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A unique prokaryotic vertical distribution in the groundwaters of deep sedimentary geological settings in Hokkaido, Japan

Ayumi Sugiyama[1*], Tetsuo Ibara[1], Kazuyo Nagaosa[2], Atsunao Marui[3] and Kenji Kato[2]

Abstract

The purpose of this study is to clarify the vertical prokaryotic distribution in groundwater in a terrestrial subsurface sedimentary environment with a geological complex. Six groundwater samples were collected from a coastal 1200-m-deep borehole in which digging strata deposited between 2.3 and 1.5 Ma in Horonobe, Hokkaido, Japan. The studied succession was divided into three vertical zones that were geochemically differentiated according to their chloride contents and water-stable isotopes. The upper zone (UZ; shallower than 500 m) primarily contained fresh water supplied by penetrating meteoric water, the connate water zone (CWZ; deeper than 790 m) contained paleo-seawater, and the diffusion zone (DZ; 500–790 m depth) located between UZ and CWZ. Fluctuations in the prokaryotic density and constituents were observed across these three zones. The prokaryotic density decreased from UZ toward DZ, and the density of DZ was two orders of magnitude lower than that of UZ and CWZ. High prokaryotic activity was observed in CWZ below DZ. The upward expansion of prokaryotic distribution from CWZ, where high prokaryotic potential expressed by biomass can be maintained almost equivalent to that in the marine environment, probably occurred on a geological timescale from 80 ka to 1.3 Ma, as shown by the groundwater age of DZ. The DZ is a zone where the geochemistry has changed drastically owing to the mixing of penetrating meteoric water and the diffusion of deep paleo-seawater, preserving a unique subsurface environment. This chemically mixed zone might be considered as a buffering zone for prokaryotes to prevent the expansion of prokaryotic density and activity provided by diffusion and their in situ growth from both above and below the zones, which is expected to be maintained over a geological timescale. This zone is considered important for using subsurface space in the deep subsurface environment of the island arc.

Keywords Prokaryotic distribution, Diffusion zone, Meteoric water, Paleo-seawater, Sedimentary environment, Island arc, Prokaryotic buffering zone

1 Introduction

The deep subsurface represents the last territory in the search for life on Earth, and the findings of these studies have rewritten our understanding of the biosphere in this planet. Intensive research on microbial abundance and the possible activity of microbes in subsurface environments has only begun in the last few decades. Deep, dark, and oxygen-limited large subsurface environments have been explored in subseafloor habitats since the pioneering work of Parkes et al. (1989; 1994). Whitman et al. (1998) and Kallmeyer et al. (2012) reported a large

*Correspondence:
Ayumi Sugiyama
sugiyama-ay@atk-eng.jp
[1] Asano Taiseikiso Engineering Co., Ltd., 2-8-7 Kitaueno, Taitou-Ku, Tokyo 110-0014, Japan
[2] Department of Geosciences, Graduate School of Science, Shizuoka University, 836 Oya, Suruga-Ku, Shizuoka 422-8529, Japan
[3] Geological Survey of Japan, National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Higashi, Tsukuba 305-8567, Japan

abundance of microbes in oceanic sedimentary environments, whereas Inagaki et al. (2015) showed that the vertical distribution of microbes extended to a depth of over 2.5 km. Microbial metabolic activity supported by penetrating seawater has attracted the interest of researchers working in the field of biogeochemistry (e.g., D'Hondt et al. 2004). Unveiled large subsurface biosphere is crucial for understanding life in extreme environments (Inagaki et al. 2006; 2015; Lipp et al. 2008; Kawagucci et al. 2018).

In contrast to marine subsurface microbial studies, terrestrial studies were launched from the hard-rock geological environment in Scandinavia with the aim of revealing biogeochemical changes mainly caused by microbial function (Pedersen 1997; 1999). Studies have revealed that the total number of microbes in subsurface granitic environments ranges from 10^3 to 10^7 cells/mL in groundwater, in the presence of a hydrogen-driven deep biosphere in crystalline bedrock. Sulfate reducing microbes are also active in the subsurface environment. Subsequently, intensive deep subsurface microbial studies were conducted in Olkiluoto, Finland, with similar objectives (e.g., Itävaara et al. 2016; Miettinen et al. 2018). These studies revealed that the density of microbes decreases with depth and that sulfate reduction and methane oxidation are the main energy sources under steady-state natural conditions.

In a continental sedimentary environment, Chandler et al. (1997) found phylogenetically diverse microbial communities at the Hanford Site of North America. The environmental complexity resulting from water–rock interactions in the terrestrial subsurface environment affects the abundance of organic compounds and microbial distribution. The path of water through fractures and pores in rocks supports microbial activity (Fredrickson et al. 1997). Kato et al. (2009) found high microbial density and associated metabolic activity at the geological boundary between sedimentary units at a depth of approximately 300 m in Horonobe. The vertical microbial distribution in this sedimentary environment was notably different from the conventional thought that microbial density decreases linearly with log-depth (e.g., Kallmeyer et al. 2012; McMahon and Parnell 2014; Magnabosco et al. 2018).

To elucidate the vertical distribution of microbes in groundwater, the chemistry of which is affected by the geological complexity in a terrestrial sedimentary environment in the island arc, we collected groundwater samples from a borehole with a depth exceeding 1200 m, constructed in a coastal area of Hokkaido Island in Japan (Fig. 1). The study area is covered by the thick Yuchi Formation, which was deposited in a shallow marine environment and overlies the Sarabetsu Formation and alluvium (Ikawa et al. 2014). The examined groundwater was further characterized by an interesting mixing zone that extended from a depth of 500 to 790 m and was formed by the penetration and diffusion of meteoric water and paleo-seawater with an age of millions of years (Ikawa et al. 2014). The upper zone (UZ), 500 m below the ground surface (bgs), primarily contains fresh water supplied by penetrated meteoric water. In this zone, the chloride concentration was primarily below 3000 mg/L (Fig. 2c). In contrast, the lower zone at a depth > 790 m bgs consisted of a connate water zone (CWZ) of

