# Preliminary Microbial Analyses of Groundwater in Heronobu Underground Research Laboratory, Hokkaido, Japan



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# Preliminary Microbial Analyses of Groundwater in Horonobe Underground Research Laboratory, Hokkaido, Japan

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**Abstract - Groundwater obtained from the Tertiary mudstones at the Horonobe Underground Research Center has been investigated for chemical and microbiological characteristics. Existence of microorganisms was ascertained by enumerating the total numbers of bacteria by epifluorescent microscopy using an acridine-orange direct-count technique. Enumeration of viable microorganisms was determined for nine bacteria indicating a reducing environment deep underground.**

## I. Introduction

As part of a series of research conducted to ascertain the existence and extent of microbiological activity deep underground, this study attempts to gain a more comprehensive perspective by supplementing microbiological examination with chemical and isotopic analyses.

Japan Nuclear Cycle Development Institute (JNC) is developing an underground research laboratory (URL) project for Neogene sedimentary rock in Horonobe, Hokkaido. The project was commenced in March 2001 to conduct systematic research and development in the actual deep underground in order to better assess the deep underground environment and to build confidence in the technical feasibility of the geological disposal. During fiscal 2001, a surface-based regional geological investigation was performed in various areas in Horonobe assumed to have sufficient thickness of the host sedimentary formation at about 500m depth. The investigation included heli-borne survey (electromagnetic, magnetic and natural radioactivity), ground geophysical survey (electromagnetic survey), borehole investigation (two boreholes of 720m each) and geological investigation.

## II. Study site and sampling

Horonobe town is located in the north-western part of Hokkaido, about 60km south of Wakkanai. The research area is located in the Teshio sedimentary basin of the Neogene period. Sedimentary rock formations from the mid Miocene to the Pleistocene periods known as the Soya coal bearing formation, Masuporo, Wakkani, Koetoi, Yuchi, and Sarobetu formations overlie on the bedrock dating from the Cretaceous and Paleogene periods.

Groundwater samples were taken from the two boreholes HDB-1 and HDB-2 which were drilled vertically for 720m from the surface. Sampling of HDB-1 was performed in section 548.00 to 563.18m which was sealed off by double packers. Groundwater of HDB-2, however, was sampled in section 344.90 to 404.90m which was sealed off by a single packer when the drilling campaign was stopped at the depth of 404.90. A hydraulic test equipment developed by Japan Nuclear Cycle Development Institute was applied for groundwater sampling. Because the hydraulic conductivity was very small, air lift method was applied instead of continuous pumping up of groundwater.

## III. Method

Microbial analyses were performed by conventional culture method. Total numbers of bacteria was enumerated by an acridine-orange direct-count technique using a epifluorescent microscopy [1].

The count for viable microorganisms was taken for the following nine types: heterotroph, anaerobic heterotroph, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, iron-oxidizing bacteria (*Thiobacillus ferrooxidans*), nitrate-reducing bacteria、denitrifying bacteria, sulfate reducing bacteria and methane-producing bacteria.

The viable count for heterotroph was observed on the culture medium of Nutrient-Agar (manufactured by Difco), using the agar plate culture method. For the viable count of anaerobic heterotroph, the gas pack method [2] was employed on the culture medium of Nutrient-Agar (Difco). The culture was maintained at 20 degrees for two weeks after which period the colony of bacteria appearing in the agar was taken count.

Viable count of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria was enumerated on the culture medium developed by [2], using the most probable number (MPN) technique. The culture temperature was set at 20 degrees over a period of 60 days.

Viable count of iron-oxidizing bacteria (*Thiobacillus ferrooxidans*) was enumerated on the culture medium developed by, using the MPN technique. The culture was set at 30 degrees. The period was set for 30 days, and the growth was checked by any change in the color of the culture medium upon the fifth, tenth, fifteenth and twentieth day.

