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Citation	Applied Geochemistry, 21(1), 48-57 <a href="https://doi.org/10.1016/j.apgeochem.2005.08.003">https://doi.org/10.1016/j.apgeochem.2005.08.003</a>
Issue Date	2006-01
Doc URL	<a href="http://hdl.handle.net/2115/7401">http://hdl.handle.net/2115/7401</a>
Type	article (author version)
File Information	AG21_1.pdf



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**Phosphatase and microbial activity with biochemical indicators in permafrost active layer sediments over the past 10,000 years**

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## Abstract

Core samples of boreal terrestrial sediments at depths from 0 to 300 cm at Rikubetsu, Hokkaido, Japan were analyzed for alkaline and acid phosphatase enzymatic activities. Enzymatic activities of alkaline phosphatase (ALP) and acid phosphatase (ACP) were greatest at the surface and decreased with depth; ALP and ACP activities were 25.5  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and 22.0  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ , respectively, with in the top 5 cm. These biological indicators were compared with measurements of microbial cell density and chemical indicators, including total organic carbon (TOC) and total hydrolyzed amino acids (THAA). The product-moment correlation coefficients ( $r$ ) for ALP and ACP versus microbial cell density were 0.949 and 0.810, respectively. The coefficients for THAA and TOC versus ALP were 0.997 and 0.995, respectively. Vertical distributions of enzymatic activity are highly consistent with observed microbial biomass profile and diagenetic organic matter in the sediment. However, the vertical profile of phosphate concentration shows a negative correlation coefficient for ALP and ACP of  $-0.937$  and  $-0.855$ , respectively. Consequently, ALP and ACP were shown to be useful biomarkers of microbial activities in the terrestrial sediment over the past 10,000 years at Rikubetsu, Hokkaido, Japan.

## 20 Keywords:

phosphatase activity, biomarkers, microbial activity, terrestrial sediment, diagenesis

## Introduction

Alkaline and acid phosphatases (orthophosphate monoester phosphohydrolases) are crucial enzymes in the catalysis of phospho-monoesterase reactions (Trowsdale et al., 1990). Since the 1960s, many phosphatases have been characterized; for example, the *Escherichia coli* alkaline phosphatases have been widely studied in terms of biosynthesis (Derman and Beckwith, 1991; Karamyshev et al., 1998; Kim and Wyckoff, 1989) and catalytic properties (Coleman, 1992). The fact that alkaline phosphatase (ALP) and acid phosphatase (ACP) are found widely in taxonomic groups ranging from bacteria to mammals, indicates their importance in fundamental biochemical processes (Posen, 1967).

Diagenesis of organic matter is largely dependent on surface and/or subterranean microbial activity in marine sediment (Deming and Barross, 1993). Sedimentary organic material is largely composed of high molecular weight compounds and particles, which are unsuitable for direct microbial utilization (Thurman, 1985). Enzyme activity is generally recognized as playing a key role in the degradation and utilization of organic polymers by these micro-organisms, as only compounds with molecular masses lower than 600 Da can pass through cell (Gottschalk, 1986; Hoppe, 1991; Meyer-Reil, 1991). Temperature has also been identified as a factor that controls enzymatic activity (Mayer, 1989), but with a few notable exceptions (Reichardt, 1988). Studying thermostable enzymes such as ALPs is not only of interest for understanding life in extreme environments, but for industrial processes as well (Pantazaki et al., 1998; Park et al., 1999; Mori et al., 1999). Psychrophillic microorganisms are found in

a number of cold habitats including rocks (Friedmann, 1982); the frozen soils of the Arctic and Alpine environments (Russel, 1992); permafrost regions (Gilichinsky et al., 1993); in polar ice, oceans, and ice-covered lakes in Antarctic lakes (McKay, 1986); on top of and in deep layers of glaciers (Abyzov et al., 1998); and putative cold-loving  
5 archaea have been detected in glacial seawaters in Antarctica (DeLong et al, 1994).

Unique biomarkers of microbial activity in Antarctic rocks have been indicated by iron-rich diagenetic mineral (Wierzchos et al., 2003). Determination of the aminopeptidase and  $\beta$ -glucosidase in the sediments could be applied as a key parameter for understanding the role of bacteria in Antarctic sediments (Fabiano and Danovaro,  
10 1998). Our goal is to determine to what degree enzymatic activities, especially phosphatase activities, are applicable as biomarkers to evaluate subterranean biological activity. Although several studies have been conducted on phosphatases in marine sediments, few biogeochemical studies of phosphatases have been conducted in terrestrial sediment. The present study shows how ALP and ACP are correlated with  
15 extremophilic microbial activity in boreal terrestrial sediment at Rikubetsu, Hokkaido, Japan. The aims of this research are (i) to determine enzymatic activities in relation to biomass and vertical distribution of organic matter and (ii) to determine the relationship between biological and chemical indicators in the diagenetic process.

## 20 **Field site and sample collection**

The core samples were collected at Rikubetsu, Hokkaido (43° 28' 0" N, 143° 44' 5" E) by the Obayashi Corporation in February 1996 (Takano et al., 2004a).

Rikubetsu, Hokkaido, one of coldest cities in Japan, is located near the center of Hokkaido in a boreal area (Figure 1). The coring site was located at an altitude of 207 m and exhibits an annual average temperature of 5.8 °C, an annual average of precipitation of 67 mm, and an average of 142.3 hours of sunshine per a month. The  
5 coring site was situated in a slightly marshy area that freezes seasonally down to a depth of 80 cm, and covered by ice during the winter. The coring was performed to a maximum depth of 300 cm.

Vertical profiles of moisture content, density, pH and particle size were investigated by the conformable test method of JIS A 1204 (1990) and JIS A 1225  
10 (2000) in Japanese Industrial Standards Committee (Table 1). The sediment core samples were analyzed for age using  $^{14}\text{C}$  radiocarbon dating (Fig. 2; Takano et al., 2004a, 2005). Because the concentration of organic carbon in sediment was very low, a bulk sample of ca. 200 g was analyzed using an accelerator mass spectrometric system (AMS) after washing with HCl by the Beta Analytic Inc, Florida, USA and Geo  
15 Science Laboratory Corporation, Japan.

