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# Growth Determinations for Unattached Bacteria in a Contaminated Aquifer

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**Growth rates of unattached bacteria in groundwater contaminated with treated sewage and collected at various distances from the source of contamination were estimated by using frequency of dividing cells and tritiated-thymidine uptake and compared with growth rates obtained with unsupplemented, closed-bottle incubations. Estimates of bacterial generation times [(ln 2)/μ] along a 3-km-long transect in oxygen-depleted (0.1 to 0.7 mg of dissolved oxygen liter$^{-1}$) groundwater ranged from 16 h at 0.26 km downgradient from an on-land, treated-sewage outfall to 139 h at 1.6 km and correlated with bacterial abundance ($r^2 = 0.88$ at $P < 0.001$). Partitioning of assimilated thymidine into nucleic acid generally decreased with distance from the contaminant source, and one population in heavily contaminated groundwater assimilated little thymidine during a 20-h incubation. Several assumptions commonly made when frequency of dividing cells and tritiated-thymidine uptake are used were not applicable to the groundwater samples.**

The persistence of organic contaminants in groundwater can be difficult to predict from laboratory microcosm experiments, in part because the mobilities and growth rates of groundwater bacteria subjected to various levels of organic contamination are not known. Although much is being learned about the distribution (8) and transport (4, 12) of bacteria in contaminated aquifers, little is known about their rates of growth. Most bacteria in groundwater habitats are attached to solid surfaces, but significant numbers of free-living (unattached) bacteria have been observed in highly contaminated groundwater (8). In such cases, growth rates of bacteria that are free to move with the groundwater may have significant effects on the rates of degradation near contaminant sources and on the degrees to which areas further downgradient become seeded with bacteria acclimated to the presence of a particular organic compound. However, the methods available for estimating bacterial growth in aquatic environments are plagued by uncertainties, and their suitabilities for groundwater application have not been tested.

The two methods commonly used to assess bacterial productivity in surface waters, frequency of dividing cells (FDC) and tritiated-thymidine uptake (TTU), were evaluated for use in estimating in situ (instantaneous) growth rates of unattached bacteria in contaminated zones of a sandy, drinking-water aquifer in Cape Cod, Mass. Results obtained by FDC and TTU were compared with those obtained by direct counts during short-term, closed-bottle incubations. The contaminant plume resulted from on-land disposal of treated sewage from a point source and was characterized by specific conductivities >80 μS and by nutrient gradients ranging from near-pristine groundwater to heavily contaminated zones, some of which contained highly refractory and toxic organic material. Because of these gradients, abundances and activities of unbound bacteria change substantially along the plume (8).

Samples of contaminated groundwater for bacterial growth determinations were taken from observation wells that lay along a 3-km-long transect through a zone of low dissolved oxygen (0.1 to 0.7 mg of dissolved oxygen liter$^{-1}$) that constituted much of the plume. Observation wells (5-cm diameter, 250-μm slot width) were sampled with a down-well submersible pump connected to Teflon (E. I. du Pont de Nemours & Co., Inc.) tubing. Samples taken for FDC analysis were fixed immediately with filtered, reagent-grade formaldehyde (final concentration, 2% wt/vol$^{-1}$) and kept on ice, along with unfixed samples for TTU analysis. Separate samples for unsupplemented-growth incubations were taken by syringe from valves placed in the intake line of a peristaltic pump and injected into sterile septated serum bottles. These samples were incubated immediately, whereas FDC calibration and TTU incubations commenced within 48 h of collection. Temperature, specific conductivity, and dissolved oxygen were measured with probes fitted into tightly sealed in-stream containers (Table 1). Low values of dissolved oxygen were verified by iodometric titration (5).

