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Growth and Metabolic Flexibility in Groundwater Bacteria

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Abstract. Groundwater bacteria isolated from an oligotrophic-saturated soil showed a mixed strategy of economized metabolism and migration when grown in a continuous-flow column system simulating poor or nutrient-amended growth conditions. The cells were generally <0.5 μm in diameter in pure groundwater, but doubled in size when the concentration of dissolved organic carbon and phosphate in groundwater was increased 10-fold. The biomass, estimated from analysis of muramic acid (MuAc) in cell wall peptidoglucans, increased at the same time by a factor of 5 when the solid support in the columns was gravel and by a factor of 10 when it was glass beads. Bacteria in pure groundwater stored 10 times more of the energy-rich polysaccharide, poly-β-hydroxybutyric acid (PHB), than bacteria in enriched groundwater, and those cells that were attached to the gravel stored 10 times as much as cells in the interstitial pore water. Once phosphate was added to groundwater, stored PHB was metabolized. The proportion of free-living to attached bacteria was 2 to 10 times higher in enriched compared with pure groundwater indicating a mass transport of cells as the carrying capacity of their habitat rose.

Introduction

Bacteria experiencing periods of carbon and nutrient depletion in subsurface environments should benefit from a strategy for survival based either on enhanced migration rate to more favorable habitats or conservative metabolic expenditures, including a low endogenous respiration and synthesis of energy-rich storage products during favorable growth conditions. Some species tend to survive even extended periods of nutrient and carbon depletion without growing and reproducing, while others can maintain growth and reproduction at a low metabolic rate [29]. The frequency and amplitude of population fluctuations, driven by the random variations in extrinsic factors, such as energy, water, and nutrient supply, should depend on the relative proportion of oligotrophic bacteria, able to grow and reproduce at low nutrient levels, and copiotrophic bacteria, that require high amounts of nutrients for growth. Variations between periods of resource depletion and excess will be smoothed out if oligotrophs are common, whereas larger abundance and activity variations are characteristic for environments dominated by copiotrophs and characterized by extended periods of depletion.

Shallow aquifer sediments are normally oligotrophic environments with <0.1% organic carbon and <10 mg carbon/liter of pore water. The rather few aquifers that, so far, have been examined are inhabited by fairly constant numbers of bacteria, 1–10 × 10^6/g dry weight, of which 1–10% are metabolically active [8, 11, 18, 41, 42]. Based on a comparison of their utilization of polymeric, nonpolymeric, and nitrogen compounds, groundwater isolates have lower activity than isolates from sewage sludge and surface waters [10]. Bacterial growth rates based on tritiated thymidine incorporation are significantly lower in subsurface soils than in marine or freshwater sediments or surface soils [38].

The indigenous groundwater bacteria are subject to resource variations that depend on the quality of the water percolating down from the soil above. The interstitial pore water becomes the most dynamic habitat, with little buffering of the water quality variations in percolation. The particle surfaces become a more stable habitat, where the carbon and nutrient supply for bacteria is determined by the net result of desorption from the particle surface and diffusion from the pore water. The population density on particles with abundant polymer and nutrient resources probably remains more independent of water percolation than on nutrient-poor surfaces, where the emigration rate is high and the cells that move eventually grow in the pore water if this is enriched by percolation water.

The purpose of this work was to examine growth and abundance of groundwater bacteria subject to variations in carbon and phosphorous concentrations in the percolation water. Four major hypotheses were tested: (1) groundwater bacteria are more numerous on particles than in the pore water, unless the oligotrophic groundwater is enriched by percolation; (2) the growth in pore water is more enhanced than the growth on particles when the percolation water becomes carbon and phosphorous enriched; (3) groundwater bacteria accumulate poly-β-hydroxybutyric acid (PHB), an energy-rich storage product, in response to nutrient depletion and use it when the water is enriched; (4) the mass response in bacteria is more pronounced than the numerical response when nutrient and carbon are supplied, because the cells become dwarfs under poor conditions.

The hypotheses were addressed in a laboratory continuous-flow column system supported by glass beads or gravel, inoculated with bacteria isolated from a saturated soil, and supplied with filter-sterilized groundwater from the same area. Abundance and biomass of bacteria were recorded by direct counting of acridine orange-stained cells and analysis of muramic acid (MuAc) [34], and their metabolic response to changes in the carrying capacity was determined by analysis of PHB.

