained at various locations and depths in the Chesapeake Bay (Table 3). The average thymidine conversion factor was (2.83 ± 1.19) \times 10^{18} \text{cells mol}^{-1}, which is similar to that commonly used (2.0 \times 10^{18} \text{cells mol}^{-1}) (5) and to that

![Fig. 2. Comparison of \(^3\)H\text{leucine} incorporation rates. The dashed line was obtained by linear regression of all values (slope = 1.10 ± 0.07; \(r = 0.96\); \(n = 35\)). The solid line has a slope of 1.](http://aem.asm.org/)
measured specifically for the Chesapeake Bay ($4.0 \times 10^{18}$ cells mol$^{-1}$; H. Ducklow, personal communication). The average leucine conversion factors were $(1.42 \pm 0.69) \times 10^{-17}$ and $(1.64 \pm 1.21) \times 10^{-17}$ cells mol$^{-1}$ for the dual- and single-label methods, respectively (Table 3).

**Application of the method to aquatic environments.** The dual-label method was applied to samples from the Chesapeake Bay during October 1986 to estimate rates of bacterial production. Leucine and thymidine incorporation rates measured by the dual-label method covared over depth. Subsequent measurements at the same station over a diel cycle showed that leucine and thymidine levels continued to covary through time and that surface values obtained by the dual- and single-label methods were in close agreement. In surface samples the ratio of $^{14}$C-leucine to $^{3}$H-thymidine incorporation was 0.80 (Table 2). Similar trends were observed in samples from Delaware Bay and the Mid Atlantic Bight. These comparisons are part of a larger data base that will be discussed in detail elsewhere (G. Chin-Leo and D. L. Kirchman, manuscript in preparation).

We applied our measured conversion factors to the depth profile of incorporation given in Fig. 5. Data on thymidine and leucine incorporation indicated that bacterial production was highest in surface and bottom waters (Table 4). The production rate in surface waters was estimated to be $13.3 \times 10^{17}$ and $10.8 \times 10^{17}$ cells liter$^{-1}$ h$^{-1}$ based on $^{3}$H-thymidine and $^{14}$C-leucine incorporation, respectively. Overall, the difference between production estimates based on thymidine and leucine incorporation was small ($<25\%$) and was within experimental errors. Since our measured conversion factor ($2.83 \times 10^{18}$ cells mol$^{-1}$) was very similar to the commonly used factor of $2.0 \times 10^{18}$ cells mol$^{-1}$ (6), bacterial production estimates from these factors were also very similar (Table 4).

**DISCUSSION**

Our results indicate that in the coastal waters tested, thymidine and leucine incorporation can be measured in a single incubation by the dual-label approach and that thymidine and leucine incorporation give similar estimates of bacterial production. Incorporation rates determined from dual-label incubations differed only slightly from those obtained from single-label incubations; for leucine, the differences can probably be attributed to the radioactivity in leucyl-tRNA which is retained in the cold-TCA-insoluble fraction. The size of this pool did not seem to increase with incubation time (Fig. 1), probably owing to rapid incorporation of radiolabeled leucine into proteins (16). Addition of two substrates in relatively high concentrations (25 nM) did not appear to stimulate bacterial production during short incubations ($<5\,\text{h}$).

An anomalous observation was that during growth experiments $^{14}$C-leucine incorporation measured by the dual-label technique (cold-TCA extraction) was two to three times higher than the single-label $^{3}$H-leucine incorporation rate (hot-TCA extraction) (Fig. 2). The ratio of $^{14}$C-leucine to $^{3}$H-leucine incorporation was high only during the initial increase in cell numbers (Fig. 3). A possible explanation is...
that during periods of increasing growth rate, assimilation of leucine into leucyl-tRNA may be faster than its incorporation into proteins, thus leading to an accumulation of radioactivity in the cold-TCA-insoluble fraction.

With the thymidine method, accurate estimates of bacterial production depend on using the appropriate conversion factor. Our growth experiments provide more evidence that the average conversion factor is near 2.0 × 10⁻⁹ cells mol⁻¹ (6). However, we also observed almost a sixfold variation in this factor. For many questions, highly accurate estimates of bacterial production are not needed, nor is it even possible to measure conversion factors for each production estimate. For example, the ability to determine the spatial and temporal variation in bacterial production over scales of hours and kilometers does not depend on knowing the absolute rate of production. Estimating conversion factors every hour or kilometer would be impossible. However, it is necessary to ensure that the observed changes in thymidine incorporation are due to real changes in bacterial production, not in the conversion factor or some artifact. Because two independent incorporation rates are measured simultaneously, the dual-label approach is more likely to separate real changes from artifacts than the single-label approach.