FDC (expressed as percentage of dividing cells), obtained by computing the fraction of the total bacterial population with clear invaginations of cell wall between dividing cells, was used to estimate growth rate by a modification of the technique described by Hagström et al. (7). Dividing and total unattached bacteria were enumerated in separate acridine-orange-stained preparations with a Dialux 20 microscope (Leitz/Opto-Metric Div. of E. Leitz Inc.), set up for epifluorescence as described by Harvey et al. (8). Where possible, at least 350 bacteria and 100 actively dividing bacteria were enumerated. A set of unfixed 12°C incubations of contaminated groundwater was used to calibrate the relationship between FDC and growth rate (μ [hour$^{-1}$]) for the contaminant plume. Changes in bacterial abundance and FDC were monitored in each incubating sample for 36 h, and growth rates were calculated by using $\mu = (\ln N - \ln N_0)/(t - t_0)$, where $N_0$ and $N$ are bacterial abundances at the beginning and end, respectively, of the incubations and $(t - t_0)$ is the elapsed time in hours. To minimize nutrient-induced changes in the makeup of bacterial populations within the incubating samples, nutrient amendments were not used to promote rapid growth as with other bacterial-growth studies that used FDC (7, 14). Rather, samples were diluted 1:3 with filter-sterilized groundwater from the same sample. In situ growth rates were then calculated from FDC

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TABLE 1. Results of analyses of contaminated groundwater samples

| Distance from outfall (km) | Sample depth[a] (m) | Dissolved oxygen (mg liter$^{-1}$) | Temp (°C) | Specific conductance (μS) | Bacterial growth rate (μ h$^{-1}$) by: | |
|---|---|---|---|---|---|---|
| | | | | | FDC[b] | TTU[c] |
| 0.01 | 13 | 0.60 | 11 | 320 | 0.016 ± 0.003 | 0.002–0.013 |
| 0.26 | 20 | 0.14 | 14 | 350 | 0.042 ± 0.005 | 0.001–0.004 |
| 0.39 | 19 | 0.10 | 14 | 196 | 0.030 ± 0.005 | NC |
| 0.56 | 15 | 0.77 | 13 | 300 | 0.020 ± 0.003 | 0.007–0.047 |
| 0.99 | 17 | 0.10 | 11 | 160 | 0.008 ± 0.001 | 0.001–0.007 |
| 1.61 | 20 | 0.07 | 12 | 240 | 0.005 ± 0.002 | NC |
| 2.14 | 26 | 0.13 | 11 | 220 | 0.013 ± 0.001 | 0.003–0.021 |
| 3.03 | 26 | 0.12 | 11 | 220 | 0.009 ± 0.004 | NC |
| Control[d] | 45 | 10.9 | 12 | 60 | —[e] | —[e] |

[a] Below land surface.
[b] Mean ± standard error (propagated).
[c] Range of values represents use of conversion factors given in the text. NC, Not calculated because of a lag in thymidine uptake.
[d] Upgradient uncontaminated groundwater from the control well.
[e] —, Below detection limit of method.

values determined from microscopic examination of fixed groundwater samples. Calculations were made by using the best-fit regression equation that was obtained by plotting calculated growth rates and corresponding FDC values for the 12°C incubation series, $\mu = 0.0032\text{FDC} + 0.0044$ ($r^2 = 0.89$ at $P \leq 0.001$; $n = 10$).

With the TTU method (2), filter-sterilized [*methyl*-$^3$H] thymidine (68 Ci mmol$^{-1}$; lot no. 24043; ICN Pharmaceuticals Inc.) solution and groundwater were added to sterile 60-ml syringes (three replicates per sample) to a final thymidine concentration of 10 nM and incubated at 12°C. Controls were incubated on ice. Subsamples (10 ml each) were withdrawn at 2, 5, 10, and 20 h and extracted with ice-cold trichloroacetic acid solution (final concentration, 5%). The total-TTU determinations were made on bacterial residue collected on 0.22-μm-pore-size filters (GSWP 02500; Millipore Corp.). The bacterial residues were air dried, dissolved in ethyl acetate, and counted in Aquasol (NEN-934; New England Nuclear Corp.). Tritium counts were made to within ±1% error on an LS3801 liquid scintillation counter (Beckman Instruments, Inc.) using the Compton edge shift method of quench factor correction.