Materials and Methods

All experiments were performed in a laboratory, autoclaved, all-glass continuous-flow system made from a 300-ml groundwater supply tank, a 50 × 70-mm column filled with glass beads or gravel as a solid support, an Ismatec model peristaltic pump to regulate the water velocity, and 100-ml septum bottles to collect the samples (Fig. 1). The groundwater used was collected from a well at the Vomb water infiltration area in southernmost Sweden, which supports the cities of Lund and Malmö with drinking water produced by infiltrating lake water into a sandy aquifer. The ground-

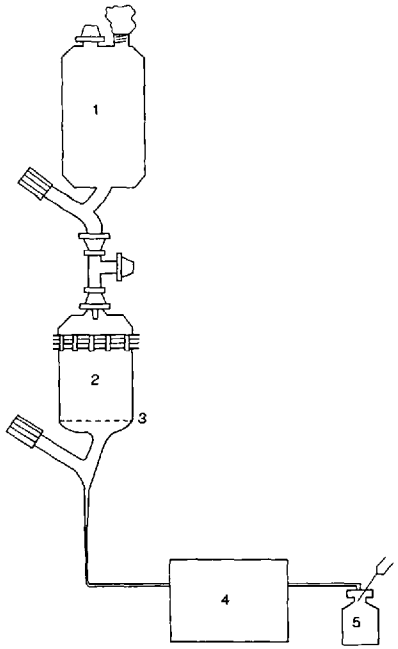

Fig. 1. Groundwater continuous flow model. 1, groundwater reservoir, 300 ml; 2, 50 × 70-mm column filled with glass beads or gravel; 3, sintered glass disc; 4, peristaltic pump; 5, sealed 100-ml serum bottle.

water was first filtered through a Whatman GF-A glass fiber filter and then a 0.45-μm Millipore HAWP filter mounted in a Millipore 100-ml stainless pressure holder applying compressed air at a pressure of about 200 kPA. Finally, the water was filtered through an autoclaved 0.2-μm Millipore GVWP filter mounted in the same autoclaved holder, applying a constant head pressure of 1 m. This low pressure was used to minimize the probability for a breakthrough of small bacterial cells through the filter. The filtered water was stored at 5°C until used within a week, and the filtering efficiency was checked by plate counts of 0.5-ml aliquots on agar plates with a poor growth medium (15 g agar, 0.5 g yeast extract, 0.5 g glucose, 0.25 g peptone, and 50 μg mineral salt solution in 1 liter of distilled water).

The cap and bottom of the glass column were equipped with a sintered glass disc to support the solid phase. The glass beads, 2 mm in diameter, were washed in chloroform, acetone, methanol, 0.1 M HCl, 0.5 M $CaCl_2$, and finally in distilled, deionized water. They were autoclaved before being packed in the glass columns. Glass beads were used to provide a smooth and regular support for bacterial attachment. Gravel (0.5–1.5 mm in diameter) from the saturated soil at the field site was washed in 2,000 ml of 1 M NaCl and rinsed in several portions of 2,000 ml distilled water to remove clay minerals and then fired at 550°C for 24 hours to remove organic compounds. After these treatments, clays and organic compounds were undetectable by spectrophotometry and total carbon analysis by a Leco model CR 12, and the surfaces looked clean in electron microscopy. The gravel treatment eliminated clay and organic carbon content as variables in the experiments and improved replicability in attachment of bacteria by >50%, as determined by preliminary experiments.

The interstitial pore water volume in the columns was ~25 ml. The Darcy flow velocity was maintained at 30 ml day^{-1}, and samples were collected from the effluent once a day. The concentration of dissolved organic carbon in the filtered groundwater was 6 mg $liter^{-1}$ and the concentration of PO_4 was 8 μg $liter^{-1}$. This was regarded as pure groundwater, which was supplied to two replicate columns for each solid support. The experiment was repeated once, so that each treatment was made with four replicates. The same number of columns were provided with amended groundwater (50 mg $liter^{-1}$ glucose and 100 μg $liter^{-1}$ PO_4).

