# Subsurface bacterial growth and grazing impact revealed by an in situ experiment in a shallow aquifer

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**ABSTRACT**

To elucidate bacterial population dynamics in an aquifer, we attempted to reveal the impact of protozoan grazing on bacterial productivity and community structure by an in situ incubation experiment using a diffusion chamber. The abundance and vertical distribution of bacteria and protozoa in the aquifer were revealed using wells that were drilled in a sedimentary rock system in Itako, Ibaraki, Japan. The water column in the wells possessed aerobic and anaerobic layers. Results of the in situ incubation experiment suggested that protozoan grazing contributes significantly to bacterial population dynamics. On August 19, 2003, the total number of bacteria (TDC) decreased from $1.5 \times 10^6$ cells $ml^{-1}$ at 2.2 m depth to $3.0 \times 10^5$ cells $ml^{-1}$ at 10 m depth. The relative contribution of the domain Bacteria to TDC ranged between 63% and 84%. Protozoa existed at a density of $4.2 \times 10^4$ to $1.9 \times 10^5$ cells $ml^{-1}$ in both aerobic and microaerobic conditions. A grazing elimination experiment in situ for 6 days brought about clearly different bacterial community profiles between the 2.2 m and 10 m samples. The bacterial composition of the initial community was predominantlyβ- and γ-*proteobacteria* at 2.2 m, while at 10 m β-, α- and γ-*proteobacteria* represented 56%, 26% and 13% of the community, respectively. The distribution of bacterial abundance, community composition and growth rates in the subsurface were influenced by grazing as well as by geochemical factors (dissolved oxygen and concentrations of organic carbon, methane and sulfate).

**Key words;** bacterial growth, subsurface, grazing impact, in situ incubation, microbial community, microaerobic environment

## INTRODUCTION

Since the geochemical and ecological importance of the existence, and thus activity, of bacteria in the subsurface environment was indicated by Ghiorse and Wilson (1988), extensive studies have been undertaken during the last decade (e.g., Fredrickson and Fletcher 2001). But the knowledge was not sufficient yet to overview the ecology of bacteria under the hidden milieu. The distribution of bacteria in subsurface environments has been highlighted by studies of sub-seafloor microbial distribution (Parks et al. 1994; Parks et al. 2005; Inagaki et al. 2006), which show that bacteria occur at up to ca. 1000 m below the sea-floor at densities of up to $10^5$ cells $cm^{-3}$. Such knowledge leads to the idea that the function of bacteria in geochemical processes in the subsurface might be greater than previous thought.

In contrast to the subseafloor studies, terrestrial studies of subsurface bacteria have mainly focused on shallow aquifers, particularly concerning the contamination of groundwater (Cho and Kim 2000; Langwaldt et al. 2005). Both molecular and culturablility studies on bacterial distribution in terrestrial subsurface environments have been intensively carried out with regard to radioactive waste concerns (Pederson et al.2000a; 2000b; Haveman and Pedersen 2002).

Detailed studies on the activity of microbes in subsurface environments were in major carried out concerning methane production and oxidation processes in both marine and terrestrial environment (Kotelikova and Pedersen 1998; Mikucki et al. 2003; Erwin et al. 2005; Newby et al. 2004). But information on bacterial growth activity in the subsurface is still limited for both environments.

Once bacteria exert activity in response to particular geochemical processes at a site where they exist, we can expect them to undergo growth. Thus questions arise as to the fate of growing bacterial populations: how fast they grow and how the growth is affected by predation. Novarino et al. (1997) reviewed protistan communities in aquifers, where protistan communities are usually dominated by heterotrophic flagellates, which are relatively small, in the range 2-3 μm. They indicated that protistan density was low in pristine uncontaminated sites, but after contamination density increased by several orders of magnitude. Kinner et al. (1997) suggested from laboratory experiments that flagellate bacterivory is an important control on free-living bacteria in organic contaminated aquifers.

The object of this study was to reveal the growth potential of bacteria by eliminating the grazing pressure of protozoa, and therefore the effect of it on the dynamics of bacterial populations, by an in situ incubation experiment. The experiment was carried out in a newly drilled well with a water column consisting of aerobic and anaerobic layers in a sedimentary rock system.

## MATERIALS AND METHODS

### *Sampling site, employed wells and sampling procedure*

The experiment was carried out at the Center for Water Environment Studies of Ibaraki University, located on an alluvial plain in Itako City adjacent Lake Kitaura, Ibaraki Prefecture, Japan. Paddy fields exist on one side and upland terraces covered by thick deposits of volcanic ash on the other. The site is located in the lower part of a catchment of ca 45 $km^2$.

Experimental wells #3A and #4-2 were drilled in 2002 and 2003 respectively, and ORP measurements showed that the deeper parts of the drilled area, i.e., below 6 m, were in a permanent anoxic condition. The depth of well #4-2, which was used for the experiment, was 11 m with a diameter of 150 mm, and the borehole casing had an open strainer from 4.5 to 10.5 m to allow entry of groundwater at the depths where groundwater flow had been previously confirmed. The depth of well #3-A was 11 m with a diameter of 65 mm, and the borehole casing had an open strainer from 4.5 to 10.5m.

The water was sampled using an ethanol-rinsed piston sampler (400mmφx500mm, 5014-B, RIGO Co. Ltd., Japan).

When the sampled water contained a significant amount of $Fe^{2+}$, EDTA was quickly added to the water to a final concentration of 6 mM to prevent the formation of $Fe_2O_3$ aggregates, which interfere with microscopic observation. Samples were either immediately fixed for the enumeration of bacteria or subsamples for the incubation experiment were filtered through the procedure described further below. Sampled water was subsampled immediately and fixed with neutralized formaldehyde (final concentration of 2%) for enumerating total bacteria and protozoa and with paraformaldehyde (final concentration of 3%) for FISH and was then filtered for incubation.