Viable count of nitrate-reducing bacteria and denitrifying

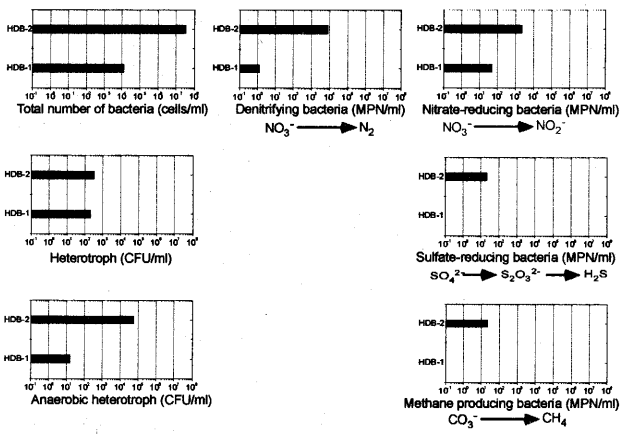


Fig.1: Total number of bacteria and viable counts of microorganisms. CFU is colony-forming units. MPN is most probable number.

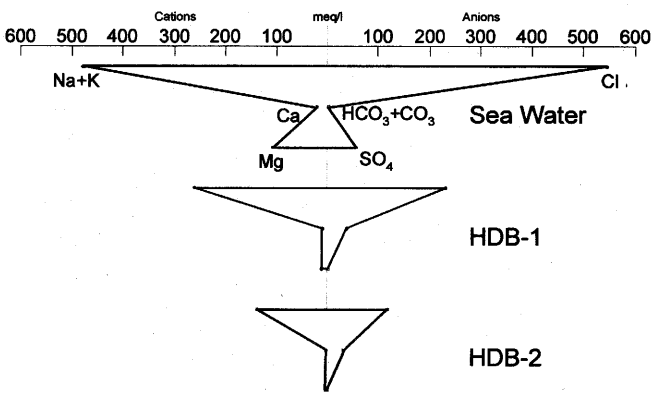


Fig.2 Hexa-diagram of groundwater of HDB-1 and HDB-2 with comparison to present sea water. The amount of $Na^+$ and $Cl^-$ is about 1/2 to 1/3 of present sea water.

bacteria was enumerated on the culture medium developed by [5] using the MPN technique. The culture temperature was set at 30 degree over a period of 10 to 14 days. Viable count of nitrate-reducing bacteria and denitrifying bacteria was calculated by assessing the number of test-tubes showing a change in color from a shade of green to dark blue of BTB. Nitrate-reducing bacteria was found in those test-tubes with the culture medium turning into dark blue. If nitrogen gas was released at the same time as changing color, the existence of denitrifying bacteria was confirmed [5].

Viable count of sulfate reducing bacteria was enumerated on an altered culture medium of Postgate developed by using the MPN technique. The culture temperature was set at 30 degrees period of 14 days.

Viable count of methane-producing bacteria was enumerated on the culture medium developed by [5], using the MPN technique. The culture was set at 25, and the growth was checked upon the 30th, 45th, 60th and 80th day. The growth was ascertained by the generation of methane gas, using gas chromatography.

## IV. Result

1) Total number of bacteria

The results of total number of bacteria were 1.3×10E4 cells/ml for HDB-1 and 3.4×10E7 cells/ml for HDB-2, respectively.

2) Enumeration of viable microorganisms

2-1 HDB-1:

Heterotroph was found to be dominant、with a viable count of 2.1×10E2 CFU/ml. Viable count of anaerobic heterotroph was 1.5×10E1 CFU/ml. Viable counts of Nitrate-reducing bacteria and denitrifying bacteria are 4.9×10 MPN/ml and 1.3 MPN/ml, respectively. Overall, the dominance of heterotrophic and reducing bacteria characterized HDB-1 and no presence of autotroph such as ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, iron-oxidizing bacteria (*Thiobacillus ferrooxidans*) was detected. The lack of sulfate reducing bacteria and methane-producing bacteria also leads to the assumption that the environment is not conductive to reaction of sulfate reduction and generation of methane.

2-2 HDB-2

Viable counts for heterotroph and anaerobic heterotroph were measured at 3.2×10E3CFU/ml and 5.8×10E4 CFU/ml, respectively. As for viable counts of other bacteria, the measurements were 2.2×10E3 MPN/ml for Nitrate-reducing bacteria、7.9×10E3 MPN/ml for denitrifying bacteria, 2.2×10E1 MPN/ml for sulfate reducing bacteria、and 2.2×10E1 MPN/ml for methane producing bacteria.

