Microbial DNA; A brand-new tracer of groundwater flow

[1,2]Ayumi Sugiyama, [3]Kenji Kato, [3]Kazuyo Nagaosa, [1]Tetsuo Ibara, [1]Kazuyoshi Takenobu,
[2]Maki Tsujimura and [4]Atsunao Marui

[1]Asano Taiseikiso Engineering Co., Ltd.,
2-8-7, Kitaueno, Taitou-ku, 110-0014, Tokyo, Japan,
[2]Faculty of Life and Environmental Sciences, University of Tsukuba
[3]Department of Geoscience, Faculty of Sciences, Shizuoka University
[4]Geological Survey of Japan, National Institute of Advanced Industrial Science and Technology (AIST)

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Abstract
To understanding better how the groundwater flow passes and its influence on the water chemistry we employ a brand-new method to analyze microbes with abundance and community constituents transported by groundwater. In the previous studies, estimation of transit time and groundwater flow path by groundwater flow analysis, estimation of averaged residence time of groundwater by chemical analysis such as various radioactive elements and stable isotopes have been widely used in hydrological studies. These methods show an averaged value of chemistry of the investigated water which was blended by various water with different sources and flow passes in subsurface environment. In order to improve the reliability of the estimation of the transport of groundwater, we focus on microbes in particular Microbial DNA. Particles represent the place from which they were transported. Transported microbes express the environment being appropriate for their growth. Interaction among rocks - groundwater - microorganisms is studied to evaluate biogeochemical influences on natural barrier of geological disposal facility for high-level-radioactive wastes. This study shows the possibility to reveal the groundwater history such as groundwater flow and the environment from which groundwater flow through analyzing the density of prokaryotes and microbial community structure analysis.

1. Introduction

In order to evaluate the long-term performance of natural barrier, it is also necessary to evaluate the stability of it on the order of a thousand years or more. Long-term assessment of geological environment has been studied such as long-term stability of geological environment (earthquake/fault, volcano/magmatism activity, uplift/erosion and topography variation) and geological environmental characteristics (geology, rocks and groundwater). In this study, we try to understand geological condition, groundwater condition and physicochemical properties through groundwater. We focus on groundwater influence on geological environment.

In order to estimate the groundwater flow paths, groundwater flow simulation and chemical tracer approach have been carried out (e.g., Kazemi et al., 2006; Tosaka et al., 2010; Hasegawa et al., 2013; Klaus and McDonnell, 2013). Although groundwater flow simulation estimates the travel time of groundwater and groundwater flow paths, some areas are difficult to adapt it. For example, the place, which heterogeneous groundwater flow is dominated or obtaining the physical information such as geology is not easy. The mean residence time of groundwater was estimated by analysis of chemical components in groundwater such as various radioactive isotopes and stable isotopes (Hasegawa et al., 2013). In particular, the ancient groundwater age (more than million years) is accurately measured by ^{4}He, ^{14}C and ^{36}Cl (e.g., Kasemi et al., 2006; Munnich, 1957; Bentley et al., 1986). However, these methods lead just an averaged value of investigated groundwater. And, the abovementioned results are different from each other because they are analyzing different substance. Although studies of relationship among rocks-groundwater-microbes are under way as an assessment of geochemical and microbial influences in natural barriers (e.g., Nagaoka et al., 2009), the method of microbial DNA for the estimation of groundwater flow paths is novel. We propose the microbial DNA as a new tracer for grasping the groundwater flow paths. Table 1 summarizes the characteristics of different methods including the microbial method proposed in this paper for clarify the groundwater flow system.

Table 1. Comparison of three different approaches to clarify groundwater flow system.

Methods/Approachs	Tracers/Softwares	Obtained informations	Trait	Refferences
Groundwater flow simulation	GETFLOWS, MODFLOW, FEFLOW etc.	Travel time Groundwater flow path	・It's inappropriate in areas where heterogeneous groundwater flow is dominant ・It's not easy to adapt it in areas where physical information such as geology is difficult to obtain	Kasemi et al. (2006) Tosaka et al. (2010)
Chemical tracer approach	Stable/radioactive isotopes, inorganic ions, CFCs, SF_6 etc.	Mean residence time	・Different tracer has different information ・It shows averaged value of chemistry of the sampled water	Hasegawa et al. (2013) Klaus and McDonnell (2013)
Microbial tracer approach	Microbes	Biogeochemical influences on natural barrier	・We focus on interaction among rocks-groundwater-microorganisms	Nagaoka et al. (2009)
	Viruses	Short mean residence time Preferential flow paths	・We focus on anthropogenic pollution	Hunt et al. (2014)
	Microbial DNA	**Groundwater flow history**	**・It expresses the environment appropriate for their growth**	**This study**

