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Applied Geochemistry 21 (2006) 48–57
Applied Geochemistry
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Phosphatase and microbial activity with biochemical indicators in semi-permafrost active layer sediments over the past 10,000 years

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Received 4 August 2004; accepted 20 August 2005
Editorial handling by G. Ferrris
Available online 14 November 2005

Abstract

Core samples of boreal terrestrial sediments from depths of 0–300 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 and 22.0 nmol min^{-1} g^{-1}, respectively, within the top 5 cm. These biological indicators were compared with measurements of microbial cell density and chemical indicators, including total organic C (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 the observed microbial biomass profile and diagenetic organic matter in the sediment. However, the vertical profile of PO_4 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 ka at Rikubetsu, Hokkaido, Japan.

1. 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

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doi:10.1016/j.apgeochem.2005.08.003

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). Enzymatic activity is generally recognized as playing a key role in the degradation and utilization of organic polymers by these microorganisms, 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 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 Fe-rich diagenetic minerals (Weirzchos et al., 2003). Determination of the aminopeptidase and β-glucosidase in the sediments could be applied as a key parameter for understanding the role of bacteria in Antarctic sediments (Fabiano and Danovaro, 1998). The present 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 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.

2. 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 (Fig. 1). The coring site was located at an altitude of 207 m and exhibits an annual average temperature of 5.8 °C, annual average of precipitation of 67 mm, and an average of 142.3 h of sunshine per a month. The coring site was situated in a slightly marshy area that freezes seasonally down to a depth of 80 cm, and is 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 (2000), the Japanese Industrial Standards Committee (Table 1). The sediment core samples were analyzed for age using ^{14}C dating (Fig. 2; Takano et al., 2004a, in press). Because the concentration of organic C 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 Science Laboratory Corporation, Japan.

3. Materials and methods

3.1. Preparation of glassware and stock solutions

All glassware used in the sampling and analysis was soaked overnight in 7 M HNO_3 and rinsed with sterile Milli-Q water. Prior to use, glassware was heated for 2 h 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

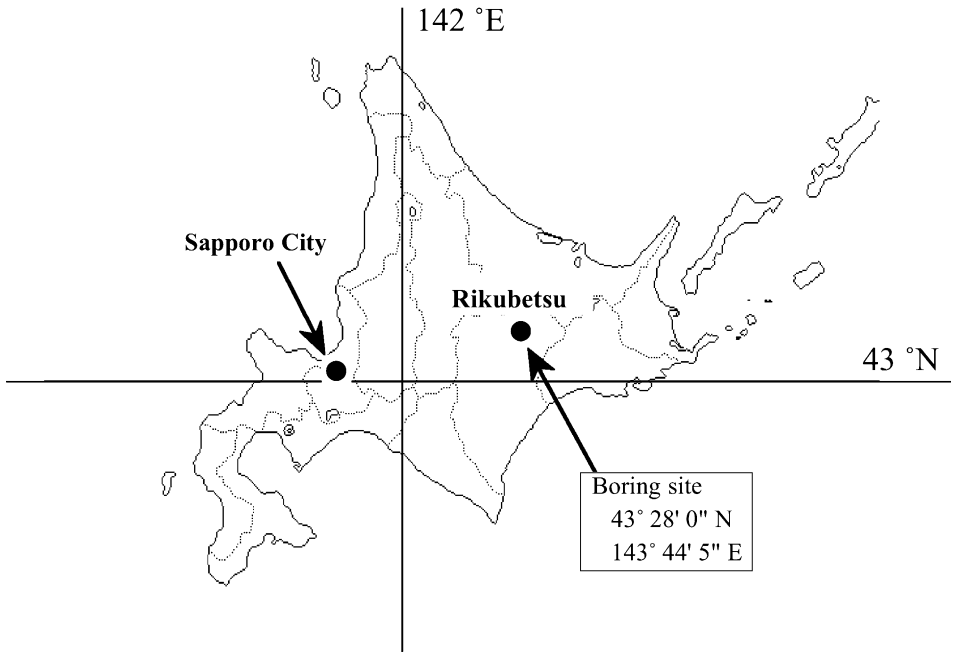

Fig. 1. The coring site is located at 143°44′5″E, 43°28′0″N in Rikubetsu, Hokkaido, Japan. From Takano et al., 2004a.

