Function of meiobenthos and microorganisms in cellulose breakdown in sediments of wetlands with different origins in Hokkaido

Kyohei Yamada ・ **Haruhiko Toyohara**

Division of Applied Biosciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Corresponding author

Haruhiko Toyohara

Tel/Fax: 81-075-753-6446

Email: toyohara@kais.kyoto-u.ac.jp

Abstract

To validate the mechanism of cellulose breakdown in cold climate wetlands, we investigated cellulase activity in sediments collected from 17 wetland sites in Hokkaido, the northern area of Japan. We evaluated cellulase activity by quantitative analysis of glucose released from carboxymethyl cellulose and found that sediments from peat fens demonstrated high activity, followed by sediments from lagoons and estuaries. Sediments from peat fens also contained greater amounts of organic matter, followed by lagoons and estuaries, thereby suggesting a strong positive correlation between organic matter content and cellulase activity. Evaluation of cellulase activity by qualitative cellulose zymographic analysis showed that various cellulases with different molecular sizes were implicated in cellulose breakdown in wetlands. Among them, cellulose breakdown in Meguma Pond (peat fen), Notsuke Gulf (peat fen) and Lake Utonai (lagoon) was potentially due to microorganism cellulase, while that in Lake Chobushi (lagoon) was ascribed to meiobenthos (Oligochaeta species) cellulase. The findings presented herein suggest that the origin and activity level of cellulase varied, depending on the types of cold climate wetlands.

Keywords: Cellulase・Cellulose・Cold district・Microorganism・Hokkaido・Meiobenthos・Sediment・Wetland

Introduction

Wetlands play ecologically important roles as breeding grounds and stopping points for migratory birds, as well as habitats for aquatic invertebrates, because of the richness of nutrients derived from rivers, lakes, and seas [1]. Cellulose, a component of plant cell walls, is a major organic material in the sediment of wetlands. Cellulose is a high-molecular-weight polysaccharide comprised of β-1,4-linked glucose residues and biochemically stable compared to starch, in which glucose residues are bound by α-1,4 linkages and α-1,6 linkages [2,3]. Cellulase, which is a general term for enzymes that belong to the glycoside hydrolase family and catalyzes the hydrolysis of the β-1,4-glycoside linkages of cellulose chains, includes endo-β-1,4-glucanase (EC 3.2.1.4) and cellobiohydrolase (EC 3.2.1.91). Endo-β-1,4-glucanase and cellobiohydrolase degrade cellulose to cellulodextrin or cellobiose, and another enzyme β-glucosidase (EC 3.2.1.21) further degrades them into glucose [4]. Cellulases from bacteria [5], filamentous fungi [6], basidiomycetes [7], myxomycetes [8], and protozoa [9] have been extensively studied. Occurrence of cellulase of which genes are encoded on chromosomes of their own have been reported from termite [10] and nematoda [11, 12]. Occurrence of these endogenous cellulases has also been reported in aquatic

animals, such as blue mussels, abalones, sea urchins [13, 14, 15], and brackish clam [16].

Cellulase and β-acetylglucosaminidase activities in sediments collected from various wetlands in Japan were measured as part of the research conducted for The International Collaborative Research on the Management of Wetland Ecosystem of the National Institute for Environmental Studies between 1998 and 2002 [17]. In this report, high cellulase activities were detected in the sediments from Lake Furen and Biwase River, located in the east area of Hokkaido Prefecture of Japan, and the activities were assumed to be derived from microorganisms. Recently, it was shown that the cellulase activities in these northern areas of Japan can be ascribed to meiobenthos, but not to microorganisms, and suggested that meiobenthos play an important role in the breakdown of cellulose, especially in cold climates [18]. Meiobenthos are defined as animal that pass through a 1-mm mesh filter and are known to be composed of a variety of fauna corresponding to 22 phyla [19].

There are many untouched wetlands in Hokkaido, which has the greatest number of wetlands on the registry of the 500 most important wetlands in Japan maintained by the Ministry of Environment [20] and Ramsar Convention [21]. Wetlands are classified as lakes, rivers, or estuaries. Hokkaido has many lakes, most of which are

classified as lagoons that were formed when a part of the sea was enclosed by land. Many lagoons are located in Hokkaido (e.g., Lake Saroma and Lake Furen). Land-derived organic matter accumulates more easily in lagoons than in estuaries, because lagoons have only a narrow mouth open to the sea [22]. Many peat fens are localized in the eastern and northern parts of Hokkaido, because cellulose breakdown by microorganisms is suppressed at low level due to low temperature throughout a year. For example, annual mean temperatures around Meguma Pond and Notsuke Gulf in 2010 were 6.7°C and 6.3°C, respectively (Japan Meteorological Agency Web: http://www.jma.go.jp/ "Accessed 19 August 2011".). Because enough amount of cellulose derived from undecayed plants in peat fens could be available, it is assumed that various cellulose consumers inhabit there [23]. Although various types of wetlands located in Hokkaido are presumed to be inhabited by diverse cellulose consumers such as microorganisms and meiobenthos, it remains unknown what types of organisms are mainly involved in cellulose breakdown in these wetlands.

In the present study, in order to evaluate cellulose breakdown in cold climate wetlands, we compared the degree of cellulose breakdown among the different types of wetlands in Hokkaido and tried to identify major cellulose consumers in these wetlands.

Materials and methods

Materials

Figure 1 shows the sampling sites and their latitude and longitude measured by a handy GPS (eTrex Vista HCx; Garmin, Olathe, KS, USA). Sampling was performed from early to mid-August 2010 and from mid-September to early October 2010. We collected sediments from 11 lagoons (Koetoi Onuma Pond, Lake Kuccharo, Lake Saroma, Lake Notoro, Lake Abashiri, Lake Furen, Mochirippu Pond, Lake Akkeshi, Pashikuru Pond, Lake Chobushi, and Lake Utonai), 2 peat fens (Notsuke Gulf and Meguma Pond), and 4 estuaries (Teshio River, Ishikari River, Mukawa River, and Saru River). Sediments from Lake Saroma, Lake Notoro, Lake Abashiri, Lake Akkeshi, Lake Furen, Notsuke Gulf, Mochirippu Pond, Pashikuru Pond, and Lake Chobushi were collected on August 9–12, 2010, and those from the other sites were collected from September 29 to October 2, 2010. We collected approximately 1 kg of sediments from a depth of 5 cm of each collecting site. We selected one collecting site apparently without plants for each wetland and transported these samples at 4°C back to the laboratory at Kyoto University. Sediment samples were stored at 4°C until analyses. Salt

Fig. 1

concentration of environmental water from each sampling site was measured by a salinometer (IS/Mill-E; AS ONE corporation, Osaka, Japan). Table 1 and Table 2 show salinity and composition of grain sizes of each wetland, respectively. Unless otherwise specified, special grades of reagents were commercially obtained from nacalai tesque (Kyoto, Japan).

