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# Comparison of Methods for Measurement of Bacterial Growth Rates in Mixed Batch Cultures†

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Eight methods of assessing growth rate constants of bacteria were compared in batch cultures of 3-μm-filtered estuarine water from the Skidaway River in Ga. Mixed assemblages of bacteria were grown under four nutrient regimes of added yeast extract ranging from 0 to 100 mg/liter. Linear and exponential growth rate constants were computed from changes in cell densities, biovolumes, and ATP concentrations. Exponential growth rate constants were obtained from the frequency of dividing cells and RNA synthesis as measured by [$^3$H]adenine uptake. Rate constants obtained during lag, exponential, and stationary growth phases depended largely on the method used. Constants calculated from changes in cell densities, frequency of dividing cells, and adenine uptake correlated most closely with each other, whereas constants calculated from changes in ATP concentrations and biovolumes correlated best with each other. Estimates of in situ bacterial productivity and growth vary depending on the method used and the assumptions made regarding the growth state of bacteria.

Several techniques for measuring the growth of the microbial assemblage of bacteria in aquatic environments have recently been introduced (3, 4, 9). For calculations of daily productivity from instantaneous or short-term (<1-h) growth measurements, assumptions are made that the microbes have balanced, steady-state growth. Changes in cell size, nutritional state, and the concentrations of intracellular components which may occur during incubation or over the extrapolated time are not understood. There is no evidence of steady-state growth in diffusion chamber experiments done by Lavoie and Sieburth (described in reference 14), and such growth has been questioned by others (1, 16).

Filtration of water samples through 3-μm filters has been used to remove predators of bacteria from natural microbial assemblages (3, 11, 15). Increases in cell density (3, 11) or ATP concentration (15) in the absence of predation are used to compute bacterial growth rates. Fuhrman and Azam (3) found good correlation between bacterial productivities calculated from increases in acridine orange direct counts (AODC), assuming linear growth, and those calculated from [$^3$H]thymidine uptake. Newell and Christian (11) used similar incubations to calibrate the frequency of dividing cells (FDC) procedure of Hagström et al. (4), assuming exponential growth. Whereas the incubation of 3-μm filtrate cultures has been used to estimate steady-state bacterial growth rates, in fact such cultures may have growth like that of batch cultures: lag, exponential, and stationary growth phases. Because knowledge of the growth state of bacteria is important in assessing growth rates and hence productivity, we examined several methods of calculating rates in 3-μm filtrate batch cultures of natural assemblages of bacteria from estuarine waters. Growth rates were determined during lag, exponential, and stationary growth phases from changes in cell densities, biovolumes, and ATP concentrations, from FDCs, and from [$^3$H]adenine uptake. Enrichments with yeast extract (YE) were used to stimulate growth and thus provide a variety of rates and growth states for comparisons of methods. Bacteria were also grown without enrichment to simulate more natural conditions.

## MATERIALS AND METHODS

**Experimental design.** Water samples were collected at high slack tide on 30 June 1980 and 1 July 1980 from the Skidaway River at the Skidaway Institute of Oceanography, Savannah, Ga. (81°59'N, 81°01'W). Water temperature was 29°C, and salinity ranged from 22.5 to 24 ppt (mg/ml). On 30 June, 1,600 ml of water was filtered through coarse glass fiber filters (P-100 Uni-Pore; Bio-Rad Laboratories) and 0.4-μm and 0.2-μm pore size polycarbonate membrane filters (Nuclepore; Nuclepore Corp.). On 1 July, a comparable volume was filtered through coarse glass fiber filters

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and 3-μm pore size polycarbonate membrane filters. Portions (400 ml) of the 0.2-μm- and 3-μm-filtered water were combined into each of four autoclaved 1-liter bottles. YE solutions were sterilized by filtration and added to three bottles (final concentrations, 25 [25YE], 50 [50YE], and 100 [100YE] mg of YE per liter). The remaining bottle was not enriched (0YE). These cultures were incubated at 29°C on a rotary shaker (100 rpm).