Bacterial growth rates were computed from control-corrected rates of TTU for the first 2 h of incubation and adjusted for partitioning of assimilated $^3$H label into protein. To account for uncertainties in assumptions, a range of μ values was obtained for each sample by using conversion factors of $2.0 \times 10^{17}$ and $1.3 \times 10^{18}$ cells produced mol of thymidine incorporated$^{-1}$ (2). Bacterial growth rates for samples that had lags in [*methyl*-$^3$H]thymidine uptake were not computed, since the precise length of the lags could not be determined. Partitioning of assimilated thymidine between intracellular pools of nucleic acids (DNA plus RNA) and protein involved additional removal of 5-ml samples at 2, 5, and 10 h, extraction in boiling trichloroacetic acid (final concentration, 5%) for 1 h, and chilling at 1°C in an ice bath before filtration. The fractions of assimilated [*methyl*-$^3$H]thymidine incorporated into bacterial nucleic acid were computed from differences in $^3$H counts between hot and cold trichloroacetic acid extractions (3).

Growth rate estimates obtained by TTU and FDC techniques are given in Table 1. Estimates determined by FDC ranged from 0.042 ± 0.005 h$^{-1}$, for groundwater 0.26 km downgradient from the outfall, to 0.005 ± 0.002 h$^{-1}$, for groundwater 1.6 km downgradient from the outfall, and correlated with bacterial abundances along the sampled transect ($r^2 = 0.88$ at $P < 0.001$; $n = 8$). Except in the sample taken 2.4 km downgradient from the outfall, thymidine uptake was most rapid in samples taken within 1 km of the outfall. The 2-day growth rates determined from closed-bottle incubations of undiluted contaminated groundwater taken 2.1 and 3.0 km from the outfall were 0.008 and 0.000 h$^{-1}$, respectively. The former growth rate (0.008 h$^{-1}$) was close to that estimated by FDC (0.013 h$^{-1}$). Growth rates could not be estimated for samples of uncontaminated groundwater from the control well, because the near-zero FDC value was outside the range of the cell growth calibration for FDC and there was a significant lag before TTU was measurable.

Differences in bacterial growth rates along a 2-km-long transect, in which dissolved organic carbon (DOC) decreased with distance downgradient from the outfall, are shown in Fig. 1. These differences reflect considerable variations in nutrient conditions. Reported levels of DOC in the contaminant plume ranged from <1 to 4 mg liter$^{-1}$ (L. Barber, M.S. thesis, University of Colorado, Boulder, 1986). The contaminant plume appeared to be in a short-term steady state, judging from results of a 2-year time variability study of chemical constituents and bacterial distribution. Although substantial changes in specific conductivities, microbial abundances, and concentrations of dissolved inorganic carbon, nitrate, ammonia, and DOC sometimes occur over several months, only slight differences have been observed over shorter times and among replicate samples (R. L. Smith, R. W. Harvey, and B. Howes, unpublished data). For example, coefficients of variation for determinations of bacterial abundances among replicate ($n = 4$) well water samples collected 0.39 and 0.56 km downgradient from the outfall were only 0.05 and 0.11, respectively. Therefore, the observed differences in growth rates of unattached bacteria along the plume appear to result mainly from differences in sample depths and distances from the contaminant source.

