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Effect of Organic Contamination upon Microbial Distributions and Heterotrophic Uptake in a Cape Cod, Mass., Aquifer

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Bacterial abundance, distribution, and heterotrophic uptake in a freshwater aquifer contaminated by treated sewage were determined from analyses of groundwater and sediment-core samples. The number of free-living (unattached) bacteria in contaminated groundwater declined steadily with increasing distance from the source of sewage infiltration, from 1.94 (±0.20) × 10^6 ml^{-1} at 0.21 km to 0.25 (±0.02) × 10^6 ml^{-1} at 0.97 km. Bacterial abundance in groundwater sampled at 0.31 km correlated strongly with specific conductance and increased sharply from 4.0 (±0.3) × 10^4 ml^{-1} at a depth of 6 m to 1.58 (±0.12) × 10^6 ml^{-1} at 14 m, then declined at 20 and 31 m to 1.29 (±0.12) × 10^6 and 0.96 (±0.12) × 10^6 ml^{-1}, respectively. A majority of the bacteria in contaminated and uncontaminated zones of the aquifer were bound to the surfaces of particulates, <60 μm in diameter. The glucose uptake rate, assayed at in situ and 5 μM concentrations, declined steadily in contaminated groundwater sampled along a transect. A preparative wet-sieving technique for use in processing core samples for bacterial enumeration is described and evaluated.

Microbial degradation of organic contaminants in groundwater has recently become a subject of considerable interest, yet little is known about it (1). The effect of microbial processes upon pollutants and leachates percolating through surface soils has been well documented. Both organic and inorganic compounds are subjected to a variety of transformation-degradation reactions by a diverse array of microbial populations present in the soil. In contrast, however, few definitive studies have dealt with the response of groundwater bacteria to significant organic contamination. It is likely that localized organic contamination of oligotrophic groundwater would result in a zone of increased bacterial biomass and activity. The extent of the zone would be dependent in part upon the concentration of readily degradable organics and could potentially serve as an indicator of the fate of these organics within the aquifer.

Much of what is known about specific subsurface bacterial populations derives from studies involving well-water samples. These studies have demonstrated the presence of diverse microbial populations in groundwater systems (4, 11, 13, 17; E. Rades-Rohkohl and P. Hirsh, Abstr. 6th Int. Symp. Environ. Biogeochem., 1983 p. 56). However, like those in soils and some aquatic habitats, a majority of the bacterial population in the saturated zone of the terrestrial subsurface is probably associated with solid surfaces (5, 18). Hence, well-water samples may not adequately reflect the total microbial population.

In this study we examined the effect of land-disposed, secondarily treated sewage upon bacterial concentration, distribution, solid-solution partitioning, and glucose uptake rates within a 4-km-long plume of contaminated groundwater in Cape Cod, Mass. Distinct differences in bacterial populations and activity were evident as a result of the contamination, whereas >95% of the bacterial biomass was associated with particulate surfaces. This has important ramifications for sampling techniques used in future microbiology studies of the subsurface.

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MATERIALS AND METHODS

Study area. The study area consisted of a sand and gravel glacial outwash on western Cape Cod, Mass., bearing a plume of sewage-contaminated groundwater (Fig. 1). The plume was formed by land disposal of treated sewage from a secondary sewage treatment plant at Otis Air Base. The plant has been operating since 1936 and has treated more than 3 × 10^{10} liters of sewage. The treated sewage is discharged to infiltration sand beds, percolates to the water table, and moves southward with the flowing groundwater toward Nantucket Sound. The resulting zone of contaminated groundwater, delineated by elevated specific conductance, is 3.4 km long, 0.9 km wide, and 23 m thick. The plume is overlain by 6 to 9 m of uncontaminated groundwater and does not penetrate into the silty sand and till that underlie the outwash. The groundwater temperature in the aquifer does not vary seasonally and ranges from 9.5 to 14°C. The plume and its hydrogeological setting are described in detail by LeBlanc (U.S. Geological Survey open file report no. 82-274, 1982).