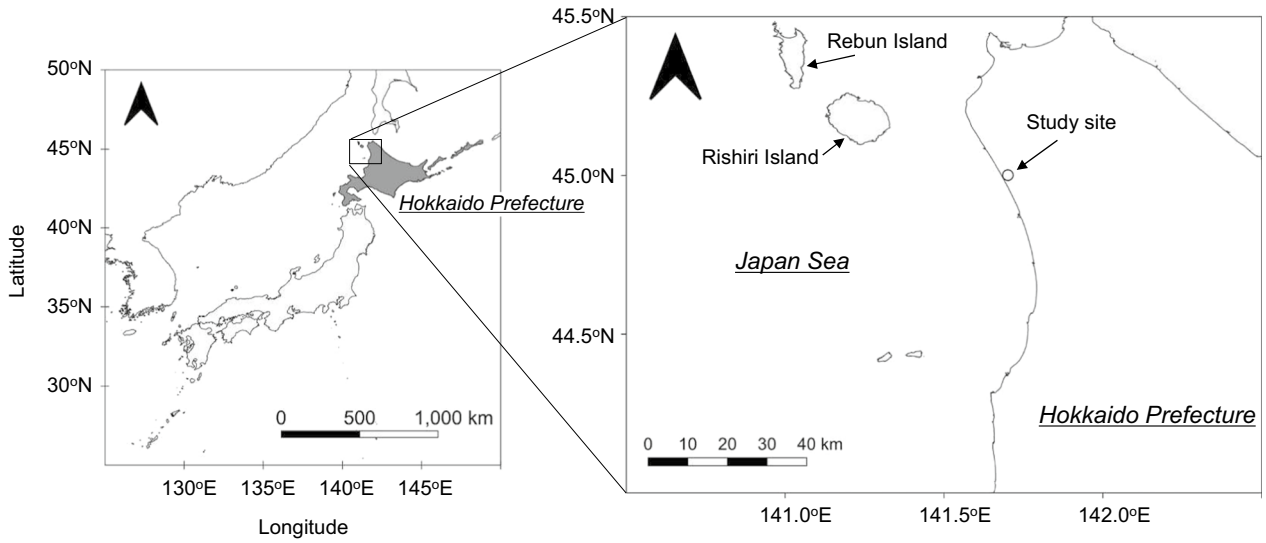

Fig. 1 Location of the study site

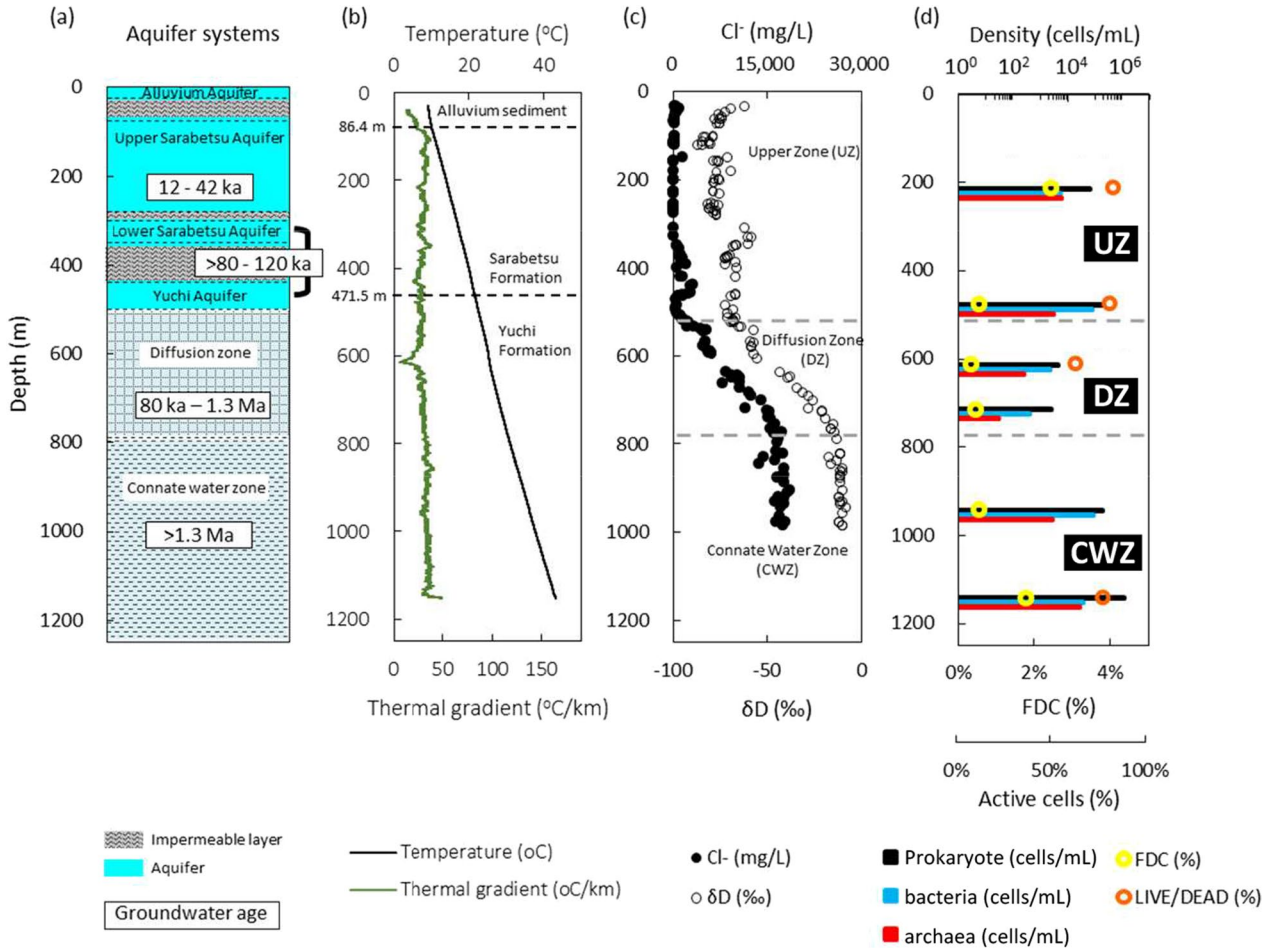

Fig. 2 Vertical geological, hydrological, and microbiological zonation of the studied borehole. **a** Geology with estimated aquifer systems based on Ikawa et al. (2014), **b** water temperature and geology, **c** chloride concentration and δD (based on Ikawa et al. 2014), **d** density of prokaryotes and FDC, relative density of bacteria and archaea estimated by FISH, and active cells detected by the LIVE/DEAD method

paleo-seawater with a high chloride concentration (maximum concentration of approximately 17,000 mg/L), which is almost equivalent to that of modern-day seawater (19,000 mg/L). The zone between these two zones at 500–790 m bgs, was defined as the diffusion zone (DZ) with an intermediate chloride concentration that differed from those of the other two zones. The DZ in the sedimentary geological setting formed over the geological timescale showed a drastic change in geochemistry.

Microbial life in this unique environment needs to be revealed not only from a physical aspect but also from the historical aspect of geology. To extend our understanding of microbial life in deep and dark subsurface habitats, it is necessary to demonstrate the microbial diversity and possible microbial activity in this complex environment. Compared to microbial studies of marine subsurface and terrestrial hard-rock environments, there are limited studies on microbial ecology in the sedimentary geological settings of the island arcs. Further understanding of microbial ecology in such diverse deep subsurface environments could provide basic information that not only enables the assessment of life under extreme environments but also enables the use of subsurface environments.