## **Materials and Methods**

### **Preparation of Glassware and Stock Solutions**

20 All glassware used in the sampling and analysis was soaked overnight in 7 M  $\text{HNO}_3$  and rinsed with sterile Milli-Q water. Prior to use, glassware was heated for 2 hours at 500°C in a high temperature oven (Yamato DR-22) to eliminate any possible organic contaminants. Modified universal buffer (MUB) stock solution was prepared

by dissolving 12.1 g of tris-hydrochloric aminomethane, 11.6 g of maleic acid, 14.0 g of citric acid and 6.3 g of boric acid in Milli-Q water into 488 ml of 0.1 M NaOH and adjusting the final volume to 1,000 ml (hereafter, MUB stock solution).

## 5 Alkaline Phosphatase (ALP) Activity

Determination of ALP activity was performed according to published methods Tabatabai and Bremner (1969) and Tabatabai (1982). Briefly, MUB stock solution (200 ml) was adjusted to pH 11 by 0.1 M ~~mol/L~~ NaOH and diluted to 1,000 ml with Milli-Q water (hereafter, MUB working solution). Next 0.928 g of *p*-nitrophenyl phosphate was dissolved in 100 ml of prepared working solution (hereafter, MUB substrate solution, pH 11). Recovered sediment core samples were then placed in sample vials, sealed with MILLI WRAP filters (Millipore Co.), freeze-dried, and gently pulverized. Powdered sample (0.25 g) was incubated in 50  $\mu$ l of toluene (ultra pure grade), 1 ml of MUB working solution, and 250  $\mu$ l of MUB substrate solution for one hour at 37°C in a water bath. The reaction was terminated with the addition of 250  $\mu$ l of 0.5 M CaCl<sub>2</sub> and 1 ml of 0.5 M NaOH, the solution was filtered through a 0.20- $\mu$ m PTFE membrane filter (ADVANTEC PTFE). The absorbance of reaction product (*p*-nitrophenol) at 410 nm was measured with a JASCO V-550 UV-VIS spectrometer.

ALP activity was calculated as

$$Z = \Delta C \times V / 1000 / \Delta t \quad [\mu\text{mol}/\text{min}] \quad (1)$$

where Z is ALP activity,  $\Delta C$  is the increase in PNP concentration ( $\mu$ mol), V is the total

volume of substrate solution (ml),  $\Delta t$  is incubation time (min).

### **Acid Phosphatase (ACP) Activity**

MUB stock solution was adjusted to pH 6.5 by 0.1 mol·l<sup>-1</sup> HCl and diluted to  
5 1,000 ml with Milli-Q (hereafter, MUB working solution, pH 6.5). Next 0.928 g of  
*p*-nitrophenyl phosphate was dissolved in 100 ml of MUB working solution (hereafter,  
MUB substrate solution, pH 6.5). The experimental procedure was performed in the  
same manner as for the ALP analysis.

### 10 **Analysis of sulfur and organic carbon**

Bulk powder samples were compressed into tablet coins under pressure of 70  
MPa by a hydraulic jack. Elemental sulfur content (wt %) was determined by X-ray  
fluorescence spectrometry. Total organic carbon concentration (wt %) of the  
sediments was described previously (Takano et al., 2004a).

15

### **Amino acid analysis**

Approximately 1.0 g of the freeze-dried sample was placed in an acid washed  
Teflon tube held in a metal vessel. The sample was digested in the presence of 10 ml  
of 5 M HF-0.1 M HCl with continuous heating at 110°C for 16 hours to extract  
20 organics from the silicate matrix. The samples were then placed on a hot plate in a  
draft chamber to evaporate the acids, and the organic residues were extracted with  
Mill-Q water by ultra-sonication. To obtain the total hydrolyzed amino acid fraction



(THAA), the aqueous fraction was filtered through a GF/A 1.6  $\mu\text{m}$  pore size glass fiber filter, freeze-dried in a glass test tube, and hydrolyzed with 2 ml of 6 M HCl on a block heater at 110°C for 2 h in a sealed tube. The hydrolysates were dried *in vacuo* using a diaphragm pump, were adjusted to pH 1 by 2 ml of 0.1 M HCl, and desalted with a  
5 AG-50W-X8 cation exchange resin column (Bio-Rad Laboratories). The amino acid fraction was eluted with 10 ml of 10%  $\text{NH}_3$  aqueous solution. The eluent was freeze-dried and redissolved in 1.0 ml of 0.1 M HCl before injection into the liquid chromatographic system.

The THAA concentrations were determined using an ion-exchange HPLC  
10 system composed of two HPLC pumps (Shimadzu LC-6A), a cation exchange column (Shimpack ISC-07/S1504, 4 mm i.d.  $\times$  150 mm), a post column derivatization system using *o*-phthalaldehyde (OPA) and N-acetyl-L-cystein (N-AcCys), and a Shimadzu RF-535 fluorometric detector (excitation wavelength: 355 nm and emission wavelength: 435 nm).

15

#### **Determination of viable microbial cell density**

Sediment specimens (100 mg) were stained with 0.5 ml of staining solution (20 mM bis-trispropane buffer, pH 6.5 with 20 mM  $\text{CaCl}_2$ , 10  $\mu\text{M}$  CFDA-AM (5-carboxyfluorescein diacetate acetoxymethylester) and 0.05 % Pluronic-F127 for 60  
20 min at room temperature (Tsuji et al., 1995). Mixtures were washed 4 times with the staining solution followed by centrifugation at  $4 \times 10^5$  g for 3 min, and the sediment was compacted and mounted on a hole glass slide. After adding the measurement

solution (20 mM bis-tris propane buffer, pH 5.0 with 20 mM CaCl<sub>2</sub>), the slides were viewed under a Zeiss Axiovert 135 M fluorescence microscope equipped with cooled CCD Cameras. Viable microorganisms were discriminated from the background based on fluorescence intensity using IP-Lab spectrum software (Signal Analytics Ltd.,  
5 USA).