Bacteria were isolated from the sandy saturated soil that was sampled at the infiltration site at

6.5 m depth by a technique described by Bengtsson [3]. The soil carbon concentration of the aquifer material was 0.2%. Bacteria were isolated from the core material by agitating 2 g of soil with 20 ml of filter-sterilized groundwater for 30 min on a hand–wrist shaker. Aliquots (0.1 ml) of serially diluted suspensions were spread on plates with the poor growth media described (above) and incubated aerobically at 12°C. Colonies that grew were transferred to fresh medium. Six different isolates, all of which were Gram-positive (g+), were mixed and suspended in filter-sterilized groundwater and then inoculated at the top of the column, just beneath the sintered glass disc. The pump was started 2 hours after the application. Two pore volumes were pumped through the column before the first effluent samples were collected. The effluent samples were analyzed for total numbers of bacteria by epifluorescence microscopy using the acridine-orange direct count technique [39].

At the end of an experiment the column was drained for interstitial pore water, and glass beads and gravel gently rinsed with 50 ml sterile groundwater to remove loosely adhered cells. Glass beads and gravel were sampled from four depths in a column. Pore water, which was centrifuged at 3,000 × *g* for 20 min to remove suspended particles, and solid supports were analyzed for total numbers of bacteria and biomass by MuAc analysis, and the physiological state was assessed by analysis of PHB. Subsamples of the particles were prepared for scanning electron microscopy (SEM).

Direct Count

Two grams of solid support were suspended in 20 ml sterilized Calgon- (0.2%) peptone (0.1%) solution and shaken for 30 min at 160 rpm. Fifty microliters of acridine orange solution (0.1%) was added after serial dilution of the suspension. The mixture was left for 2 min at room temperature and then slowly filtered through a black 0.22-μm filter (Millipore-MF dyed with Ebony black). The number of fluorescent cells was then counted in a Zeiss 2 Fl microscope equipped for phase-contrast observations.

Muramic Acid Analysis

Two grams of solid support were suspended in 20 ml of sterilized 0.1% sodium pyrophosphate and treated by ultrasonication for 10 min. The suspension was centrifuged at 3,000 × *g* for 10 min. The supernatant was removed, the extraction repeated once, and the supernatants combined. The samples were heated at 60°C for 10 min and centrifuged at 1,000 × *g* for 10 min. The cells were washed twice in phosphate-buffered saline, suspended in distilled water, frozen overnight, thawed, and ultrasonicated to disintegrate the cells. The cell walls were then prepared by centrifugation at 25,000 × *g* for 20 min. The supernatant was discarded and the cell walls washed in distilled water. The peptidoglucans were extracted with 1 ml formamide at 150°C for 15 min [43] and freeze-dried. The peptidoglucans were hydrolyzed with 1 ml of 6 M HCl in teflon-lined screw-cap test tubes under nitrogen at 100°C for 4 hours. The hydrolyzate was diluted, freeze-dried, and dissolved in 100 μl of 2.5% acetic acid. Norleucine was added as internal standard. The solution was applied to a Dowex (H^+) cation exchange column at a rate of 1 ml min^{-1}. The resin was washed with 10 ml deionized water and MuAc eluted with 4 ml of 4 M HCl [40]. The eluate was diluted and freeze-dried. The MuAc was derivatized with iso-butanol-HCl and heptafluorobutyric anhydride (HFBA) and analyzed by gas chromatography according to the method of Bengtsson et al. [2].

A calibration curve of MuAc was prepared from a stock solution in 0.1 M HCl (Sigma, St. Lous, Missouri, USA).

PHB Analysis

Pore water and solid support material were freeze-dried and extracted with a 2-ml mixture of chloroform-methanol-water (1:2:0.8) [4] in a screw-cap test tube. The sample was sonicated for

15 min and then extracted for 2 hours in a heating block so that the chloroform was nearly boiling. The tubes were centrifuged at 4,000 × g, the supernatant transferred to another test tube, and the residue washed once with 0.5 ml of solvent, which was combined with the supernatant. One milliliter of distilled water and 1 ml of chloroform were mixed with the supernatant solution on a Vortex mixer. The mixture was centrifuged at 4,000 × g, the aqueous phase discarded, and the chloroform phase evaporated with a stream of nitrogen gas.