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

Depth (cm)	Moisture content (%)	Grain density (ρs)	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	2.8	6.2	9	19	40	6	13
75–100	32	2.7	6.1	9	23	49	7	3
100–125	25	2.6	6.5	9	21	43	7	8
125–150	32							
150–175	32							
175–200	39							
200–250	43	2.8	6.5	7	23	52	4	2
250–300	40							

Milli-Q water in 488 mL of 0.1 M NaOH and adjusting the final volume to 1000 mL (hereafter, MUB stock solution).

3.2. 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 NaOH and diluted to 1000 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 μL of toluene (ultra pure grade), 1 mL of MUB working solution, and 250 μL of MUB substrate solution for 1 h at 37 °C in a water bath. The reaction was terminated with the addition of 250 μL of 0.5 M $CaCl_2$ and 1 mL of 0.5 M NaOH, the solution was filtered through a 0.20-μm PTFE membrane filter (ADVANTEC PTFE). The absorbance of reaction

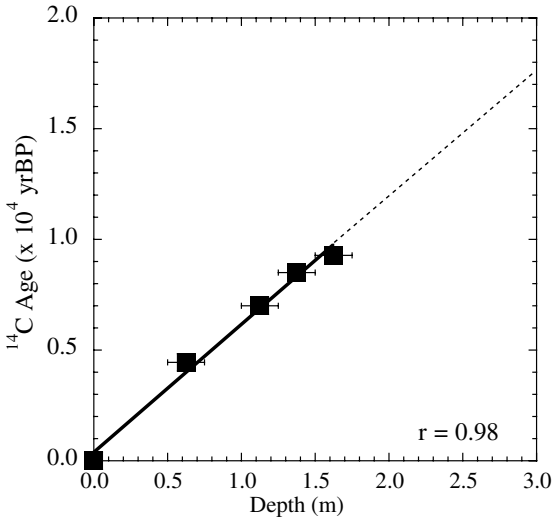

Fig. 2. Age profile of core sediment from ^{14}C radiocarbon dating by accelerator mass spectrometry (data set taken from Takano et al., in press).

product (*p*-nitrophenol) at 410 nm was measured with a JASCO V-550 UV–VIS spectrometer.

ALP activity was calculated as

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

where Z is ALP activity, ΔC is the increase in PNP concentration (μmol), V is the total volume of substrate solution (mL), Δt is incubation time (min).

3.3. Acid phosphatase (ACP) activity

MUB stock solution was adjusted to pH 6.5 by 0.1 mol L^{-1} HCl and diluted to 1000 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.

3.4. Analysis of sulfur and organic carbon

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

3.5. 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 h to extract 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 μm 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 AG-50W-X8 cation exchange resin column (Bio-Rad Laboratories). The amino acid fraction was eluted with 10 mL of 10% 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 system composed of two HPLC pumps (Shimadzu LC-6A), a cation exchange column (Shimpack ISC-07/S1504, 4 mm i.d.× 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).

3.6. Determination of viable microbial cell density

Sediment specimens (100 mg) were stained with 0.5 mL of staining solution (20 mM bis–tris propane buffer, pH 6.5 with 20 mM $CaCl_2$, 10 μM CFDA-AM (5-carboxyfluorescein diacetate acetoxymethylester) and 0.05% Pluronic-F127 for 60 min at room temperature (Tsuji et al., 1995). Mixtures were washed four times with the staining solution followed by centrifugation at 4×10^5 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 $CaCl_2$), 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., USA).

3.7. Analysis of water extractable cations and anions

Bulk powdered samples (1.0 g) were shaken with 10 mL of ion-exchanged water for 6 h. The mixture was centrifuged at 3000 rpm for 1 h, and the supernatant was filtered through a 0.20-μm PTFE membrane filter. Anions and cations were measured using a Shimadzu ion chromatography system. Phosphate (PO_4^{3-}) was determined by the molybdate blue method. Analysis reagent was prepared by mixing 50 mL of 2.5 M H_2SO_4, 2.7 g L^{-1} of potassium antimonyl tartrate, 15 mL of 40 g L^{-1} molybdate ammonium, and 30 mL of 17.6 g L^{-1} 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 PO_4^{3-} measurements. However, the concentration of the PO_4^{3-} was not adjusted for the absorbance in this study.