Table 1

Table 2

Measurement of sediment cellulase activity by quantitative analysis

Cellulase activity of sediments was measured within 2 weeks of collection, according to the method of Hayano et al. [24], by using tetrazolium as a coloring agent [25]. Five grams (wet weight) of sediment, passed through a 2 mm-filter, was collected in a 50 ml-conical tube and added to 0.5 ml toluene for sterilization. Next, 10 ml of 0.2 M acetate buffer (pH 5.9) and 10 ml of 1% sodium carboxymethyl cellulose (CMC; Sigma, St Louis, MO, US) were added and incubated in a water bath at 30°C for 24 h with shaking. The same reaction mixture containing water instead of CMC was used as a control. After incubation, tubes were centrifuged at 8,000 × *g* for 5 min, and 100 µl of supernatant was added to a 1.5-ml tube. One milliliter of blue tetrazolium was added to the tube and heated at 100°C for 4 min in a block incubator (Block Incubator BI-525;

ASTEC, Fukuoka, Japan), and the absorbance at 660 nm was measured by a spectrophotometer (UV mini 1240; Shimadzu Corporation, Kyoto, Japan) after cooling. The value of the absorbance was converted to glucose concentration by using a standard curve of glucose (0–180 μg/ml) created at the same time. The pellet obtained by centrifugation was dried in a dryer (PS-420; ADVANTEC, Tokyo, Japan) at 60°C overnight to determine the dry weight. Cellulase activity was represented as the amount of glucose released from CMC per 1 g sediment (dry weight) per 1 h.

Isolation of meiobenthos

Meiobenthos were isolated alive from sediments within 1 week of collection. Meiobenthos were recovered in the fraction that included materials small enough to pass through a 1-mm mesh filter but too large to pass through a 63-μm mesh filter. Each meiobenthos was isolated under observation with a microscope (S2X12; Olympus, Tokyo, Japan). Classification of meiobenthos was performed at the level of Class according to Robert et al. [19] except for nematoda due to the difficulty in classification of this species. Classification of arthropods was performed according to Joei et al. [26]. We used single body of meiobenthos for qualitative cellulase assay and two bodies for

quantitative assay.

Cellulase activity of oligochaeta from Notsuke Gulf was measured quantitatively according to the modified method of Niiyama and Toyohara [27]. Briefly, two bodies of living oligochaeta were homogenized with cold 110 µl phosphate-buffered saline (PBS, containing 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Then, 3 µl of meiobenthos extract, 3 µl of 1 M sodium acetate buffer (pH 5.9), and 24 µl of 1% CMC solution were mixed. Reactions were carried out at 30°C and 4°C for 1, 3,7,12, and 24 h with shaking. After incubation, the mixtures were heated at 100°C for 3 min in the block incubator described above to terminate the enzyme reaction. The amount of reducing sugar produced was measured by the tetrazolium blue method [25]. The absorbance at 660 nm was measured with a UV-mini 1240 spectrophotometer.

Preparation and culture of cellulose breakdown microorganisms

Sediment was spread on an agar plate (1.5% agar containing 0.5% CMC, 0.15% $Ca(NO_3)_2$, 0.05% $MgSO_4$, 0.05% K_2HPO_4) and cultured at 25°C for 1 week. Autoclaved 0.1% soft agar was then added to the cultured plate, and the surface of the

plate containing microorganisms was scraped with a bacteria spreader. Soft agar containing cultured microorganisms was added to a liquid culture medium (0.5%CMC, 0.15% $Ca(NO_3)_2$, 0.05% $MgSO_4$, and 0.05% K_2HPO_4) and cultured at 25°C for 1 week. Culture medium was then filtered through paper filter (No. 1; Toyo Roshi Kaisha, Tokyo, Japan), and the filtrate was used for SDS-PAGE zymographic analysis.

Measurement of cellulase activity by qualitative analysis with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) zymography

An aliquot of sediment and a 1/5 volume of 6 × SDS sample buffer (containing 0.6 M Tris-HCl (pH 6.8), 60% glycerol, 6% SDS, and 0.06% bromophenol blue) were mixed with a homogenizer (HandySonic UR-20P; TOMY SEIKO, Tokyo, Japan), incubated on ice for 2 h, and centrifuged at 8,000 × *g* for 5 min. The supernatant was used for SDS-PAGE zymographic analysis.

Meiobenthos were picked up from the sediments one by one using a pair of tweezers under a binocular microscope (S2X12; Olympus, Tokyo, Japan), and each was then homogenized alive with cold 20 µl PBS to prepare a meiobenthos extract for SDS-PAGE zymographic analysis. Approximate lengths of each meiobenthos are as

follows. A nematoda obtained from Meguma Pond is 2-3 mm long and that from Lake Utonai is 4 mm long. An oligochaeta species from Meguma Pond, Lake Notoro and Lake Utonai is 1-2 mm long, 4 mm long, and 8 mm long, respectively. A polychaeta species from Lake Utonai is 1-2 mm long. Maxillopoda species from Meguma Pond is 1 mm long.

Cellulase zymographic analysis was performed using 7.5% SDS-PAGE gels containing 0.1% CMC. After electrophoresis, the gels were soaked in 10 mM acetate buffer (pH 5.5) containing 0.1% TritonX-100 for 30 min to remove SDS from the gels. The gels were transferred to 10 mM acetate buffer (pH 5.5), incubated at 37°C or 4°C overnight, and then stained with 0.1% Congo Red. In case of sediment of Notsuke Gulf, the gel was incubated for 4 days because of low activity. The gels were destained using 1 M NaCl. The active bands were detected as nonstained bands.

Measurement of organic component ratio

Dried sediment obtained as mentioned above was heated in a mantle heater (KCA-10A; Koito, Tokyo, Japan) at 600°C for 3 h [28]. Organic component ratio (%)

was calculated according to the formula below.

Organic component ratio (%) = [(dry weight – ignition weight)/(dry weight)] × 100

Results

Comparison of cellulase activity level by quantitative cellulase analysis

Among 17 wetland sites in Hokkaido, Meguma Pond showed the highest cellulase activity (peat fen, 737.88 nmol/gh, Table 1), followed by Notsuke Gulf (peat fen, 92.39 nmol/gh), Lake Utonai (fresh water lagoon, 44.45 nmol/gh), Lake Saroma (lagoon, 28.48 nmol/gh), Lake Akkeshi (lagoon, 21.42 nmol/gh), and Lake Notoro (lagoon, 13.86 nmol/gh), as summarized in Table 1. Sediments from the estuaries of the Teshio River, Ishikari River, Mukawa River, and Saru River showed little or no cellulase activity.

Qualitative analysis of cellulases by SDS-PAGE zymography

Fig. 2

Among 17 wetlands in Hokkaido, active cellulase bands were detected in all

samples by SDS-PAGE zymographic analysis, except for sediments from Pashikuru Pond, Mukawa River, Saru River, and Lake Abashiri (data not shown). For meiobenthos, active cellulase bands were detected in the Oligochaeta species in Meguma Pond (Fig. 2), Notsuke Gulf (Fig. 2), Lake Notoro and Lake Abashiri (data not shown), Lake Chobushi (Fig. 2), Lake Utonai (Fig. 2), Ishikari River, and Koetoi Onuma Pond (data not shown); Malacostraca species in Lake Kuccharo (data not shown); Nematoda species in Lake Saroma (data not shown); Foraminifera species in Lake Akkeshi (data not shown); and Polychaeta species in Teshio River (data not shown).