The dual-label approach is useful as a method for measuring bacterial production only when thymidine and leucine incorporation covary, which occurs frequently (see above; Chin-Leo and Kirchman, in preparation). However, instances when thymidine and leucine incorporation failed to covary have also been observed. Specific problems with either method may explain this lack of covariance. Methodological problems of the thymidine procedure that may lead to erroneous production estimates include incorporation of thymidine into protein and RNA (18), extensive catabolism of thymidine in oxic surface waters (12a), and a significant fraction of the growing bacterial assemblage not taking up thymidine (3, 19). A potential source of artifact of the leucine procedure is turnover of protein, although rates in natural assemblages published to date have been low (17). Furthermore, although a high percentage (ca. 90%) of labeled leucine has been reported to be incorporated into protein (14), more studies are needed to test the generality of this pattern. Finally, isotope dilution by internal and external pools of leucine and thymidine can alter estimates of bacterial production by either technique.

Alternatively, lack of covariance may be explained by periods of unbalanced growth when rates of macromolecular synthesis are uncoupled (D. L. Kirchman, S. Y. Newell, and R. E. Hodson, Int. Symp. Microb. Ecol. 1985, in press). This phenomenon has been observed in pure cultures and studied extensively (13); it generally occurs when bacteria shift from one growth rate to another. For example, during an increase in growth rate, the rate of RNA synthesis first increases; this is followed by increases in protein and DNA synthesis, leading eventually to increases in cell division. Since the thymidine and leucine methods estimate DNA and protein synthesis, respectively, lack of covariance may reflect unbalanced growth.

In conclusion, the dual-label procedure greatly reduces the time and materials needed to routinely perform simultaneous measurements of thymidine and leucine incorporation while minimizing errors associated with repeated subsampling and sample manipulation. Coincident measurements of leucine incorporation provide an independent check of the thymidine method and thus aid in circumventing the uncertainties associated with unknown variations in the conversion factor. This qualitative approach may be useful when studying variations in bacterial production over time and over small distances where conversion factors may vary. Furthermore, lack of covariance between thymidine and leucine incorporation may indicate periods of unbalanced growth when rates of macromolecule syntheses are uncoupled (10, 11). These events may reflect periods of shifting growth conditions, and their detection can be useful in the study of the metabolism of bacteria and its regulation by environmental conditions (14; Chin-Leo and Kirchman, in preparation).

### Table 3. Comparison of conversion factors calculated from growth experiments in the Mid Atlantic Bight and the Chesapeake Bay

<table>
<thead>
<tr>
<th>Date and location (depth)</th>
<th>Conversion factor° (cells mol⁻¹) for:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[³H]Tdr (dual label)</td>
</tr>
<tr>
<td>May 87</td>
<td></td>
</tr>
<tr>
<td>MAB° (2 m)</td>
<td>(3.24 ± 1.25) × 10⁻⁹</td>
</tr>
<tr>
<td>CB° (2 m)</td>
<td>(5.65 ± 2.62) × 10⁻⁹</td>
</tr>
<tr>
<td>July 87</td>
<td></td>
</tr>
<tr>
<td>CB° (2 m)</td>
<td>(1.42 ± 0.67) × 10⁻⁹</td>
</tr>
<tr>
<td>CB° (28 m)</td>
<td>(1.00 ± 0.23) × 10⁻⁹</td>
</tr>
</tbody>
</table>

° Average conversion factors (± standard error) were (2.83 ± 1.19) × 10⁻⁹ for [³H]thymidine ([³H]Tdr), (1.42 ± 0.69) × 10⁻⁷ for [¹⁴C]Leu, and (1.64 ± 1.21) × 10⁻⁷ for [³H]Leu.

### Table 4. Estimates of bacterial production calculated from incorporation rates of [³H]thymidine and [¹⁴C]leucine°

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>Production (10⁷ cells liter⁻¹ h⁻¹) calculated from incorporation of:</th>
<th>[\text{[³H]Tdr} \ (\text{ref. 6})]</th>
<th>[\text{[³H]Tdr} \ (\text{this study})]</th>
<th>[\text{[¹⁴C]Leu/\text{[³H]Tdr}}]</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>9.4</td>
<td>13.3</td>
<td>10.8</td>
<td>0.81</td>
</tr>
<tr>
<td>6</td>
<td>5.2</td>
<td>7.4</td>
<td>7.0</td>
<td>0.95</td>
</tr>
<tr>
<td>15</td>
<td>2.5</td>
<td>3.6</td>
<td>3.8</td>
<td>1.10</td>
</tr>
<tr>
<td>18</td>
<td>5.0</td>
<td>7.1</td>
<td>6.0</td>
<td>0.85</td>
</tr>
<tr>
<td>24</td>
<td>10.0</td>
<td>14.0</td>
<td>8.0</td>
<td>0.57</td>
</tr>
</tbody>
</table>

° Conversion rates were measured by the dual-label method with samples obtained at station 848 in the Chesapeake Bay in October 1986.

*Conversion factors used were 2 × 10⁶ cells per mol for [³H]thymidine ([³H]Tdr) (obtained from reference 6), 2.83 × 10⁹ cells per mol for [³H]thymidine (this study), and 1.42 × 10⁹ cells per mol for [¹⁴C]leucine (this study).

† Ratios were calculated using the thymidine and leucine conversion factors obtained in this study. The mean ratio (± standard deviation) was 0.86 ± 0.19.
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LITERATURE CITED