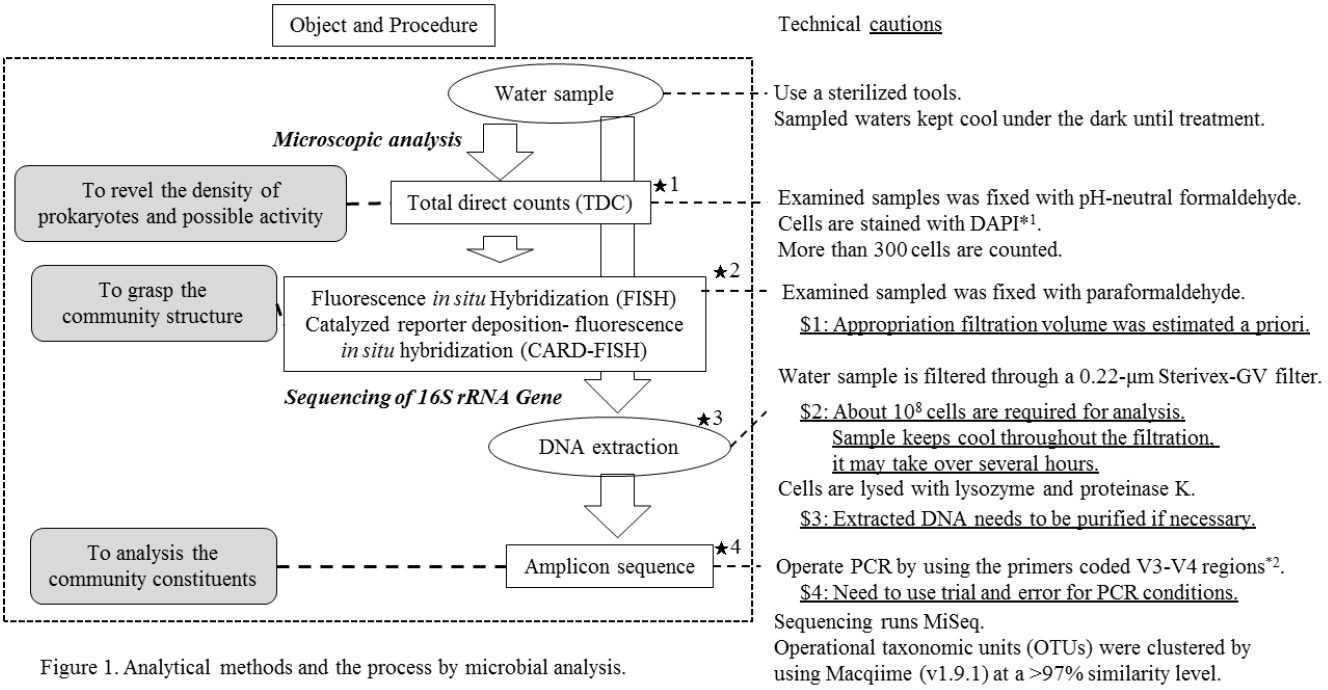

Figure 1. Analytical methods and the process by microbial analysis.

*1 DAPI; 4',6-diamidino-2- phenylindole
*2 V3-V4 regions; Pro341F (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN BGC ASC AG-3') / Pro805R (5'-GTC TCG TGG GCT CGG AGA TGT 10 GTA TAA GAG ACA GGA CTA CNV GGG TAT CTA ATC C-3') primer set.
★1: Porter and Feig, 1980 ★2: DeLong et al., 1989; Amman et al., 1990; Pernthaler et al., 2002; Teire et al., 2004; Stahl and Amann, 1991
★3: Kimura et al., 2007; Somerville et al., 1989 ★4: Claesson et al., 2010; Klindword et al., 2012; Takahashi et al., 2014

Advantages of employing microbial DNA for the groundwater flow system studies are shown in the following,

1) It is possible to estimate the 'State of the place' such as physicochemical condition by microbial community structure analysis.
2) It is possible to suggest the existence of a specific groundwater flow path.
3) It is possible to estimate with high reproducibility to use the different kind of tracers.

Therefore, in this paper, we introduce a case study of estimating the groundwater flow paths by applying the microbial DNA analysis in groundwater.