As shown in Fig. 2a, sediment from Meguma Pond demonstrated activity as a broad smear above 38 kDa. For meiobenthos, Oligochaeta species showed an active band at 48 kDa, but Nematoda species and Maxillopoda species showed no activity. However, culture medium of microorganisms showed an active band of high molecular weight (above 199 kDa).

Figure 2b shows the cellulase activity from the Notsuke Gulf sample. Sediment exhibited intensive active bands at 33 and 87 kDa and faint active bands at 49, 146, 172 and 244 kDa, while Oligochaeta species showed at 26, 29 and 30 kDa. On the other hand, culture medium of microorganisms showed active bands at 108, 146, 172 and 244 kDa.

Figure 2c shows the cellulase activity from the Lake Notoro sample. Sediment showed weak active bands at 24, 30, and 58 kDa. Oligochaeta species showed a strong active band at 28 kDa and a weak active band at 29 kDa. Ostracoda species demonstrated a weak active band at 27 kDa, while the culture medium of microorganisms showed active bands at 49, 108, and 230 kDa.

Figure 2d shows results from the Lake Chobushi sample. Sediment showed active bands at 33, 59, and 62 kDa, while Oligochaeta species showed active bands at 30, 33, 36, 38, 43, 59, and 62 kDa. Although smear active bands were detected by 24 h-incubation because of the intensive cellulase activity of Oligochaeta species, sharp bands could be detected by 10 h-incubation.

Figure 2e shows the results from the Lake Utonai sample. Sediment showed active bands at 46, 65, and 105 kDa. Nematoda species showed no activity, while Oligochaeta species showed an active band at 68 kDa.

Demonstration of cellulase activity of meiobenthos at low temperature

Fig. 3

As shown in Fig.3, Oligochaeta species demonstrated the substantial cellulase activity bands at 4°C in zymographic analysis, of which activity levels were corresponded to those at 37°C. Oligochaeta species in Notsuke Gulf showed 29 and 30

kDa active bands, while those in Lake Chobushi showed 36, 38, 43 and 59 kDa active bands.

Figure 4 shows the cellulase activity of oligochaeta species in Notsuke Gulf. Fig.4 Higher activity was detected at 30°C than at 4°C. It should be stressed that the activity level at 4°C was almost corresponded with 30% of that at 30°C.

Relationship between the amount of organic matter and cellulase activity level

As shown in Table 1, sediment from peat fens such as Meguma Pond and Notsuke Gulf contained large amounts of organic matter, 66.6% and 16.9%, respectively. Sediments from lagoons such as Lake Saroma, Lake Akkeshi, and Lake Utonai contained 1.5%, 6.4%, and 1.5% organic matter, respectively. Sediments from the estuaries of the Teshio River, Ishikari River, and Saru River contained 1.0%, 0.1%, and 0.1% organic matter, respectively. There was a strong positive correlation (r = 0.96) between the amount of organic matter and the cellulase activity level among sediments collected from 17 wetlands.

Discussion

We measured cellulase activity in sediments collected from 17 wetlands in Hokkaido to evaluate cellulose breakdown in cold climates. According to our quantitative analysis (Table 1), sediments from peat fens showed the highest cellulase activity, followed by those from lagoons and estuaries so far as measured on August and September in the specific collecting site.

SDS-PAGE zymographic analysis revealed that the molecular size of active cellulase bands in sediments from Notsuke Gulf (peat fen) corresponded with those from culture medium of microorganisms. To confirm microorganism cellulases actually act at cold temperature, we measured activity at 4°C. As shown in Fig. 3(a), culture medium of microorganisms showed active bands of 146 and 172 kDa, suggesting that microorganism cellulases might play any function in cellulose breakdown in Notsuke Gulf. The molecular sizes of active cellulase bands in the sediments of Lake Chobushi (lagoon) corresponded with those from meiobenthos. These findings suggest that microorganisms and meiobenthos play important roles in cellulose breakdown, especially in these wetlands in Hokkaido. However, the possibility that the molecular sizes of cellulase active bands of sediments and microorganisms/meiobenthos apparently coincided is not completely ruled out. Further

immunological analysis is needed to validate that the active bands of sediments were derived from microorganisms or meiobenthos.

Oligochaeta showed a strong active band that did not coincide with any bands in the sediment samples from Lake Notoro (Fig. 2c). Despite the fact, it is assumed that Oligochaeta species could play any function in cellulose breakdown in Hokkaido, together with the fact that oligochaeta played an important role in Lake Chobushi as described above. As shown in Fig.3, Oligochaeta species demonstrated the substantial cellulase activity at 4°C in qualitative analysis. Oligochaeta species in Notsuke Gulf actually demonstrated the activity at 4°C almost corresponded with 30% of that at 30°C (Fig.4), suggesting that meiobenthos might play any role to degrade plant residues at low temperature. Since same active bands were demonstrated at 4°C and 37°C, these Oligochaeta species were assumed to possess cellulases active at broad temperature range.

As shown in Table 1, a strong positive correlation was observed between the amount of organic matter and the cellulase activity level. Based on the following facts; (i) organic matters are assumed to be derived from plant residues [29], (ii) in Meguma Pond and Notsuke Gulf where high content of organic matters are detected in sediments, cellulase activity of sediments was derived from microorganisms (Figs. 2a and b), and

(iii) microorganisms secrete cellulases extracellularly[30], (iv) Liu and Toyohara reported that fungal cellulase actually bound to plant residues [31], it seems likely that cellulases secreted from microorganisms would bind to plant residues and degrade them in the wetlands of peat fen sediments. In our preliminary experiments, cellulases from *Corbicula japonica* bound to plant residues similar to fungal cellulases (data not shown), meiobenthos cellulases would function as sediment-binding form in sediment of Hokkaido wetlands.

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References

1. Beukema JJ (1979) Biomass and species richness of the macrobenthic animals living on a tidal flat area in the Dutch Wadden sea- effects of a severe winter. Neth

J Sea Res 13:203-223

2. Cosgrove DJ (2005) Growth of the plant cell wall. Nat Rev Mol Cell Biol 6:850-861

3. Vries RP, Visser J (2001) *Aspergillus* enzymes involved in breakdown of plant cell wall polysaccharides. Microbiol Rev 65:497-522

4. Watanabe H, Tokuda G (2010) Cellulolytic systems in insects. Annu Rev Entomol 55:609-632

5. Olson DG, Tripathi SA, Giannone RJ, Lo J, Caiazza NC, Hogsett DA, Hettich RL, Guss AM, Dubrovsky G, Lynd LR (2010) Deletion of the Cel48S cellulase from *Clostridium thermocellum*. Proc Natl Acad Sci 107:17727-17732

6. Trinci APJ, Davies DR, Gull K, Lawrence MI, Nielsen BB, Rickers A, Theodorou MK (1994) Anaerobic fungi in herbivorous animals. Mycol Res 98:129-152

7. Chow CM, Yague E, Raguz S, Wood DA, Thurston CF (1994) The *cel3* gene of *Agaricus-bisporus* codes for a modular cellulase and is transcriptionally regulated by the carbon source. Appl Environ Microbiol 60:2779-2785