Sampling. Water samples for microbial assays were obtained from a network of screened (250-μm slot width) polyvinyl chloride observation wells (5.0 cm in diameter) located along the path of the contaminant plume (Fig. 1). At three sites, located 0.21, 0.31, and 2.9 km from the sewage infiltration beds, clusters of wells screened at different depths were sampled to obtain depth profiles of the contaminant plume. A stainless steel submersible pump (model SP81; Keck Geophysical Instruments, Inc., Okemos, Mich.) connected to Teflon tubing was used to sample groundwater from the observation wells. Samples were taken in sterile glass and plastic bottles after 3 to 5 well volumes had been pumped and specific conductance and pH had stabilized. Plastic gloves were worn during the sampling procedure to reduce the possibility of sample contamination from skin-associated flora. Samples used for total bacterial counts were fixed immediately with glutaraldehyde (final concentration, 0.2% [wt/vol]); all samples were kept on ice during transport and stored at 4°C in the laboratory. When possible, samples were processed within 48 h of collection.

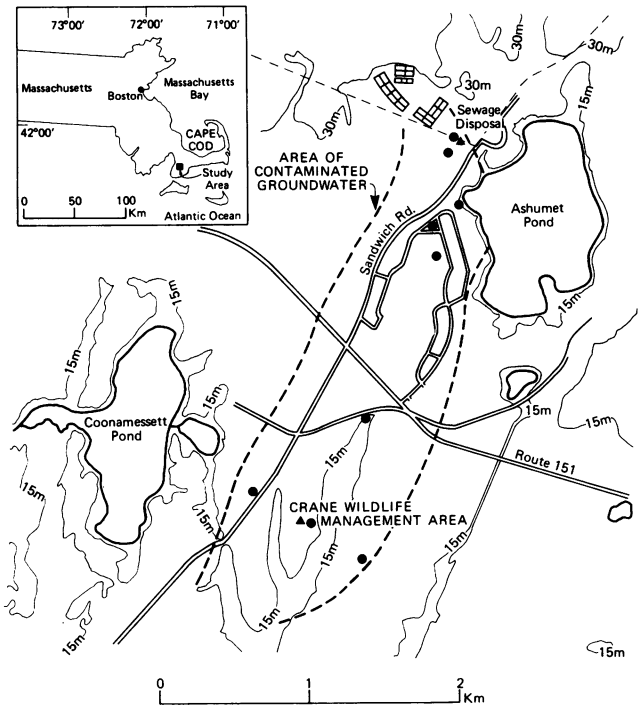

FIG. 1. Groundwater study area in Cape Cod, Mass. The dashed line delineates the path of the contaminant plume caused by infiltration of treated sewage into the aquifer. Symbols: ▲, sites where core samples were taken; ●, sampled observation wells.

Six core samples of aquifer sediments were taken at depths ranging from 12 to 32 m at 0.21 and 2.93 km from the source of contamination, respectively. This was accomplished by means of a split-spoon sampling device lowered down the hollow stem of an auger drilling rig and driven 0.3 to 0.6 m below the bottom of the augers. The split-spoon–hollow-stem auger method was chosen because drilling fluids are not required and contamination problems are minimized (12). However, cores obtained in this manner are likely to receive more contamination than those taken with the shallow coring device described by Wilson et al. (18) and may not be suitable for bacterial isolation work. Approximately 0.5 kg of solid aquifer material was stored in sterile 500-ml bottles and kept on ice until processed. Data obtained from core samples were compared with those obtained from groundwater sampled from adjacent wells at equivalent depths.

Bacterial enumeration. Free-living bacteria in well-water samples were enumerated by an acridine orange direct-counting procedure (9). Since quantities of abiotic suspended particulates in sampled well water were low, up to 40 ml of undiluted sample could be used for each acridine orange preparation, depending on bacterial abundance. Particle-bound bacteria constituted <5% of the total population in well-water samples and were not enumerated. A Leitz Dialux 20 microscope, fitted for epifluorescence (HBO 100-W Hg lamp; H-2 barrier-exciter filter system with K480 quenching filter; 100× [1.32 numerical aperture] NPL Fluotar iris objective), was used to enumerate bacteria at a total magnification of 1,000×.