***Geological characteristics of the sediment***

The core retrieved from drilling of well #4-4 in 2004 at a distance of 2.2 m from well #4-2 was analyzed for geological constituents. The sediments were of Holocene origin, consisting mainly of silt and sandy silt. The abundance of sand increased between 4 m and 10 m depths. The water level at the site fluctuates seasonally under the influence of precipitation; the water table was at 4.05 m depth when coring was carried out. The core at 2.1 m was water-saturated and showed a bluish color suggesting the presence of sulfide. The sediment was a silty texture with an admixture of undecomposed organic matter. Mottling at this depth indicates the fluctuation of oxidation-reduction potential. The core at 3.6 m was water-saturated and had a bluish gray color and sandy-silt texture. Below this, the texture of the sediment becomes coarse. The diffusion of air into this sandy layer from oxic gravel or from water moving through fissures could be expected. The total organic carbon content (TOC) was a moderate 18.2 mg $g^{-1}$ and soluble ferrous iron ($Fe^{2+}$) content was 1.6 mg.$g^{-1}$ soil (oven dry weight) at 0.25 m and 1.75 m depths. The TOC content at 3.6 m deep was a very low 1.0 mg.$g^{-1}$ and $Fe^{2+}$ content was higher at 2.7 mg $g^{-1}$. At 6.1 m deep, TOC was very low (1.2 mg $g^{-1}$) and $Fe^{2+}$ was 2.3 mg $g^{-1}$.

***Measurement of biogeochemical parameters***

Water temperature, pH, conductivity, Eh and dissolved oxygen (DO) were measured using portable meters (pH: HM-12P, TOA Co., Ltd., Tokyo, Japan, EC: CM -14P, TOA Co., Ltd., Tokyo, Japan, Eh: RM-12P, TOA Co., Ltd., Tokyo, Japan, Eh and DO: Troll 8000, In-Situ Inc., Wyoming, U.S.A.). $Fe^{2+}$ concentration was analyzed by ferrorigin immediately after sampling (Stookey 1970; Viollier et al. 2000). Total organic carbon (TOC) was analyzed with a Total Organic Analyzer, TOC-5000 (Shimadzu, Co Ltd, Japan). A 5 ml sample of collected groundwater was placed in triplicate vials (27 ml) containing 5 ml of saturated NaCl for laboratory measurement of dissolved methane. The vials were sealed with Teflon septa and aluminum caps, shaken well then stored at 4 ℃ until analysis. Vials were brought to room temperature before measurement. The headspace was analyzed on a GC-14B gas chromatograph (Shimadzu, Japan) equipped with a flame ionization detector and a Molecular Sieve 13X-s 2 mm ID X 2 m SS column. The temperatures employed for measurement were as follows: injector, 110 ℃; column, 100 ℃; detector, 160 ℃. Nitrogen was used as the carrier gas (40 ml $min^{-1}$).

***In situ incubation experiment***

Bacterial productivity was estimated from in situ growth rate measured directly using a diffusion chamber (Figure 1b, 1c). Grazers were eliminated by filtration through a 3.0 μm Nuclepore filter and the chambers then immersed at the depth where the water was collected. The 33 ml capacity diffusion chambers consisted of Nuclepore filters (0.2 μm in pore size) at both ends of each chamber to enable dissolved molecules to diffuse into the chambers but

preventing the passage of bacteria. The diffusion rate is shown in Figure 1a, which was measured using cytochrome c (12,000 dalton). The diffusion chamber was immersed into a plastic beaker filled with cytochrome c stained with dark blue. Figure 1a shows that cytochrome c diffused swiftly into the chamber. The relative concentration of cytochrome c inside the chamber became one-third within 10 hours. Subsamples were filtered through a 3.0 μm Nuclepore filter to eliminate any small coexisting bacterial grazers. The diffusion chambers were placed in the well at depths of 2.2 m (surface of the water level), 6 m and 10.5 m and the chambers were retrieved after several days incubation.

The experiments were carried out in August 2003 and December 2003 in well #4-2. During the August, 2003 experiment, water measurements made on August 19 showed that temperature did not change vertically, which led to a similar DO concentration from the top of the water (2 m depth) to 10 m depth, being from 0.40 to 0.20 mg $l^{-1}$. A similar experiment was performed in well #3-A in July 2004. In this case, the concentration of ferric ions increased sharply at 5.5 m where DO was 0.43 mg $l^{-1}$, while DO at the surface was 2.80 mg $l^{-1}$ at the surface. The DO at 5.5 m in well #3-A in July 2004 was the same as that observed in well #4-2 on 19 August, 2003.

***Total direct count (TDC) of bacteria and fluorescence in situ hybridization (FISH)***

Microbial cells were collected by filtration of 0.7 to 2 ml samples of water and stained with acridine orange to a final concentration of 0.1% (Hobbie et al. 1977). The bacterial cells were observed by epifluorescence microscopy (BX50, Olympus Corp., Japan) with 20 microscopic fields for each sample.

Fixed well samples were filtered on 0.2 μm Nuclepore filter (Whatman, UK) within 24 h. Cells on the filter were rinsed three times with phosphate-buffered saline (pH 7.2, Dulbecco's PBS(-), Nissui, Japan) and dehydrated in 1 ml of 50%, 80% and 99.5% ethanol for 3 minutes. Filters were then air-dried. Filters were stored at -20℃ until hybridization. The 16S rRNA targeted oligonucleotide probes employed in the study were domain-specific probes for Bacteria (EUB338; Amman et al. 1990) and Archaea (ARC915; Stahl and Amman 1991) and phylogenetic-group-specific probes for δ-*proteobacteria* (DEL338; Amman et al. 1990). These probes were labeled with tetramethyl rhodamine isothiocyanate.

Hybridizations were carried out at 46℃ for 90 min on filters placed on slides coated with gelatin with hybridization buffer containing 0.9 M NaCl, 20 mM Tris·HCl (pH 7.4), 0.01% SDS, formamide (20% for EUB338, 30% for ARC915 and DEL) and 5 ng $\mu l^{-1}$ of the respective labeled probe. Each filter was washed at 48 ℃ for 15 min in pre-warmed washing buffer containing NaCl (0.025 mM for EUB338, 70 mM for ARC915, 80 mM for DEL), 20 mM Tris·HCl (pH7.4), EDTA(0.5 mM for EUB338 and DEL, 5 mM for ARC915) and 0.01% SDS, then rinsed with milliQ (Direct-Q Millipore, US) water. The filters were air-dried and then stained with 0.1 ug $ml^{-1}$ DAPI on glass slides for 5 min.
The preparations were observed with a BX50-FLA epifluorescence microscopic system equipped with a 3CCD camera (C5810, Hamamatsu Photonics, Japan).