The highest growth rates, 0.042 h$^{-1}$ (by FDC; 0.26 km from the outfall) and 0.047 h$^{-1}$ (by TTU; 0.56 km from the outfall), are comparable to growth rates reported in several bacterial-productivity studies for coastal waters (9, 18) and lakes (1, 10) in which FDC, TTU, or unsupplemented-growth incubation techniques were used. Data on bacterial growth rates in polluted groundwaters are scarce. However, Marxsen (11) reported that maximum values of glucose uptake in samples collected from contaminated groundwater in the Federal Republic of Germany were similar to those reported in at least one estuary (21), but lower than those

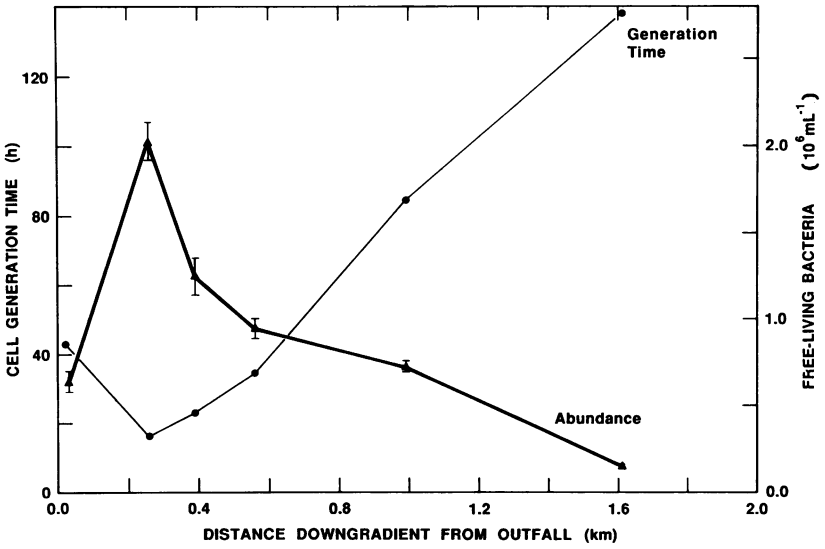


FIG. 1. Changes in bacterial abundances and average generation times [(ln 2)/$\mu$] of free-living (unattached) populations along a 2-km-long transect through the contaminant plume. Generation times were calculated by FDC. Bars indicate standard errors among replicate fields. Bacterial abundances and generation times for samples collected from the zone contaminated with alkylbenzene sulfonate detergents were (0.77 ± 0.03) × $10^6$ ml$^{-1}$ and 63.7 ± 6.9 h$^{-1}$, respectively, at 2.1 km downgradient and (0.07 ± 0.01) × $10^6$ ml$^{-1}$ and 78.8 ± 34.0 h$^{-1}$, respectively, at 3.0 km downgradient.

reported for unpolluted streams. Relative to growth rates in most lakes and rivers, growth rates in downgradient sections of the plume (>0.6 km from the outfall) were low, i.e., 0.005 to 0.013 h$^{-1}$ (by FDC) and 0.001 to 0.021 h$^{-1}$ (by TTU). Bacterial growth rates were lowest at the point farthest from the outfall, judging from data obtained from both the unsupplemented-growth incubation assays and TTU analyses (Fig. 2).

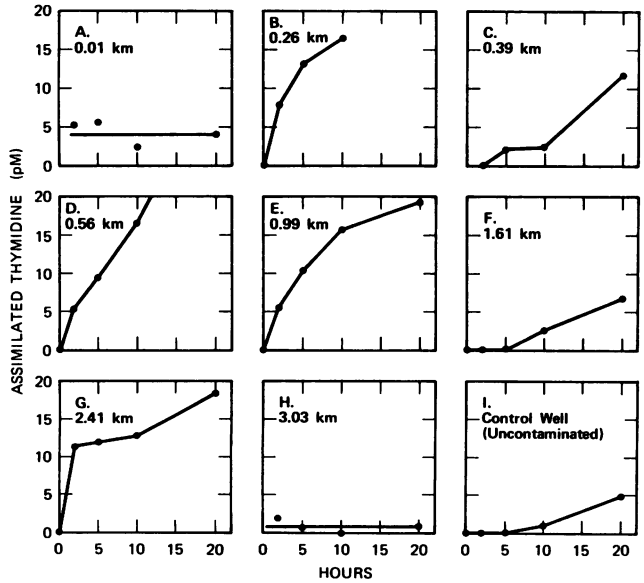


FIG. 2. Uptake of added thymidine by bacteria in contaminated groundwater versus time. Samples were collected along a 3-km-long transect of the plume and from an uncontaminated control well. Values represent the control-corrected means of three replicate incubations. Relative errors among replicates vary from 1%, for the sample taken at 0.56 km downgradient from the outfall, to 24%, for the sample taken at 3.0 km downgradient from the outfall. Distances are from the outfall.