8. Ronsness PA (1968) Cellulolytic enzymes during morphogenesis in *Dictyostelium discoideum*. J Bacterial 96:639-645

9. Bera-Maillet C, Devillard E, Cezette M, Jouany JP, Forano E (2005) Xylanases and

carboxymethylcellulases of the rumen protozoa *Polyplastron multivesiculatum*, *Eudiplodinium maggii* and *Entodinium* sp. FEMS Microbiol Let 244:149-156

10. Watanabe H, Noda H, Tokuda G, Lo N (1998) A cellulase gene of termite origin. Nature 394:330-331

11. Smant G, Stokkermans PWGJ, Yan Y, Boer de MJ, Baum JT, Wang XH, Hussey RS, Gommers FJ, Henrissat B, Davis EL, Helder J, Schots A, Bakker J (1998) Endogenous cellulases in animals: Isolation of β-1,4-endoglucanase genes from two species of plant-parasitic cyst nematodes. Proc Natl Acad Sci USA 95:4905-4911

12. Kikuchi T, Shibuya H, Jones TJ (2005) Molecular and biochemical characterization of an endo-β-1,3-glucanase from the pinewood nematode *Bursaphelenchus xylophilus* acquired by horizontal gene transfer from bacteria. Biochem J 389:117-125

13. Xu BZ, Janson JC, Sellos D (2001) Cloning and sequencing of a molluscan endo-beta-1,4-glucanase gene from the blue mussel, *Mytilus edulis.* Eur J Biochem 268:3718-3727

14. Suzuki K, Ojima T, Nishita K (2003) Purification and cDNA cloning of a cellulase from abalone *Haliotis discus hannai.* Eur J Biochem 270:771-778

15. Nishida Y, Suzuki K, Kumagai Y, Tanaka H, Inoue A, Ojima T (2007) Isolation and

primary structure of a cellulase from the Japanese sea urchin *Strongylocentrotus nudus.* Biochimie 89:1002-1011

16. Sakamoto K, Touhata K, Yamashita M, Kasai A, Toyohara H (2007) Cellulose digestion by common Japanese freshwater clam *Corbicula japonica*. Fish Sci 73:675-683

17. National Institute for Environmental Studies, Japan (2003) International collaborative research on the management of wetland ecosystem (in Japanese). Report of special research from the National Institute for Environmental Studies, Japan, pp 8-13

18. Toyohara H, Park Y, Tsuchiya K, Liu W (2011) Cellulase activity in meiobenthos in wetlands. Fish Sci 78:133-137

19. Robert PH, Hjalmar T (1988) Introduction to the study of meiofauna. Smithsonian Institution Press, Washington, D.C, pp 243-354

20. Nature Convention Bureau in Japanese Ministry of the Environment (2002) Five hundred important wetlands in Japan (in Japanese). Nature Convention Bureau, Tokyo

21. Ramsar Convention Secretariat (2011) The list of wetlands of international importance. Ramsar Convention Secretariat, Ramsar, pp 21-22

22. Wit R, Stal LJ, Lomstein BA, Herbert RA, van Gemerden H, Viaroli P, Cecherelli VU, Rodriguez-Valera F, Bartoli M, Giordani G, Azzoni R, Schaub B, Welsh DT, Donnelly A, Cifuentes A, Anton J, Finster K, Nielsen LB, Pedersen AGU, Neubauer AT, Colangelo MA, Heijs SK (2001) The role of buffering capacities in stabilising coastal lagoon ecosystems. Continent Shelf Res 21:2021-2041

23. Artz RRE, Anderson IC, Chapman SJ, Hagn A, Schloter M, Potts JM, Campbell CD (2007) Changes in fungal community composition in response to vegetational succession during the natural regeneration of cutover peatlands. Microbiol Ecol 54: 508-522

24. Soil Microbiological Society (1992) Experimental methods for soil microbiology (in Japanese). Yokendo, Tokyo, pp 370-371

25. Chong K J, Peter N L (1985) Determination of reducing sugars in the nanomole range with tetrazolium blue. J Biochem Biophys Methods 11:109-115

26. Joei WM, Gorge ED (2001) An updated classification of the recent crustacean. Natural History Museum of Los Angeles Country, Los Angeles, pp 1-124

27. Niiyama T, Toyohara H (2011) Widespread distribution of cellulase and hemicellulase activities among aquatic invertebrates. Fish Sci 77: 649-655

28. Hwang I H, Ouchi Y, Matsuto T (2007) Characteristics of leachate from pyrolysis

residue of sewage sludge. Chemosphere 68:1913-1919

29. Nguyen LM (2000) Organic matter composition, microbial biomass and microbial activity in gravel-bed constructed wetlands treating farm dairy wastewaters. Ecol Eng 16:199-221

30. Barnet CC, Berka RM, Fowler T (1991) Cloning and amplification of the gene encoding an extracellular bold β-glucosidase from *Trichoderma reesei*: Evidence for improved rates of saccharification of cellulosic substrates. Nat Biotechnol 9:562-567

31. Liu W, Toyohara H (2012) Sediment-complex-binding cellulose breakdown in wetlands of rivers. Fish Sci. Doi: 10.1007/s12562-012-0471-y

Figure captions

Figure 1 Sampling sites of wetlands in Hokkaido. Geological types of wetlands are classified into 3 types: lagoon, peat fen, or estuary. Letters indicating sampling sites correspond to those in Table 1 and Table 2.

Figure 2 Qualitative analysis of cellulase activity by SDS-PAGE cellulose zymography at 37°C. (a) Meguma Pond: Lane 1, sediment; lane 2, Nematoda; lane 3, Oligochaeta; lane 4, Maxillopoda; lane 5, microorganisms. (b) Notsuke Gulf: Lane 1, sediment; lane 2, Oligochaeta; lane 3, microorganisms. (c) Lake Notoro: Lane 1, sediment; lane 2, Oligochaeta; lane 3, *Ostracoda*; lane 4, microorganisms. (d) Lake Chobushi: lane 1, sediment; lane 2, Oligochaeta (24h-incubation); lane 3, Oligochaeta (10 h-incubation). (e) Lake Utonai: Lane 1, sediment; lane 2, Nematoda; lane 3, Oligochaeta; lane 4, microorganisms. Note that active bands of each animal do not reflect the enzyme activity level correctly. *Asterisks* mean that the animal belongs to meiobenthos.

Figure 3 Qualitative analysis of cellulase activity of oligochaeta species from Notsuke Gulf (a) and Lake Chobushi (b). (a) Notsuke Gulf: Lane 1, sediment; lane 2,

Oligochaeta; lane 3, microorganism. *Asterisks* mean that the animal belongs to meiobenthos.

Figure 4 Cellulase activity of oligochaeta species in Notsuke Gulf at 4°C and 37°C as a function of time. Values are mean ± standard deviation (n=3).