## V. Discussion

1) Contamination of drilling fluid

Borehole HDB-1 was drilled without using drilling mud until the depth of 275.5m, then continued drilling from 275.5m to 720m using drilling mud. The drilling mud was made of bentonite (Kunigel V1) mixed into drilling fluid.

As an index to measuring the impact of the drilling fluid, Sodium-naphtionate (4-Amino-1-naphthalenesulfonic Acid Sodium Salt) were added to form a concentration of 10 ppm (100 g of Sodium-naphtionate per $10m^3$ drilling fluid) for tracing purposes.

At the time of obtaining groundwater sampling for analysis, the tracer concentration was measured to be 1.09ppm at borehole HDB-1and between 0.7 and 0.9ppm at HDB-2.

As a result, the present groundwater samplings are not completely free of influence from drilling fluid. Assessing the extent of the drilling fluid is one of the tasks to be addressed in the future.

2) According to the hexa-diagram (Fig.2), the groundwater has a conspicuously high percentage of Na (+K) and *Cl* among dissolved ions. The high figures for dissolved ions

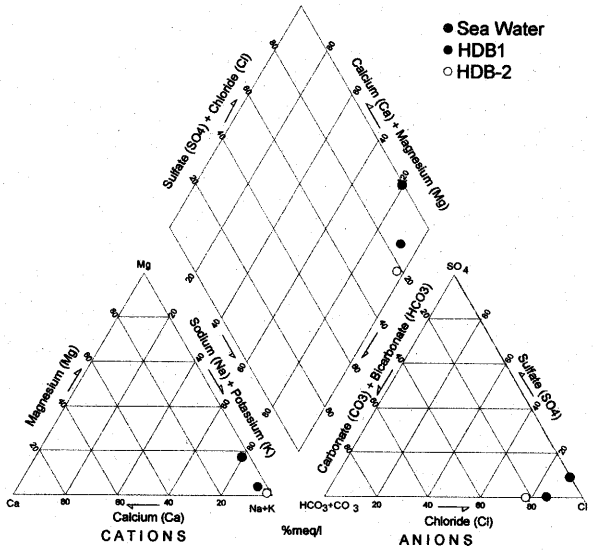


Fig.3 Tri-linear diagram of HDB-1 and HDB-2 with comparison to present sea water

especially for Na and C*l*, for the groundwater obtained from HDB-2 points to a strong influence of fossil seawater. As supporting evidence, fossil seawater is known for having lower concentration of $SO_4$ as compared to present seawater.

As can be seen from Fig.2, the comparison with present seawater indicates that the amount of $SO_4$ is lower for the groundwater sample. The same is obvious from the tri-linear diagram of Fig.3 where the rate of $SO_4$ is low among the list of anions. It is also known that fossil seawater is usually found in the deep underground of reducing environment. In a reducing environment, $SO_4$ concentration diminishes in the following manner:

$$SO_4^{2-} + 2C + 2H_2O \longrightarrow 2HCO_3 + H_2S$$

As a result, the concentration of $SO_4$ in fossil seawater is lower than in present seawater. In the groundwater of HDB-2, sulfate reducing bacteria is confirmed at $2.2\times10$ MPN/ml and thus is considered to have triggered the lowering of $SO_4$ concentration under the reducing environment.