4. Results and discussion

4.1. 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 and 22.0 nmol min^{-1} g^{-1}, respectively, at the surface, but exponentially decreased with depth (Fig. 3). As seen in Fig. 3, two pathways of degradation (Belluomini et al., 1986) are plausible: at first, the degradation pathway for labile phosphatases under oxidative surface environment conditions proceeds exponentially, next the degradation pathway for inactive phosphatases proceeds asymptotically with time after burial below the surface layer. As shown in Figs. 3 and 5, the vertical profiles of total organic C and total S 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 N and 10–15% of total organic C 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 between TOC, THAA and TS. A positive correlation between TS and TOC in the sediments suggests that the mineral formation processes depend on the amount of organic matter in these environments which are rich in Fe and SO_4^{2-} (Wicks et al., 1991). The main source of S in sediments is microbially reduced SO_4^{2-}, which also contains organic S (Mitchell et al., 1984; Vairavamurthy et al., 1994). In anoxic subterranean

Table 2
The vertical profiles of total organic C (TOC), total S (TS), enzymatic activities, microorganisms and amino acid profiles in the sediment of Rikubetsu, Hokkaido, Japan

Depth (cm)	TOC (wt%)	TS (wt%)	Enzymatic activities		Microorganisms	Amino acid analogs		
			ALP (nmol/min/g)	ACP (nmol/min/g)	Cell density (×10^8 cells/g)	THAA (μmol/g)	Asp (μmol/g)	D/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 D/L ratio of aspartic acid (Asp) are cited from Takano et al., 2004a. Counting microorganisms as done by fluorescence microscopic method (Tsuji et al., 1995). Abbreviations: ALP, alkaline phosphatase; ACP, acid phosphatase.

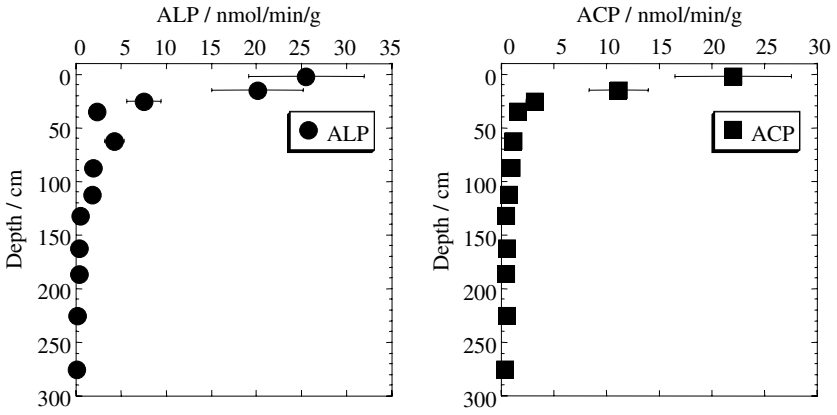

Fig. 3. Vertical distributions of alkaline phosphatase (ALP) and acid phosphatase (ACP) in sediment core samples at Rikubetsu, Hokkaido, Japan. Error bars represent the standard deviation of triplicate analysis.

environments, SO_4^{2-} (as well as Fe and Mn oxides) is an important electron acceptor in microbial oxidation of organic matter (Canfield, 1994).

4.2. Correlation between phosphatase activities and inorganic ions

Nitrate concentration also drastically decreased in concentration at lower depths by two orders of magnitude while PO_4^{3-} gradually increased with depth (Table 3). Subterranean microbial activity also produces NH_3 in the detritus component, and its free analogs may be converted to NH_4^+ ions and then finally oxidized to form NO_3^-. Similarly, there were notable increases in the relative abundances of β-alanine and γ-aminobutyric acid to THAA with depth. It has been reported that diagenesis in sediments causes decomposition of amino acids via decarboxylation (Ratcliff et al., 1974); for example, aspartic acid is converted to β-alanine by decarboxylation at the α-C (Andersson et al., 2000; Takano et al., 2004a). Another modification of sedimentary organic compounds is the deamination 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 P as PO_4^{3-}. Ecosystems have a high requirement for nutrients such as inorganic P and N (Kobayashi et al., 1987). As shown in Fig. 4 and Table 4, concentration of PO_4^{3-} is negatively correlated with ALP and ACP which suggests PO_4^{3-} is one of limiting factors for microbial growth. When PO_4^{3-} is insufficient to support growth, organisms may produce more alkaline phosphatase in order quickly to obtain a supply of PO_4^{3-}. On the other hand, when P concentrations are sufficiently high to meet the requirements of the organisms, the element key to the ecosystem is another nutrient element (Kobayashi et al., 1987).