森岡克司先生
前略
このたびはご審査賜りありがとうございました。1 名の審査員のコメントに対し下記のように対応いたしました。審査員の指示に従い追加実験を行ったため，訂正原稿の提出が遅れたことをお詫びいたします。ご審査のほど，よろしくお願いいたします。
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Major points

1. Fig. 2 について

1-1 バンドパターンについて
前回から変更されたようですが、今一つ明瞭なバンドが見えているようには感じません。特に Fig. 2b の sediment のレーンの 121 と 172kDa のバンドはどう見ても（PC 画面上でも印刷しても）はっきりとは見えません。この図ではとても読者を納得させることはできませんので、sediment のレーン添加量を増やすか、反応時間を長くすることにより明瞭なバンドを提示してください。Fig. 2e についてもゲル上部にスタックしているという意味では sediment と microorganism のセルラーゼは同じ性質をもつのかもしれませんが、これで両者を同じものであると類推するのは無理があると感じます。上記二つのデータは line252-253 に記述されているように、本論文中で非常に重要な論拠となるデータですので、Fig. 2b は再試を奨めます。Fig. 2e は再試で良い結果が出ないようであれば、sediment の高分子量のバンドを microorganism に帰着させる記述を削除した方が良いと思います。

―ご指摘に従い Fig. 2b の野付湾底泥については 4 日間の反応を行うことで、明確な活性バンドを検出することができたので、そのデータと差し替えました。訂正した部分は以下の通りです。

L186-187:In case of sediment of Notsuke Gulf, the gel was incubated for 4 days because of low activity. The gels were destained using 1 M NaCl. The active bands were detected as nonstained bands.

L226-230:Sediment exhibited intensive active bands at 33 and 87 kDa and faint active bands at 49, 146, 172 and 244 kDa, while Oligochaeta species showed at 26, 29 and 30 kDa. On the other hand, culture medium of microorganisms showed active bands at 108, 146, 172 and 244 kDa.

―Fig.2eについては再試を行ってもバンドがスタックしてしまったためsedimentの高分子バンドをmicroorganismに帰着させる記述を削除しました。訂正した部分は以下の通りです

L273-275:SDS-PAGE zymographic analysis revealed that the molecular size of active cellulase bands in sediments from Notsuke Gulf (peat fen) corresponded with those from culture medium of microorganisms.

1-2　分類に関して

・Nematoda, Oligochaeta, Harpcitcoida, Ostracoda,Polychaetaはそれぞれmeiobenthosに属する種類であり、microorganismに比して解析していることは図中に矢印、もしくは括弧等で示された方が分かりやすいと思います。

―ご指摘に従いそれぞれの生物がMeiobenthosであることが分かり易くなるように図中に＊で示しました。またそれに伴いFigure captionに記述を加えました。訂正した部分は以下の通りです。

L418: *Asterisks* mean that the animal belongs to meiobenthos
L422-423:*Asterisks* mean that the animal belongs to meiobenthos

・またHarpcitcoidaとOstracodaは斜体になっています。一般に分類表記で斜体は学名の属、種に使うもので、Harpcitcoida（ソコミジンコ目）、Ostracoda（カイムシ下綱）等には使わないように感じます。もしなんらかの理由があるなら説明が必要かと思います。

―ご指摘の通り、表記を訂正しました。訂正した部分は以下の通りです。

L233:Ostracoda species demonstrated a weak active band at 27 kDa.
L414-415:(c) Lake Notoro: Lane 1, sediment; lane 2, Oligochaeta; lane 3, Ostracoda; lane 4, microorganisms.

L180:Maxillopoda species from Meguma Pond is 1 mm long.
L223:but Nematoda species and Maxillopoda species showed no activity.
L411-412:Meguma Pond: Lane 1, sediment; lane 2, Nematoda; lane 3, Oligochaeta; lane 4, Maxillopoda; lane 5, microorganisms.

・さらに、これは単なる suggestion ですが、上記の Nematoda（線形動物門）、Oligochaeta（貧毛綱）、Polychaeta（多毛綱）、Harpcitcoida（ソコミジンコ目）、Ostracoda（カイムシ下綱）は門、綱、目と分類の階層がまちまちです。Meiobenthos の分類は非常に難解なようですが、統一された方が良いようにも思います。これに関して Introduction 中で meiobenthod に関する良い詳細な説明があった方が良いと思います

―ご指摘の通り、網(class)レベルに統一しました。節足動物門の分類体系は Joei らの分類に従い(26)、Harpacticoida （目)は Maxillopoda(綱)へ、Tanaidacea は Malacostraca （綱)へと表記を訂正しました。Ostracoda は Joei らの分類では網であるため、そのまま表記しました。Nematoda に関しましては網レベルの分類が非常に困難であるため、例外として門レベルで書きその旨を 138 行目に書き加えました。訂正した部分は以下の通りです。
また 62-64 行目にメイオベントスの定義、説明及び引用文献を加えました。訂正した部分は以下の通りです。

L62-64:Meiobenthos are defined as animal that pass through a 1-mm mesh filter and are known to be composed of a variety of fauna corresponding to 22 phyla [19].
L138-139:Classification of meiobenthos was performed at the level of Class according to Robert et al. [19] except for nematoda due to the difficulty in classification of this species. Classification of arthropods was performed according to Joei et al. [26].
L180:Maxillopoda species from Meguma Pond is 1 mm long.
L223:but Nematoda species and Maxillopoda species showed no activity.
L411-412:Meguma Pond: Lane 1, sediment; lane 2, Nematoda; lane 3, Oligochaeta; lane 4, Maxillopoda; lane 5, microorganisms.
L218: Malacostraca species in Lake Kuccharo (data not shown);

2.　　Fig. 3 について
決まった温度の影響を定性的に見ていることに非常に疑問を持ちます。
・4℃と 30℃での酵素活性を定量化することはできませんか？

―野付湾については新たに 4℃と 30℃で定量的に活性を測定し、その経時的変化を図４として追加致しました。この追加に伴い、測定法について新たに下記の文章を追加致しました。長節湖については試料が残っていないため前回と同じく定性的評価のみに留めました。
追加した部分は以下の通りです。

L142-152: Cellulase activity of oligochaeta from Notsuke Gulf was measured quantitatively according to the modified method of Niiyama and Toyohara [27]. Briefly, two bodies of living oligochaeta were homogenized with cold 110 µl phosphate-buffered saline (PBS, containing 140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Then, 3 µl of meiobenthos extract, 3 µl of 1 M sodium acetate buffer (pH 5.9), and 24 µl of 1% CMC solution were mixed. Reactions were carried out at 30°C and 4°C for 1, 3,7,12, and 24 h with shaking. After incubation, the mixtures were heated at 100°C for 3 min in the block incubator described above to terminate the enzyme reaction. The amount of reducing sugar produced was measured by the tetrazolium blue method [25]. The absorbance at 660 nm was measured with a UV-mini 1240 spectrophotometer.
L251-252: Figure 4 shows the cellulase activity of oligochaeta species in Notsuke Gulf. Higher activity was detected at 30°C than at 4°C. It should be stressed that the activity level at 4°C was almost corresponded with 30% of that at 30°C.
L291-295: As shown in Fig.3, Oligochaeta species demonstrated the substantial cellulase activity at 4°C in qualitative analysis. Oligochaeta species in Notsuke Gulf actually demonstrated the activity at 4°C almost corresponded with 30% of that at 30°C (Fig.4), suggesting that meiobenthos might play any role to degrade plant residues at low temperature.
L425-426: Figure 4 Cellulase activity of oligochaeta species in Notsuke Gulf at 4°C and 37°C as a function of time. Values are mean ± standard deviation (n=3).