### **Analysis of water extractable cations and anions**

Bulk powdered samples (1.0 g) were shaken with 10 ml of ion-exchanged water for 6 hr. The mixture was centrifuged at 3,000 rpm for 1 hour, and then the  
10 supernatant was filtrated through a 0.20 µm PTFE membrane filter. Anions and cations were measured using a Shimadzu ion chromatography system. Phosphate (PO<sub>4</sub><sup>3-</sup>) was determined by the molybdate blue method: Analysis reagent was prepared by mixing 50 ml of 2.5 M H<sub>2</sub>SO<sub>4</sub>, 2.7 g/l of potassium antimonyl tartrate, 15 ml of 40  
15 g·l<sup>-1</sup> molybdate ammonium, and 30 ml of 17.6 g·l<sup>-1</sup> of ascorbic acid. This was mixed with 0.25 g of powdered sample, and then filtered through a 0.20 µm PTFE membrane filter (ADVANTEC). Absorbance was measured at 700 nm with a JASCO V-550 UV-VIS spectrometer. The core sample may contain humic acid analogs which interfere with the phosphate measurements. However, the concentration of the phosphate was not adjusted for the absorbance in this study,

20

## **Results and Discussion**

### **Vertical profiles of phosphatase activities and other biochemical indicators**

Vertical profiles of ALP, ACP, and viable numbers of microorganism are summarized in Table 2. ALP and ACP showed maximum values of 25.5  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  and 22.0  $\text{nmol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ , respectively, at the surface, but exponentially decreased with depth (Figure 3). As seen in Figure 3, two steps of degradation stage (Belluomini et al., 1986) are plausible: at first, degradation pathway for labile phosphatases under oxidative surface environment proceeds exponentially, next degradation pathway for inactive phosphatases proceeds asymptotically with time after burial below the surface layer. As shown in Figure 3 and 5, the vertical profiles of total organic carbon and total sulfur also correlated with the two enzymatic parameters. Enzymatic activity showed a decrease with increasing sediment depth, indicating vertical shifts in both availability and nutritional quality of degradable organic matter. Enzymes are essential proteins associated with living organisms, and proteins, peptides and free amino acids account for 30–40% of the total nitrogen and 10–15% of total organic carbon in marine surface sediments (Burdige and Martens, 1988; Cowie and Hedges, 1992).

Previous studies of diagenetic organic matter (Takano et al., 2004a) in the same core samples showed positive correlations among TOC, THAA and TS. A positive correlation between TS and TOC in the sediments suggest that the mineral formation processes depend on the amount of organic matter in these environments which are rich in iron and  $\text{SO}_4^{2-}$  (Wicks et al., 1991). The main source of sulfur in sediments is microbially reduced  $\text{SO}_4^{2-}$ , which also contains organic sulfur (Mitchell et al., 1984; Vairavamurthy et al., 1994). In anoxic subterranean environments,  $\text{SO}_4^{2-}$  (as

well as Fe and Mn oxides) are important electron acceptors in microbial oxidation of organic matter (Canfield, 1994).

### **Correlation between phosphatase activities and inorganic ions**

5 Nitrate concentration ( $\text{NO}_3^-$ ) also drastically decreased in concentration at lower depths by two orders of magnitude while phosphate ( $\text{PO}_4^{3-}$ ) gradually increased with the depth (Table 3). Subterranean microbial activity also produces ammonia in the detritus component, and its free analogs may be converted to  $\text{NH}_4^+$  ions and then finally oxidized to form nitrate ( $\text{NO}_3^-$ ). Similarly, there were notable increases in the  
10 relative abundances of  $\beta$ -alanine and  $\gamma$ -aminobutyric acid to THAA with the depth. It has been reported that diagenesis in sediments causes decomposition of amino acids *via* decarboxylation (Ratcriff et al., 1974); for example, aspartic acid is converted to  $\beta$ -alanine by decarboxylation at the  $\alpha$ -carbon (Andersson et al., 2000; Takano et al., 2004a). Another modification of sedimentary organic compounds is the deamination  
15 process; for example, asparagine and glutamine are converted to aspartic acid and glutamic acid, respectively, by deamination reactions.

Phosphorus is one of the most important nutrient elements. Microorganisms utilize phosphorus as orthophosphate ( $\text{PO}_4^{3-}$ ). Ecosystems have a high requirement for nutrients such as inorganic phosphorus and nitrogen (Kobayashi et al., 1987). As  
20 shown in Figure 4 and Table 4, concentration of  $\text{PO}_4^{3-}$  is negatively correlated with ALP and ACP which suggest phosphate is one of limiting factors for microbial growth. When orthophosphate is insufficient to support growth, organisms may

produce more alkaline phosphatase in order quickly obtain a supply of orthophosphate. On the other hand, when phosphorus concentrations are sufficiently high to meet the requirements of the organisms, the element key to the ecosystem is another nutrient element, such as nitrogen (Kobayashi et al., 1987).

5

### **Phosphatase activity as a biomarker**

Table 4 shows the correlations among the distributions of ALP, ACP, microbial cell density and organic matter. The product-moment correlation coefficients ( $r$ ) for ALP and ACP versus microbial cell density are 0.949 and 0.810, respectively. Images of sediment core sample slides show microbial cells as fluorescent spots (Figure 5). Also, any fibrous microorganisms are observed at the surface, and many of these seem to be fungi based on their sizes, which is consistent with aerobic requirements at these bacteria. Many cells are aggregated at the core surface, while they are sparsely distributed in deeper fractions, this is likely to reflect the heterogenous distribution of organic matter, such as plant detritus at the surface, compared to digested and fragmented organic matter at depth, which well result in the homogeneous distribution of heterotrophic cells (Meyer-Reil, 1987).

The product-moment correlation coefficients ( $r$ ) for total hydrolyzed amino acids (THAA) and total organic carbon (TOC) versus ALP were 0.985 and 0.992, respectively. Vertical distributions of enzymatic activity are consistent with the subterranean microbial biomass and diagenetic organic matter in the sediment. On the other hand, vertical distributions of phosphoric acid showed negative correlation

coefficients for ALP and ACP of  $-0.855$  and  $-0.940$ , respectively. Comprehensive evaluation regard to ALP and ACP in the sediment has been also been required from the point of view of soil science (Tabatabai and Bremner, 1969; Tabatabai, 1982). The highly positive correlations of ALP and ACP with microbial cell density in the present study provide good evidence that phosphatase activity is a plausible new biomarker of subterranean microbial activity.

Microbes associated enzymes can be considered labile organic matter (LOM) in the surface environment. Generally, LOM proceed to semi-labile organic matter (Semi-LOM) in the early diagenesis during sedimentation. Continuously the semi-LOM proceeds biologically inactive refractory organic matter (ROM) in the next step. In the present study, sub-surface zone of upper -50 cm are in LOM stage. Around – 40 cm are semi-LOM which is a transition state between LOM and ROM. Consequently asymptotical decreasing zone below – 40 cm are biologically very poor activities and occupied ROM components.

15

## Conclusions

The present results suggest that ALP and ACP degrade exponentially in the early stages of diagenesis, and that the enzymes decrease asymptotically with increased burial time. The positive and negative correlations provide good evidence for the relationship between the population of subsurface microorganisms and enzymatic activities with regards to the available organic matter. Consequently, we clarified the importance of determining enzymatic activity, especially ALP and ACP, as these can be

used as biomarkers of subterranean microbial activity and organic matter in the sediments.