2 Results

2.1 Groundwater geochemical conditions

The groundwater temperature increased with depth from 9.3 to 43.2 °C at 1152 m bgs (Fig. 2b), with an average thermal gradient of 29.8 °C/km, ranging from 29.0 °C/km for the depth range between 100 and 580 m bgs to 33.0 °C/km for the 620–1152 m bgs depth range. All the examined groundwater samples exhibited a dissolved oxygen concentration of 0 mg/L.

At 214 m in UZ, the values of pH, electric conductivity (EC), and Eh were 7.94, 0.08 S/m, and − 228.3 mV, respectively (Additional file 3: Table S1). At 476 m in UZ, the EC and Eh values were markedly different from those at

214 m (2.68 S/m and − 55.0 mV, respectively); however, the pH value was not notably different between the two depths.

In DZ, at 613 m, the values of pH, EC, and Eh were 7.21, 2.91 S/m, and − 261.3 mV, respectively; at 715 m, the values were 7.45, 4.66 S/m, and − 341.1 mV, respectively. Thus, the groundwater at 715 m was more saline and less saline than that at 613 m.

At 943 and 1143 m in CWZ, the values of pH were 6.86 and 6.65, respectively, which were slightly lower than those of the other samples because of the dissolution equilibrium of hydrogen carbonate. EC was 5.79 S/m and Eh was − 289.1 mV at 943 m, and at 1143 m EC was 4.58 S/m and Eh was − 293.0 mV (Additional file 3: Table S1).

2.2 Vertical distribution of prokaryotes

The density and frequency of dividing cells (FDC) in prokaryotes were determined in the groundwater samples collected from six different depths (Fig. 2d; Additional file 3: Table S2). The density of prokaryotes in UZ was $6.71\times10^4\pm0.23\times10^4$ cells/mL at 214 m and $1.98\times10^5\pm0.16\times10^5$ cells/mL at 476 m. The sample collected from 214 m showed the highest FDC value (2.44%) among the examined samples. FDC, an indicator of the growth potential of a given population, was low in DZ and at 943 m. LIVE/DEAD staining, which was used to estimate the contribution of living cells to the entire prokaryotic density, was greater than 60% in all samples, with the highest value of 80–82% in UZ. This suggests that these prokaryotes have the potential to exert their beneficial effect.

The prokaryotic density sharply decreased in DZ by approximately one or two orders of magnitude relative to the values in UZ, being $4.75\times10^3\pm0.20\times10^3$ cells/mL at 613 m and $2.68\times10^3\pm0.03\times10^3$ cells/mL at 715 m.

The CWZ exhibited a high density of prokaryotes, with $1.87\times10^5\pm0.09\times10^5$ cells/mL at 943 m and $1.15\times10^6\pm0.06\times10^6$ cells/mL at 1143 m. FDC increased at 1143 m to 1.79%, from 0.56% at 943 m. The density of bacteria was as high as 9.1×10^4 cells/mL at 943 m and 3.8×10^4 cells/mL at 1143 m, and that of archaea increased from 2.9×10^3 cells/mL at 943 m to 2.9×10^4 cells/mL at 1143 m (Fig. 3). Although the estimates of *Methanomicrobiales* and *Methanobacteriales* by fluorescence in situ hybridization (FISH) contributed to the total prokaryotic cell counts of FISH small (0.79% and 0.63%, respectively, at 1143 m, Additional file 3: Table S2), these results suggest that these groups might be active because they were detectable by RNA staining.

2.3 Community constituents

Next-generation sequencing (NGS) retrieved between 535 and 1,553 operational taxonomic units (OTUs) belonged to 23–42 orders from the examined samples. *Clostridiales* (Nos. 31–43, Fig. 3, Additional file 3: Table S3), *Desulfuromonadales* (represented by No. 18), and *Pseudomonadales* (Nos. 7–11) were retrieved from every depth and comprised 52–84% of the total retrieved sequences. *Clostridiales*, *Desulfuromonadales*, and *Pseudomonadales* accounted for 11% (613 m) to 45% (943 m), 1% (1143 m) to 47% (715 m), and 2% (715 m) to 42% (613 m) of sequences, respectively, at the order level. However, the constituents of *Clostridiales* differed at the genus level at different depths.

The vertical distribution of taxa at the order level exhibited some variation; in DZ, where the density was low, *Pseudomonadales* was dominant at 613 m, whereas the dominant bacterial group at 715 m was *Desulfuromonadales*. These depths are clearly differentiated by the chloride concentration, from approximately 500 mg/L in the shallowest part (approximately 500 m) of DZ to approximately 16,800 mg/L at approximately 790 m (Ikawa et al. 2014).

2.3.1 Upper zone

The NGS data analyzed at the genus level are plotted in Fig. 3, and detailed data are provided in Additional file 3: Table S3. The order *Clostridiales*, comprising 22% of the total retrieved sequences at 214 m, was represented by *Acetobacterium*, some genera of *Eubacteriaceae*, and some genera of *Clostridium* (Nos. 38, 39, and 43 in Additional file 3: Table S3). *Acetobacterium* are strictly anaerobic bacteria with an optimal temperature range of 27–30 °C for mesophilic species, and 20–30 °C for psychrotolerant species. They oxidize H_2 by reducing CO_2 or fermentation (Simankova and Kotsyurbenko 2009). In addition, some constituents of *Desulfuromonadales* were retrieved from UZ, specifically, some genera of *Pelobacteraceae* (No. 18), which comprised 10% of the community. *Pseudomonas* (No. 8) comprised 8% of the retrieved sequences.

Some genera of *Lachnospiraceae* (No. 36; order *Clostridiales*) dominated the prokaryotic community at 476 m, accounting for 32% of the total retrieved sequences. After *Clostridiales*, some genera of *Pelobacteraceae* (No. 18) from the order *Desulfuromonadales* contributed 14% of the total sequences. In the order *Pseudomonadales*, *Pseudomonas*, *Acinetobacter*, and some genera of *Pseudomonadales* (Nos. 8, 10, and 11) were retrieved from 476 m and together accounted for 16% of the total. In addition to these major constituents, *Oleibacter* (order *Oceanospirillales*, No. 12) was retrieved from a depth of 476 m in the lower part of UZ.