**Sampling.** Samples (18 ml) were removed from each bottle at 0, 6, 9, 12, 15, 18, and 19 h and placed into a sterile vial containing 1 ml of 37% formaldehyde. AODC (7, 11) and FDC (4, 11) were determined from these samples. The biovolume of bacteria also was determined at 0, 6, 12, and 18 h in 0YE and 50YE by epifluorescence microscopy at a magnification of 2,000× (11).

Samples (25 ml) were removed from each bottle at 0, 6, 12, and 18 h to determine RNA synthesis (9) from [$^3$H]adenine uptake. [$^3$H]adenine (10 μCi; specific activity, 15.2 μCi/mmol) was added to each sample. After 45 min at 29°C, 10 ml was filtered through a 0.4-μm Nuclepore filter. RNA, protein, and DNA were extracted in 5% trichloroacetic acid (TCA). The remaining 15 ml was similarly filtered, and ATP was extracted in 5 ml of boiling Tris buffer. The filters with the TCA precipitate were placed in 0.3 N KOH at 37°C for 18 h to hydrolyze RNA and to solubilize DNA and protein. Longer hydrolization time did not increase the breakdown of RNA or the solubilization of DNA and protein. The hydrolysate was then neutralized with an equivalent amount of 3 N HCl, made to 5% TCA, kept on ice for 30 min, and filtered through 0.4-μm Nuclepore filters. Radioactivity on the filters represented labeled DNA and protein produced from adenine breakdown products entering biosynthetic pathways. The filtrate containing labeled RNA was counted by liquid scintillation. TCA precipitate on the filters was hydrolyzed in 10 ml of 5% TCA at 85°C for 30 min. The hydrolysate was kept on ice for 30 min, and the precipitate was filtered through 0.2-μm Nuclepore filters. The precipitated labeled protein on the filters and the DNA in the filtrates were counted by liquid scintillation. Carrier RNA and DNA (final concentration, 50 μg/ml) were added to minimize loss.

[$^3$H]ATP and total ATP were measured by thin-layer chromatography and bioluminescence, respectively (9). Radioactivity eluted from the chromatographic plates was measured by liquid scintillation and corrected for quench with an internal $^3$H source. The specific activity of the intracellular ATP pool was calculated from the disintegrations per minute per sample and ATP per sample (nanograms) and expressed relative to the reconstituted sample (250 μl). Rates of bacterial RNA synthesis were corrected for the specific activity of the ATP pool and picomoles of adenine incorporated into RNA per milliliter per minute.

**Calculations of growth rate constants.** Changes in cell densities, biovolumes, and ATP concentrations over time were used to compute specific growth rate constants, assuming either linear or exponential growth. Growth is considered in its broadest sense as a change in any measure of cell number or biomass. The specific or instantaneous growth rate constants (i.e., $\Delta t \rightarrow 0$) were used in lieu of doubling time constants because linear ($\Delta$ h$^{-1}$) and exponential ($\mu$ h$^{-1}$) growth rate constants are more directly comparable in this form and because productivities are computed from these parameters. Linear growth rate constants were computed as follows:

$$\Delta = (X_{t_1} - X_{t_0})/[(X_{t_0})(t_1 - t_0)] \quad (1)$$

where $X$ is cell density, biovolume, or ATP concentration at specific times in hours ($t_1$ or $t_0$). Exponential growth rate constants were computed as follows:

$$\mu = (\ell n\, X_{t_1} - \ell n X_{t_0})/(t_1 - t_0) \quad (2)$$

where the symbols calculated are as given above. The exponential growth rate constant calculated from FDC was computed as follows:

$$\mu = e^{(0.299\ \mathrm{FDC}\ -\ 4.961)} \quad (3)$$

by the equation of Newell and Christian (11). The exponential growth rate constant calculated from adenine uptake was computed as follows:

$$\mu = \text{ng [dry weight] synthesized ml}^{-1}\ \text{h}^{-1}/\text{ng [dry weight] ml}^{-1} \quad (4)$$

The numerator was obtained from the picomoles of adenine incorporated into RNA, based on approximations that RNA is 25% AMP and cell dry weight is 15% RNA (9). The dry weight of cells was computed from ATP, assuming that there was 200 g (dry weight) per 1 g of ATP (5, 12). Pearson correlation coefficients were calculated for all combinations of two growth rate constants.

