Estimations of Bacterial Growth Rates in Natural Waters

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Specific growth rates as low as 0.005 hr\(^{-1}\) (generation times of 20 to 200 hr) of aquatic bacteria in natural waters have been calculated from significant differences between dilution rates and washout rates in a chemostat. The measured growth rates were affected by the treatment of the water samples (type of sterilization) and by competition with the natural microflora for the unknown growth-limiting substrate.

In most natural waters, freely suspended bacterial cells are assumed to exist under starving conditions. Growth will be limited primarily by low concentrations of a suitable carbon and energy source. Part of the bacterial population may be present in resting and dormant stages, but it is not inconceivable that continuous growth occurs at extremely low rates.

Novick (10, 11) observed definite minimum growth rates in chemostat experiments. A tryptophan-requiring mutant of *Escherichia coli* in well-supplemented media ceased to grow below a generation time of 15 hr at 37°C. He assumed that unbalanced and discontinuous growth occurred at shorter retention times in the chemostat, preventing the establishment of a steady state. Since then, Postgate and Hunter (15) and Tempest et al. (17) found steady states at far longer retention times with cultures of *Aerobacter aerogenes* in studies on the survival of bacteria.

The present paper reports on indirect determinations of growth rates from washout rates of bacterial populations in continuous culture. The technique was studied with the intention to estimate rates of microbial growth and transformations in natural waters. Postgate (13) emphasized the scarcity of such data and their relevance for water bacteriology and microbial ecology.

In earlier experiments, attempts were made to apply the mathematical relationships among growth rate, population density, and concentration of the limiting substrate at steady state in a chemostat with the aim of assessing bacterial growth in natural water as a medium of unknown composition. Sterilized seawater was run through a chemostat inoculated with a test strain of a marine isolate. The establishment of a steady state at a particular dilution rate would indicate the potential population density that can be produced by the unknown growth-limiting substrate at the growth rate imposed. If the concentration of the particular limiting substrate is too low to maintain a constant population at a deliberate dilution rate, lowering of this dilution rate will permit seeking for possible steady states at lower concentrations of the unknown limiting substrate (2).

Except in heavily contaminated inshore seawater, steady states could never be achieved at dilution rates \(D\) as low as 0.042 hr\(^{-1}\) (retention time \(R = 1/D = 24\) hr). During these studies, however, a large amount of data on washout rates at various dilution rates were recorded. In many cases, the test strains ceased to grow altogether when the dilution rate exceeded their maximal growth rate under the conditions given. In other words, the washout rate of these organisms equaled the dilution rate of the chemostat. In other cases, however, the test strains continued to grow while washed out from the chemostat at dilution rates higher than their maximal growth rate. If the washout rate of these organisms reached constancy, the calculation of the actual growth rate was possible. This technique and some pertinent data are discussed in the present paper.

Theoretical. The technique used is based on the simple principle of calculating growth rates from washout rates of a test strain in a chemostat fed with natural water as the medium.

The population density \(x\) of the test strain during transient state in a chemostat changes according to Herbert et al. (2):

\[
\frac{dx}{dt} = \mu x - Dx
\]
or

\[ x = x_0 e^{(\mu - D)t} \]  

(2)

where \( x_0 \) = population density (cell number or dry weight/ml) at zero time \((t = 0)\), \( \mu \) = growth rate \((\text{hr}^{-1})\), and \( D \) = dilution rate \((\text{hr}^{-1})\). Equation 2 can be solved for the growth rate:

\[ \mu = D + \frac{1}{t} \ln \left( \frac{x}{x_0} \right) \]  

(3)

where

\[ -A = \frac{1}{t} \ln \left( \frac{x}{x_0} \right) \]  

(4)

represents the washout rate. If the population of the test strain does not approach a steady state and the decrease of cell numbers reaches a constant rate (95% confidence intervals) at a given dilution rate, the growth rate can be estimated by use of equation 3.

Theoretically, four different cases of constant washout rates are conceivable (Fig. 1): (i) the concentration of the growth-limiting substrate in the water permits the inoculated population to double within one retention time and a steady state will be established; (ii) the inoculated population does not grow and will be washed out at a rate equal to the dilution rate; (iii) the washout rate is lower than the dilution rate; and (iv) the washout rate is higher than the dilution rate (possibly a consequence of loss of viability, lysis, bactericidal effects, or grazing). Examples for all four of these cases have been found in the experiments.