The residue was hydrolyzed for 30 min at 80°C with 1 ml of 15% NaOH in 50% methanol [24]. One milliliter of pentane was added and the mixture shaken and centrifuged, and the pentane phase discarded. The aqueous phase was acidified with 25% HCl to yield the free acids and then freeze-dried. Traces of water were removed by 50 μl methylene chloride, which was evaporated with dry nitrogen gas. Twenty-five microliters of pentafluorobenzylbromide (2% in acetone) (Fluka Chemie AG, Buchs, Switzerland) and 10 μl of pyridine (previously diluted 1:3 with acetone) were added, and the mixture was shaken and heated at 60°C for 15 min. Diesterification of the hydroxy acids was performed by adding 30 μl HFBA, shaking, and heating the mixture at 60°C for 5 min. Fifty microliters of pentane was added and shaken with the mixture. The sample was treated with 100 μl of 0.1 M HCl to extract interfering substances into the aqueous phase, which was then discarded. The washing procedure was repeated with 100 μl of 0.1 M NaOH. After 30 min at room temperature the hexane layer was removed and made up to 100 μl with a Hamilton syringe.

A calibration curve was prepared from rac-3-hydroxy-butyric acid (Fluka).

Gas chromatography was performed on a Varian 3700 equipped with electron capture detection. A 25-m fused silica capillary column coated with SE54 as stationary phase was used with 2.0 ml min^{-1} N_2 as carrier gas. The injector temperature was 250°C and detector temperature was 300°C. The oven temperature was linearly increased by 60°C min^{-1} from 85 to 220°C. Splitless injections were made (split closed for 30 sec).

Scanning Electron Microscopy

Solid support samples were prefixed in 2% glutaraldehyde in 0.2 M cacodylate buffer (pH 7.2) for 2 hours. The particles were then subjected to repeated washings in distilled water and dehydrated for 10 min in acidic dimethoxypropane.

After repeated washings in absolute ethanol the particles were immersed in ethanol/freon TF (3:1) for two periods of 15 min each, in ethanol/freon (1:1) for two periods of 30 min each, and finally in pure freon TF overnight. The particles were air-dried, glued on a metal holder, coated with gold/palladium, and viewed with a Nanolab 2000 scanning electron microscope.

Statistical Analysis

The treatments were compared by analysis of variance with 3° of freedom. A mean was calculated for each replicate from all sampling observations and treated as a replicate observation in the statistical analysis. Standard deviation was calculated for the mean of each sampling occasion.

Results

The number of cells in the column effluent remained virtually constant in each treatment throughout the experiment, with a coefficient of variation that was usually well below 0.2% (Fig. 2). The cell numbers resulting from each individual treatment were significantly different from all other treatments ($P < 0.01$); gravel was a more favorable substratum than glass beads, and groundwater supplied with glucose and phosphate supported higher cell numbers than