Enumerations of adherent bacteria in core samples were made for four particle size fractions. Core subsamples (ca. 100 g) were weighed and aseptically wet sieved through a sequential series of mesh filters with progressively smaller mesh openings: 105 (polypropylene), 60 (nylon), and 20 μm (nylon) (Spectrum Medical Industries, Inc.). These three mesh filters were substituted with a single Teflon filter (74 μm mesh size) in subsequent assays of the abundance of adherent bacteria in which the distribution of bacteria among particulate size fractions was not determined. Sieved particulates were collected in sterile 400-ml Pyrex beakers containing ca. 100 ml of water. Particles retained on each mesh filter were voided of remaining interstitial water by a gentle rinse (ca. 50 ml, pH 6.5) and collected in 250-ml polypropylene bottles which were then filled with filter-sterilized water. All preparative operations were performed with a laminar flow hood (>99.9 efficient at 0.3 μm), surgical gloves, sterile apparatus, and filter-sterilized (0.2 μm pore size), Milli-Q purified water (resistance, 18 Mohm). Levels of contamination introduced in sample processing were accounted for with a control blank, which was prepared by repeated washings of a subsample with 0.1 N NaOH before the preparative procedure. Microscopic observation showed that adherent bacteria were completely removed in the presence of NaOH. The use of NaOH to remove adherent bacteria is described by Corpe (2). Levels of bacterial contamination introduced during the wet sieving procedure accounted for <1% of the total counts.

Well stirred 250-ml suspensions of size-fractionated particulates in the three smaller size fractions were sampled by Eppendorf pipette (100 to 1,000 μl) and assayed for solid content and population density of adherent bacteria. A 5-ml quantity of filter-sterilized dilution water was added to each suspension immediately before filtration. For the largest size fractions, which consisted of sand grains >105 μm in diameter, adherent bacteria were enumerated on individual grains embedded in 0.1% agar by using 500× magnification. Calculated concentrations of adherent bacteria were expressed both in terms of dry weight of size-fractionated and total solid material and per unit volume of aquifer material. Values of porosity used in these calculations were estimated from the amount of water which could be added to dry, weighed core material occupying a known volume. The wet sieving procedure was compared with a preparative settling technique described in an earlier study of subsurface microbes (18). In the latter study, a 2-min settling procedure was used to void core material suspensions of the largest sand grains, which interfere with the enumeration procedure.

Glucose uptake measurements. Uptake of tritiated glucose by groundwater bacteria was measured in four well-water samples after storage for 5 days at 4°C. Samples were obtained along a 0.6-km-long transect of the contaminant plume. Four 50-ml portions of each sample were placed in plastic 50-ml syringes, which were then sealed with rubber-stoppered injection hubs. One syringe was injected with NaOH (final concentration, 0.1 N) and served as a killed control. A second syringe was amended with glucose (final concentration, 5 μM). All syringes were injected with similar quantities of [^{3}H]glucose (14.5 Ci/mmol, 3.6 μCi total; New England Nuclear Corp.), shaken, and incubated anaerobically without headspaces at 12°C. The third and fourth syringes were assumed to contain near in situ concentrations of glucose, since the final concentration of added [^{3}H]glucose was only 5.0 nM. A 10-ml subsample from each syringe was filtered through a Gelman Metricel filter (0.2 μm pore size, 25 mm diameter) after 0, 5, 10, 22, and 48 h of incubation. The filters were then clarified in 10 ml of Aquasol, dark adapted, and assayed for ^{3}H activity by liquid scintillation