For archaea, some genera of *Methanobacteriaceae* (No. 57) were retrieved from 214 m, accounting for 8% of

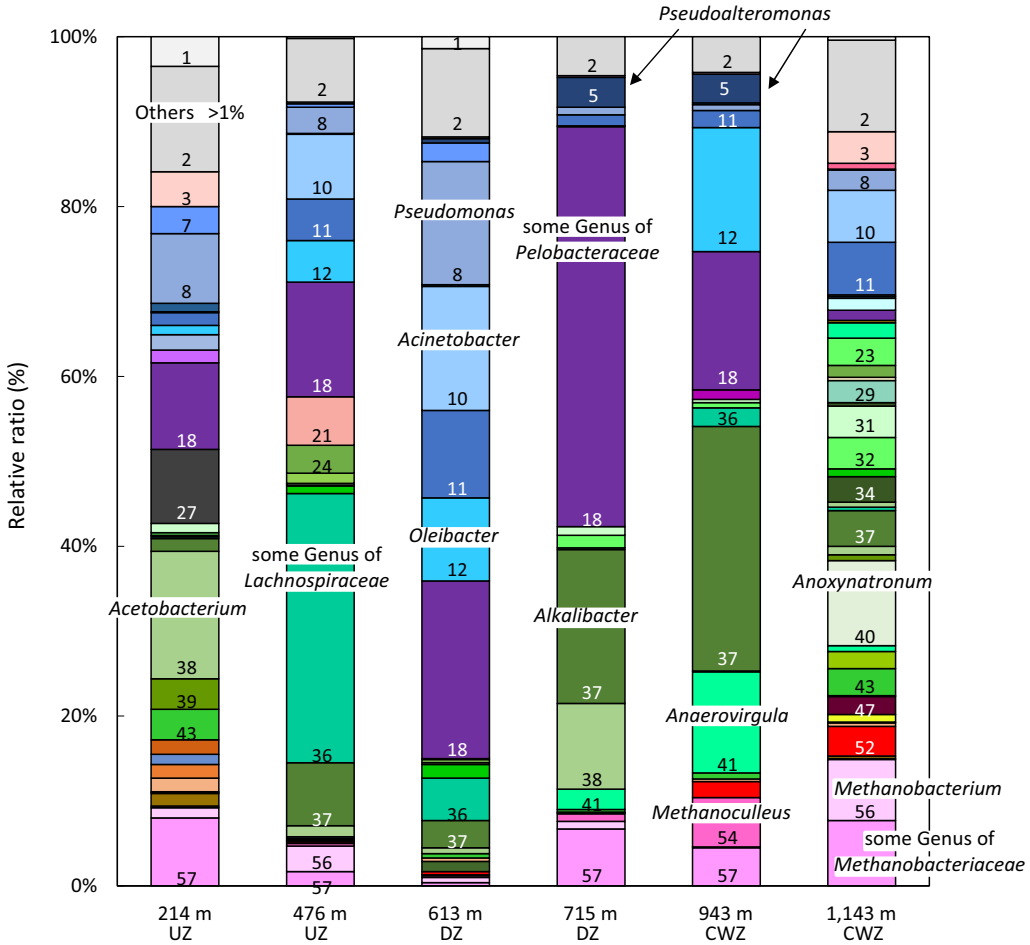

Fig. 3 Relative contribution of prokaryotic constituents revealed by NGS analysis at genus level. The abundances of each fraction and captions are provided in Additional file 3: Table S3

the total. At the depth of 476 m, the contribution to the entire community was 1.7%.

2.3.2 Diffusion zone

The community constituents of DZ, where the prokaryotic density was very low, markedly differed between 613 and 715 m, reflecting environmental changes along a sharp physicochemical gradient. The major constituents at 613 m were some genera of *Pelobacteraceae* (No. 18) of the order *Desulfuromonadales*, which contributed 22% of the total retrieved sequences. *Pseudomonas*, *Acinetobacter*, and some genera of *Pseudomonadales* (Nos. 8, 10, and 11) accounted for 39% of the total sequences. These bacteria also occurred at the depth of 476 m with a relative abundance of 16%. *Oleibacter* (*Oceanospirillales*, No. 12) contributed 10% of the total retrieved sequences at 613 m and 5% at 476 m. The lowest detected proportion of *Clostridiales*, comprising up to 11% of the total community, was found at 613 m, whereas this group was the major member of the community at 476 m.

Members of *Pelobacteraceae* (No. 18) comprised 47% of the total retrieved sequences at the depth of 715 m in the deeper part of DZ. These bacteria are strictly anaerobic, mesophilic, chemolithoheterotrophs or chemoorganotrophs (Kuever et al. 2005). *Alkalibacter*, *Acetobacterium*, and *Anaerovirgula* (Nos. 37, 38, and 41) were the major constituents of *Clostridiales* at 715 m, comprising 34% of the total retrieved sequences. *Alkalibacter* are strictly anaerobic, obligately alkaliphilic, and halotolerant bacteria with an optimal growth temperature of 35 °C (Garnova and Zhilina 2009a). *Pseudoalteromonas* (order *Vibrionales*, No. 5) was retrieved from depths of 715 and 943 m, but was not a major member of the assemblages at other depths (maximum 0.5% at 613 m). Another feature of the community at 715 m was the presence of archaea and members of the family *Methanobacteriaceae* (Nos.

56 and 57), whereas at 613 m, the contribution of these methanogens was very small.

2.3.3 Connate water zone

For the order *Clostridiales*, *Alkalibacter* and *Anaerovirgula* (Nos. 37 and 41) were retrieved from 943 m, with relative abundances of 29% and 12% of the total sequences, respectively. In addition to *Clostridiales*, some genera of *Pelobacteraceae*, *Oleibacter*, and *Pseudoalteromonas* (Nos. 18, 12, and 5) were retrieved from 943 m. Some methanogens, such as *Methanobacterium*, *Methanocorpusculum*, and *Methanoculleus* (Nos. 53–57), were detected at 943 m, accounting for 10% of the total retrieved sequences. A relatively high density of archaea compared to the overlying zones was also detected by FISH analysis (Fig. 2d).

The deepest sample obtained (1143 m depth) showed a clearly different community structure with a large diversity of prokaryotes. A typical feature of this sample was the presence of various genera of *Clostridiales*, such as *Soehngenia*, *Dethiosulfatibacter*, *Desulfitobacter*, *Alkalibacter*, *Anoxynatronum*, *Thermotalea*, and *Clostridium* (Nos. 31, 32, 34, 37, 40, 42, and 43), which are anaerobic, alkaliphilic, mesophilic, or thermophilic bacteria (Parshina and Stams 2009; Garnova and Zhilina 2009a; b). Among these genera, *Anoxynatronum* is capable of fermentative metabolism. For the order *Pseudomonadales*, *Pseudomonas*, *Acinetobacter*, and some genera of *Pseudomonadales* (Nos. 8, 10, and 11) were retrieved from 1143 m. Some genera of *Pelobacteraceae* (No. 18), which were the major constituents of DZ, contributed minimally to the total reads at 1143 m, relative to samples from higher and lower depths. The sample from 1143 m exhibited the largest proportion of methanogens among the examined groundwater samples, accounting for 15% of the total retrieved sequences.