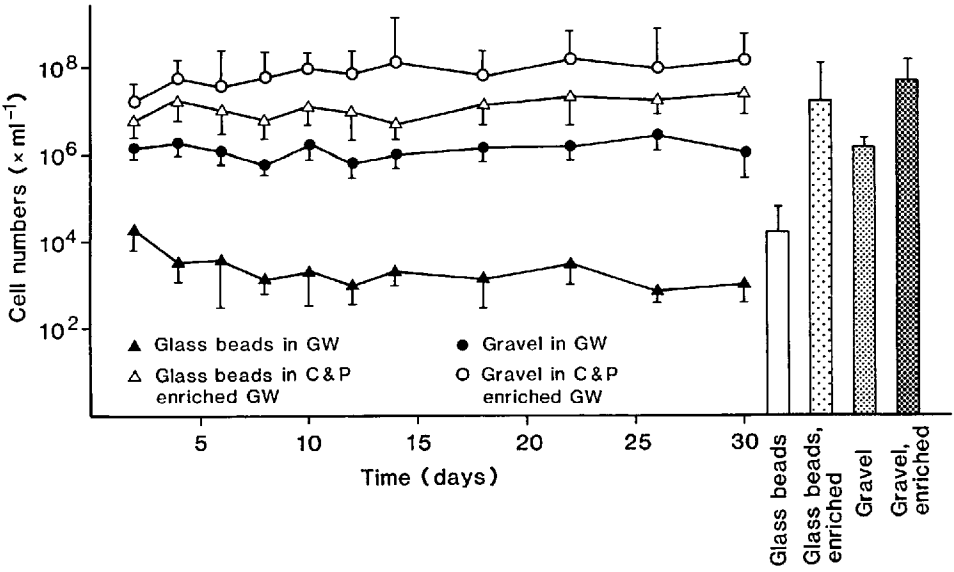

Fig. 2. Cell abundance in column effluents (solid lines) during incubation for 30 days and in interstitial pore water at the end of the experiments (bars). GW, groundwater; C & P, groundwater enriched with carbon and phosphate. Standard deviation of the mean is indicated for the column effluent data and standard error of the mean for the pore water data.

pure groundwater. Since columns with glass beads and gravel received the same pure groundwater, the difference in cell abundance by three orders of magnitude was due to differences in attachment and growth support at the two kinds of solids.

The greatest cell abundance was observed in the effluent from enriched gravel columns. With the 10-fold increase in glucose and PO_4 concentration the cell numbers increased from about 10^6 ml^{-1} to about 10^8 ml^{-1}. Similarly, the cell abundance became 10^4 times greater in the effluent from the glass bead columns when the pure groundwater was enriched.

The same relative order of ascending cell abundances was observed in the interstitial pore water at the end of the experiment, although the differences among the treatments were smaller (Fig. 2). The cells were generally very small, <0.5 μm, in the pure groundwater, but were clearly larger, >1.0 μm, in the gravel columns with enriched groundwater.

Hence, three conclusions can be drawn from these observations: (1) if the prewashed gravel is accepted as a reasonably natural substratum, glass beads have habitat properties that are clearly artificial, due to reduced interfacial areas or toxic properties, so that differences observed in laboratory experiments may be more qualitative than quantitative; (2) cell numbers in the effluent of continuous flow laboratory models are representative for cell abundance in the interstitial pore water at equilibrium; (3) the growth of bacteria in the pure groundwater studied was limited by the concentration of organic carbon, phos-

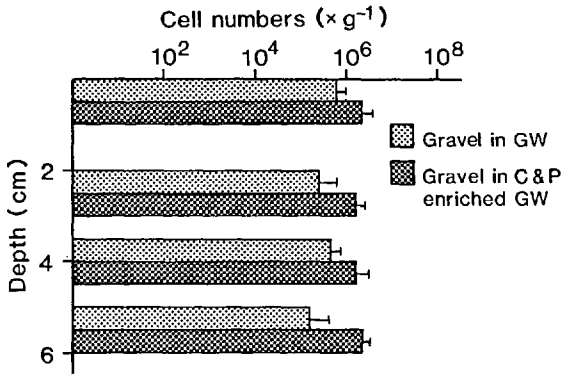

Fig. 3. Cell abundance on gravels at different depths of the groundwater columns at the end of the experiments. Standard error of the mean is indicated.

phate, or a combination of both. Normal carbon and phosphate concentrations limit the population size by at least a factor of 35.

The number of cells that adhered to gravel in the pure groundwater was about four times fewer at the bottom of the columns than at the top, whereas the cell abundance remained constant with the depth when carbon and phosphate was added to the water (Fig. 3). The number of attached bacteria was influenced significantly less by the enrichment than the number of free-living bacteria; their abundance increased four times at the column top and 10 times at the column bottom. Since the numbers became equal at all depths by the enrichment, carbon and PO_4 concentrations were probably more than sufficient for growth at the solid support and factors other than energy and nutrient supply became growth limiting. Whereas attached bacteria were 2.5 to 10 times fewer than free-living in the pure groundwater, the difference increased to more than 20 times when the water was enriched, clearly demonstrating the importance of the energy and nutrient conditions for the relative abundance of groundwater bacteria in the solid and liquid phases. Cells viewed by SEM were irregularly and sparsely distributed on the surface of the glass beads and tended to be associated with loosely adhered fragments of organic coatings in enriched columns. The distribution was more aggregated, especially to different kinds of depressions, on the gravel surface, but many single cells were still found.