From the point of view of chemical evolution and the frontiers of the biosphere, enzymatic activities are of interest and the distribution of subterranean  
5 (Takano et al., 2003) and submarine hydrothermal sub-vent ecosystem (Takano et al., 2004b). In order to construct a consolidated model of the extreme environments in submarine hydrothermal vents and the interactions between the sub-vent biosphere, the Archaeal Park Project, which integrates geology, biology and chemistry, is now in progress (Urabe et al., 2001).

10

### **Acknowledgement**

The authors express their sincere thanks to two anonymous reviewer for constructive reviewing comments which helped to improve the earlier version of the manuscript. The authors also thank to associate editor Dr. F. Grant Ferris, Department  
15 of Geology, University of Toronto, for the manuscript handling and invaluable suggestions. The authors would like to thank Dr. Yukishige Kawasaki, Mitsubishi Kagaku Institute of Life Science, Mr. T. Ebashi and Mr. T. Ujiie, Institute for Marine Resources and Environment, National Institute of Advanced Industrial Science and Technology for their experimental help and discussion. This research was partly  
20 funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan through the Special Co-ordination Fund for the Archaeal Park Project; an international research project on the interaction between the sub-vent biosphere and the

geo-environment. The corresponding author (Y.T.) is supported in part by a 21st Century Center of Excellence (COE) Program on "Neo-Science of Natural History" (Program Leader: Hisatake Okada) at Hokkaido University financed by the MEXT of Japan.

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## FIGURE LEGENDS

**FIGURE** The coring site is located at 143° 44' 5"E, 43° 28' 0" N in Rikubetsu, Hokkaido, Japan. Referred from Takano et al., 2004a.

5

**FIGURE 2** Age profile of core sediment from  $^{14}\text{C}$  radiocarbon dating by accelerator mass spectrometric system (Data set taken from Takano et al., 2005).

**FIGURE 3** Vertical distributions of alkaline phosphatase (ALP) and acid phosphatase (ACP) in sediment core samples at Rikubetsu, Hokkaido, Japan. Error bars represent the standard deviation of triplicate analysis.

10

**FIGURE 4** Vertical distributions of total organic carbon (TOC), total sulfur (TS), phosphate ( $\text{PO}_4^{3-}$ ), and nitrate ( $\text{NO}_3^-$ ) in the sediment core samples at Rikubetsu, Hokkaido, Japan.

15

**FIGURE 5** Microbial cell density in sediment samples determined by fluorescence microscopy. The fluorescent bright spots represent subterranean microorganisms in the sediment at Rikubetsu, Hokkaido, Japan.

20

## **TABLE LEGENDS**

TABLE 1 The vertical profiles of moisture content, density, pH and particle size distribution of the core samples in Rikubetsu, Hokkaido, Japan.

5 TABLE 2 The vertical profiles of total organic carbon (TOC), total sulfur (TS), enzymatic activities, microorganisms and amino acid profiles in the sediment of Rikubetsu, Hokkaido, Japan.

TABLE 3 The vertical profiles of inorganic anions and cations in the sediment of  
10 Rikubetsu, Hokkaido, Japan.

TABLE 4 The correlation of microbial cell density, alkaline phosphatase, acid phosphatase, total hydrolyzed amino acids, total organic carbon, phosphate (orthophosphate), D/L ratio of amino acid, molar ratio of non-proteinous amino acid.