***Protozoa enumeration***

Protozoa enumeration was performed by modifying the method of Lim et al. (1993; 1996). Fixed samples were filtered on 0.6 μm Nuclepore filter within 48 h. Cells on the filter were rinsed three times with PBS(-) and dehydrated in 1 ml of 50%, 80% and 99.5% ethanol for 3 minutes. The filter was then air-dried. Filters were stored at -20 ℃ until hybridization. The following probes that were labeled with fluorescein isothiocyanate (FITC) were used for detection and enumeration of protista cells: Euk 1209 (Amman et al. 1990), Euk 502 (Giovannoni et al. 1988), and Euk 309 (Sogin and Gunderson 1987). These probes are

complementary to regions on the small-subunit (SSU) rRNA that are conserved for all eukaryotes.

Preincubation was performed at 40°C for 45 minutes on filters placed on slides coated with gelatin with 5×SET buffer containing 0.75 M NaCl, 100 mM Tris·HCl (pH 7.8), 5mM EDTA and 0.1% SDS. Three labeled probes (final concentration 5 ng $\mu l^{-1}$ respectively) were added to each filter, hybridization was performed at 40°C for 180 minutes. Each filter was washed at 45 °C for 10 minutes in pre-warmed 0.2×SET buffer containing 30mM NaCl, 4 mM Tris·HCl (pH7.8) and 0.2mM EDTA, then rinsed with milliQ water. The filters were air-dried and then stained with 0.1 $\mu g\ ml^{-1}$ DAPI on glass slides for 5 minutes.

***Cloning and sequencing***

Groundwater samples in volumes of 100 to 500 ml were filtered through a Nuclepore filter (pore size, 0.2 μm; Whatman, UK). Cells were lysed using a bead beater (Mini-BeadBeaterTM, BioSpec Products, Inc., US) and bacterial DNA was extracted using phenol-chloroform-isoamylalcohol (Zwart et al. 1998) and purified with UltraClean Soil DNA Isolation Kit (MoBio Lab Inc., US). To generate near full-length 16S rDNA clones, the extracted DNA was subjected to PCR amplification with primers 27F and 1492R (Lane, 1991). The PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen Corp., UK) before being ligated into a pCR 4Blunt-TOPO vector (Invitrogen Corp.) and transformed into competent *E. coli* TOPO10 cells (Invitrogen Corp.). Clone libraries of bacterial 16S rDNAs were then constructed. The sequences of the representative 16S rDNA clones were determined using a capillary DNA sequencer (CEQ2000XL DNA Analysis System, Beckman Coulter Inc., US) with the 16S rDNA-specific primer 341F (Muyzer et al. 1993) used for sequence reactions.

***Phylogenetic analysis***

Sequence data were aligned using the CLUSTAL W package (Thompson et al. 1994), with clones having similarities of 97% or above grouped into operational taxonomic units (OTUs). The clones were homology-searched using BLAST and the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/). Phylogenetic trees were produced using the neighbor-joining algorithm of the NJ plot program (Saitou and Nei, 1987).

**RESULTS**

***Biogeochemical parameters***

The vertical profiles of physical and chemical parameters shown in Figure 2 were obtained from well #4-2 on 19 August, 2003. The dissolved oxygen (DO) concentration at the surface was 2.2 mg $l^{-1}$ and decreased with depth to 0.4 mg $l^{-1}$ at 6 m deep. Corresponding to the decrease in dissolved oxygen, $Fe^{2+}$ concentration increased sharply at 6 m deep. Water temperature was comparatively stable vertically ranging from 13.5 to 17.5 °C. The pH was stable and close to neutral at about 6.49 to 6.78 throughout the observation. TOC concentration was high from 28.24 to 31.84 mg $l^{-1}$. Methane was not found in this well, though it was observed in well #3-A, which had a diameter about half the size of that of well #4-2. Sulfate was observed in well #4-2 at low concentrations of 0.80–1.11 μm throughout the water column from 3 to 10 m deep.

***TDC and FISH***

Figure 3 shows the vertical distribution of bacteria in well #4-2 on August 19, 2003. TDC was high at 1.5 x $10^6$ ±3.6 x $10^5$ cells $ml^{-1}$ at the surface of the water column (2 m below the well head). The number of bacteria decreased with depth to 5.8 x $10^5$±8.5 x $10^4$ and 3.0 x

$10^5 \pm 5.5 \times 10^4$ cells ml$^{-1}$ at 6 m and 10 m, respectively.

FISH analysis of bacterial constituents showed that at the surface the domain Bacteria comprised 84% of TDC whereas the domain Archaea comprised only 0.9%. At 6 m and 10 m deep, the domain Bacteria comprised 63% and 66% of TDC, though the proportion of TDC consisting of the domain Archaea increased to 6.8% and 4.1% at 6 m and 10 m, respectively. The number of δ-*proteobacteria,* which includes sulfate-reducing bacteria (SRB), was $1.4 \times 10^4 \pm 7.2 \times 10^3$ (0.9% of TDC) and $1.5 \times 10^3 \pm 1.1 \times 10^2$ cells ml$^{-1}$ (0.5% of TDC) at 2.2 m and 10 m, respectively. No δ-*proteobacteria* were found at 6 m deep by the method employed of filtering 1 ml through a 0.2 μm filter of 15 mm diameter.