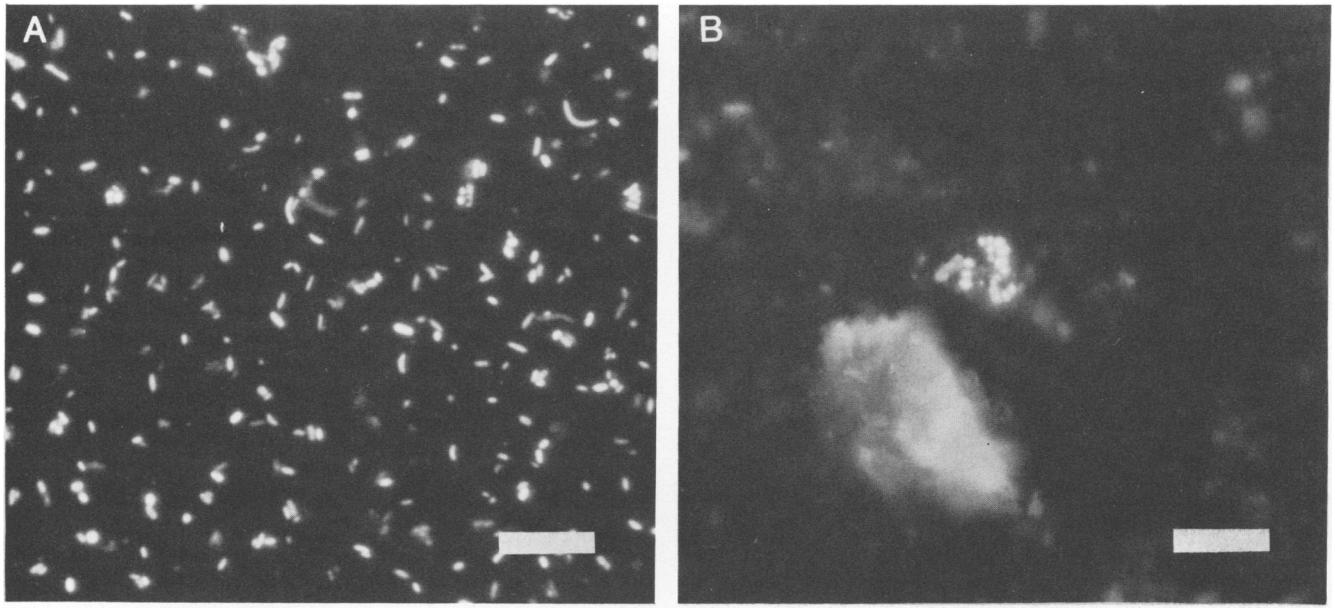

FIG. 2. Reflective fluorescence photomicrographs of acridine orange-stained bacteria in a sewage-contaminated zone of the Cape Cod aquifer. A, Free-living bacteria from a well-water sample; B, adherent bacteria from a core sample. Note the small colony of bacteria in the center of the photo. Bars, 10 μm.

counting with the internal standard addition ([^{3}H]toluene) method for quench-factor correction.

RESULTS

Acridine orange-stained bacteria in well-water samples examined by epifluorescence microscopy were predominantly single and unattached, with an average cell length of <1 μm (Fig. 2A). Relative to those found in many freshwater environments, little variation in average cell size or morphology was observed among samples. Particle-bound bacteria in core samples (Fig. 2B) were, on average, somewhat larger and more clustered than free-living bacteria in well-water samples. A majority of the adherent bacteria were ≤1.5 μm in length. Microcolonies of between 10 and 100 bacteria were common features on particle surfaces. Substantial morphological diversity was observed within samples. However, as observed in previous studies of the subsurface (5, 18), eucaryotic microorganisms, commonly associated with near-surface soils, were not detected in any of the samples examined. Concentrations of groundwater bacteria along the path of the contaminant plume are depicted as a function of distance from the sewage infiltration beds in Fig. 3. Bacterial numbers (from acridine orange direct counting) in groundwater sampled from the middle of the contaminant plume declined sharply from 1.94 (±0.02) × 10^6 to 0.25 (±0.02) × 10^6 ml^{-1} at 0.21 and 0.93 km, respectively. Little variation in bacterial abundance was observed for contaminated groundwater samples taken beyond 1 km. However, bacterial counts in contaminated groundwater from the three more distant sampling locations ($\bar{x}$ = 0.21 [±0.02] × 10^6 ml^{-1}) were significantly higher than that for uncontaminated groundwater (4.0 [±0.5] × 10^4 ml^{-1}) sampled in the same region but at a shallower depth.