3 Discussion

3.1 Vertically unique distribution of prokaryotes

Previous studies have reported that the density of prokaryotes in the subseafloor and terrestrial subsurface environments decreases almost linearly with log-depth from 10^8 to 10^3 cells for the examined sample volume at depths ranging from near the surface to over 3 km (Kallmeyer et al. 2012; McMahon and Parnell 2014; Parkes et al. 2014; Magnabosco et al. 2018). The vertical distribution of prokaryotes in the deep groundwater of the sedimentary geological setting was observed herein, wherein a low density was found at the intermediate depth named DZ, which contained paleo-seawater that had infiltrated upward from 790 to 500 m depth, while the density of prokaryotes increased again in the deeper zone at depths of 943 and 1143 m. The density of prokaryotes in DZ was of the order of 10^3 cells/mL, which was two orders of magnitude lower than that in UZ; however, it increased again to 10^5 cells/mL at 943 m in CWZ composed of paleo-seawater. This density of prokaryotes in CWZ demonstrates that the deep, old groundwater retained a biomass similar to that of the ocean surface environment (Murakami et al. 1999; Karner et al. 2001; Salka et al. 2008). The deeper sample collected at 1143 m exhibited a higher prokaryotic density and possible growth potential, as suggested by the FDC, than the sample collected at 943 m in the upper part of CWZ. This finding can be partly explained by the relatively high archaeal density detected by NGS and FISH at 1143 m. The groundwater temperature increased linearly from 9.3 °C at the surface to 42 °C at 1143 m. The prokaryotic distribution found in this terrestrial sedimentary geological setting differed from that reported for a hard-rock geological setting. This vertical prokaryotic profile has not been previously described and is unique.

The most interesting finding was the apparent low density of prokaryotes in DZ compared to the high density observed in CWZ. Thus, a low prokaryotic density was observed in DZ between UZ and CWZ. The density and growth potential suggested by the FDC of the prokaryotic communities in DZ were very low compared to those at shallower (476 m) and deeper (943 m) depths. The unique vertical profiles of prokaryotes may have resulted from differences in water chemistry with depth. The chloride concentration and stable isotope signatures indicate that the groundwater of UZ was supplied by meteoric water, whereas the deeper zone of CWZ, below 790 m, was filled with paleo-seawater with an age of approximately 1.3 Ma (Ikawa et al. 2014). The chloride concentration and δD changed markedly with the depth in DZ (Fig. 2c), thus suggesting a drastic vertical change in the chemical condition for prokaryotes caused by the mixing of meteoric water with paleo-seawater that had gradually diffused vertically over the geological time. The difference in prokaryotic constituents between the upper and deeper DZ may result from changes in chemical conditions, as discussed below. These major environmental changes resulted in the observed distribution of prokaryotes in DZ, which was different from that reported previously wherein a linear decrease in the density of prokaryotes was solely governed by an increase in temperature until more than 45 °C (e.g., Heuer et al. 2020; Dai et al. 2021) or a sharp decrease in the density occurred due to high salinity (Nisson et al. 2023). The age of the groundwater in DZ ranges from 80 ka to 1.3 Ma (Ikawa et al. 2014); thus, the mixing of meteoric and paleo-seawater restricted the distribution and density of prokaryotes over a long geological timeframe.

3.2 Characteristics of community constituents

To elucidate the drastic vertical changes in prokaryotic abundance in DZ, community similarity analysis at the genus level was conducted using the Jaccard index (Jaccard 1912) and nonmetric multidimensional scaling (NMDS) (Borcard et al. 2011). In the Jaccard analysis, the community similarity of the prokaryotic constituents of samples from 613 and 715 m depths in DZ was low. However, the similarities between those from 613 and 476 m depths and between those from 715 and 943 m depths were high (Additional file 3: Table S5). The NMDS analysis classified the examined prokaryotic communities into three different clusters: Cluster A, consisting of prokaryotic components at 214 and 476 m in UZ and at 613 m in the upper part of DZ; Cluster B, consisting of prokaryotes at 715 m in the lower part of DZ and at 943 m in the upper part of CWZ; and Cluster C, consisting solely of prokaryotes at 1143 m in CWZ (Fig. 4). The similarity of the examined prokaryotic community constituents in groundwater may reflect the responses of prokaryotes to environmental changes caused by the mixing of meteoric water and paleo-seawater. Details are provided as follows:

The major prokaryotic constituents of Cluster A at the order level were *Clostridiales*, *Desulfuromonadales*, and *Pseudomonadales*. These prokaryotes were also retrieved from seawater-based formations in the Minami-Kanto gas field (Ishii et al. 2019) and the deep groundwater of an accretionary prism in Japan (Matsushita et al. 2016). Genus-level analysis revealed that some genera of *Lachnospiraceae* (No. 36) dominated the order *Clostridiales* at 476 and 613 m. Some genera of *Pelobacteraceae* (No. 18) appeared at all depths in Cluster A with a similar

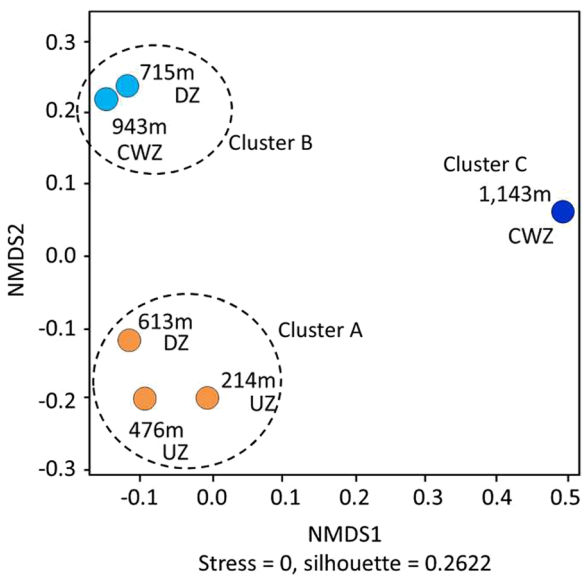

Fig. 4 Genus-level NMDS analysis of the prokaryotic community

contribution to the community. *Pseudomonas*, *Acinetobacter*, and some genera of *Pseudomonadales* (Nos. 8, 10, and 11) were the major constituents of the 613 m community and were also retrieved from 214 and 476 m. *Pseudomonas* grows aerobically in general, but in some cases, they can grow anaerobically using nitrate as an electron acceptor (Palleroni 2009). *Acinetobacter* is frequently present in soil and water environments (Juni 2005). *Oleibacter* (No. 12) was retrieved from both 476 and 613 m, as well as from 214 m, although its relative abundance was low at 214 m. They are mesophilic (Teramoto 2015) and an optimum growth temperature for the type species *O. marinus* are 25–30 °C (Teramoto et al. 2011). The small contribution of *Oleibacter* at 214 m probably resulted from low temperatures. DZ exhibited drastic changes in its chemistry, which were reflected by the differences in prokaryotic constituents between the two samples from the upper (613 m) and lower (715 m) parts of DZ. The prokaryotic constituents in the upper part of DZ resemble those in UZ.