The system used here resembles, in principle, a dual-stage chemostat as suggested by Novick (12) in which the dilution rate of the second vessel exceeds, by means of an additional medium inflow, the maximal growth rate of the culture. In the more simple system used in the present study, measurements are made during the transient state of the culture. Under this condition, decrease of the population density may affect growth during prolonged washout periods.

The present study disregards growth of aquatic bacteria attached to particles.

**MATERIALS AND METHODS**

A series of 0.5-, 1.0-, or 2.0-liter chemostats connected to a joint 20-liter reservoir were used as described earlier (4). The dilution rates were controlled independently for each chemostat by varying the volume of the growth vessel, the length of the thermostated capillary resistance tubing, and the height of the pressure head (overflow vessel). This system gave satisfactory constancy of flow rates as low as 0.1 ml/min for periods as long as 4 to 5 months. The temperature was controlled by internal heaters or cooling coils connected to thermoregulators. Back-contamination of the medium from the chemostat was prevented by heating part of the inlet tube (2.0 mm diameter) with a 0.1 ohm/cm nichrome resistance wire 15 cm long (5).

In shipboard experiments (R/V ATLANTIS II), raw seawater was piped directly into the pressure head of a dual continuous-culture system and prefiltered (10 μm average pore size) to remove detritus particles and larger plankton organisms. If, in these experiments, the seawater was supplemented, sterile medium was metered into the chemostat from a separate reservoir, the final dilution rate being calculated from the combined flow rates (5).

In laboratory experiments, the seawater was sterilized by filtration or was autoclaved immediately after sampling and kept in 20-liter bottles to be connected to the pressure heads of the chemostats. In one set of experiments, the seawater was supplemented with 0.01 or 0.1 mM glycerol.

The tests reported here were made with isolates from our culture collection: Spirillum sp. (103), Pseudomonas sp. (201), Achromobacter sp. (317), and Serratia marinaodura. The strains were grown at 18 C in a seawater medium (0.01% peptone, 0.001% yeast extract; both Difco products), filtered, washed, and resuspended in sterile unsupplemented seawater for 48 hr. After a quick estimation of the cell concentration in a Petroff-Hausser chamber, part of the cell suspension was added to a chemostat to arrive at a final concentration of about 10^7 cells/ml. Samples for plate counts were taken from the chemostat at intervals of at least half a retention time and streaked on a seawater-agar medium (0.5% peptone, 0.05% yeast extract). The confidence intervals of the data were computed by linear regression according to a program by Shannon (16).
RESULTS

During attempts to establish steady states of test organisms in chemostats run with sterile seawater, a substantial amount of data on washout rates has been collected over the past 4 years. In 41 of 65 experiments, the washout rates reached constancy and growth rates could be calculated. In the remaining experiments, the washout rates increased with time and with decreasing population densities. This phenomenon, in addition to the fact that several test strains did not show any growth at dilution rates higher than their maximal growth rates, suggests that the calculated growth rates cannot be regarded as maximal growth rates. Additional experiments were designed to examine (i) the reproducibility of the data, (ii) the growth response upon filter-sterilization as compared with heat-sterilization of seawater, and (iii) competition for a supplemented carbon source by the natural microbial population.

The statistical treatment of the data given in Fig. 2 showed that the washout rate was distinctly different from the dilution rate. In Table 1, the results of 22 such experiments are compiled, demonstrating the degree of reproducibility of the calculated growth rates given in generation times (1/μ). When the chemostat was operated at a 12-hr retention time (R = 1/D) instead of a 6-hr retention time, the calculated growth rate was slightly lower in six of eight cases. With the exception of Spirillum, autoclaving appeared to promote growth considerably more than did filter-sterilization of the seawater (Table 1).

The colonies of Spirillum as well as of Serratia could be readily distinguished from colonies of other bacteria with a stereomicroscope. Therefore, these strains could be used for competition experiments. The data given in Tables 1 and 2 demonstrate a distinct competition for the natural substrate as well as for supplemented glycerol by the natural microflora. A steady state was ob-

![Graph showing population density over time with calculated growth rates A and D.](http://jb.asm.org/)

**TABLE 1. Generation times of test strains in unsupplemented seawater**

<table>
<thead>
<tr>
<th>Organism</th>
<th>Retention time</th>
<th>Generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hr</td>
<td>Unsteril. seawater</td>
</tr>
<tr>
<td>Spirillum sp. (103)</td>
<td>6</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>78</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>6</td>
<td>110, 98</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>130</td>
</tr>
<tr>
<td>Achromobacter sp. (317)</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>0</td>
</tr>
</tbody>
</table>

*Woods Hole Oceanographic Institution dock, samples 19 to 24-67. Since growth measurements are based on viable counts, generation time (G = 1/μ) is given rather than doubling time (td = ln2/μ). Parallel experiments were done in six cases. Achromobacter sp. was not grown in unsterilized seawater.