The generally higher rates of growth obtained by FDC compared with those obtained by TTU in this study are consistent with those reported in other studies (6, 16,). The reasons for the poor correlation between results obtained by FDC and by TTU are unclear. Both FDC and TTU have recognized shortcomings, although these methods have often been used in the determination of instantaneous bacterial growth rates in surface water investigations. Growth rates estimated by FDC strongly correlated with abundances of free-living bacteria and, in a vertical transect through the plume, with values of glucose uptake (D. R. LeBlanc et al., manuscript in preparation) and generally decreased with distance downgradient from the source of contamination. Since such correlations were not observed for growth rates estimated by TTU, additional modifications of the TTU technique may be necessary for more accurate application of the method to groundwater samples from our site.

An advantage of FDC is that, once the correlation between FDC and $\mu$ has been determined, estimation of bacterial growth does not necessitate sample incubation. However, the FDC assay is not suitable for use with particle-bound populations, since adherent bacteria often remain in close contact long after the termination of cell division. Also, there are some factors and assumptions that can affect the accuracy of FDC-determined estimates, e.g., temperature, portion of bacteria that is metabolically active, range and magnitude of growth rates being estimated, and the manner in which the calibration relationship of FDC versus $\mu$ is determined.

Although there is a dearth of data on the relationship between $\mu$, FDC, and temperature (16), the FDC assay appears to be most accurate for water between 5 and 15°C (7). This may not be a major problem at our site, since there is little temporal or spatial variation in the groundwater temperature, which ranges between 11 and 14°C (Table 1). Large numbers of inactive groundwater bacteria represent a potential source of error in FDC-estimated growth rates,

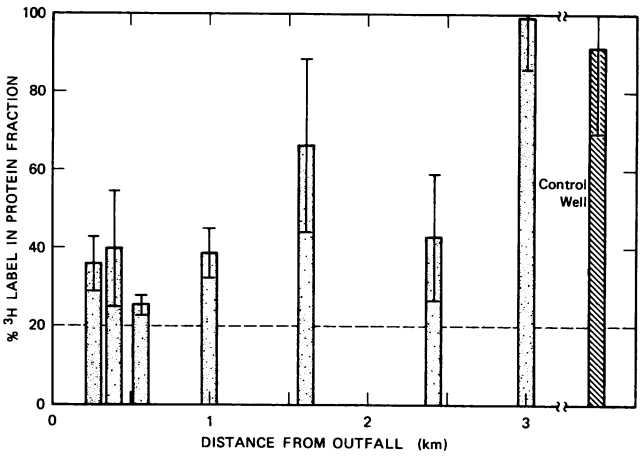


FIG. 3. Proportion of tritium in bacterial protein as operationally defined by hot and cold trichloroacetic acid extraction procedures (7) for [*methyl*-$^3$H]thymidine-amended groundwater. The heights of the columns represent the mean values for replicate incubations of groundwater (▨) and control (▧) samples. Bars indicate standard errors among replicates. – – –, Assumed value (20%) of tritium from [*methyl*-$^3$H]thymidine incorporated into bacterial proteins.

since the FDC assay assumes all bacteria to be metabolically active (16). It has often been assumed that many aquifer bacteria are inactive. However, a recent study suggests that significant portions of bacterial populations in at least some nutrient-poor groundwater may be metabolically active (19), judging from their ability to reproduce on diluted soil extract agar medium. The assumption that FDC is linearly related to $\mu$ is based on the further assumption that the time between constriction of the cell wall and physical separation of dividing cells remains constant over a range of growth rates (14). Since this does not appear to be the case for $\mu$ values $<0.69$ $h^{-1}$ (20), estimates of $\mu$ for aquifer bacterial populations will generally fall in the nonlinear range. The apparent linearity of our calibration data for FDC versus $\mu$ may be the result of the narrow range of growth rates (between 0.01 and 0.10 $h^{-1}$) used for calibration and may not apply to wider ranges of $\mu$. Therefore, estimation of bacterial growth rates in highly contaminated groundwater immediately adjacent to the outfall would necessitate another calibration.