Cluster B, in which *Alkalibacter* and some genera of *Pelobacteraceae* (Nos. 37 and 18) were the major prokaryotic constituents, was retrieved from depths of 715 and 943 m. Some genera of *Pelobacteraceae* were retrieved from every examined depth; however, their contributions were particularly high in the community of prokaryotes in Cluster B. *Anaerovirgula* and *Pseudoalteromonas* (Nos. 41 and 5), which are marine bacteria, were the characteristic constituents of Cluster B. *Anaerovirgula* is obligately anaerobic, alkaliphilic, and mesophilic, and an optimum growth temperature for the type species *A. multivorans* is 35 °C (Pikuta et al. 2006), which was slightly higher than the temperature at 715 m. *Pseudoalteromonas* is anaerobic and halophilic bacteria (Bowman and McMeekin 2005); this genus was not retrieved from UZ. The increasing temperature and salinity through the chemical diffusion zone formed with a geological time length in the depth interval of 715 to 943 m might provide appropriate conditions for the development of prokaryotic communities in Cluster B. Another characteristic of the components in Cluster B was reflected by the low values of the Chao 1, Shannon, and Simpson diversity indices (Additional file 3: Table S4). All indices were the lowest at 715 m, followed by those at 943 m. This indicates that these depths are in a transitional state from paleo-seawater to dilution by penetrating meteoric water.

Cluster C consisted solely of prokaryotes at 1143 m in CWZ. The Shannon and Simpson indices of the 1143 m depth sample were the highest among the examined samples (Additional file 3: Table S4). The major prokaryotes in Cluster C belonged to the order *Clostridiales* (Nos. 31, 32, 34, 37, 40, 42, and 43). In addition, the relative contribution of methanogens, represented by

Methanobacteriaceae (Nos. 57 and 56), which utilize H_2/CO_2 or formic acid (e.g., Maestrojuán et al. 1990; Skerman et al. 1980), was as high as 15% of the total sequences. These prokaryotes are characterized by the deep groundwater of an accretionary prism and various hot springs retrieved from depths greater than 1 km (Matsushita et al. 2020; Sugiyama et al. 2021a).

Geochemical analysis showed that more than 90% of the dissolved gases at 1143 m were methane, which was shown to be biogenic based on its stable carbon isotope value (Agency for Natural Resources and Energy 2018). The presence of methanogens has been demonstrated not only by DNA analysis, but also by RNA detection through FISH analysis (Amann et al. 1995). These findings suggest that methanogenic activity may have occurred in the paleo-seawater environment. Substrates for methanogenesis, namely H_2/CO_2 or formic acid, can be produced by *Clostridiales*. The number of reads retrieved from NGS suggested a high correlation between methanogens and *Clostridiales* (*t*-test; t [10] = 0.833, not significant). Thus, a syntrophic consortium of H_2-producing fermentative bacteria and H_2-utilizing methanogenic archaea may be present for an accretionary prism, as inferred by Matsushita et al. (2020). The abundance of methanogens estimated from the NGS decreased upward from CWZ, became very low at 613 m depth in DZ, and increase again upward. Some genera of *Methanobacteriaceae*, such as *Methanobacterium* (No. 56), which can perform hydrogenotrophic methanogenesis, have been detected in deep marine sediments (Shlimon et al. 2004; Ishii et al. 2019) and terrestrial surface or subsurface environments (Zhu et al. 2011; Nyyssönen et al. 2014; Sugiyama et al. 2021a). The reason for the decrease in the relative abundance of methanogens from 476 to 613 m depth through the geologically changing environment from the sedimentary boundary of the Sarabestu and Yuchi formations at 471.5 m to the chemical diffusion zone of approximately 500 to 790 m is not yet fully understood.

3.3 Microbiological studies in extension of utility for subsurface environment

Environmental and social needs have developed the focus of studies on deep subsurface environments, from searching for natural resources to the utilization of space for carbon dioxide capture and storage and radioactive waste (Pedersen 1999; Morozova et al. 2011; Momper et al. 2017). Global warming and climate change concerns have triggered global efforts to reduce atmospheric carbon dioxide, and carbon dioxide capture and storage. One of the major strategies for reducing atmospheric carbon dioxide is to embed it into subsurface space (Leung et al. 2014; Kumar et al. 2018). In addition, constructing a high-level radioactive waste repository for geological disposal is one of the most urgent requirements for utilizing geological space.

Microbes present in reduced subsurface environments pose potential risks associated with geochemical instability (Parkes et al. 2014; Miettinen et al. 2018). Regarding the function of microbes in the subsurface environment in relation to the subsurface space, the following possible microbial effects should be considered: (1) methanogenesis and H_2-producing metabolic processes resulting in the release of explosive gases; (2) metabolic activity that changes the environmental redox potential, leading to enhanced metal corrosion (Yoshikawa et al. 2009; Kato et al. 2020); and (3) geochemical reactions and migration of embedded chemical species (Utsunomiya et al. 2020). Elucidating the potential activities of methanogenesis and H_2-producing processes is important subjects among them, because explosive gases are released into the environment. Possibility remains that the geological structure plays a role as a cap rock to trap the methane and hydrogen gases produced below (Caesar et al. 2019; Drake et al. 2019). Thus, the biogeochemical effect of the subsurface environment is governed by the strength of microbial activity (Whitman et al. 1998; Parkes et al. 2014). However, it is necessary to clarify the in situ concentration of related chemical compounds, such as hydrogen, CO_2, and methane to estimate the activity of methanogenesis, as the analysis of microbial DNA and RNA does not show this activity.