# FIG.1

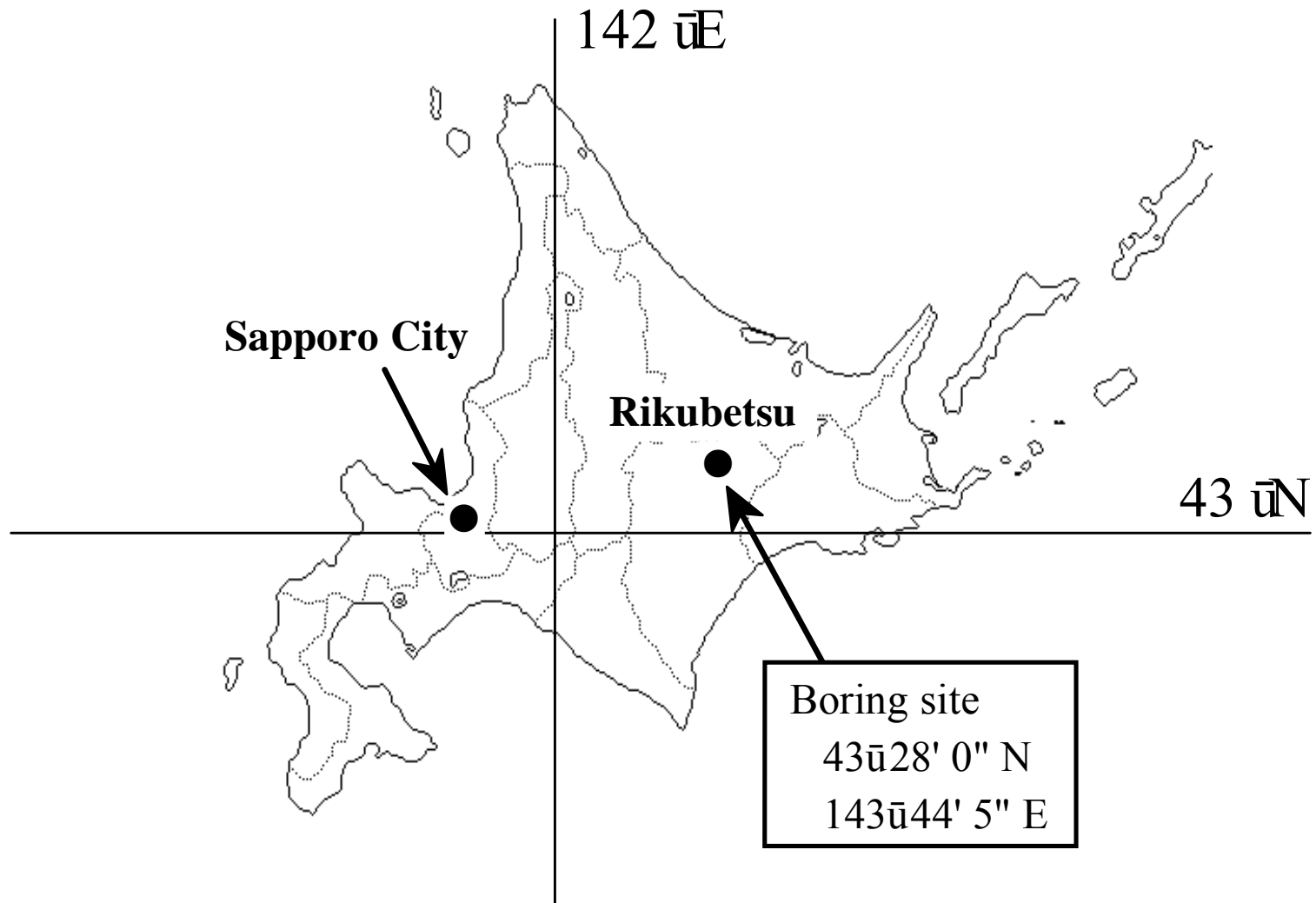
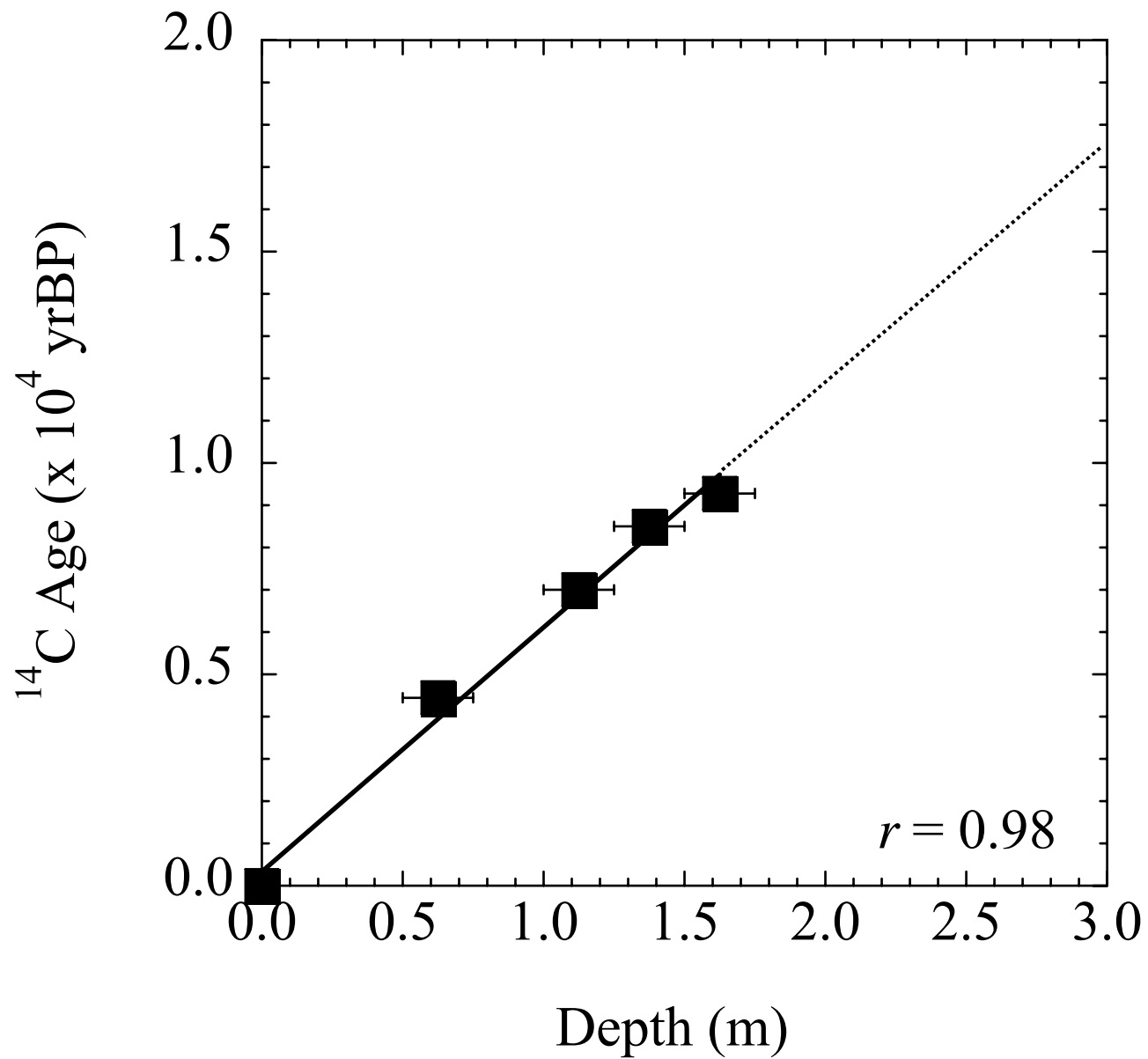
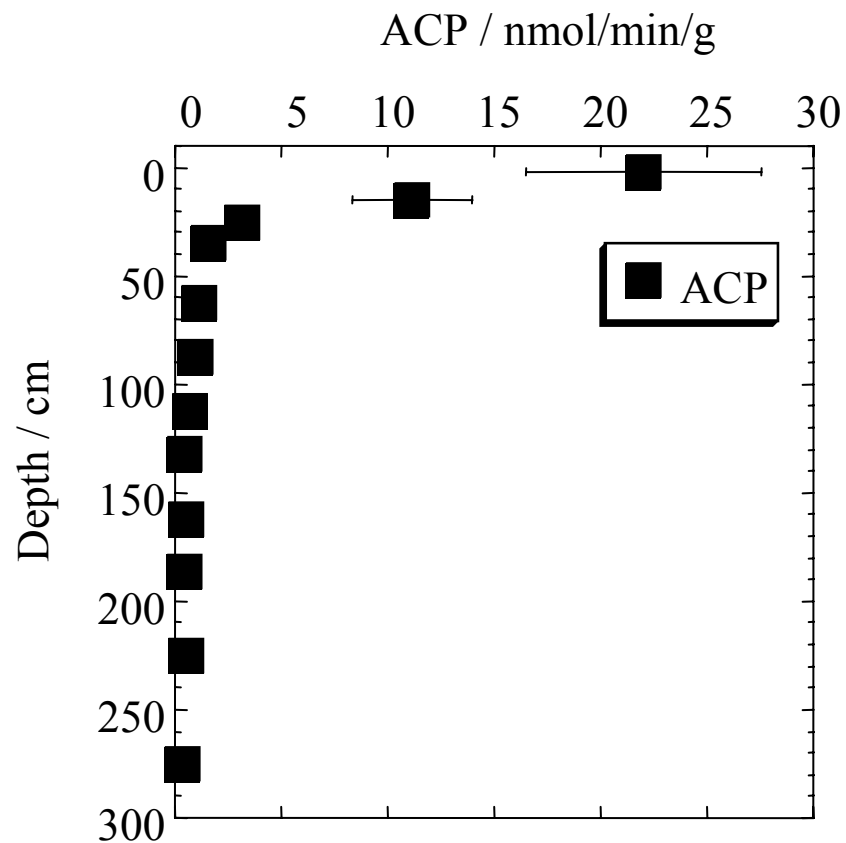
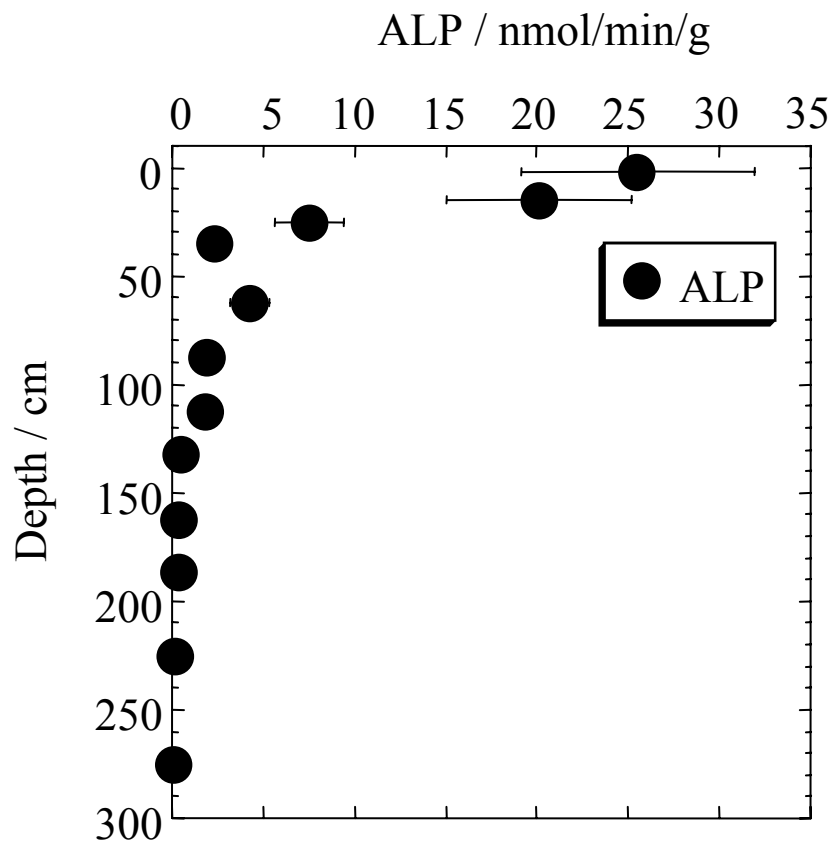