## RESULTS AND DISCUSSION

Growth curves for bacteria as measured by AODC in the four cultures (0YE, 25YE, 50YE, 100YE) are shown in Fig. 1. Bacterial assemblages in all cultures displayed a lag in growth for the first 6 h. Growth rates in the next 3 h (6 to 9 h) were rapid and increased with increasing enrichment. Prolonged exponential growth occurred in the culture with no YE, whereas the stationary phase occurred after 9 h in cultures with YE. Maximum densities of cells increased with increasing YE concentration.

Cell volume at the start of the experiment was 0.06 μm$^3$ and increased to 0.17 and 1.21 μm$^3$ by 6 h in the 0YE and 50YE cultures, respectively. The volumes per cell in 0YE were 0.17 and 0.18 μm$^3$ at 12 and 18 h, respectively, whereas the volumes per cell in 50YE decreased to 0.41 μm$^3$ by 12 h and then increased to 0.44 μm$^3$ by 18 h. Previously, Newell and Christian (unpublished data) found that cell volumes were similar at any given incubation time in various YE enrichments. Thus, cell volumes were not recorded for 25YE and 100YE cultures.

Concentrations of total ATP increased during the lag phase and reached maxima at 12 h in all cultures (Fig. 2A). The concentrations of total and labeled ATP were actually determined at the end of a 45-min incubation beyond the time noted. The concentrations of labeled ATP were highest at 18 h in three of the four cultures (Fig. 2B). Productivities (as picomoles of adenine

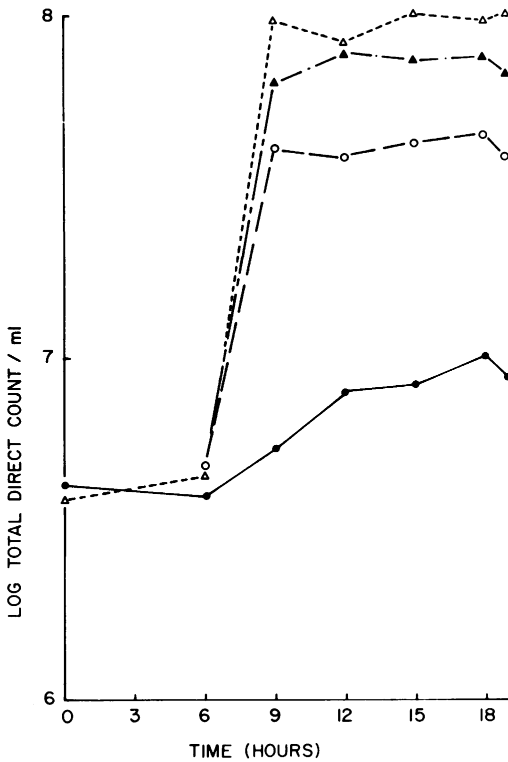


FIG. 1. Growth curves for a mixed bacterial assemblage under four enrichment conditions as measured by AODC. Symbols: ●, growth in control water; ○, growth in 25YE; ▲, growth in 50YE; △, growth in 100YE. Coefficients of variation (not shown) of the direct counts averaged 25% and ranged from 10 to 40% of their respective means.

uncorporated per minute per milliliter) were highest at 6 h in 25YE and 50YE and at 12 h in 0YE and 100YE (Fig. 2C). Figures 2D, 2E, and 2F show the amount of label in RNA, DNA, and protein, respectively. Most label was found in RNA in all cases, but the proportions found in the other fractions were larger than those described by Karl (9).

Fuhrman and Azam (3) assumed that bacteria grew linearly in unenriched 3-μm filtrate cultures, whereas Newell and Christian (11) assumed that bacteria grew exponentially in both unenriched and enriched 3-μm filtrate cultures. In Table 1 we compare these two types of growth rates calculated from changes in cell densities. When growth was slow (i.e., lag and stationary phases and unenriched exponential growth phase), the calculated rate constants were similar, but when cell division rates were more rapid, the differences between the two calculated rate constants were greater. Theoretically, positive linear growth rate constants calculated from any variable set are always higher than positive exponential growth rate constants. Thus, knowledge of the mathematical characteristics of growth is important in calculating growth rates and productivity. We assumed that bacteria grew exponentially in the lag and exponential growth phases and linearly in the stationary growth phase because of characteristic shapes of growth curves (Fig. 1). There is still a possibility that linear growth occurs in unenriched waters where growth is slow (3).