**TABLE 2. Generation times of Spirillum sp. (103) in untreated and in autoclaved seawater with and without the addition of glycerol**

<table>
<thead>
<tr>
<th>Glycerol conc</th>
<th>Seawater treatment</th>
<th>Generation time</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>hr</td>
</tr>
<tr>
<td>1 M</td>
<td>Unsterilized</td>
<td>127</td>
</tr>
<tr>
<td></td>
<td>Autoclaved</td>
<td>98</td>
</tr>
<tr>
<td>0.1</td>
<td>Unsterilized</td>
<td>81</td>
</tr>
<tr>
<td></td>
<td>Autoclaved</td>
<td>17</td>
</tr>
<tr>
<td>3.0</td>
<td>Unsterilized</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>Autoclaved</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Seawater: Eel Pond, Woods Hole, sample 4-67. Retention time in all experiments 6 hr.
tained when *Spirillum* sp. (103) was grown in autoclaved seawater supplemented with 0.1 mM glycerol (Table 2). The maximal growth rate in that particular case was estimated from the washout rates of cells when the dilution rate was increased to 0.3 hr⁻¹.

A more detailed presentation of results and discussion of ecological aspects will be published elsewhere.

**DISCUSSION**

It has been observed by several authors that growth of certain bacteria cannot be slowed down beyond a certain minimum rate in the chemostat. Novick's (10) interpretation that the organisms are "forced into lag" implies that lowering the dilution rate to a certain point produces a state similar to that observed during the lag phase of a batch culture. His formulation does not preclude anyone of several possible mechanisms leading to the cessation of growth at a low rate of nutrient supply. It has been shown, for example, that endogenous respiration and maintenance metabolism may be responsible for the decline of the growth rate when the population is limited by the carbon and energy source (1, 7). Furthermore, growth might be strongly affected by minimum population densities in suboptimal media (3, 6, 9) independently of the nature of the growth-limiting factor.

Postgate and Hunter (15) observed an increasing "death rate" with decreasing growth rate of steady-state populations of *A. aerogenes* that eventually led to washout of the population at a minimum dilution rate. Their lowest growth rate of the surviving cells was calculated as 0.0101 hr⁻¹, a generation time of nearly 100 hr. At that state of the culture, about 60% of the cells were nonviable as established by a slide culture technique (14). By lowering the temperature, Tempest et al. (17) were able to increase the viability of slow-growing steady-state populations. They found that nitrogen-limited populations exhibited lower proportions of nonviable cells than carbon-limited populations at comparable growth rates.

In the present experiments, changing proportions of nonviable to viable cells were not recorded since no microscopic counting technique was used. In a few cases, however, washout rates were lower than the dilution rate; this could be attributed only to a loss of viability under the particular conditions of growth. The data given in Fig. 3 indicate approach to a steady state after a washout rate of 0.122 hr⁻¹ had been established for about six retention times. The leveling-off of the counts ceased abruptly, however, after another 11 retention times when the population had reached a density of about 8 × 10⁴ cells/ml.

The subsequent washout proceeded at a rate (A’ in Fig. 3) lower than the dilution rate. In other words, the calculated growth rate had a negative value (−μ = 0.005 hr⁻¹) and can be defined as a death rate (15).

The most plausible explanation for the occurrence of two different washout rates may be found in a population effect similar to that observed in continuous culture experiments in which the concentration of the growth-limiting substrate was lowered stepwise at an unchanged dilution rate. When a certain minimum population density was reached, growth ceased and complete washout occurred (3, 6, 8). This observation suggests the production of growth-stimulatory materials by the cells, as a result of which growth is population-dependent. A negative growth rate was not recorded in these experiments. The cessation of growth of some of our isolates at dilution rates higher than their maximal growth rates might be attributed to a similar population effect.

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**LITERATURE CITED**