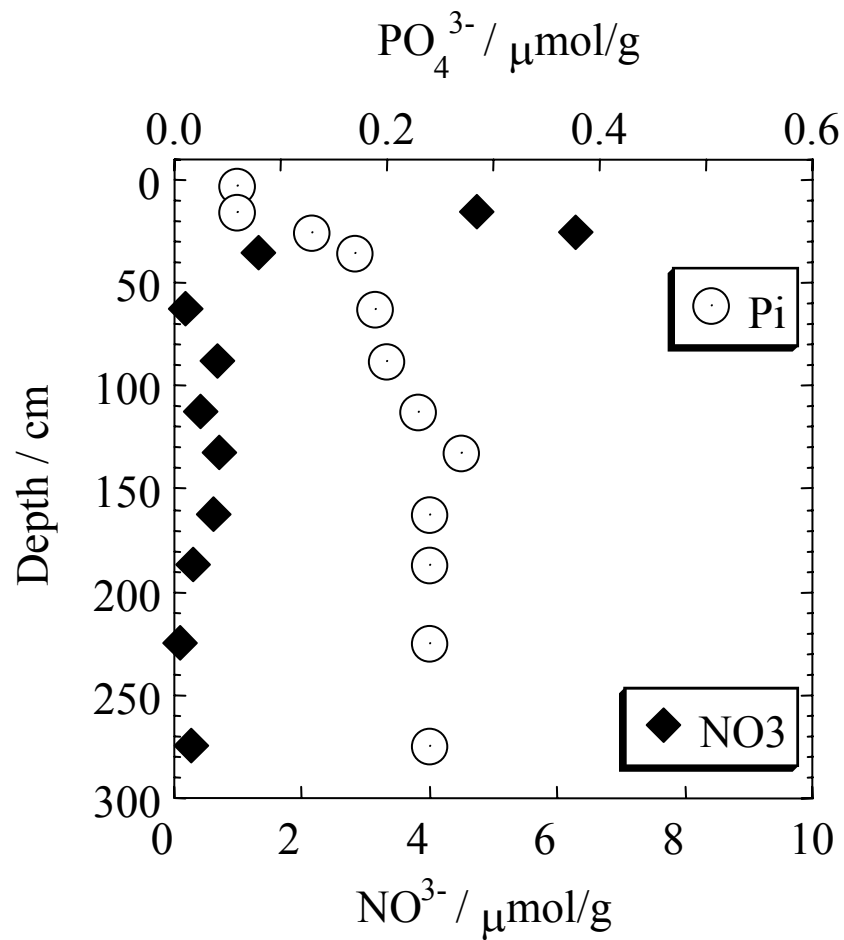
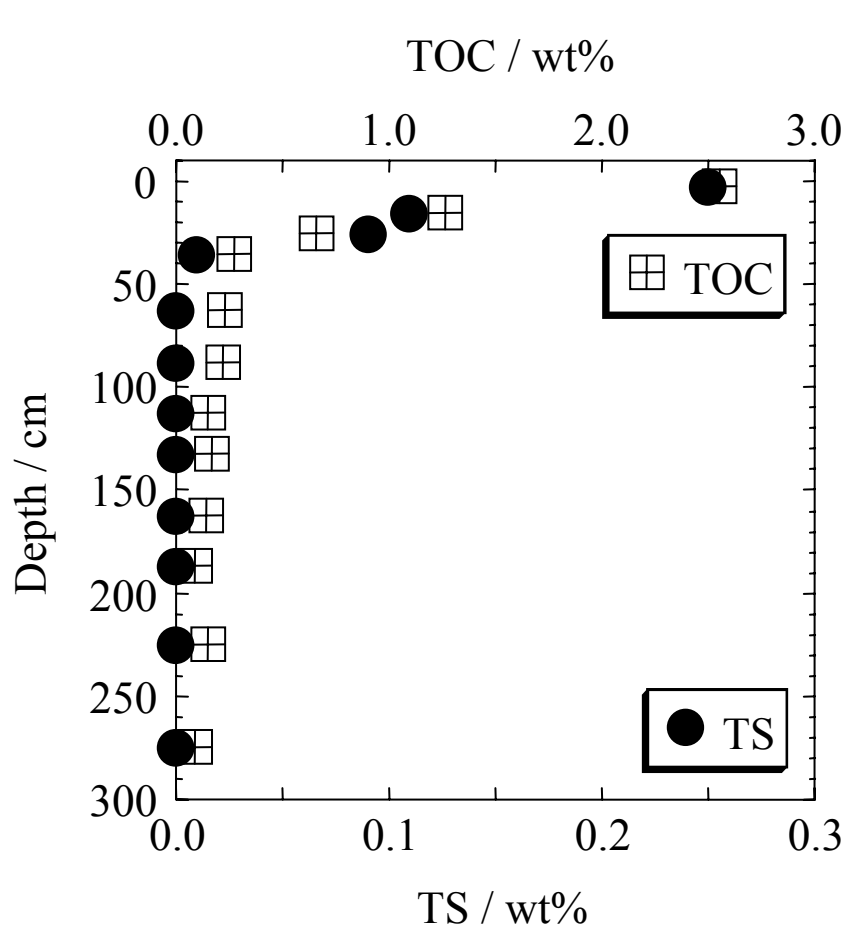
FIG.2