2. Methods

Analytical methods and the process are shown in Figure 1, and the cautions of the analytical techniques are also shown. Details of the method of microbial analyses are given in Sugiyama et al. (submitted to Biogeosciences).

Water samples were collected from boreholes and spring water. After counting total direct counts (TDC), microbial community was identified by using fluorescence *in situ* hybridization (FISH) and Next-generation sequencing.

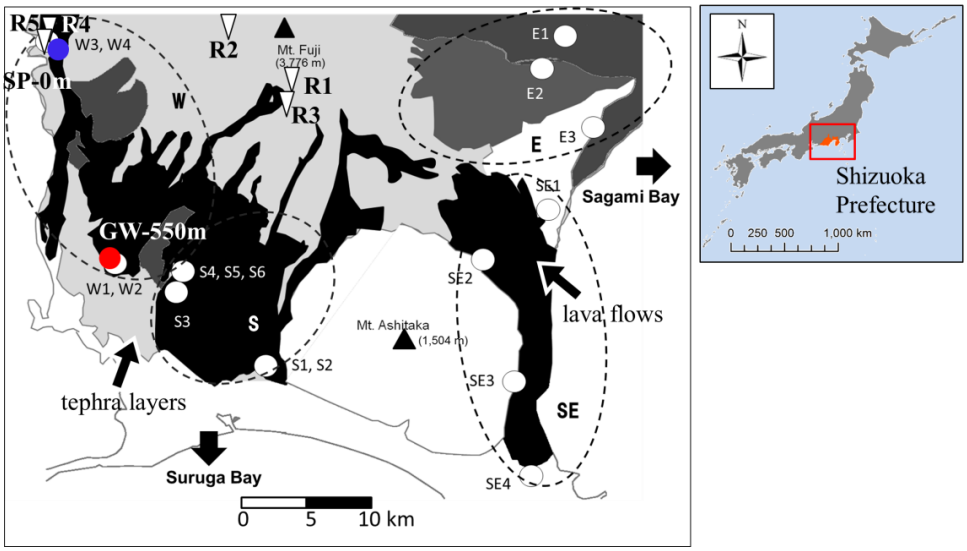

Figure 2. Geological map of Mt. Fuji (Segawa et al., 2015 and Sugiyama et al., 2017).
We collected the samples, which are rainwater (R1 to R5) and groundwater (SP-0m and GW-550m). SP-0m is spring water and GW-550m is well water with deep groundwater (the depth is 550 m).

3. Results and Discussion

3.1. Case of Mt. Fuji

3.1.1. Background

A huge amount of groundwater is stored in subsurface environment of Mt. Fuji (3,776 m a.s.l.), which is the largest volcanic mountain in Japan (Figure 2). The groundwater characteristics are described as follows (Sugiyama et al., 2017).

1. Temperature of groundwater ranges from 10 to 15 ^{o}C in the foot of Mt. Fuji.
2. Almost saturated dissolved oxygen was included in groundwater.
3. Major reservoir is in the lava where the height is about 100~200m.
4. The density of prokaryotes was very low (10^3 cells/mL), although the density of prokaryotes of rainwater and soil were 10^4 to 10^5 and 10^8 to 10^9 cells/mL, respectively.
5. Microbial components of groundwater were different from that of rainwater.
6. Assuming piston flow transport an apparent residence time was estimated to about 30 years by ^{36}Cl/Cl ratio (Tosaki et al., 2011).

3.1.2. Suggestion of the depth information of groundwater

Finding thermophilic bacterial DNA (e.g., *Allobaculum stercoricanis*) in spring water, whose temperature was as low as 10 to 15^{o}C throughout the year, suggested at least some of the groundwater source was expected at a depth of 600 m or greater, based on a temperature gradient of 4^{o}C/100 m, because thermophilic prokaryotes are optimally adapted to temperatures > 40^{o}C. This depth is far below the lava layer that was taken to be a substantial pool of groundwater. Thus, analysis of microbial DNA can suggested the depth information of groundwater (Segawa et al., 2015).

3.1.3. Possibility to reveal the groundwater flow paths

Multiple analyses (stable isotope analysis, chemical analysis and microbial analysis) performed to elucidate the groundwater flow paths under the heavy rainfall for groundwater (SP-0m and GW-550m; details were shown in Figure 2).