Bacterial numbers in the well water as a function of the depth of the well screen at two sites, 0.21 and 0.31 km from the infiltration beds, are given in Fig. 4. At both sites, bacterial abundance was lowest in samples taken from the shallowest well, increased at the next depth, and decreased in the two deepest wells. At 0.31 km, bacterial abundance increased from 4.0 (±0.3) × 10^4 ml^{-1} at 6 m below land surface to 1.58 (±0.12) × 10^6 ml^{-1} at 14 m, a 40-fold increase within 8 m. The specific conductance increased over four-fold in these same samples from 68 to 300 μS, respectively.

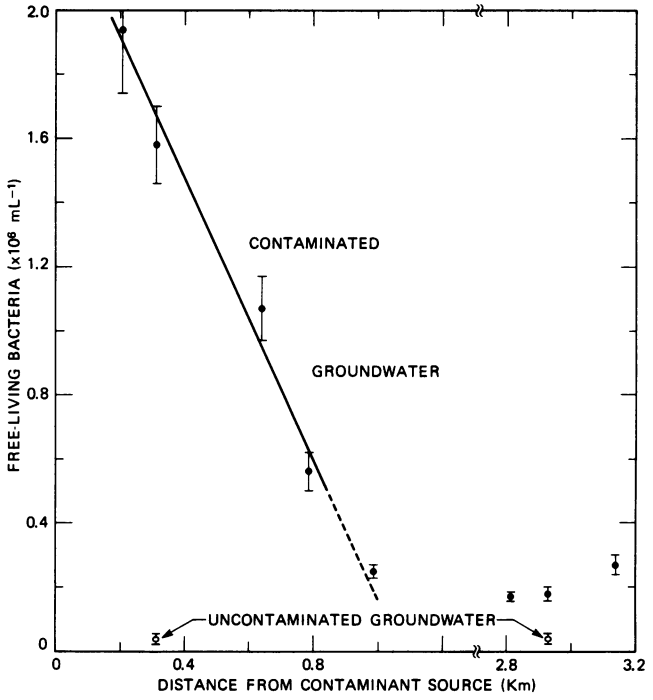

FIG. 3. Plot of the number of free-living bacteria (± standard deviation) in groundwater versus the distance from sewage disposal beds. Symbols: ●, samples taken from contaminated groundwater; ○, samples from uncontaminated groundwater (as determined by specific conductance). The straight line indicates the best fit by linear regression (r^2 = 0.98 at $P \leq 0.05$).

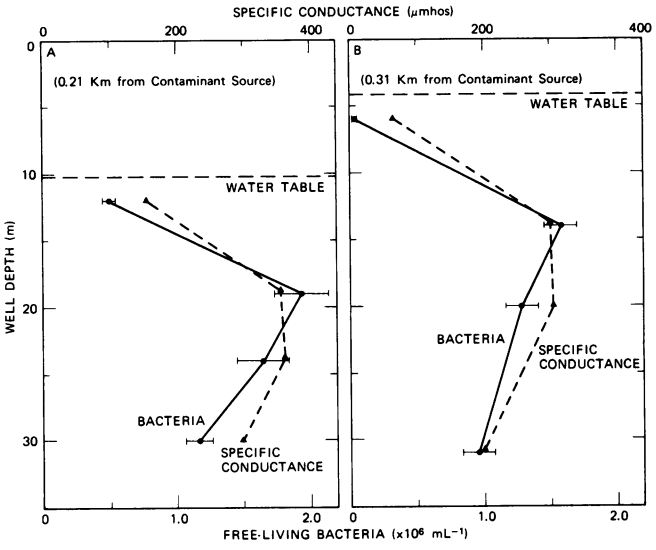

FIG. 4. Plot of the number of free-living bacteria (●) and specific conductance (▲) versus depth below the land surface for two clusters of observation wells located 0.21 (A) and 0.31 km (B) from the treated sewage disposal area. Apparent differences in water table depths (horizontal dashed lines) between the two well clusters are due to topographical variation.

The number of bacteria at 20 and 31 m decreased to 1.29 (±0.12) × 10^6 and 0.96 (±0.12) × 10^6 ml^{-1}, respectively. The bacterial abundance at both sites correlated strongly with specific conductance. The coefficient of determination (r^2) was 0.91 and 0.96 ($P \leq 0.05$) for the two sampling sites, respectively.