***In situ incubation experiment***

Figure 4 shows the changes in TDC of 3.0 μm filtrate (a) and non-filtrate (b) during the in situ incubation experiment that was carried out in well #4-2 from the 19th to 25th August, 2003. Irrespective of filtration treatment, the TDC increased during the incubation. Apparent bacterial growth occurred in the 3.0 μm filtrate for the samples at 6 m and 10 m. The measured growth rate (μ, day$^{-1}$) of 3.0 μm filtrate bacterial population was 0.269, 0.410 and 0.509 day$^{-1}$ for 2.2 m, 6 m and 10 m, respectively. The calculated doubling time was thus 2.58, 1.69 and 1.40 days for 2.2 m, 6 m and 10 m deep, respectively. The increase in bacterial number was significant irrespective of filtration. The differences between non-filtrate and 3.0 μm filtrate can be considered an estimate of bacterial in situ growth, as some incubation artifacts were not taken to be neglected. If this artifact of filtration treatment were compensated the estimated actual growth rate was 0.092, 0.099, and 0.131 day$^{-1}$ for 2.2 m, 6 m and 10 m, respectively. The doubling time of 5.3 days at 10 m was fast compared to the 7.5 days and 7.0 days observed at 2.2 m and 6 m, respectively. It was revealed that bacterial in situ growth activity of the microaerobic layer was higher than that of the aerobic layer in this well environment. These findings did not largely differ from the estimates obtained from similar experiment carried out from 19 to 23 December, 2003; the obtained doubling time for bacteria less than 3.0 μm was 8.32 and 2.62 days for 2.2 m and 10 m deep, respectively. A relatively large growth rate in the deeper layer, which contained very low dissolved oxygen, was also found in the experiment in well #3-A from 10 to 17 July, 2004. The estimated growth rate of bacteria less than 3.0 μm was 0.098, 0.138 and 0.238 day$^{-1}$ with doubling times of 7.06, 5.02 and 2.90 days at 2.2 m, 6 m and 10 m, respectively.

***Phylogenic analyses***

Altogether 173 clones of 16S rDNA were analyzed to elucidate grazing impact on bacterial community structure for the experiment performed from 19 to 25 August, 2003. Our sequences fell into 11 and 18 OTUs for in situ water at 2.2 m and 10 m, and 16 and 9 OTUs for water samples after incubation at 2.2 m and 10 m, respectively. When compared vertically, the bacterial diversity of in situ water, as measured by abundance of OTUs, was lower at 2.2 m than at 10 m (Table 3). Grazing elimination caused the number of OTUs to increase at 2.2 m and decrease at 10 m.

Figure 5 shows the constituents of the bacterial community as revealed by clone analysis. Before the incubation, β- and γ-*proteobacteria* comprised 58% and 30% of the initial community at 2.2 m, while at 10 m deep β-, α- and γ-*proteobacteria* were present in proportions of 56%, 26% and 13%, respectively before the incubation. The in situ grazing elimination experiment for 6 days brought about clearly different profiles in the bacterial community as α-*proteobacteria* and *Actinobacteria* comprised almost 50% of the total community while β-*proteobacteria* decreased to 26% at 2.2 m. In contrast, at 10 m the contribution of α-*proteobacteria* to the whole bacterial community increased to over 60% after incubation, while β-*proteobacteria* decreased from 56% to 27%.

The phylogenetic tree for the 54 OTUs detected from sequencing of about 500 bp from positions 374 through 875 (*E. coli* 16S rRNA numbering) is shown in Figure 6. The 54 OTUs fall into four phyla of the domain Bacteria, and bacteria belonging to *Proteobacteria* were divided into four classes, α-, β-, γ- and δ- *proteobacteria*. More than half of the OTUs were grouped into α- and β-*proteobacteria*, while δ-*proteobacteria* consisted of one OUT, which was found in the in situ water at 10 m. On the contrary, bacteria belonging to the phylum *Actinobacteria,* which consisted of two OTUs, appeared after incubation at 2.2 m. Many OTUs detected from both layers were related to unidentified clones found in contaminated groundwater, but mostly belonged to β-*proteobacteria*. A remarkable finding resulting from the in situ incubation experiment was the increase in the number of OTUs belonging to α-*proteobacteria* (Figure 6).

Results of detailed analysis are shown in the phylogenetic tree in Figure 6. OTU I-Top-50, with the largest number of clones, 15, was very close to *Aquaspirillum delicatum* (Wen et al. 1999) with 99% similarity. This OTU accounted for one-third of all clones sequenced from the water before incubation at 2.2 m. *A. delicatum*, a chemoorganotroph of the β-*proteobacteria* class, is known to occur both in aerobic and microaerobic conditions (Pot and Gillis 2005), but we did not find them after incubation. Another clone of *A. delicatum* shown by I-Bottom-39 (98% similarity) consisting of two clones was found at 10 m before incubation but not after.

I-Top-49, the second largest OTU at 2.2m consisted of nine clones and was related, but with only 92% similarity, to *Methylococcus* sp., a methane-oxidizer (Dedysh et al. 2004). A similar bacterial group existed at 10 m as I-Bottom-22, consisting of four clones. I-Top-33, which consisted of three clones, was assumed to be *Ferribacterium limneticus* (99% similarity), an iron-reducer (Cummings et al. 1999). A similar bacterial group was also found before incubation at 10 m by I-Bottom-02 (two clones). Following these, I-Top-24, consisting of two clones, had 98% similarity and was thus assumed to be another methane-oxidizer, *Methylobacter* sp. (Wartiainen et al. 2006), a member of the γ-*proteobacteria*. I-Top-31 consisted of three clones and was related to *Methylovorus mays*, a methylotrophic bacteria (Doronina et al. 2005), but with a low similarity of 95%.

Bacteria of in situ water at 10 m were represented mainly by I-Bottom-13, which consisted of nine clones with 99%similarity to the β-*proteobacteria Ralstonia pickettii* strain TRW, which is able to degrade vinyl chloride as a sole carbon source (Elango et al. 2006). OTU I-Bottom-49 consisted of eight clones similar to a bacteria isolated from arsenite-oxidizing biofilm (Salmassi et al. 2006). OTU I-Bottom-09 (five clones) was identified as a *Rhizobium* sp. (α-*proteobacteria*) which was isolated from mine tailings (Macur et al. 2001). Other OTUs of α-proteobacteria identified at 10 m were *Bradyrhizobium* sp. (I-Bottom-43), *Methylobacterium aminovorans* (I-Bottom-20) and *Methylobacterium organophilum* (I-Bottom-10). Other characteristic bacteria were shown by I-Bottom 42, which consisted of one clone and was related to *Pelobacter propionicus,* a δ-*proteobacteria,* with a similarity of 97%. *P. propionicus* has been suggested to be a sulfur reducing bacteria that is possibly able to oxidize methane simultaneously (Evers et al. 1993). A γ-proteobacteria, I-Bottom-48, consisting of two clones, existed at 10 m and was identified as *Yersinia aldovae* with a similarity of 99%. This is an enterobacteria occurring in various natural environments (Bottone et al. 2005).