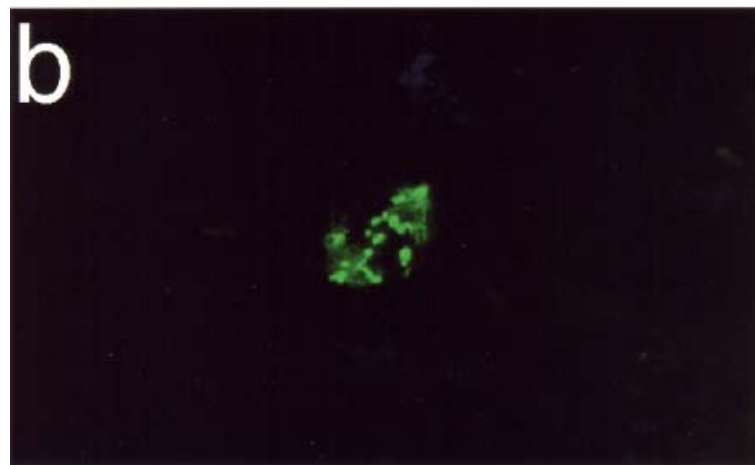
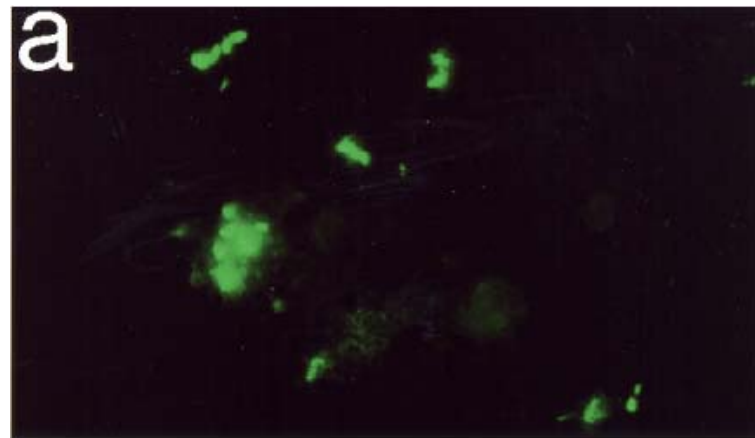
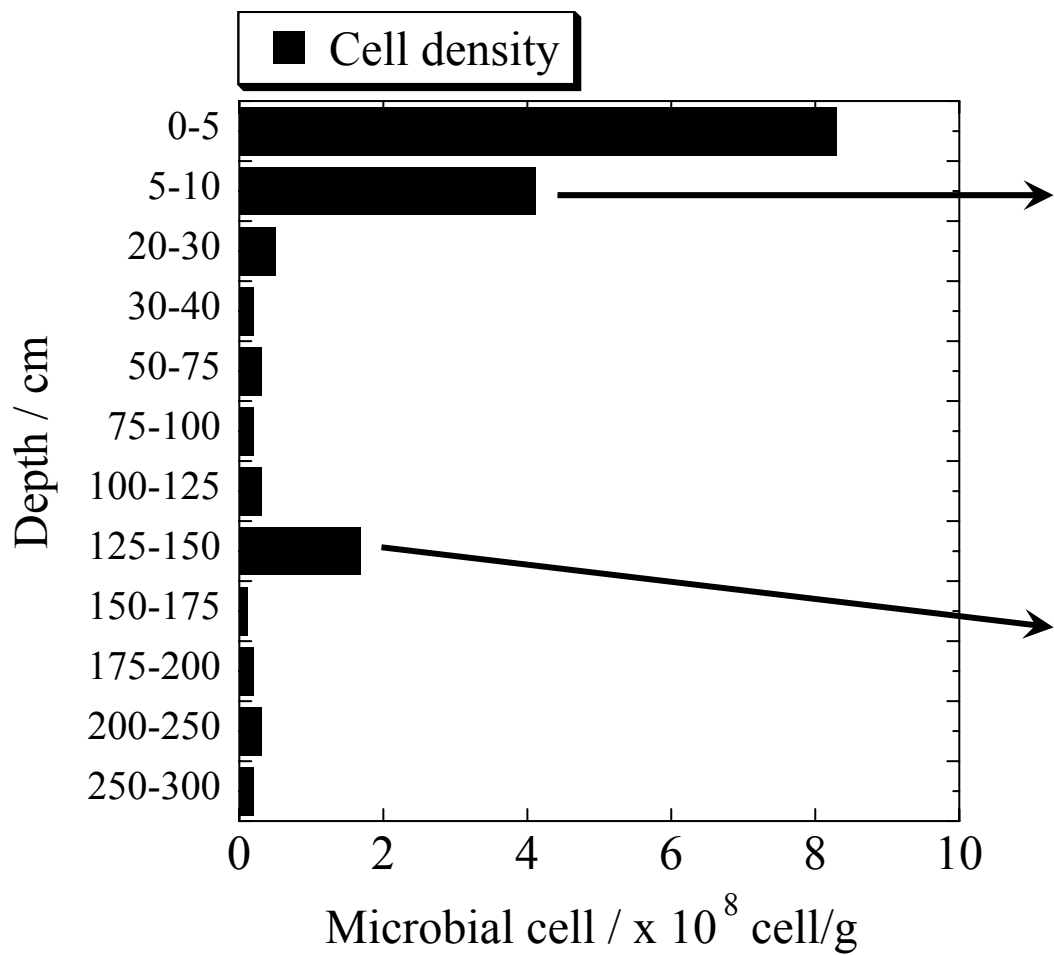
# FIG.3