・また line266 にあるように Oligochaeta が 4℃のセルロース分解において重要であるという論調にするならば、Fig.2b を 4℃で行い、sediment と microorganism のバンドも同時に考察すべきです。

―ご指摘に従い sediment、Oligochaeta、microorganism について 4℃でザイモグラフィーを行い、その結果を Fig.3a として元のものと差し替えました。

Fig３a に示すように、Oligochaeta は 4℃で活性バンド（29,30 kDa）を示したことから、低温度においてもセルロース分解を有しており、底泥中のセルロース分解になんらかの役割を果たしていることが推測されます。しかし、底泥自体のセルロース分解バンド（172,146 kDa）と Oligochaeta のバンドのサイズは一致しないことから、野付湾底泥において Oligochaeta のセルラーゼは主役ではないと考えられます。したがって旧原稿で 267 行目に記述しました「重要な」という表現は正確ではないので、新たな原稿では下記のように訂正いたしました。

L294-295:, suggesting that meiobenthos might play any role to degrade plant residues at low temperature.
L273-279:SDS-PAGE zymographic analysis revealed that the molecular size of active cellulase bands in sediments from Notsuke Gulf (peat fen) corresponded with those from culture medium of microorganisms. To confirm microorganism cellulases actually act at cold temperature, we measured activity at 4°C. As shown in Fig. 3(a), culture medium of microorganisms showed active bands of 146 and 172 kDa, suggesting that microorganism cellulases might play any function in cellulose breakdown in Notsuke Gulf.

3.　　Sediment とセルラーゼの関係について
・審査員 1 も指摘しているように sediment 中のセルラーゼと meiobenthos、microorganism のセルラーゼの関係が今一つ不鮮明です。meiobenthos、microorganism から分泌されたセルラーゼが sediment 中の因子に結合しているという記述（出来れば引用文献）があった方がよいと思います。

―最近私どもの研究室から菌のセルラーゼが底泥成分、特に植物残渣に強く吸着するということを示す論文を発表いたしました（参考文献 31）。また予備実験ではありますが、ヤマトシジミのセルラーゼが同様に植物残渣等の底泥成分に吸着性を示す結果も得ております。これらの内容を踏まえ新たに、行目に「北海道の泥炭湿地において、微生物由来のセルラーゼが底泥成分に吸着して活性を発現している可能性があること、及びメイオベントス由来のセルラーゼも同様に北海道湿地帯において底泥成分に吸着した形で活性を発現している可能性があること」を示す文章を追加致しました。新たに挿入した部分は以下の通りです。

L303-309:(iv) Liu and Toyohara reported that fungal cellulase actually bound to plant residues [31], it seems likely that cellulases secreted from microorganisms would bind to plant residues and degrade them in the wetlands of peat fen sediments. . In our preliminary experiments, cellulases from *Corbicula japonica* bound to plant residues similar to fungal cellulases (data not shown), meiobenthos cellulases would function as sediment-binding form in sediment of Hokkaido wetlands.

Minor points

Line 25
--Lake Utonai (lagoon) was potentially due to fungal cellulose
→ Lake Utonai (lagoon) was potentially due to microorganism cellulose
―ご指摘に従い訂正いたしました。訂正した箇所は以下の通りです。

L25-26: Lake Utonai (lagoon) was potentially due to microorganism cellulose

Line 59
Recently, we showed
→ Recently, it was shown （文献 18 に本論文著者の名前なし）
―ご指摘に従い訂正いたしました。訂正した箇所は以下の通りです

L59-62: Recently, it was shown that the cellulase activities in these northern areas of Japan can be ascribed to meiobenthos, but not to microorganisms, and suggested that meiobenthos play an important role in the breakdown of cellulose, especially in cold climates [18].

和文要旨

成因が異なる北海道の湿地帯底泥におけるセルロース分解に果たすメイオベントスと微生物の役割

山田京平，豊原治彦（京大院農）

寒冷地湿地帯のセルロース分解機構を明らかにする目的で，北海道の湿地帯 17 か所の底泥のセルロース分解活性を測定した。その結果，泥炭湿地が特に活性が高く，海跡湖，河口域の順に活性は低下した。活性の定性分析の結果，メグマ沼（泥炭湿地），野付湾（泥炭湿地）及びウトナイ湖（海跡湖）では微生物が，長節湖（海跡湖）ではメイオベントスが分解に関わっていることが示された。以上の結果から，寒冷地湿地帯底泥のセルロース分解には微生物がやメイオベントス由来のセルラーゼが重要な働きを果たしていることが示唆された。