The biomass of bacteria, estimated from the MuAc analyses of cell wall peptidoglucans, was also dependent on the quality of the solid support and the carbon and nutrient supply. The gravel supported a larger biomass, especially in pure groundwater, than the glass beads, and the biomass increased five (gravel) to 10 (beads) times on addition of glucose and PO_4 (Fig. 4). The changes in biomass and cell abundance in relation to soil depth and nutrient conditions were essentially identical (i.e., a decrease by a factor of 4 with the depth, an increase by a factor of 4–5 at the top, and by a factor of 10 at the bottom when the water was enriched), although the biomass was still unevenly distributed in the enriched soil. As a consequence, the amount of MuAc per cell remained virtually the same (3.8–4.7 per 10^6 cells) on the gravel regardless of nutrient conditions.

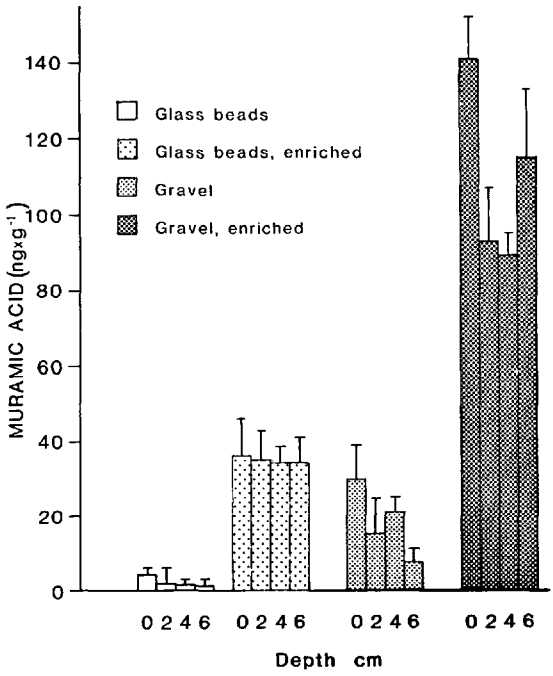

Fig. 4. Concentration of muramic acid at the gravel surface at different depths of the groundwater columns at the end of the experiments. Mean and standard error are given.

Two different patterns of PHB distributions were observed. The concentration in pore water and on solids at the column bottom were lowest in pure groundwater and highest in enriched water. The opposite occurred on solids at the column top (Fig. 5). The concentrations were always higher in gravel columns than in glass bead columns. Also the PHB concentrations demonstrated that glass beads were a more unfavorable growth support than gravel and that the free-living bacteria were capable of producing more PHB than the attached, especially in enriched water. The PHB concentrations in soil columns were almost identical in pore water and on bottom solids, which in comparison with the actual cell numbers (see Figs. 2 and 3) indicate, that attached cells stored about 10 times more PHB per cell (Table 1). When PHB quantities were compared on a per cell basis rather than per gram or per ml, it was observed that cells in pure groundwater stored about 10 times more PHB than cells in enriched water (Table 1). The same proportions were found when PHB was calculated from the concentrations of MuAc (Table 1). According to both calculation procedures the cells on gravel at the column bottom stored about twice as much PHB as cells at the column top. All these comparisons indicate that PO_4 rather than carbon was most growth-limiting in the pure groundwater. There was sufficient carbon to accumulate in the cells, and relatively more carbon was stored as nutrient conditions became worse (e.g., on particles as compared to pore water and at the bottom as compared to the top of the columns). Once PO_4 was added to the water, stored PHB was metabolized,

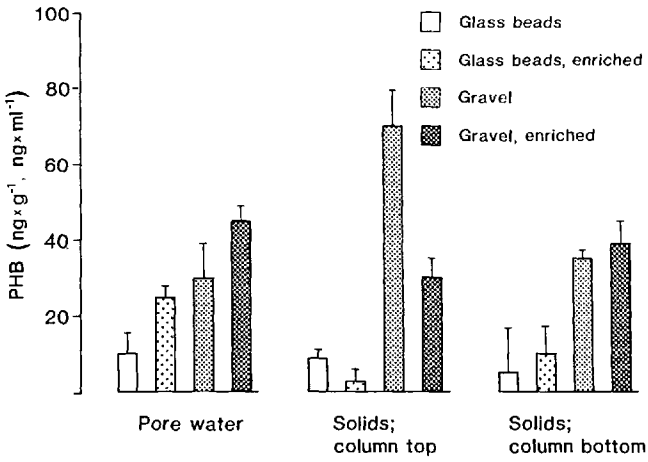

Fig. 5. Concentration of poly-β-hydroxybutyric acid (PHB) in pore water and attached bacteria in groundwater columns at the end of the experiments. Mean and standard error are given.

although cells at the column bottom still behaved as if their growth conditions were less balanced than cells at the column top.