Previous studies have confirmed that sedimentary geological settings promote the abundance and growth of a diverse range of microbial species compared to hard-rock systems (Parkes et al. 2014; Inagaki et al. 2015). In the present study, we discovered a zone of reduced microbial activity, i.e., DZ, where environmental conditions changed drastically; this zone was located above a deeper zone with higher microbial activity provided by paleo-seawater. The DZ exhibited a marked fluctuation in the vertical distribution of microbes, which was associated with differences in community constituents. The upward expansion in the distribution of microbes from CWZ, where the high microbial potential can be maintained equivalent to that in the marine environment in the examined paleo-seawater, probably occurred through a geological timescale shown by the groundwater age of DZ from 80 ka to 1.3 Ma (Ikawa et al. 2014). The DZ is characterized by drastic changes in geochemistry because of the mixing of penetrating meteoric water and the diffusion of deep paleo-seawater, preserving a unique subsurface environment over a long period of time. This zone mitigates the high microbial activity in the deeper zone with high temperatures in the presence of anoxic paleo-seawater, which reduces the possible risk of microbial hydrogen and methane gas production.

Therefore, this zone could be regarded as a buffering zone for prokaryotes, preventing the expansion of their density and activity in deep subsurface sedimentary environments. The findings from this zone indicate a unique vertical prokaryotic profile in a terrestrial sedimentary geological setting. We suggest that this zone is important for planning the future use of subsurface environments in the island arcs.

4 Conclusions

We found a unique prokaryotic vertical distribution in groundwater at intermediate depths of meteoric water and paleo-seawater from 500 to 790 m, named the diffusion zone, DZ, in a sedimentary geological setting in Hokkaido, Japan. The examined groundwater was obtained from a borehole with a depth exceeding 1200 m, which was constructed as part of a pre-experimental study of the sedimentary terrestrial subsurface environment for radioactive waste treatment.

The density of DZ prokaryotes was of the order of 10^3 cells/mL, which was two orders of magnitude lower than that of the upper zone, UZ. The density increased again to 10^5 cells/mL at 943 m in the connate water zone, CWZ, composed of paleo-seawater > 1.3 Ma. The observed unique vertical profile of prokaryotes is suggested to result from the differences in water chemistry and physics with depth.

The prokaryotic community constituents in the upper and lower DZ differed, indicating a significant change in the groundwater environment. However, the estimated community constituent of the upper part of DZ was similar to that of UZ and that of the lower part of DZ was not different from that of CWZ. This suggests that community constituents remained in the major profiles of the upper and lower zones. Although prokaryotic density was suppressed, the major community constituents maintained their abundance within a given condition.

Unlike the understanding of prokaryotes in hard-rock environments, the vertical distribution of prokaryotes in the groundwater constrained by both meteoric water and paleo-seawater is not simple. These findings led us to hypothesize that the DZ could act as a buffering zone for prokaryotic density and activity over a geological timescale. Sedimentary microbiology is expected to be more complex than that of hard-rock systems. Thus, we need to expand our understanding of the microbial ecology in sedimentary geological environments for the future use of subsurface environments.

5 Methods/experimental

5.1 Study site

The examined groundwater was sampled from an observation well (DD-1) drilled from 5.2 m above sea level to 1205 m bgs in a sedimentary geological setting in Horonobe, Hokkaido, Japan. The well is located 300 m from the coastline. The details of DD-1 are described by Ikawa et al. (2014) based on HRISE (2005). The location of the study site and its geology are shown in Figs. 1 and 2b, respectively. The geological formations encountered in this study are the Yuchi, the Sarabetsu, and Alluvium formations. The Yuchi Formation is estimated to be approximately 1100-m thick, and consists mainly of fine sand, clay, and silt, indicating that the unit was deposited in a shallow marine environment. The formation was deposited at 2.3 Ma, and is overlain by the Sarabetsu Formation (1.5 Ma and younger). In well DD-1, the boundary between the Yuchi and the Sarabetsu formations is located at 471.5 m bgs. The geological succession is topped by alluvium from 86.4 m bgs upward. Ikawa et al. (2014) divided the studied aquifer down to 1004 m into several systems-Alluvium Aquifer, Upper Sarabetsu Aquifer, Lower Sarabetsu Aquifer, Yuchi Aquifer, DZ, and CWZ-based on differences in the geological setting inferred from measurements of chloride concentration and δD of pore water. The age of each aquifer was estimated from δD and $\delta^{18}O$ values of groundwater to be 12–42 ka for the Upper Sarabetsu Aquifer, > 80–120 ka for the Lower Sarabetsu Aquifer and Yuchi Aquifer, 80 ka to 1.3 Ma for DZ, and > 1.3 Ma for CWZ (Fig. 2a).

5.2 Sample collection and field measurements

We examined groundwater samples obtained from the three zones of borehole DD-1 between February 2017 and December 2018: 214 m and 476 m, in UZ, 613 m and 715 m, in DZ, and 943 m and 1143 m, in CWZ. Each groundwater sample was collected by using a packer system (Asano Taiseikiso Engineering Co., Ltd., Tokyo, Japan) and sterilized bottles. The sampling procedures and microbial analytical processes are summarized in the flow chart of Sugiyama et al. (2021b). The microbial analysis methods are described in the following section. The individual screen depths and environmental parameters measured in situ are listed in Additional file 3: Table S1. The examined water sample at a depth of 214 m was obtained from the Sarabetsu Formation and at depths of those at 613, 715, 943, and 1143 m from the Yuchi Formation. A water sample at 476 m was taken from the boundary between the Sarabetsu and Yuchi formations.

Temperature logging was performed to identify aquifers between 30 and 1152 m (Fig. 2b). Preliminary drainage was performed prior to groundwater sampling to confirm the pH and EC stability and attenuation of Amino G acid (7-amino-1,3-naphthalene disulfonic acid mono potassium salt; $C_{10}H_8KNO_6S_2$) added during drilling to ensure the in situ water quality of the studied depth.

Environmental parameters, such as water temperature, pH, EC, and oxidation–reduction potential (ORP), were measured in situ by using Ocean Seven 303 (IDRONAUT S.r.l., Italy). The ORP values were transformed into Eh (STD) values (H conversion) by using Eq. (1):

$$\begin{aligned}\text{Eh (STD)[mV]} =&(\text{Eh[mV]} + 206)\\ &- \left(0.7 \times \left(\text{Temperature[°C]} - 25\right)\right)\ldots\end{aligned} \quad (1)$$

Dissolved oxygen was measured by using Ocean Seven 303 for ground-pumped water. Amino G acid concentration was measured using a spectrophotometer (FP-8000, JASCO Corp., Japan).

5.3 Microbial analysis

The microbial communities were elucidated though cell counts, LIVE/DEAD staining, FISH to evaluate specific population dynamics. In addition, DNA analysis was performed to elucidate the community constituents using NGS of the 16S rRNA gene. Microbial DNA has been shown to suggest groundwater flow paths based on differences in the physiological characteristics of the retrieved DNA, which resulted from the environmental distribution of microbes (Segawa et al. 2015; Sugiyama et al. 2018; Schilling et al. 2023).