In contrast to the in situ bacterial community occurring before incubation, the grazer elimination experiment provided different community profiles. The predominant bacteria at 2.2 m such as I-Top-50 (β-*proteobacteria*), I-Top-49 (γ-*proteobacteria*), I-Top-33 (β-*proteobacteria*) and I-Top-24 (γ-*proteobacteria*), declined and were replaced by several α-proteobacteria that appeared after the grazing elimination experiment. The appearing α-proteobacteria included *Paracoccus marcusii* (A-Top-06, four clones, 99% similarity),

*Methylobacterium organophilum* (A-Top-43, three clones, 99% similarity) and *Brevundimonas vesicularis* (A-Top-13, two clones, 98% similarity). The contribution of β-*proteobacteria* decreased through the incubation and were represented by *Ralstonia pickettii* (A-Top-46, eight clones, 99% similarity). It is interesting that they were found at 10 m but not at 2.2 m before the incubation. The *Actinobacteria* characteristic phylum appeared only after incubation at 2.2 m and was represented by *Propionibacterium acnes* (A-Top-39, eight clones, 99% similarity). The fourth largest contribution at the subclass was γ-proteobacteria, which was comprised of *Pseudomonas* spp. (A-Top-14, three clones, 99% similarity; A-Top-41, one clone, 99% similarity) and *Escherichia coli* (A-Top-47, one clone, 99% similarity). *Firmicutes* appeared after the incubation at 2.2 m as *Streptococcus mitis* (A-Top-37, two clones, 100% similarity) and *Staphylococcus* sp. (A-Top-04, two clones, 100% similarity). It is remarkable that none of the OTUs detected at 2.2 m after the incubation were found before the incubation at the same depth. In addition to this, three OTUs (A-Top-27, A-Top-43, A-Top-46) that appeared after the incubation at 2.2 m were found in situ at 10 m before the incubation.

The pattern of change in the bacterial community at 10 m was different to that at 2.2 m. More than half of the OTUs in the water after incubation at 10 m were α-*proteobacteria,* consisting of *Paracoccus* sp. (A-Bottom-01, 12 clones, 98% similarity), *Bradyrhizobium* sp. (A-Bottom-48, six clones, 100% similarity), *Methylobacterium organophilum* (A-Bottom-08, four clones, 99% similarity), *Brevundimonas* sp. (A-Bottom-10, two clones, 99% similarity) and *Methylobacterium hispanicum* (A-Bottom-08, four clones, 99% similarity). Most of the β-*proteobacteria* detected in the water before the incubation disappeared from the OTUs, but *Ralstonia* sp. (A-Bottom-31, two clones, 99% similarity) was found both before and after the incubation at 10 m. One of the remarkable findings provided by the incubation at 10 m was the appearance of *Hydrogenophaga* sp., a β-*proteobacteria* (A-Bottom-09, nine clones, 98% similarity), which was not found in the in situ well water before the incubation. The number of OTUs belonging to γ-proteobacteria decreased after incubation, expect for *Yersinia aldovae* (A-Bottom-37, one clone, 99% similarity), which was detected both before and after the incubation at 10 m.

## DISCUSSION

Grazing elimination in the in situ incubation resulted in a significant increase in the growth of bacteria both at the 2.2 m and 10 m depths of the well. Consistent with this we observed a high frequency of dividing cells (FDC) of over 8%, which exceeded the high number of 0.6 to 6% found in an algal bloom in the Baltic Sea (Hagstrom et al. 1979). The increases in cells observed in these systems are, therefore, presumed to be those that are normally eliminated by protisian grazers.

It is remarkable that at 10 m, where dissolved oxygen was 2.2 mg $l^{-1}$, the bacterial growth rate was large. The number of protozoa amounted to 1.9 x $10^5$ cells $ml^{-1}$, 1.6 x $10^5$ cells $ml^{-1}$ and 4.2 x$10^4$ cells $ml^{-1}$ at 2.2 m, 6 m and 10 m, respectively, while TDC was 1.6 x $10^6$ cells $ml^{-1}$, 5.8 x $10^5$ cells $ml^{-1}$ and 3.0 x$10^5$ cells $ml^{-1}$ in the respective corresponding layers (Figure 3). Thus, the ratio of the number of protozoa to the number of bacteria was almost 1:10 in the system studied. The abundance of protozoa in various aquatic systems, including marine systems, lakes, rivers and sediments, suggests that protozoa exist at about one-thousandth the number of bacteria (Berninger et al. 1991; Gasol and Vaque 1993). One of the possible reasons for the very high abundance of protozoa in the subsurface water obtained in this study could be the very high concentration of organic compounds measured as total organic carbon (TOC), which ranged from 28.2 mg $l^{-1}$ to 31.8 mg $l^{-1}$, almost ten-times higher than that of marine and lake systems (Wetzel 1983). The high concentration of TOC may indicate fragmental organic particles supplied from the surroundings, which can possibly be a food

source for protozoa. Another possible reason might be high grazing pressure rapidly depleting the number of free-living bacteria, as was suggested by Kinner et al. (1997) in their experimental study using a column in a contaminated aquifer.

It was confirmed that one-third of the community was comprised of several OTUs that appeared in both layers in situ. This suggests that the water in the well was mixed to some extent, but the bacterial diversity, as represented by the number of OTUs, was lower at 2.2 than at 10 m, where the dissolved oxygen concentration was as low as 0.2 mg $l^{-1}$. The grazer elimination experiment, however, brought about a higher number of OTUs at 2.2 m and a less diverse bacterial community at 10 m.