A major disadvantage of TTU in estimating bacterial growth in groundwater is that a number of assumptions (2) may not be applicable. In particular, the assumptions that the fraction of assimilated thymidine incorporated into DNA is a constant 80% and that all (nonphotosynthetic) bacteria take up thymidine quickly at measurable rates were not supported by our data. For example, partitioning of assimilated $^3$H label between nucleic acid and protein varied significantly among samples, as has been found in recent bacterial-productivity studies of two California estuaries (J. T. Hollibaugh, Abstr. Annu. Meet. Am. Soc. Limnol. Oceanogr. 1985; J. T. Hollibaugh and R. W. Harvey, Abstr. Annu. Spring Meet. Pacific Estuarine Soc., 1985). Most assimilated thymidine in samples taken within 1 km of the outfall was incorporated into DNA and RNA. However, partitioning into nucleic acids decreased with increased distance from the outfall (Fig. 3). Most assimilated labels in samples from the distant parts of the plume and in the uncontaminated control were associated with what were assumed to be proteins. This suggests that in at least some nutrient-depleted groundwater, only a small fraction of assimilated thymidine may be incorporated into new DNA.

Although six of nine samples showed significant thymidine incorporation within the first 5 h of incubation, two samples (including the uncontaminated control) exhibited a lag of at least 5 h before net bacterial assimilation of thymidine (Fig. 2). The population 3 km from the outfall assimilated little thymidine over the entire 20-h incubation. Ironically, DOC at this distant location (20- to 25-year transit time in groundwater) was among the highest in the contaminant plume (3 to 4 mg $liter^{-1}$). However, much of the DOC consisted of methylene-blue-active substances, primarily breakdown products of alkylbenzene sulfonate detergents (17). The lack of thymidine assimilation in the alkylbenzene sulfonate-contaminated groundwater, which also contains dichlorobenzene, may have resulted from substances inhibitory to bacterial metabolism or simply from an inability of that bacterial population to assimilate thymidine within the time of the incubations. In one investigation involving pure cultures, it was observed that some pseudomonads do not incorporate added thymidine into DNA (15), apparently because of a lack of the enzyme required for transport of thymidine into the cell. There is also evidence that other bacteria, particularly those with very limited nutrient requirements, may lack thymidine transport systems (13). This presents a potential problem for the use of the TTU method with nutrient-poor groundwater, which can have limited bacterial abundance and diversity relative to most surface waters. Therefore, when the TTU method is applied to contaminated groundwater, it may be necessary to monitor the immediate assimilation of added thymidine and to correct for the incorporation of $^3$H into protein in every sample. Concentration of the samples by tangential-flow filtration may also be helpful. When long (>2-h) incubations are necessary, it is suggested that cycloheximide be added to inhibit protozoa that may otherwise complicate interpretations of resulting TTU data.

In summary, a great deal of caution must be exercised in applying the methods for estimating bacterial growth rates to aquifer samples. In addition to the recognized problems of obtaining representative subsurface samples and of the methods themselves, a number of inherent assumptions may not be applicable to groundwater samples. If possible, several methods should be used to obtain a range of estimates, and such estimates should be used for comparative purposes. However, our data do suggest that bacterial populations in groundwater contaminated over long periods with organic material can adapt to the contamination, with growth rates comparable to those in productive surface waters. The time required for this adaptation is not known, and more research is needed to assess and develop methods for measuring in situ bacterial growth in aquifer sediments.

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