5.3.1 Microscopic analysis

The density of prokaryotes and the community structure were analyzed by total direct count (TDC) and FISH, following Porter and Feig (1980) and DeLong (1992), respectively, with some modifications (Kato et al. 2009; Segawa et al. 2015; Sugiyama et al. 2018). If the sampled water contained Fe^{2+}, ethylenediaminetetraacetic acid was immediately added to the sample at a final concentration of ~ 6 mM to minimize the formation of Fe_2O_3 aggregates, which interfered with the microscopic observations (Nagaosa et al. 2008). The concentration of Fe^{2+} was determined using the ferrozine method (Viollier et al. 2000). The sampled water was immediately subsampled and fixed with neutralized formaldehyde (final concentration 2%) for TDC. The subsamples for FISH were fixed with paraformaldehyde (final concentration 3%) and collected by the filtration on a nuclepore filter (0.2 μm pore size; GE Healthcare UK Ltd.). The water samples for TDC were stored at 4 °C and the filtered samples for FISH were stored at − 20 °C until analysis.

The subsample of groundwater for TDC was collected by the filtration of 1–100 mL on a nuclepore filter (0.2 μm pore size). Cells were stained with 4′,6-diamidino-2-phenylindole (DAPI, Nacalai Tesque Inc., Kyoto, Japan; final concentration, 0.01 μg/mL) and more than 20 microscopic fields, and totally more than 300 prokaryotic cells were counted under an epifluorescence microscope (BX51-FLA; Olympus Corp., Tokyo, Japan) (Kato et al. 2009). Dividing cells were counted to determine the FDC, which indicates the growth potential of a given population (Newell and Christian 1981). Counting by microscopy was performed in triplicate, and standard deviations were obtained. Hybridization was performed by using the bacteria-specific EUB338 probe or the archaea-specific ARC915 probe and non-ARC915 probe (listed in Additional file 3: Table S6). *Methanomicrobiales* and *Methanobacteriales* were analyzed in the sample at 1143 m by using probes MG1200b and MB311, respectively. The ratio of bacteria to archaea was counted and calculated based on images captured using an epifluorescence microscope (BX51-FLA) equipped with a digital camera (DP71, Olympus) (Additional file 2: Fig. S2).

To estimate the living cell ratio, LIVE/DEAD staining was performed using a LIVE/DEAD Bac Light Viability Kit consisting of two fluorescent nucleic acid stains: SYTO9 (green) and propidium iodide (PI; red) (Auty et al. 2001). SYTO9 permeates both viable and non-viable cell membranes, whereas PI only permeates damaged cell membranes, which in turn negates the SYTO9 fluorescence. Viable prokaryotic cells fluoresce in green, and those with a damaged or non-viable membranes fluoresce in red. A subsample of groundwater amount was collected on a nuclepore filter (0.2 μm pore size). The cells was stained with SYTO9 (final concentration 0.5 μM) and PI (3 μM), and the numbers of cells were counted under an epifluorescence microscope (BX51-FLA). In this study, the ratio of cells with viable prokaryotic cell membranes (defined here as living cells) among all cells was calculated.

5.3.2 DNA extraction

The sampled groundwater was filtered with a 0.22-μm Sterivex-GV filter (EMD Millipore) to collect more than 10^8 cells. Bulk DNA was extracted as described previously (Somerville et al. 1989). Prokaryotic cells were lysed using a solution of lysozyme and proteinase K in Sterivex-GV. Bulk DNA was extracted using phenol. As some substances originating in the geological environment interfere with the PCR reaction, bulk DNA was purified using a PowerClean Pro DNA Clean-Up Kit (MO BIO) and subsequently extracted using a one-step PCR inhibitor removal kit (Zymo Research). The concentrations of the extracted DNA were determined using a Qubit and dsDNA HS Assay Kit (Thermo Fisher Scientific).

5.3.3 16S rRNA gene sequencing

The 16S rRNA genes were amplified using 2-step tailed PCR by a primer coded V3-V4 regions 341f/805r primer set. A negative control that did not contain the template DNA was used to confirm that there was no contamination in the PCR. The PCR conditions are shown in Additional file 1: Fig. S1. Sample libraries for sequencing were prepared according to the Synergy H1 and QuantiFluor dsDNA System, and checked using a Fragment Analyzer and dsDNA 915 Reagent Kit (Advanced Analytical Technologies). NGS was run by MiSeq. OTUs were clustered using QIIME at a 97% similarity level. Chimeric sequences were detected using the Qiime. Individual OTUs were assigned based on representative sequences from the GreenGenes database. The accession number is DRA012929.

5.3.4 Statistical analysis

The Chao 1, Shannon, and Simpson indices were calculated according to Chao (1984), Shannon (1948), and Simpson (1949), respectively. Community similarity between the two samples was estimated using the Jaccard index (Jaccard 1912), which converts the assemblage data to presence/absence. The NMDS analysis was used to evaluate the relative similarity among the prokaryotic communities (Borcard et al. 2011). NMDS analysis was applied to use the similarity index directly because the process of normality and linearity obtained by principal components analysis is incongruous for analyzing biological communities. Cluster analysis was performed using k-medoids (Gülagiz and Sahin 2017), which are more robust than k-means (Borcard et al. 2011).

Abbreviations

TDC	Total direct count
FDC	Frequency of dividing cells
FISH	Fluorescence in situ hybridization
NGS	Next-generation sequencing
OTUs	Operational taxonomic units
NMDS	Nonmetric multidimensional scaling

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40645-023-00604-9.

Additional file 1: Fig. S1. Details of the reaction mixture and PCR conditions.

Additional file 2: Fig. S2. FISH image of groundwater sample at 476 m. Yellow arrows indicate hybridized cells (living cells) and white arrows indicate not hybridized cells (not living cells).

Additional file 3: Table S1. Observed in situ environmental parameters at the six sample depths. **Table S2.** Results of microbial analysis for the six studied depths. **Table S3.** Genus-level taxa and their relative ratios (%) in the prokaryotic community obtained by NGS analysis. **Table S4.** Results of NGS and calculated diversity indices. **Table S5.** Results of the community similarity analysis using the Jaccard index. **Table S6.** Oligonucleotide probes used in this study for target groups.

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Author contributions

AS proposed the topic, conceived, and designed the study. AS, IT, KN, and KK joined sample collection. IT performed groundwater sampling. KN conducted the experimental study and analyzed the data. AM and KK collaborated with the corresponding author (AS) in the interpretation of the data and construction of manuscript. All the authors have read and approved the final version of the manuscript.

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Availability of data and material

The datasets supporting the conclusions of this article are included within the article and its additional files.

Declarations

Competing interests

The authors declare that they have no competing interest.

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