Discussion

The maintenance of a low population density and subsequent storage of PHB during extended incubations in groundwater and the rapid metabolic response of the groundwater isolates to carbon and PO_4 enrichment is consistent with the assumption that natural groundwaters are unstable oligotrophic environments where some bacteria are adapted to track even random rises and falls in the carrying capacity. These bacteria can survive for long periods of time at very constant, low numbers (see Fig. 2), promoted by their capability to store PHB under nutrient limitations and probably by other survival mechanisms as well. However, they may proliferate when growth conditions become more favorable, especially in the interstitial pore water.

The movement of cells increased as a result of enhanced population density in the pore water (Fig. 2). The migrating cells may colonize and explore new resources and eventually absorb to a solid surface in the soil matrix. Hence, within the bacterial consortia, in a saturated soil, strategies for enhanced survival and migration seem to occur together, even so that some species alternately use both strategies.

Cells that can survive by maintaining a low endogenous metabolism synthesize protein, DNA, and storage polymers [9, 25, 26, 31] for subsequent degradation during energy and nutrient depletion [1, 17, 30]. The small size of cells in pure groundwater would improve the nutrient uptake efficiency [29]. The increase of the cell diameter from <0.5 μm in pure groundwater to >1.0 μm in amended water reduces the surface area-to-volume ratio by $>50\%$. Also attachment to solid surfaces would be advantageous, if adsorbed nutrients support growth more than the diffusion rate through the hydrodynamic boundary layer limits it, and if the profits gained from protection from shear forces

Table 1. The amount of poly-β-hydroxybutyric acid (PHB) in gravel columns in relation to cell abundance and biomass measured as muramic acid (MuAc)

Sample	PHB/cells (ng × 10^{-5})	PHB/MuAc (ng ng^{-1})
Column top		
GW	10	2.3
GW + CP	1.0	0.2
Column bottom		
GW	17	4.4
GW + CP	1.3	0.4
Pore water		
GW	1.5	—[a]
GW + CP	0.1	—

GW, pure groundwater; GW + CP, pure groundwater amended with 50 mg $liter^{-1}$ of glucose and 100 μg $liter^{-1}$ of PO_4

[a] —, not determined

and from high turnover rates of substrata outweigh the loss of cell surface area occupied for attachment.

Though the ratio of particle bound to free-living bacteria was <1, the pure groundwater had more attached bacteria than the amended water (see Fig. 3), where the relative numbers of attached went down considerably. This is in agreement with observations by Jannasch and Pritchard [14] of a number of bacterial cultures that would grow attached to particles at low nutrient concentrations and were mainly free-living at high nutrient concentrations. The flexibility in growth to variations in nutrient conditions may vary among species. The growth of one of the most common species isolated from well water by Stetzenbach et al. [37], *Acinetobacter* sp., was stimulated by more than two orders of magnitude by addition of glucose, acetate, pyruvate, or succinate (100 and 1,000 μg carbon/liter), whereas another isolate, *Moraxella* sp., was unaffected by amended well water.

In general, bacteria bound to particles in ocean and lake waters are only 1–30% of the numbers of free-living bacteria [12, 13, 16, 28], but their activity measured as substrate uptake usually surpasses that of free-living cells [5, 12, 16, 27, 35, 36]. However, when exogenous nutrients were supplied to a population of *Vibrio* sp., free-living cells became more active than attached [15], although most of the lower substrate uptake activity of the attached cells could be explained by loss of cell surface area.