Bacterial abundances per unit volume of aquifer are listed in Table 1. The numbers of surface-attached bacteria enumerated in six core samples and free-living bacteria enumerated in groundwater collected from adjacent wells screened at corresponding depths were adjusted for measured aquifer porosities. A majority of the bacteria in all samples were attached to particulates. Portions of the total sample population associated with solid surfaces ranged from 98% at 0.21 km (distance from the contaminant source) and 19 m (depth) to ca. 100% at 2.93 km and 24 m. Population densities of adherent bacteria ranged from 1.84 (±0.22) × 10^7 to 4.04 (±0.38) × 10^7 cm^{-3} of aquifer material, whereas population densities of free-living bacteria varied from 1.59 (± 0.19) × 10^4 to 6.42 (±0.66) × 10^5 cm^{-3}. Variations in the abundance of adherent bacteria at 0.21 km correlated with changes in specific conductance, as did the number of free-living bacteria in adjacent well samples (Fig. 4). However, no significant differences were found between the abundances of adherent bacteria for the two coring sites.

Average distributions of the adherent bacterial population among particle size fractions in the six core samples are listed in Table 2. A majority of the adherent bacteria (67 ± 7%) were associated with particles <20 μm in diameter, which constituted only ca. 1% of the total particulate mass of the sample. In contrast, the largest particulate size-fraction constituted a majority (97 ± 2%) of the total particulate mass but harbored an insignificant portion of the adherent bacterial population.

Glucose uptake as a function of time is illustrated in Fig. 5 for four groundwater samples taken between 0.64 and 1.8 km from the infiltration beds. Significant increases in uptake at both in situ and 5 μM glucose concentrations were observed as the distance from the source of contamination decreased. Uptake rates in glucose-amended (5-μM) samples were 10.6 and 35.9 nM/day for contaminated groundwater at 1.80 and 0.64 km, respectively. Uptake rates were more comparable when corrected for differences in the size of the bacterial populations. Specific uptake rates in amended samples taken at 1.80 and 0.64 km were 3.36 × 10^{-17} and 2.47 × 10^{-17} mol per bacterium per day, respectively.

DISCUSSION

The strong correlation between the abundance of free-living groundwater bacteria and the proximity to the source of contamination (Fig. 3) within the first kilometer of the plume suggests an influence from contaminant organics. This is also corroborated by data on glucose uptake rates (Fig. 5), which were generally higher for contaminated groundwater samples taken closer to the source of contamination. However, much of the observed difference in glucose uptake rates among glucose-amended samples may be explained by differences in the size of the bacterial populations. Since [^{3}H]glucose autoradiography was not performed, uptake rates could not be expressed in terms of the number of bacteria involved in active uptake of glucose. Nevertheless, these data suggest that within the contaminant plume, bacterial populations closer to the treated-sewage infiltration beds may utilize readily degraded organic compounds at higher rates than populations further down the gradient.

The lack of correlation between the number of free-living bacteria in contaminated groundwater beyond 1.0 km and the proximity to the treated-sewage infiltration beds (Fig. 3) may reflect the more refractory nature of dissolved organic material in this portion of the contaminant plume. Although the dissolved organic material assayed in contaminated

TABLE 1. Bacterial numbers and distribution in contaminated and uncontaminated zones of the Cape Cod aquifer

Distance to contaminant source (km)	Sample depth (m)	Porosity	Groundwater specific conductance (μS)	No. of bacteria (per cm^3 of aquifer material)	
				Free living[a]	Particle bound[b] (% bound)
0.21	12	0.18	158	1.87 (±0.19) × 10^5	1.84 (±0.22) × 10^7 (98.9)
0.21	19	0.16	355	6.42 (±0.66) × 10^5	1.94 (±0.29) × 10^7 (96.8)
0.21	25	0.15	365	5.38 (±0.62) × 10^5	4.04 (±0.38) × 10^7 (98.7)
0.21	31	0.16	300	3.98 (±0.34) × 10^5	2.29 (±0.18) × 10^7 (98.3)
2.93	24	0.19	70	1.59 (±0.19) × 10^4	3.09 (±0.46) × 10^7 (100)
2.93	32	0.19	265	6.98 (±0.74) × 10^4	2.43 (±0.17) × 10^7 (99.7)

[a] Determined from well-water bacterial abundance and porosity data.
[b] Determined from porosity data and abundances of adherent bacteria in core samples taken adjacent to screens of sampled wells.