Comparison of the dominant bacteria before and after incubation and between the two layers leads to a somewhat complicated discussion. However, this provides knowledge of the subsurface bacterial distribution and their actual in situ activity. *Aquaspirillum delicatum*, a common soil bacterium (Pot and Gillis 2005), contributed almost one-third of the in situ bacterial community, but was not detected after the incubation. If it existed in association with soil particles, the possibility exists that it might have been eliminated from the sample by the filtration procedure using a 3.0 μm filter, as 33% of the total bacteria were lost though filtration. Various methane oxidizing bacteria found both at 2.2 m and 10 m showed a relatively wide vertical distribution, and this might suggest that they possess various adaptations to a range of oxygen concentrations. In addition, anaerobic iron-reducing and sulfur-reducing bacteria were found in the in situ water at 10 m.

The bacteria that were detected in abundance only after incubation suggests that these bacteria are normally maintained at low numbers by the selective grazing of protozoa. This may explain why the number of OTUs after incubation was larger than that of in situ water at 2.2 m.

Some of the bacteria found after incubation at 2.2 m (A-Top-27, A-Top-43, A-Top-46) were detected before incubation at 10 m. This suggests that they might have existed at 2.2 m before the incubation but were not detected, probably due to low abundance.

Some of the bacteria detected at 10 m (I-Bottom-13, I-bottom-49 and I-Bottom-09) were related to clones obtained from contaminated sites, but they did not grow through the incubation. In contrast, some of the bacteria such as *Propionibacterium acnes* (A-Top-39), which is an aerotolerant anaerobic (Cummins and Johnson 1986), and *Streptococcus mitis* (A-Top-37), which is a facultative anaerobic and chemoorganotroph with fermentative metabolism (Hardie 1986), grew through the incubation, probably exerting their aerotolerancy and facultative capability, respectively.

The relatively high clone number of *Methylobacterium organophilum* (A-Bottom-08) found at 10 m after incubation suggests that this bacterium utilizes oxygen as an electron accepter, but exhibits high activity under low oxygen conditions. Hydrogen-oxidizing bacteria (A-Bottom-09), which grow either chemolithoautotrophically or chemoorganotrophically (Willems and Gillis 2005) were also found after the incubation. Kotelnikova (2002) suggested that methanotrophs may produce hydrogen by using formate dehydrogenase as a source of reductant for biosynthesis of NADH. These methane-oxidizing and hydrogen-oxidizing bacteria which were found at 10 m after incubation might drive the anaerobic methane oxidizing process. Raghoebarsing et al. (2006) suggested the possibility that the anaerobic oxidation of methane by bacteria may be coupled with the denitrification of nitrate. After the incubation at 10 m we detected many clones of *Paracoccus* sp., which is able to reduce nitrate to nitrogen (van Spanning et al. 2005). This supports the possibility that metabolic coupling occurred in the incubation experiment. Various bacterial groups were detected at 10 m before the incubation, but only some of them exhibited growth after incubation. This indicates that although bacteria may occur widely in space, their activity may be sharply constrained and they may behave differently in different conditions, as clearly

shown by this subsurface system with a gradient crossing from oxic to anoxic condition.

The bacterial growth and grazing losses shown here suggest that bacterial growth may support protozoa in microaerobic conditions and that protozoan grazing of bacteria may control the detectable constituents of bacterial communities. Bacteria are transported to and dispersed through the subsurface environment by the permeation of rainfall and the flow of river, drainage and groundwater. Thus they occur widely in the subsurface environment. But as their activity and proliferation are constrained by the given conditions, the survival of bacteria needs to be tested carefully when considering in situ bacterial activity in subsurface environments.

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**Table 1** Sequence of probes used for FISH and Protozoa enumeration.

| Probe | Specificity | Sequence (5'-3') | Target site and location* | Source |
|---|---|---|---|---|
| EUB338 | *domain Bacteria* | GCTGCCTCCCGTAGGAGT | 16S rRNA, 338-355 | Amman et al. 1990 |
| ARCH915 | *domain Archaea* | GTGCTCCCCCGCCAATTCCT | 16S rRNA, 934-915 | Stahl and Amman 1992 |
| DEL (SRB) | δ-*proteobacteria* | CGGCGTCGCTGCGTCAGG | 16S rRNA, 385-402 | Amman et al. 1990 |
| Euk 1209 | domain Eukarya | GGGCATCACAGACCTG | 18S rRNA, 1431-1446 | Giovannoni et al. 1988 |
| Euk 502 | domain Eukarya | ACCAGACTTGCCCTCC | 18S rRNA, 502-516 | Amman et al. 1990 |
| Euk 309 | domain Eukarya | TCAGGCTCCCTCTCCGG | 18S rRNA, - | Sogin and Gunderson 1987 |

* Location refers to the location on the *Escherichia coli* rRNA sequence.

**Table 2** Sequence of 16S rDNA-targetted primers.

| Primer | Specificity | Sequence (5'-3') | Location* | Source |
|---|---|---|---|---|
| 27F | domain Bacteria | AGAGTTTGATCCTGGCTCAG | 8 - 27 | Lane 1991 |
| 1492R | Universal | CGCTACCTTGTTACGACTT | 1510 -1492 | Lane1991 |
| 341F | domain Bacteria | CCTACGGGAGGCAGCAGCAG | 341-357 | Muyzer et al. 1993 |

* Location refers to the location on the Escherichia coli rRNA sequence.