During the lag phase (0 to 6 h), exponential growth rate constants ranged from −0.01 to 0.63 (Table 1). Cell densities changed little during the first 6 h, but cell volumes increased. Thus, exponential growth rate constants calculated from changes in cell density ($\mu$ cell density) were much less than exponential growth rate constants calculated from changes in biovolume ($\mu$ biovolume). Exponential growth rates calculated from changes in ATP concentration ($\mu$ ATP) corresponded more closely with $\mu$ biovolume than with $\mu$ cell density. As growth is unbalanced during this induced lag phase (13), productivities computed from changes in ATP concentration or biovolume for this period would be higher than those computed from changes in cell density. The exponential growth rate constants calculated from changes in FDC ($\mu$ FDC) and adenine uptake ($\mu$ adenine uptake) were related more to the changes in cell density than to the changes in ATP concentration or biovolume. This is expected as the equation used for conversion of FDC to exponential growth rate constant is based on cell densities (11), and for adenine uptake, only initial concentrations of ATP (0 h) were used in the calculations.

Growth rate constants during exponential growth (6 to 9 h) increased with increasing YE concentration, with the exception of $\mu$ FDC and $\mu$ adenine uptake. FDCs for the enriched cultures (15.29, 15.31, and 12.03% for 25YE, 50YE, and 100YE, respectively) were larger than those for the unenriched culture (4.64%) but did not increase with increasing YE concentration. The instantaneous measure of FDC occurred at the transition from lag to exponential growth. Measurements between 6 and 9 h may have provided a better relationship between FDC and change in cell density (11). For the enriched cultures, the $\mu$ cell density was higher than the $\mu$ biovolume because of a decrease in cell size during exponential growth. The $\mu$ adenine uptake was larger than any other calculated exponential growth rate constant in three of the four cultures. The uptake of [$^3$H]adenine was determined after 45-min incubations. During this phase of growth, cell density increased during incubation, even though we assumed that there was a linear uptake of label and a constant cell density when we did the calculations. Errors in these assump-

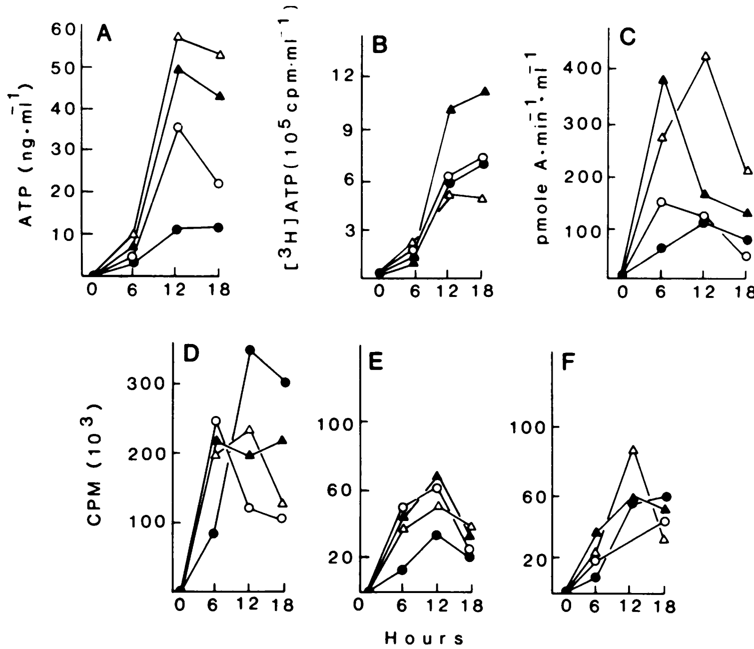


FIG. 2. Incorporation of [$^3$H]adenine into various cellular components of estuarine bacteria. Estuarine water was prefiltered through 3-μm Nuclepore filters and incubated with 0YE (●), 25YE (○), 50YE (▲), and 100YE (△). (A) Concentration of ATP; (B) radioactivity of [$^3$H]ATP in sample spotted on the chromatographic plate; (C) rate of adenine (A) incorporation into RNA; (D) radioactivity in the extracted RNA; (E) radioactivity in the extracted DNA; (F) radioactivity in the TCA precipitate (protein) after RNA and DNA extraction. All radioassays were converted to disintegrations per minute for calculating rates of adenine incorporation into RNA. Datum points are means of duplicate samples.