キーワード：寒冷地，菌類，湿地帯，セルロース，セルラーゼ，底泥，北海道，メイオベントス

Figure

Fig.1

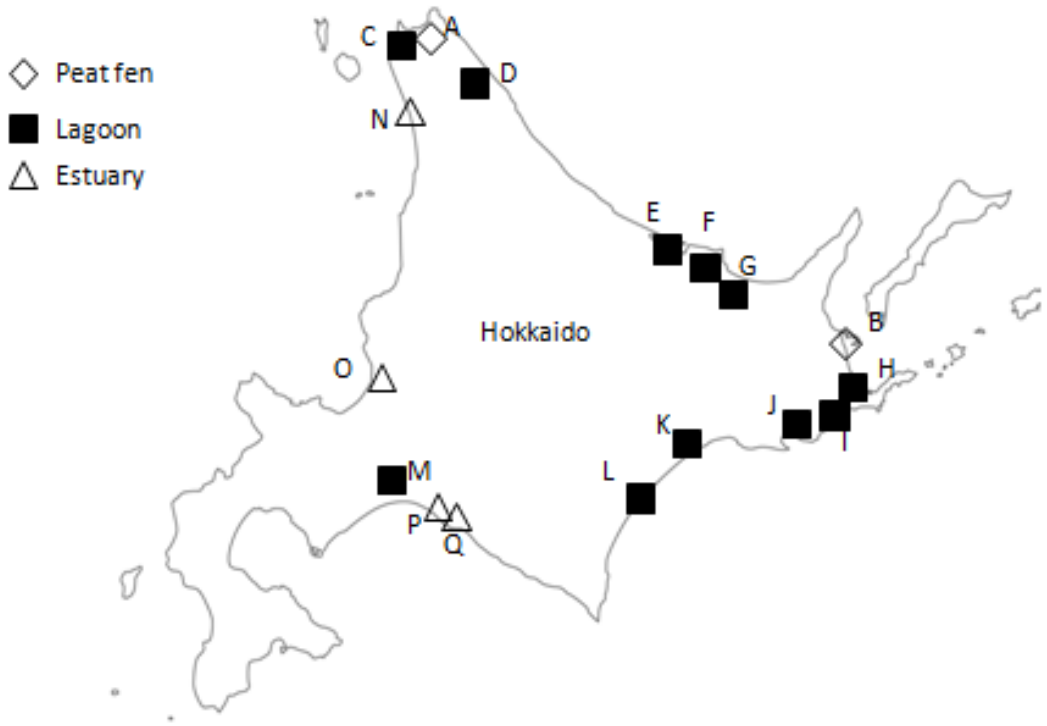


Fig.2

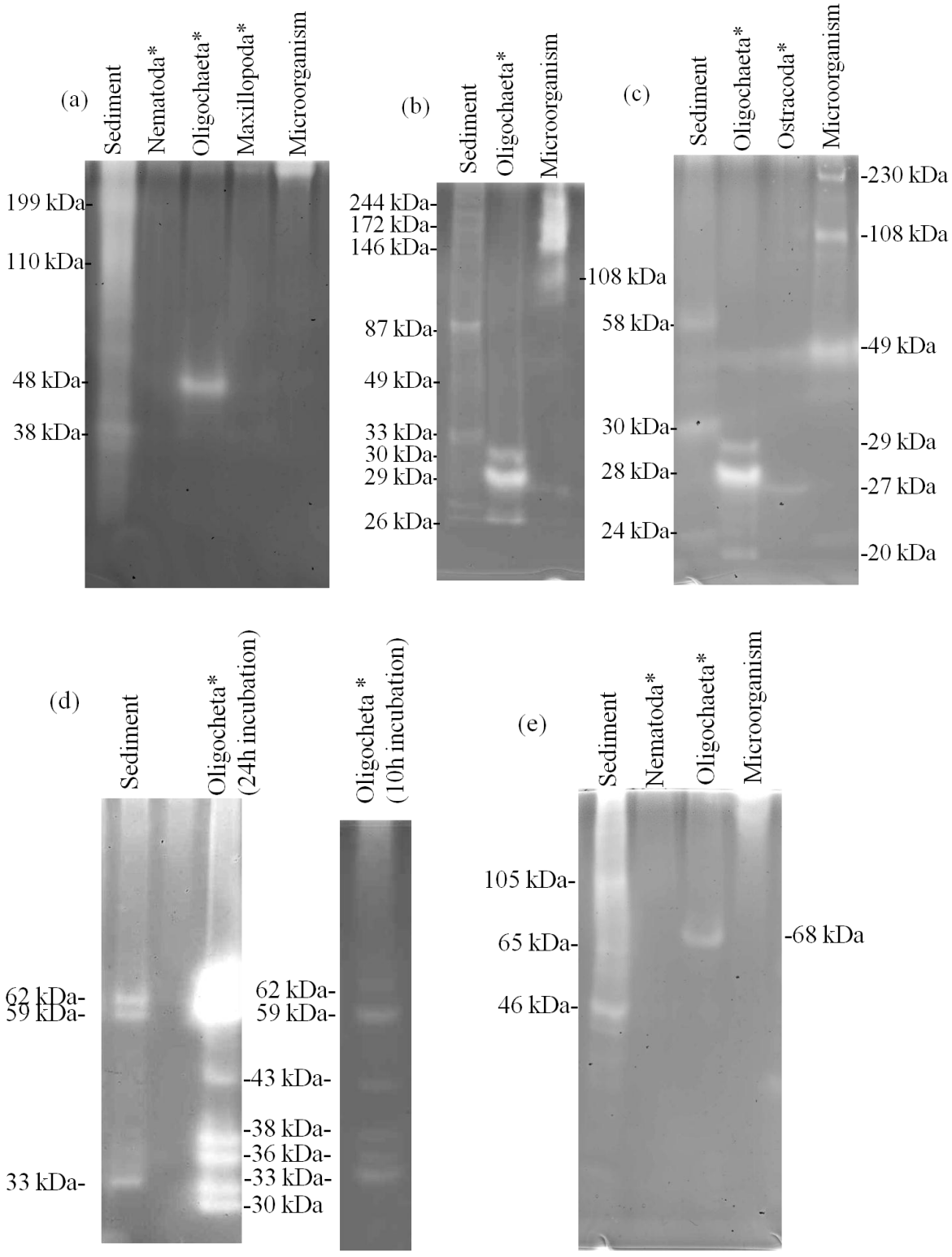

(a)
Sediment
Nematoda*
Oligochaeta*
Maxillopoda*
Microorganism
199 kDa-
110 kDa-
48 kDa-
38 kDa-
(b)
Sediment
Oligochaeta*
Microorganism
244 kDa-
172 kDa-
146 kDa-
87 kDa-
49 kDa-
33 kDa-
30 kDa-
29 kDa-
26 kDa-
(c)
Sediment
Oligochaeta*
Ostracoda*
Microorganism
-108 kDa
58 kDa-
30 kDa-
28 kDa-
24 kDa-
-230 kDa
-108 kDa
-49 kDa
-29 kDa
-27 kDa
-20 kDa
(d)
Sediment
Oligocheta* (24h incubation)
Oligocheta * (10h incubation)
62 kDa-
59 kDa-
33 kDa-
62 kDa-
59 kDa-
-43 kDa-
-38 kDa-
-36 kDa-
-33 kDa-
-30 kDa
(e)
Sediment
Nematoda*
Oligochaeta*
Microorganism
105 kDa-
65 kDa-
46 kDa-
-68 kDa