**Table 3** Characterization of the examined water by OTU abundance and dominant bacterial group

| | In situ | After incubation |
|---|---|---|
| 2.2m (Top) | OTU: less abundant (11)<br>β/γ-*proteobacteria* | OTU: abundant (16)<br>Several groups appeared without any predominance |
| 10m (Bottom) | OTU: abundant (18)<br>(Small in clone number of each OTU)<br>β-*proteobacteria* | OTU: less abundant (9)<br>α-*proteobacteria* |

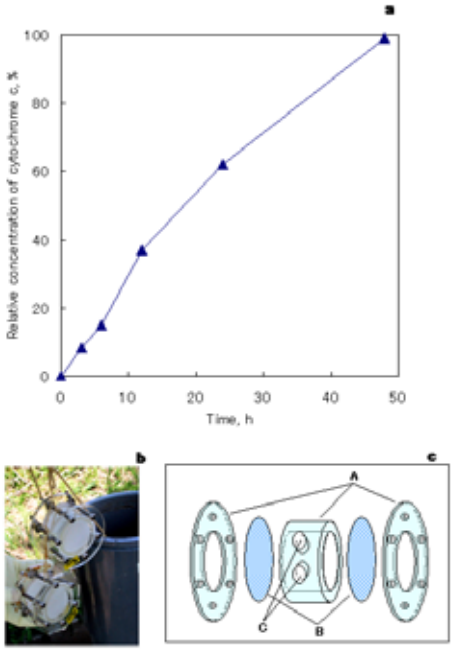


Fig. 1. Concentration of cytochrome c inside the diffusion chamber relative to the concentration outside the chamber (a). Photo of the diffusion chamber (b). Structure of the diffusion chamber (c), A: acrylic parts; B: 0.2 μm Nuclerporefilters; C: sampling holes.

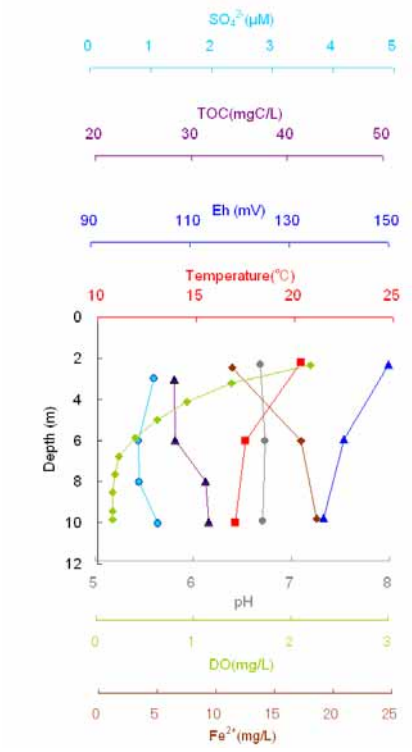


Fig. 2. Environmental profiles of #4-2 well water on August 19, 2003.

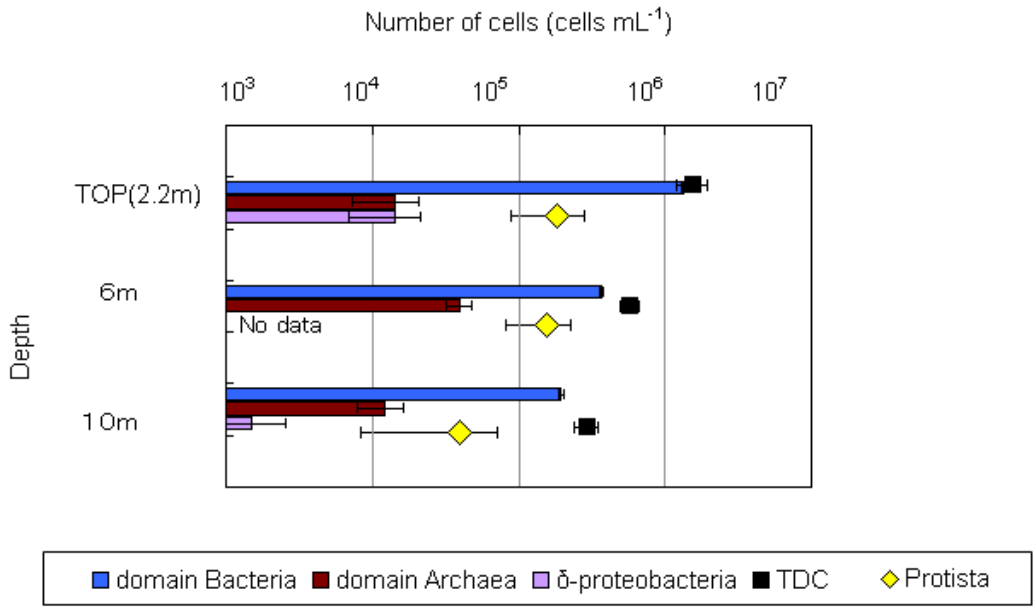


Fig. 3. Distribution of bacteria and Protista in well #4-2 on August 19, 2003.

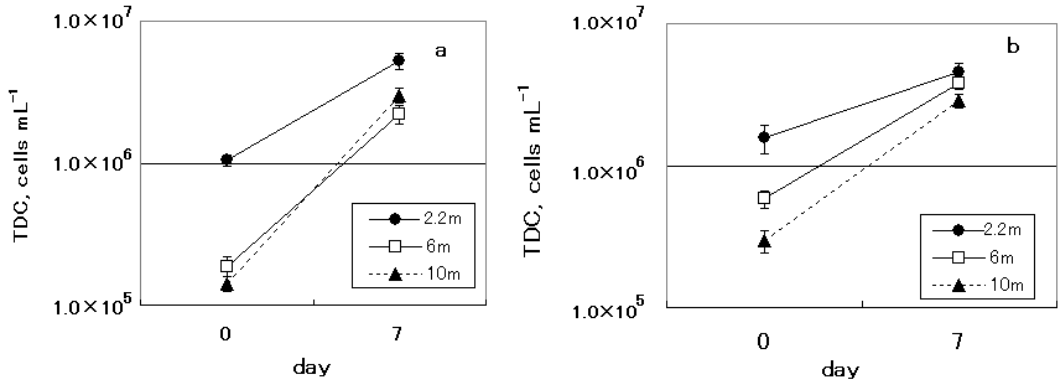


Fig. 4. Change in the total number of bacteria during in situ incubation of samples of 3 μm filtrate (a) and non-filtrate (b). The experiment was carried out from 19 to 25 August, 2003.