# FIG.4



# FIG.5



**TABLE 1** The vertical profiles of moisture content, density, pH and particle size distribution of the core samples in l

Depth /	moisture content (%)	density ( $\rho$ )	pH	particle size distribution					
				coarse sand (%)	fine sand (%)	silt (%)	clay (%)	others (%)	
0-5	133	}	2.6	6.4	5	19	53	8	1
5-10	82								
20-30	47								
30-40	37	}	2.7	5.7	6	16	55	9	6
50-75	22								
75-100	32	}	2.8	6.2	9	19	40	6	13
100-125	25								
125-150	32								
150-175	32	}	2.7	6.1	9	23	49	7	3
175-200	39								
200-250	43	}	2.6	6.5	9	21	43	7	8
250-300	40								
		}	2.8	6.5	7	23	52	4	2

**TABLE 2** The vertical profiles of total organic carbon (TOC), total sulfur (TS), enzymatic activities, microorganisms and amino acid analogs in the sediment of Rikubetsu, Hokkaido, Japan.

Depth / cm	TOC (wt %)	TS (wt %)	Enzymatic activities		Microorganisms	Amino acid analogs		
			ALP (nmol / min / g)	ACP	cell density (x 10 <sup>8</sup> cells / g)	THAA ( $\mu$ mol / g)	Asp ( $\mu$ mol / g)	/L ratio of Asp -
0-5	2.55	0.25	25.5	22.0	8.3	61.8	9.23	0.02
5-10	1.26	0.11	20.1	11.1	4.1	28.6	5.47	0.06
20-30	0.66	0.09	7.56	3.12	0.5	11.8	1.76	0.07
30-40	0.27	0.01	2.33	1.56	0.2	2.85	0.40	0.12
50-75	0.23	0.00	4.26	1.13	0.3	0.84	0.14	0.17
75-100	0.22	0.00	2.00	0.95	0.2	1.86	0.27	0.18
100-125	0.15	0.00	1.88	0.72	0.3	1.21	0.22	0.25
125-150	0.17	0.00	0.56	0.40	1.7	1.30	0.27	0.25
150-175	0.14	0.00	0.44	0.50	0.1	0.54	0.09	0.24
175-200	0.09	0.00	0.43	0.44	0.2	0.66	0.13	0.28
200-250	0.15	0.00	0.19	0.53	0.3	0.71	0.14	0.29
250-300	0.09	0.00	0.11	0.32	0.2	0.65	0.13	0.31

Total organic carbon (TOC), cell density, total hydrolyzed amino acids (THAA), concentration of aspartic acid (Asp), and acid (Asp) were cited from Takano et al., 2003. Counting microorganisms were done by fluorescence microscopic method. Abbreviations; ALP: Alkaline phosphatase, ACP: Acid phosphatase.

**TABLE 3** The vertical profiles of inorganic anions and cations in the sediment of Rikubetsu, Hokkaido, Japan.

Depth / cm	Inorganic anions			Inorganic cations	
	PO <sub>4</sub> <sup>3-</sup>	NO <sub>3</sub> <sup>-</sup>	Cl <sup>-</sup>	Na <sup>+</sup>	K <sup>+</sup>
	(μmol / l)			(μmol / l)	
0-5	0.06	32.2	1.96	1.60	2.25
5-10	0.06	4.73	0.48	0.88	0.10
20-30	0.13	6.30	0.72	1.21	0.10
30-40	0.17	1.33	0.26	1.16	0.07
50-75	0.19	0.18	0.11	1.10	0.09
75-100	0.20	0.67	1.03	1.84	0.14
100-125	0.23	0.40	0.16	1.11	0.12
125-150	0.27	0.69	0.51	1.10	0.15
150-175	0.24	0.62	0.20	1.26	0.23
175-200	0.24	0.30	0.14	0.84	0.13
200-250	0.24	0.09	0.27	0.97	0.18
250-300	0.24	0.27	0.15	0.80	0.17

TABLE 4

	Cell Densit	ALP	ACP	THAA	THA	TOC	TS	D/L	NPA
Cell Density		0.949	0.810	0.976	0.930	0.968	0.931	-0.594	-0.564
ALP	0.949		0.918	0.997	0.863	0.995	0.947	-0.758	-0.702
ACP	0.810	0.918		0.923	0.954	0.937	0.971	-0.901	-0.837
THAA	0.976	0.997	0.923		0.966	0.998	0.985	-0.725	-0.657
THA	0.930	0.863	0.954	0.966		0.964	0.957	-0.691	-0.683
TOC	0.968	0.995	0.937	0.998	0.964		0.988	-0.761	-0.692
TS	0.930	0.947	0.971	0.985	0.957	0.988		-0.797	-0.788
D/L	-0.594	-0.758	-0.901	-0.725	-0.691	-0.761	-0.797		0.962
NPA	-0.564	-0.702	-0.837	-0.657	-0.683	-0.692	-0.788	0.962	

The product-moment correlation coefficient ( $r$ ) was calculated as,

$$r = \frac{\sum_i \{ (x_i - \bar{x}) (y_i - \bar{y}) \}}{\left\{ \left[ \sum_i (x_i - \bar{x})^2 \right] \left[ \sum_i (y_i - \bar{y})^2 \right] \right\}^{1/2}}$$

Abbreviations; ALP: Alkaline phosphatase, ACP: Acid phosphatase, THAA: Total hydrolyzed amino acids, THA: Total TOC: Total organic carbon, TS: Total sulfur, D/L: Ratio of D-and L-aspartic acid, NPA: Molar ratio of subtotal of non- $\alpha$  ( $\beta$ -alanine and  $\gamma$ -aminobutyric acid).