## VI. Summary and conclusion

1. The groundwater samples were found to have a different composition from that of present seawater.

2. The results of the examinations indicate possible influence of fossil seawater.

3. As for dissolved gas, there was a high concentration of CO2 and CH4 in borehole HDB-1, and of CH4 in borehole HDB-2.

Table 1 Chemical and microbiological analyses

| Boreholes | HDB-1 | HDB-2 |
|---|---|---|
| Sampling depth (m) | 548.0-563.2 | 344.9-404.9 |
| pH | 7.7 | 7.9 |
| Temperature (℃) | 22.2 | 20.8 |
| EC (mS/m) | 2450.0 | 1490.0 |
| Na (mg/*l*) | 5010 | 3120 |
| K (mg/*l*) | 174.0 | 51 |
| NH4 (mg/*l*) | 120.0 | 53 |
| Li (mg/*l*) | 9.10 | 2.7 |
| Ca (mg/*l*) | 180 | 24.0 |
| Mg (mg/*l*) | 111.0 | 36.0 |
| Sr (mg/*l*) | 2.8 | 1.20 |
| Mn(□) (mg/*l*) | 0.45 | 0.51 |
| Fe(□) (mg/*l*) | <0.01 | 0.03 |
| Total Fe (mg/*l*) | 0.02 | 0.03 |
| SiO2 (mg/*l*) | 47 | 45 |
| Al (mg/*l*) | <0.1 | <0.1 |
| F (mg/*l*) | <0.1 | <0.1 |
| Cl (mg/*l*) | 7330 | 4180 |
| Br (mg/*l*) | <0.1 | 19 |
| NO3 (mg/*l*) | <0.1 | <0.1 |
| NO2 (mg/*l*) | 0.2 | <0.1 |
| SO4 (mg/*l*) | 98 | 2.4 |
| H2S (mg/*l*) | <0.1 | <0.1 |
| P alkalinity CaCO3(mg/*l*) | -- | -- |
| M alkalinity CaCO3(mg/*l*) | 1660 | 1740 |
| HCO3 (mg/*l*) | 2056 | 2120 |
| CO3 (mg/*l*) | -- | -- |
| TOC (mg/*l*) | 13 | 44 |
| IC (mg/*l*) | 360 | 400 |
| Fumic acid (mg/*l*) | 0.94 | 0.34 |
| Fulubic acid (mg/*l*) | 680 | 380 |
| Acetic acid (mg/*l*) | <0.5 | <0.5 |
| Formic acid (mg/*l*) | <0.5 | <0.5 |
| Se (mg/*l*) | <0.001 | <0.001 |
| P (mg/*l*) | <0.05 | 0.01 |
| I (mg/*l*) | 16 | 12 |
| T (T.U) | 1.4±0.1 | 0.6±0.1 |
| δD (‰) | -20 | -29 |
| $\delta^{18}O$ (‰) | +1.2 | +0.4 |
| Total numbers of bacteria (cells/m*l*) | +1.3E+1 | 3.4E+07 |
| Heterotroph (CFU/ m*l*) | 2.1E+2 | 3.2E+03 |
| Anaerobic heterotroph (CFU/ m*l*) | 1.5E+01 | 5.8E+04 |
| Ammonia-oxidizing bacteria(MPN/ m*l*) | N.D. | N.D. |
| Nitrite-oxidizing bacteria(MPN/ m*l*) | N.D. | N.D. |
| Iron-oxidizing bacteria(MPN/ m*l*) | N.D. | N.D. |
| Nitrate-reducing bacteria (MPN/ m*l*) | 4.9E+01 | 2.2E+03 |
| Denitrifying bacteria (MPN/ m*l*) | 1.3 | 7.9E+03 |
| Sulfate reducing bacteria (MPN/ m*l*) | N.D. | 2.2E+01 |
| Methane producing bacteria (MPN/ m*l*) | N.D. | 2.2E+01 |
| Dissolved Gas | | |
| $O_2$ (%) | 13.3 | 1.43 |
| $N_2$ (%) | 54.1 | 5.26 |
| Ar (%) | 2.36 | 0.718 |
| $CO_2$ (%) | 17.5 | 0.058 |
| $CH_4$ (%) | 8.27 | 91.3 |
| $C_2H_6$ (%) | 0.01 | 0.138 |
| $C_3H_8$ (%) | 0.01 | 0.024 |
| i-$C_4H_{10}$ (%) | 0 | 0.01 |
| n-$C_4H_{10}$ (%) | 0 | 0.01 |
| He (%) | 0.001 | 0.0044 |
| CO (%) | 0.01 | 0.01 |
| Sum | 95.561 | 98.9624 |
| Tracer (ppm) | 1.1 | 0.9 |

4. In terms of microorganisms, nitrate-reducing bacteria and denitrifying bacteria were found to exist in HDB-1, while sulfate-reducing and methane-producing activities were not detected. In HDB-2, sulfate- reducing bacteria and methane-producing bacteria were found , in addition to nitrate-reducing bacteria and denitrifying bacteria, indicating the possibility of sulfate-reducing and methane-producing activities.

## Acknowledements

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