TABLE 1. Comparison of calculated growth rate constants of mixed assemblages of bacteria

| Calculation at indicated phase | Growth rate constant ($h^{-1}$) of bacteria in: | | | |
|---|---|---|---|---|
| | 0YE | 25YE | 50YE | 100YE |
| Lag phase (0 to 6 h) | | | | |
| Δ Cell density[a] | 0.00 | 0.03 | 0.02 | 0.03 |
| μ Cell density | −0.01 | 0.03 | 0.02 | 0.03 |
| μ Biovolume | 0.16 | 0.53 | 0.52 | 0.53 |
| μ ATP | 0.61 | 0.48 | 0.63 | 0.58 |
| μ FDC | 0.03 | —[b] | — | 0.02 |
| μ Adenine uptake | 0.06 | 0.00 | 0.00 | 0.00 |
| Exponential growth phase (6 to 9 h) | | | | |
| Δ Cell density | 0.12 | 2.50 | 4.34 | 6.69 |
| μ Cell density | 0.10 | 0.71 | 0.88 | 1.02 |
| μ Biovolume | 0.11 | 0.36 | 0.52 | 0.66 |
| μ ATP (6 to 12 h) | 0.20 | — | — | — |
| μ FDC | 0.03 | 0.68 | 0.68 | 0.26 |
| μ Adenine uptake | 0.46 | 1.32 | 1.49 | 0.80 |
| Stationary phase (12 to 15 h) | | | | |
| Δ Cell density | 0.02 | 0.03 | −0.02 | 0.08 |
| μ Cell density | 0.01 | 0.03 | −0.02 | 0.07 |
| ΔBiovolume | 0.02 | 0.05 | 0.00 | 0.10 |
| Δ ATP (12 to 18 h) | 0.01 | −0.08 | −0.02 | −0.01 |
| μ FDC | 0.11 | 0.02 | 0.03 | 0.05 |
| μ Adenine uptake | 0.27 | 0.09 | 0.09 | 0.21 |

[a] Δ Cell density, linear growth rate constant calculated from changes in cell density.
[b] —, Data were not sufficient to calculate growth.

TABLE 2. Correlation analyses of results to eight procedures used to calculate bacterial growth rate constants[a]

| Constant | Correlation between procedures | | | | | | |
|---|---|---|---|---|---|---|---|
| | Δ Cell density[b] | Δ Biovolume[c] | Δ ATP[d] | μ Cell density | μ Biovolume | μ ATP | μ FDC |
| μ Adenine uptake | 0.756[e] | −0.201 | −0.609 | 0.885[e] | 0.316 | −0.512 | 0.965[e] |
| μ FDC | 0.657[f] | 0.105 | −0.348 | 0.818[e] | 0.540 | −0.341 | |
| μ ATP | −0.165 | 0.751[f] | 0.943[f] | −0.150 | 0.827[e] | | |
| μ Biovolume | 0.591[f] | 0.827[e] | 0.735[f] | 0.569 | | | |
| μ Cell density | 0.968[e] | 0.027 | −0.301 | | | | |
| Δ ATP | −0.317 | 0.662 | | | | | |
| Δ Biovolume | 0.073 | | | | | | |

[a] A total of 9 comparisons were done for Δ ATP and μ ATP, 10 were done for μ FDC, and 12 were done for all other constants.
[b] Δ Cell density, linear growth rate constant calculated from changes in cell density.
[c] Δ Biovolume, linear growth rate constant calculated from changes in biovolume.
[d] Δ ATP, linear growth rate constant calculated from changes in ATP concentration.
[e] $P \leq 0.001$.
[f] $P \leq 0.05$.

tions or in conversion factors used may lead to inaccuracies in growth rate constants.