The oxygen isotopic ratio of spring water (SP-0m) was increased and silica concentration was decreased during just a couple of weeks after the heavy rainfall exceeding 300 mm/event. The density of *Bacteria* also sharply increased. This suggests that heavy rainfall promotes shallow subsurface flow contributing to the discharge in addition to the groundwater in the deep aquifer.

On the other hand, although deep groundwater at GW-550m did not show the signatures, which are oxygen isotopic ratio and silica concentration, density of *Archaea* was increased. Due to the density of *Archaea* increased with depth in both terrestrial subsurface environments (Kato et al., 2009) and marine (Karner et al., 2001), this suggests that strengthened piston flow resulting from the heavy rain transported archaeal particles from the deep geologic layer along the groundwater flow.

Intensive observation suggests the presence of short time-scale flow pattern (contribution of shallow groundwater flow) and long time-scale flow pattern (contribution of deep groundwater flow) rather than mean residence time, which can help us to reveal the groundwater flow paths. Though chemical analysis of groundwater shows an averaged value of the investigated water which was blended by various water with different sources and routes in subsurface environment, microbial DNA analysis may suggest the place where they are originated, which may give information of the source and flow paths of the investigated water.

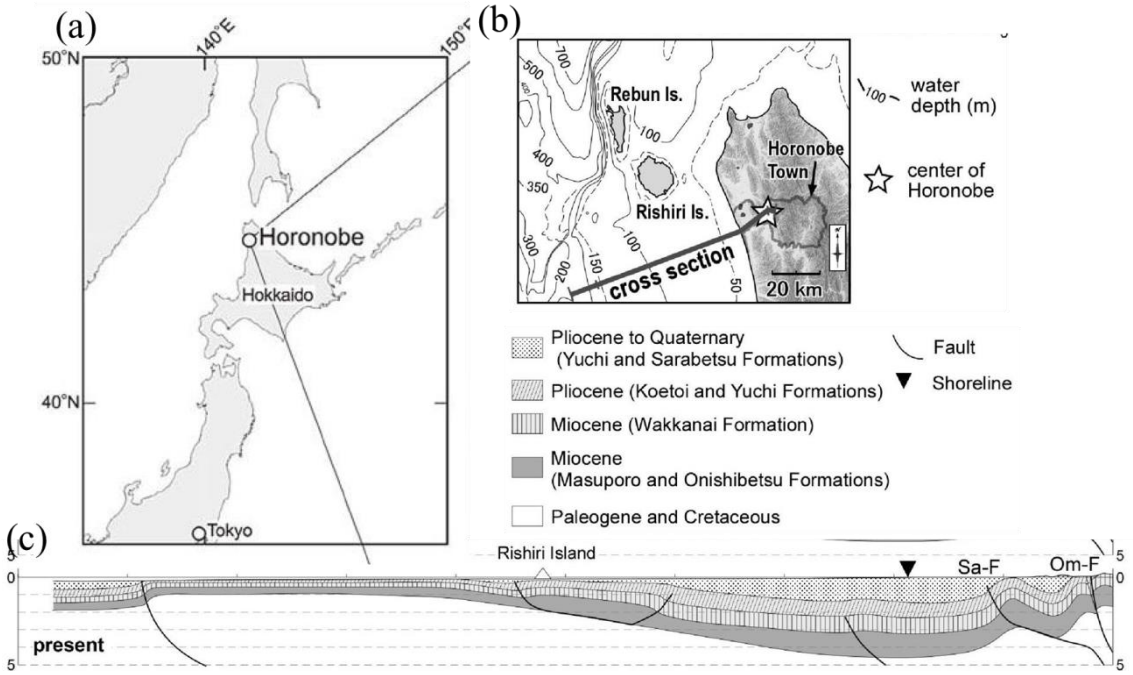

Figure 3. Location of Horonobe area in Hokkaido (Iwatsuki et al., 2009 [a)]; Niizato et al., 2007 [b) and c)]).

Table 2. Density of prokaryotes in the three geological formations (*Kato et al., 2009).

Site (borehole)	Formation	Sampling depth (m)	TDC (cells/mL)	FDC (%)	Bacteria (%)	Archaea (%)
DD-1	Yuchi	714.5-715.5	$2.68x10^3$	0.46	14.9	1.1
DD-1	Yuchi	942.5-943.5	$1.87x10^5$	0.56	48.8	1.5
H17*	Koetoi	35.5-37.5	$2.57x10^6$	0.55	7.51	2.6
HDB-6*	Koetoi-Wakkanai	281-312	$2.20x10^5$	5.63	61.9	3.5
HDB-6*	Wakkanai	364-409	$4.61x10^4$	1.08	-	-
HDB-4*	Minor fault	224-233	$8.36x10^5$	0.83	14.0	10.3
HDB-4*	Minor fault	281-291	$5.06x10^6$	1.82	6.41	10.9
HDB-4*	fault	475-482	$1.13x10^6$	0.45	12.8	19.1

Total Direct Count: TDC, Frequency of Dividing Cells: FDC.