Fig.3

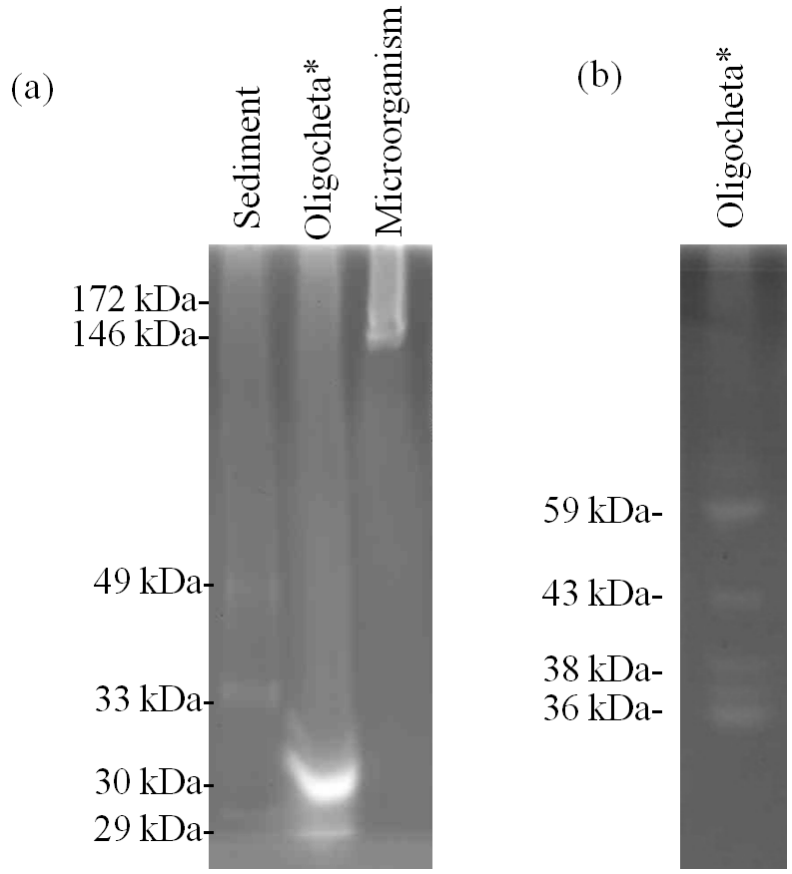
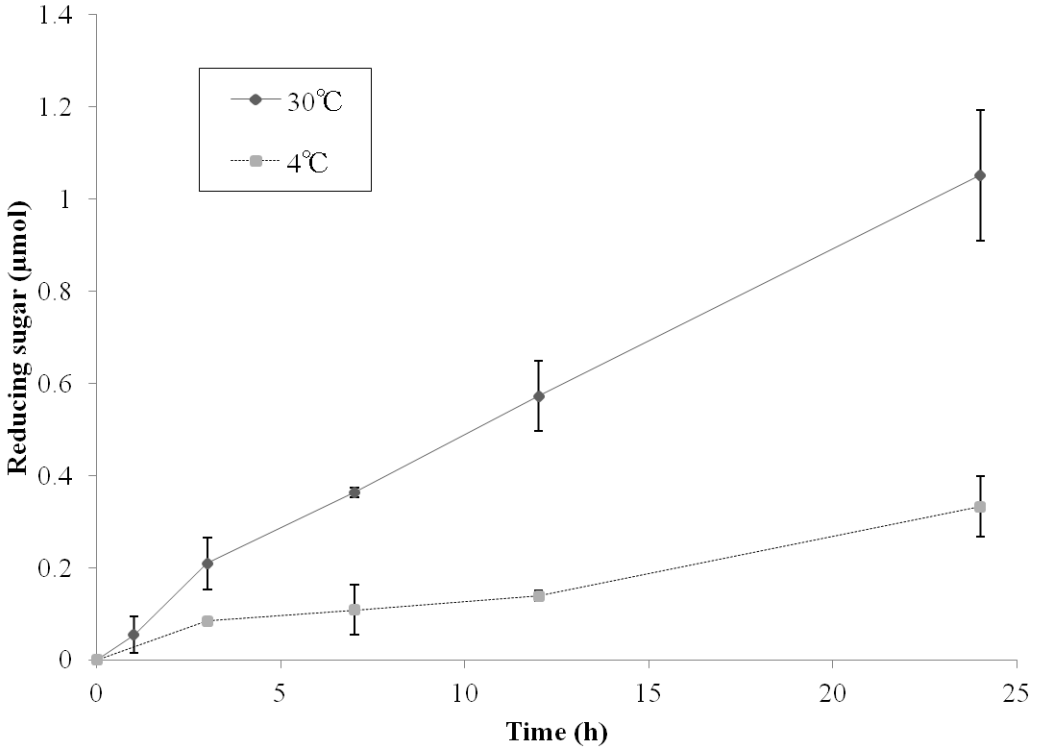

(a)
Sediment
Oligocheta*
Microorganism
172 kDa-
146 kDa-
49 kDa-
33 kDa-
30 kDa-
29 kDa-
(b)
Oligocheta*
59 kDa-
43 kDa-
38 kDa-
36 kDa-

Fig.4



Table

Table 1 Comparison of cellulose activities among wetlands in Hokkaido. Cellulase activity was determined by the quantitative assay as described in the text

Site	Wetland	Location	Geological type	Cellulase activity (nmol/gh)[a]	Organic component ratio (%)[a]	Salinity (‰)
A	Meguma Pond	45°24’ N 141’49 E	peat fen	737.88 ± 35.69	66.62	0
B	Notsuke Gulf	43°61’ N 145°27’ E	peat fen	92.39 ± 0.79	16.85	26
C	Onuma Pond	45°23’ N 141°46’ E	lagoon	6.74 ± 1.28	0.96	9
D	Lake Kuccharo	45°13’ N 142°25’E	lagoon	6.31 ± 0.29	1.07	14
E	Lake Saroma	44°08’ N 143°57’ E	lagoon	28.48 ± 0.66	1.48	25
F	Lake Notoro	44°06’ N 144°10’ E	lagoon	13.86 ± 0.81	1.84	23
G	Lake Abashiri	43°59’ N 144°13’ E	lagoon	2.80 ± 0.26	0.78	0
H	Lake Furen	43°18’ N 145°19’ E	lagoon	4.22 ± 0.69	16.68	17
I	Mochirippu Pond	43°01’ N 145°01’ E	lagoon	4.31 ± 0.35	6.65	26
J	Lake Akkeshi	43°03’ N 144°51’ E	lagoon	21.42 ± 1.11	6.45	20
K	Pashikuru Pond	42°92’ N 144°00’ E	lagoon	6.65 ± 1.32	0.65	0
L	Lake Chobushi	42°65’ N 143°61’ E	lagoon	1.58 ± 0.23	1.69	3
M	Lake Utonai	42°70’ N 141°70’ E	lagoon	44.45 ± 2.00	1.49	0
N	Teshio River	44°54’ N 141°43’ E	estuary	5.88 ± 0.50	1.04	0

O	Ishikari River	43°15’ N 141°22’ E	estuary	2.58 ± 0.58	1.23	2
P	Mukawa River	42°33’ N 141°55’ E	estuary	0	1.41	0
Q	Saru River	42°30’ N 142°00’ E	estuary	0	1.48	0

[a] Cellulase activity and organic component ratio showed a strong positive correlation (r = 0.96). p value was calculated as 8.78×10^{-10}, which was statistically significant (P<0.01). Thus, null hypothesis that the coefficient is zero is completely excluded.

Table 2 Composition of grain size of 17 wetlands in Hokkaido

Site	Wetland	Composition by weight of grain size (%)				
		>1 mm	1 mm-500 µm	500 µm-250 µm	250 µm - 63 µm	63 µm>
A	Meguma Pond	ND[a]	ND[a]	ND[a]	ND[a]	ND[a]
B	Notsuke Gulf	14.34	11.12	40.38	9.58	24.58
C	Onuma Pond	46.36	19.40	25.54	7.16	1.54
D	Lake Kuccharo	40.30	10.34	25.54	21.98	1.84
E	Lake Saroma	26.90	57.60	12.58	1.50	1.42
F	Lake Notoro	6.96	39.60	37.18	15.42	0.84
G	Lake Abashiri	18.15	47.76	26.71	7.38	0
H	Lake Furen	40.94	23.96	24.98	8.66	1.46
I	Mochirippu Pond	15.58	36.64	36.96	10.24	0.58
J	Lake Akkeshi	18.80	28.26	22.24	25.40	5.30
K	Pashikuru Pond	11.38	10.10	39.28	38.76	0.48
L	Lake Chobushi	76.50	13.60	7.15	2.75	0
M	Lake Utonai	58.40	28.64	8.39	4.56	0
N	Teshio River	9.44	35.61	48.17	6.78	0
O	Ishikari River	0	2.35	88.06	9.59	0
P	Mukawa River	25.40	48.74	12.06	13.38	0.42
Q	Saru River	2.08	10.90	64.92	21.46	0.64

[a] ND: not determined