Although there is a general agreement in the literature that the numbers of attached bacteria are low, certain advantages should favor the association of groundwater bacteria with the solid support: (1) diffusion and adsorption of nutrients to the particles creates substrate gradients with local surface accumulations, from which the cells should benefit; (2) most particles have patches of adsorbed polymeric organic matter, that can be accessed with extracellular enzymes; (3) a free-living bacterium behaves as a colloid, moving with a parcel

Table 2. The concentration of muramic acid (MuAc) and cell abundance on gravel at two depths in the columns, and the concentration of MuAc calculated on a single cell basis

Depth (cm)	MuAc (pmol/g)		Abundance (cells/g)		MuAc (pmole × 10^{-5}/cell)	
	GW	CP	GW	CP	GW	CP
0–2	10.2	48.1	8×10^5	3×10^6	1.3	1.6
6–8	2.7	39.2	2×10^5	3×10^6	1.4	1.3

GW, pure groundwater; CP, groundwater amended with 50 mg liter^{-1} of glucose and 100 μg liter^{-1} of phosphate.

of water that eventually becomes depleted of nutrients, whereas an attached bacteria is faced with new parcels exchanging with a rate determined by the hydrodynamics of the boundary layer. Little is known to explain why the majority of groundwater bacteria are not attached. The system seems to be saturated with particles, with a sufficient concentration of organic matter to facilitate growth (0.2% at the particles sampled for this study), and it is quite often oligotrophic. A number of factors may be important in limiting bacterial population size, such as particle surface area [44], the topography of particles [19, present study], carbon and nitrogen content of the surface coating [6, 7, 33], the concentration of dissolved organic matter [13], and cell surface hydrophobicity [32]. Undoubtedly, the groundwater bacteria were disfavored by the topography of the glass beads and the removal of the surface bound polymers from the gravel, so the numbers attached may be larger in the aquifer.

Attached bacteria in pure groundwater also synthesized 10 times more PHB than free-living cells (Table 1), supposedly due to a higher specific uptake of exogenous carbon diffusing into the boundary layer and eventually adsorbing to the solid surface. In this respect, PHB synthesis of attached cells faced with the nutrient enriched pore water was reduced to the same level as cells desorbed in pure groundwater. However, the cells in the poorest groundwater environment, at the column bottom, produced 1.7 times as much PHB as the cells at particles in the column top. The difference in PHB production by attached cells at the column top in pure and amended groundwater was more than 10% larger when expressed in relation to biomass, measured as the concentration of MuAc, simply because the MuAc concentration per cell was 23% higher in amended groundwater (Table 2).

The concentration of MuAc in these groundwater bacteria, about 4 ng/10^6 cells (Table 2), comes close to the numbers presented by Millar and Casida [20] for Gram-positive, laboratory grown, soil bacteria but several orders of magnitude lower than the MuAc concentration they found in various top soils. The concentration of MuAc per gram of sediment was about three orders of magnitude lower than that found in marine sediments [21, 22] and the values per cell about 10 times higher than those reported by the same authors for a Gram-negative pseudomonad culture. This is in agreement with the general observation that Gram-positive bacteria have more MuAc than Gram-negative bacteria [20, 23].

The bacteria in the pure nutrient-poor water had about six times more PHB per cell (150 ng/10^7 cells; see Table 1) than the marine g-S14 isolate used by Malmcrona-Friberg et al. [17] in starvation experiments. Their bacteria were, however, raised in media with ample carbon (g/liter) and 10 mg/liter of phosphate. During the onset of starvation in a phosphorus-free medium with 2 g/liter of glucose the PHB concentration increased by more than 20 times and exceeded the concentration found in the pure groundwater, indicating that PHB was synthesized under these conditions of unbalanced growth due to deprivation of PO_4. The opposite phenomenon was found when the groundwater was supplemented with PO_4; the PHB concentration in cells went down to about 10 ng/10^7 cells, which was three times lower than that recorded in the S14 cells.

Two of the four hypotheses addressed in the introduction were apparently rejected based on the documentation shown. Although attachment should be advantageous, especially in an oligotrophic environment, for a number of reasons already given, most of the bacteria remained in the aqueous phase, and their biomass/abundance ratio was constant, although the average cell size doubled when the pure groundwater became enriched. The data here and in the literature are still insufficient to explain and predict the distribution of cells in the aqueous and solid groundwater habitats and their investment in biomass increase and reproduction. Both of these areas need further research.

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