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

Depth (cm)	Inorganic anions (μmol/l)			Inorganic cations (μmol/l)	
	PO_4^{3-}	NO^{3-}	Cl^-	Na^+	K^+
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

4.3. Phosphatase activity as a biomarker

Table 4 shows the correlations between 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 (Fig. 5). Also, many fibrous microorganisms are observed at the surface, and

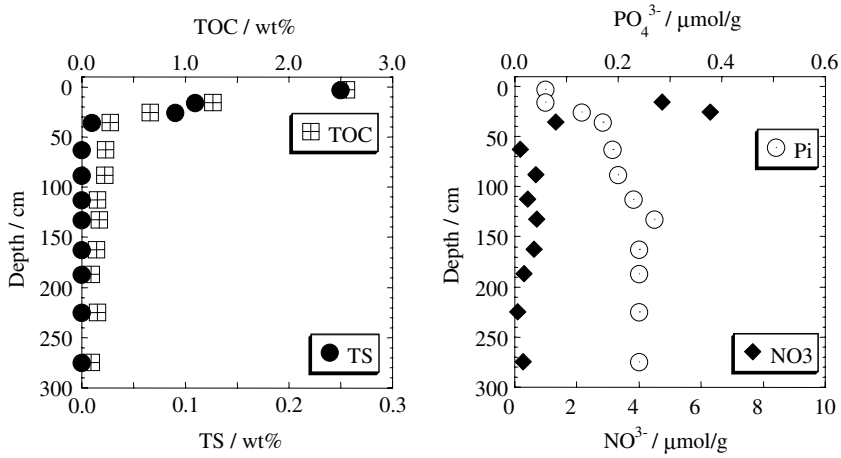

Fig. 4. Vertical distributions of total organic C (TOC), total S (TS), phosphate (PO_4^{3-}), and nitrate (NO^{3-}) in the sediment core samples at Rikubetsu, Hokkaido, Japan.

Table 4
The correlation of subterranean microbial cell density and several biomarkers in the sediment at Rikubetsu, Hokkaido, Japan

	Cell density	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	

Each value shows product–moment correlation coefficients (r).
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 hexosamine; TOC, total organic C; TS, total S; D/L, ratio of D- and L-aspartic acid; NPA, molar ratio of subtotal of non-protein amino acid (β-alanine and γ-aminobutyric acid).

many of these seem to be fungi based on their sizes, which is consistent with the aerobic requirements of 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 will 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 C (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

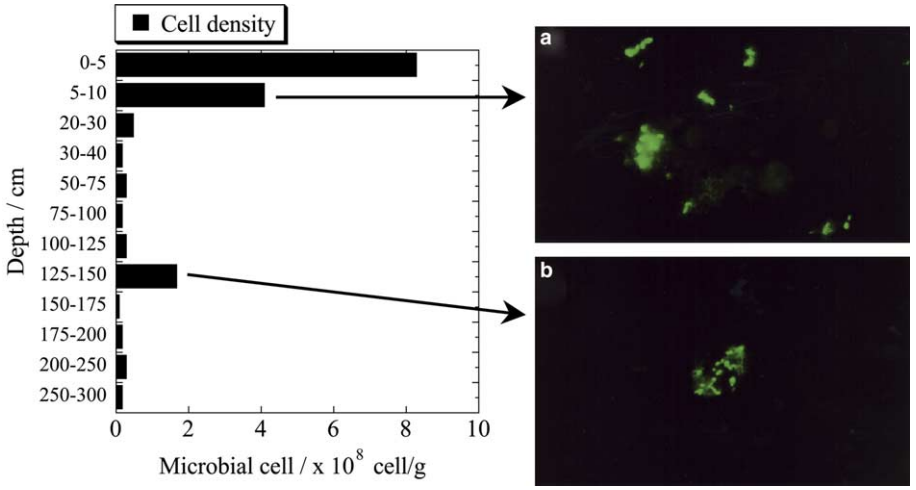

Fig. 5. Microbial cell density in sediment samples determined by fluorescence microscopy. The fluorescent bright spots represent subterranean microorganisms in the sediment at Rikubetsu, Hokkaido, Japan.

with 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 provides good evidence that phosphatase activity is a plausible new biomarker of subterranean microbial activity.

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

5. Conclusions

The present results suggests 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, the authors 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 at the frontiers of the biosphere, enzymatic activities are of interest for the distribution of the subterranean (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 Archaean Park Project, which integrates geology, biology and chemistry, is now in progress (Urabe et al., 2001).

Acknowledgements

The authors express their sincere thanks to two anonymous reviewers for constructive comments which helped to improve the earlier version of the manuscript. The authors also thank Associate Editor Dr. F. Grant Ferris for handling the manuscript and invaluable suggestions. The authors 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 funded by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan through the Special Co-ordination Fund for the Archaean 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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