3.2. Case of Horonobe

3.2.1. Background

The Horonobe coastal area, which is located along northwestern coast of Hokkaido, is an alluvial plain. It is a sedimentary layer that formed from the latter half of the Paleogeneity to the present. Sedimentary rocks and Quaternary strata are distributed. Sedimentary rocks are formed including Soya coal-bearing Formation, Onishibetsu Formation, Masuporo Formation, Wakkanai Formation and Koetoi Formation. Quaternary strata are formed including Yuchi Formation, Sarabetsu Formation, terrace deposits and alluvium deposits.

Kato et al. (2009) performed analysis of microbes in groundwater collected by boreholes drilled into sedimentary rock within two formations (Wakkanai Formation and Koetoi Formation). The density of prokaryotes was between 4.61 x 10^4 and 5.06 x 10^6 cells/mL, which is similar to the numbers observed at the marine subsurface.

3.2.2. Distribution of prokaryotes in deep groundwater

In this study, we performed microbial analysis of deep groundwater taken from different depth (Yuchi Formation at 943m and 715m) in order to understand the investigated groundwater environment and perform the scientific discussions from different viewpoint based on previous studies.

Table 2 shows density of prokaryotes in three geological formations and fault. Although density of prokaryotes at 943m depth ($1.87x10^5$ cells/mL) was not high as a sedimentary environment, which is similar to the numbers published in Kato et al. (2009), density at 715m depth was low ($2.68x10^3$ cells/mL) as a sedimentary environment. Sample of 943m depth in comparison to that of 715m depth shows high number of prokaryotes and high ratio of *Bacteria* (48.8%) next to Koetoi-Wakkanai bounder (61.9%). The vertical distribution of the prokaryotes did not show a simple decrease in abundance with increasing depth.

Characteristics of detected major genus level prokaryotes are absolutely anaerobic or anaerobic bacteria accounted for the majority. The characteristics are mesophilic or thermophilic, halophilic or halotolerant and alkalophilic bacteria. We interpreted these prokaryotes live in the seawater environment which is adaptive and inhabitable in that environment. *Methanoculleus* and some of *Methanobacteriaceae*, which produce methane under the absolutely anaerobic environment is detected. This suggests the metabolism of deep subsurface environment.

It is important to clarify the presence of microorganisms in the given subsurface environment because cells with significant activity are observed in the sample, which promotes a better understanding of the current environment.

4. Conclusions

Summary of this study are shown in Table 3. Though chemical analysis of groundwater shows an averaged value of the investigated water which was blended by various water with different sources and routes in subsurface environment, microbial DNA analysis may suggest the place where they are originated, which may give information of the source and flow paths of the investigated water. It is important to clarify the presence of microorganisms in the given subsurface environment because cells with significant activity are observed in the sample, which promotes a better understanding of the current environment. However, it is necessary to consider some analytical techniques according to each environment, and further accumulation of data is necessary in the future.

The distribution of prokaryotes and their possible functions are clearly constrained by the geological properties of deep terrestrial subsurface environments. Consequently, given the potential influence of microbial activity on the geochemistry of a given geological setting, particularly within the area of influence of radioactive waste disposal candidate site, it is necessary to consider the microbial community composition and the geological attributes of the sites at which they are the most prevalent.

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Table 3. Summary of interpreted of groundwater flow paths.

Study area	Comment on the methods spesific on the site	Obtained information spesific on the site
Mt. Fuji	・(Due to the low biomass,) A lot of sample are needed for filteration.	・Analysis of microbial community constituents suggests the depth information of groundwater. ・Intensive observation suggests the presence of short and long time-scale flow pattern rather than mean residence time.
Horonobe	・Filtration was hindered by many obstacles. ・A lot of sample and time are needed for filteration. ・Extracted DNA needs to be purified.	・The vertical distribution of the prokaryotes does not show a simple decrease in abundance with increasing depth. ・Analysis of microbial community constituents suggests seawater mixture and deep subsurface environmental metabolism.

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