TABLE 2. Average distribution of aquifer solid material and adherent bacteria among four particle size fractions

Size fraction (μm)	No. of particulates (% of total mass)[a]	No. of adherent bacteria (% of total population)[a]
<20	0.97 ± 0.65	67.3 ± 7.61
20–60	0.95 ± 0.66	22.8 ± 5.75
60–105	1.03 ± 0.65	9.85 ± 3.93
>105	97.1 ± 1.89	0

[a] Average and standard deviation computed for six core samples taken from contaminated and uncontaminated zones in the aquifer.

groundwater sampled from the more distant observation wells was at comparable levels to those observed in the more proximal wells, it consisted largely of highly refractory detergents (E. M. Thurman et al., manuscript in preparation). However, the contaminant plume may still have a measureable effect upon microbial biomass at 2 to 3 km from the sewage disposal area, since bacterial abundances in these samples are still significantly higher than those observed in uncontaminated samples from the same region.

The variation in specific conductance and bacterial abundance in groundwater sampled in the two depth profiles (Fig. 4) illustrates the three-dimensional nature of the plume within the aquifer. The linear relationship between the specific conductance and the abundance of free-living bacteria appears to be indirect. Since the specific conductance of the treated sewage (ca. 400 μS) is four to five times higher than that for uncontaminated groundwater (50 to 80 μS), observed variations in specific conductance within vertical transects through the plume reflect the degree of dilution with uncontaminated groundwater. Therefore, specific conductance indirectly reflects dilution of other contaminants which can influence bacterial abundance. The 4.1 (±0.3) × 10^4 bacteria ml^{-1} observed in the uncontaminated groundwater sample (specific conductance, 68 μS) taken at 0.21 km distance and 6 m depth were similar to the number and specific conductance found in another uncontaminated well sample (specific conductance, 70 μS, free-living bacterial counts, 4.2 [±0.5] × 10^4 ml^{-1}) taken at 2.93 km distance and 24 m depth. These data suggest that bacterial abundance in uncontaminated groundwater of this region may be relatively low, i.e., <10^5 ml^{-1}. Reported bacterial abundances (from acridine orange direct counting) for other uncontaminated groundwaters range from 1.1 × 10^3 to 2.0 × 10^6 ml^{-1} (10, 13). These data were reported for samples taken at 1,200 to 1,800 and 1.5 m depth, respectively, and thus may represent rather extreme values of bacterial numbers for groundwater, as sampled from observation wells.