All calculated growth rate constants decreased during the stationary phase (12 to 18 h). Again, the highest rates were indicated when the adenine uptake procedure was used. Adenine uptake, changes in biovolume, and FDC yielded only positive growth rate constants, whereas changes in ATP concentration and cell density yielded some negative values. Positive growth rate constants calculated from changes in biovolume were a result of the increased sizes of cells as they entered the stationary phase. Adenine uptake and FDC cannot give negative net growth rates. The FDC procedure is based on a logarithmic equation which cannot yield a negative exponential growth rate constant. The adenine uptake procedure involves incorporation of label into RNA without correction for decreases in the total RNA pool; thus, with this procedure, negative incorporation and, hence, negative net growth cannot be measured.

It is apparent that the different methods of calculation and procedures of analysis provided a variety of growth rate constants at any given time period and enrichment. To determine which growth rate constants were proportional to one another, we carried out Pearson correlation analyses (Table 2). μ Adenine uptake and μ FDC correlated best with each other and with changes in cell density. In 9 of 10 cases, however, μ adenine uptake was greater than μ FDC, and in 8 out of 12 cases, μ adenine uptake was greater than μ cell density. Linear regression analysis between μ cell density and μ adenine uptake yielded an μ adenine uptake of 1.34 (μ cell density) + 0.08. Analysis between μ cell density ($x$) and μ FDC ($y$) yielded a slope of 0.58 and a $y$ intercept of 0.03 when all values were used. When μ FDC in 100YE at 6 to 9 h was omitted, the slope rose to 0.84, and the $y$ intercept fell to 0.01. Changes in biovolume correlated best with changes in ATP concentration, although changes in ATP concentration during exponential growth in enriched cultures were not used. No ATP measurements were made at 9 h, and cell density curves (Fig. 1) indicated that the stationary phase had been reached by 12 h.

The bacterial assemblages we studied were released from predation pressure by selective filtration and subjected to various nutrient regimes. This usually results in alterations in cell size, ATP concentration, rate of RNA synthesis per cell, and division cycle characteristics (2, 6, 9, 10, 17, 18). In our study, these alterations resulted in different growth rates calculated from the different procedures for given times and nutrient conditions. For example, during the exponential growth phase, estimates for 0YE ranged from 0.03 for μ FDC to 0.46 for μ adenine uptake. We do not know if bacterial growth in nature approximates steady-state exponential or linear growth or is a series of synchronized or unsynchronized brief lag, exponential, stationary, and death phases of individual populations (micropopulations within microniches) within the assemblage (1, 8, 14, 16). If in situ growth approximates the steady-state, future intercalibration studies may need to be concerned only with exponential growth. If bacteria grow linearly in aquatic systems, great care must be taken in extrapolating from studies such as ours. If more complicated conditions exist, more information will be required on the transitional states of bacterial growth.

We cannot judge from these experiments which technique is best for measuring in situ bacterial growth, if in fact one technique is

capable of doing such. Each method measures a different aspect of growth. Only under the most rigid condition of balanced, steady-state growth might one expect close correspondence. This may not be achieved in nature and, obviously, was not achieved in our batch cultures. However, some characteristics of each method are noted here, and others have been noted in the references cited in this paragraph. Fuhrman and Azam (3) used cell density increases in unenriched filtrate cultures to estimate productivity. Potential complications with this approach include the presence of a lag phase, change in cell size, and the question of linear versus exponential growth. The FDC procedure (4, 11) does not involve incubation of samples but must be calibrated with another technique. It appears to be sensitive to rapid changes in the growth state and, by the equation proposed by Newell and Christian (11), does not demonstrate negative growth. With the conversion factors used, the [$^3$H]adenine uptake procedure (9) often gave higher growth rates than those found for $\mu$ FDC and $\mu$ cell density and is incapable of demonstrating negative growth. The [$^3$H]thymidine uptake procedure (3), not investigated here, would not demonstrate negative growth either. More intercalibration studies are necessary for the evaluation of these methods. Results of batch culture studies such as the one presented need to be compared with those using continuous culture studies and observations from natural systems extending over a wide range of environmental conditions.

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