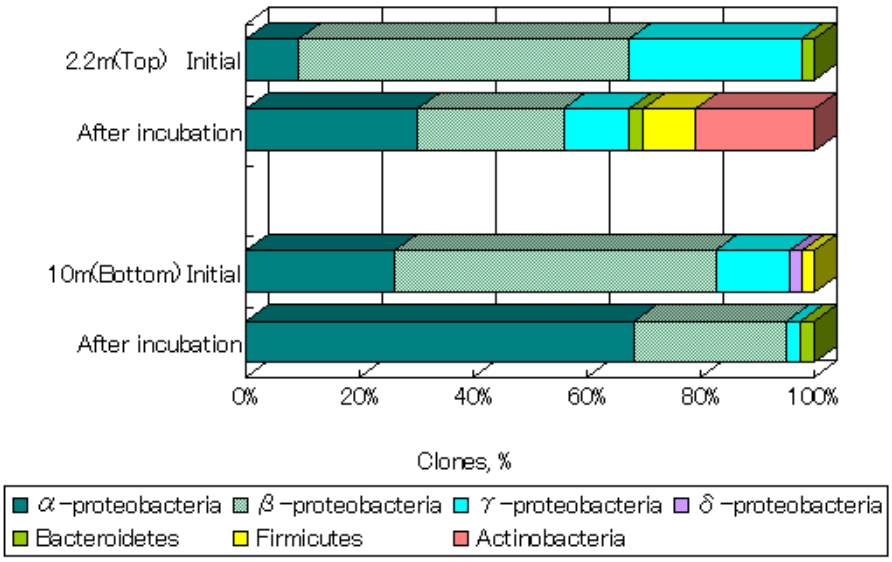


Fig. 5. Constituents of the bacterial community of water in well #4-2 as revealed by clone analysis. The in situ experiment was carried out from 19 to 25 August, 2003.

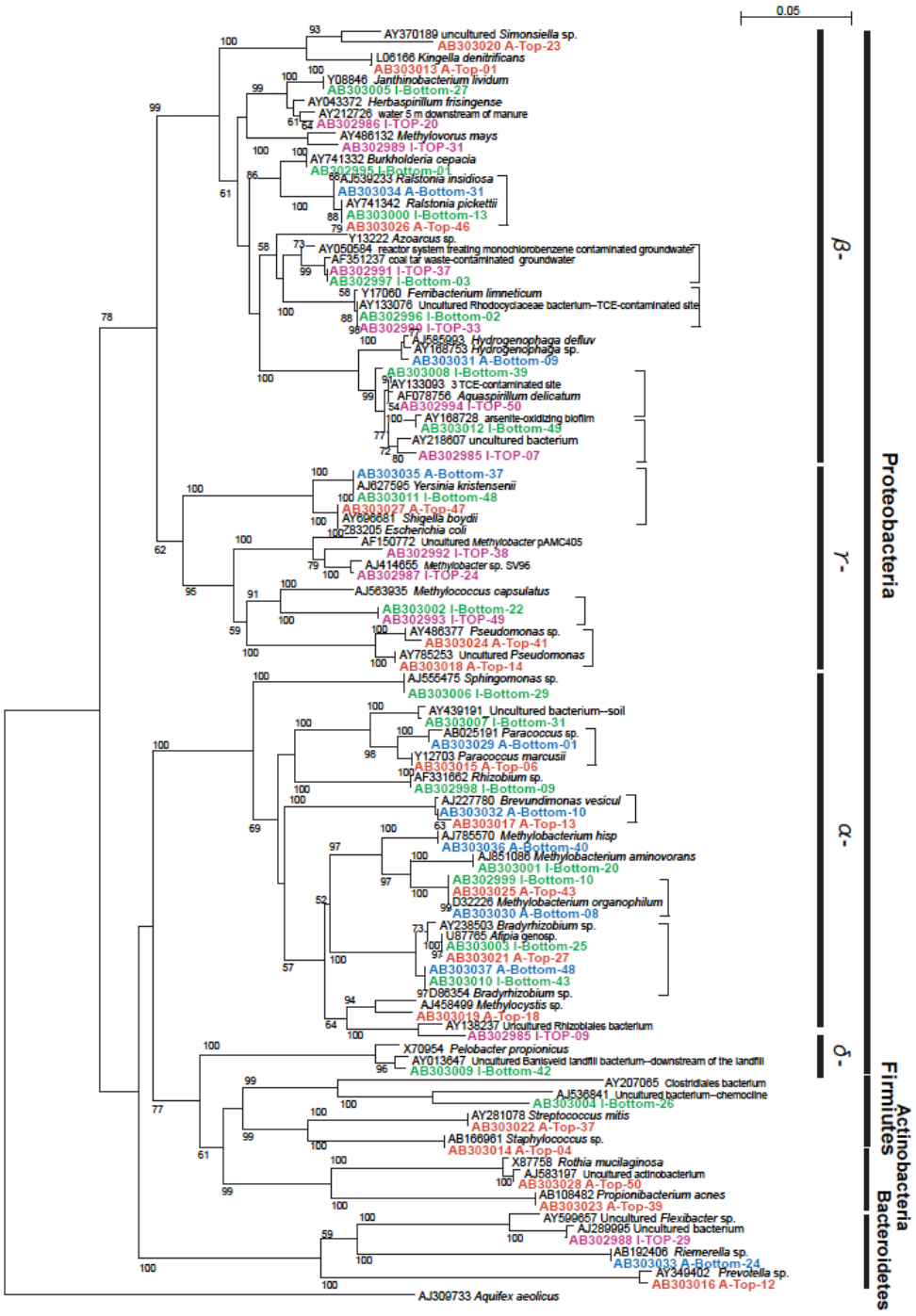


Fig. 6. Phylogenetic tree based on the 16S rRNA gene of the clones detected from the in situ incubation experiment carried out from 19 to 25 August, 2003 in well #4-2 indicating the phylogenetic relationships between the members of some lineages. Numbers at nodes refer to bootstrap values. Only bootstrap values above 50% are shown. The sequence of *Aquifex pyrophilus* was used as the outgroup to root the tree. Scale bar = 5% nucleotide substitution.