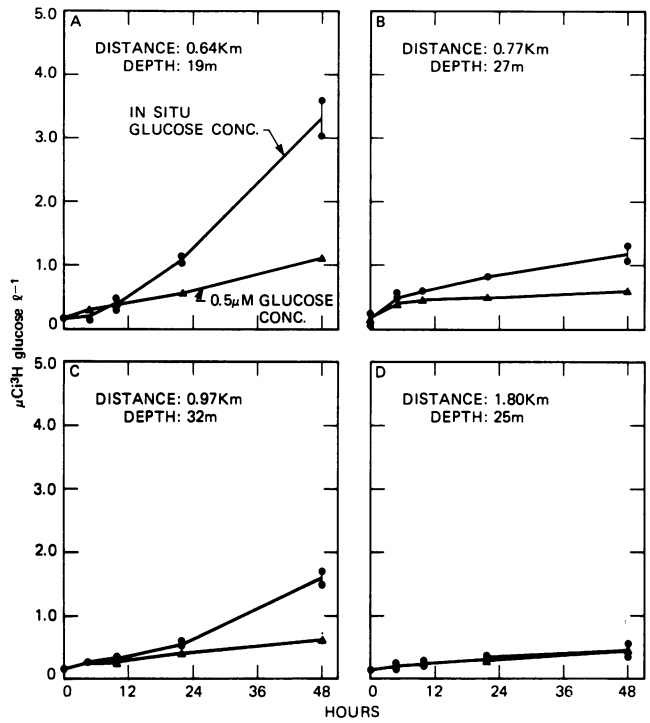

FIG. 5. Uptake of [^{3}H]glucose versus time for four contaminated groundwater samples taken along the path of the contaminant plume from 0.64 to 1.80 Km from the treated sewage disposal area. Symbols: ▲, subsamples amended with glucose (5 μM total concentration); ●, replicate subsamples incubated at assumed in situ concentrations. Uptake of glucose in killed controls (not shown) was not significant.

The microbial population in contaminated and uncontaminated areas of the saturated zone (Table 1) appeared to be dominated by adherent bacteria. Bacterial distributions between groundwater and particulate surfaces have not been reported for freshwater aquifers. However, large fractions of total bacterial populations associated with particulate surfaces in other particle-laden environments have been well documented (6–8, 15), and there is strong evidence that this is also the case for the terrestrial subsurface (5, 18). Expressed in terms of particulate dry weight, the range of adherent bacteria reported here (1.1 [±0.1] × 10^7 to 3.4 [±0.3] × 10^7 g^{-1}) are somewhat higher than the range reported for six uncontaminated cores taken from the unsaturated and saturated subsurface at 1.2 to 5.1 m depth in Oklahoma (3.4 [±2.6] × 10^6 to 9.8 [±1.3] × 10^6 g^{-1}) (18) and Louisiana (1.6 [±5.1] × 10^6 to 1.5 [±0.3] × 10^7 g^{-1}) (5). These differences may reflect differences in nutrient availability. Also, a settling procedure was used to separate out the larger-sized particles in the studies by Wilson et al. (18) and Ghiorse and Balkwill (5); this which may have resulted in some underestimation of adherent bacteria as a result of settling. Numbers of bacteria reported in these three studies for the saturated zone are 3 to 4 orders of magnitude lower than those reported for estuarine surface sediments (3, 14), probably reflecting substantial differences in available nutrients and conditions.

It appears that most bacteria in the aquifer are associated with fine silt particles less than 20 μm in diameter, judging from bacterial distribution data (Table 2). Hence, future design of subsurface samplers for microbiological studies of the saturated zone would benefit from improvements in retention of interstitial water and associated fine silt particles. Furthermore, microscope observations indicate the presence of a large number of bacterial clusters, similar to those described by D. L. Balkwill and W. C. Ghiorse (Proc. 6th Int. Symp. Environ. Biogeochem., 1984), within the smaller-size particle fractions (Fig. 2B). This suggests a high degree of heterogeneity on a microscopic scale. It is possible that small colonies of bacteria associated with the silt-sized particle fraction may break down a significant quantity of the contaminant organics degraded in the aquifer, although further study is clearly needed. Since the wet sieving procedure used to process our core samples definitively separates out larger particles of a predetermined size class, direct comparisons may be made between bacterial abundance data from a variety of aquifer sediments. Use of this prepara-

tive technique in subsurface bacterial enumerations appears to be a potential alternative to the widely used settling procedure developed for soils microbiology (16). However, in subsurface bacterial distribution studies, wet sieving may be most useful for aquifer sediments with the fewest numbers of easily dislodged bacteria.

In summary, the presence of organic contaminants in the groundwater of Cape Cod significantly affects the abundance and uptake potential of the free-living bacterial population. The number of free-living bacteria in well-water samples varied with the distance from the contaminant source and correlated with both specific conductance and glucose uptake rates. Most of the bacteria in sampled portions of the aquifer appear to be associated with solid surfaces, as has been observed in other particle-laden systems. Although there is considerable heterogenity in the abundance of adherent bacteria on a microscopic scale, surprisingly little variation was observed among core samples. Clearly, more work is needed to better characterize the population of particle-bound bacteria in the aquifer and to delineate its role in the degradation of organic contaminants. Such studies would greatly benefit from improvements in the sampling techniques used for obtaining intact aquifer material from a variety of depths in terrestrial subsurface.

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