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Distribution of putative denitrifying methane oxidizing bacteria in sediment of a freshwater lake, Lake Biwa

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Running title: Denitrifying methanotrophs in a freshwater lake

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Abstract

Methane oxidation coupled to denitrification is mediated by '*Candidatus* Methylomirabilis oxyfera', which belongs to the candidate phylum NC10. The distribution of putative denitrifying methane oxidizing bacteria related to "*M. oxyfera*" was investigated in a freshwater lake, Lake Biwa, Japan. In the surface layer of the sediment from a profundal site, a phylotype closely related to "*M. oxyfera*" was most frequently detected among NC10 bacteria in PCR analysis of the 16S rRNA gene. In the sediment, sequences related to "*M. oxyfera*" were also detected in a *pmoA* gene library. The presence of NC10 bacteria was also confirmed by catalyzed reporter deposition fluorescence in situ hybridization. Denaturing gradient gel electrophoresis (DGGE) and quantitative real-time PCR indicated that the abundance of "*M. oxyfera*"-related phylotype was higher in the upper layers of the profundal sediment. The horizontal distribution of the putative methanotrophs in sediment of the lake was also analyzed by DGGE, which revealed that their occurrence was restricted to deep water areas. These results agreed with those in a previous study of another freshwater lake, and suggested that the upper layer of the profundal sediments is major the habitat for denitrifying methanotrophs.

Key words: methane oxidation, denitrification, freshwater lake, sediment, NC10 bacteria

INTRODUCTION

Methane oxidation coupled to denitrification was first demonstrated in an enrichment culture [14]. To date, the organism responsible for this reaction has been identified as '*Candidatus* Methylomirabilis oxyfera', which belongs to the candidate phylum NC10. Although it has not yet been isolated in a pure culture, the genome sequence of "*M. oxyfera*" is now available [2]. It has been shown that this bacterium has the same enzyme system for methane oxidation as that used by well-known aerobic methane oxidizing bacteria. Under anoxic conditions, "*M. oxyfera*" generates oxygen from nitrite to drive this system [2].

With its unique ability to generate molecular oxygen under anoxic conditions, "*M. oxyfera*" can couple denitrification to methane consumption. Anaerobic methane oxidation independent of sulfate has been overlooked, and thus not taken into consideration in the global methane budget. Presently, the distribution of denitrifying methane-oxidizing bacteria in natural environments is hardly known. As major phylogenetic clusters in candidate the phylum NC10, group *a* and group *b* have been recognized [3], and involvement of group *a* in methane oxidation has been suggested from studies of enrichment cultures [2, 5, 6, 10]. In a previous study performed in an oligotrophic freshwater lake, Lake Constance, nitrate-dependent methane

oxidation and an overwhelming dominance of a specific phylotype among NC10 bacteria were observed in the profundal sediment [1]. In that study, sequences of the particulate methane monooxygenase gene (*pmoA* gene) related to "*M. oxyfera*" were also detected. On the basis of these results and comparison with those of the littoral sediment, it had been deduced that the dominant phylotype, belonging to group *a*, was responsible for the observed anaerobic methane oxidation and that it possessed the detected *pmoA* gene [1].

In the present study, the distribution of putative denitrifying methanotrophs was investigated in a mesotrophic freshwater lake, Lake Biwa, Japan, where one of the closest relatives of "*M. oxyfera*" was detected in a previous study [8]. We used DGGE, quantitative PCR, and CARD-FISH to study horizontal and vertical distributions of NC10 bacteria in lake sediment to decuce habitat preference of denitrifying methanotrophs.

MATERIALS and METHODS

Sample collection

Samples were obtained from Lake Biwa, a mesotrophic freshwater lake in Japan. In September 2004, sediment samples were obtained from two sites, site A and site Sh [9, 15]. Sediment samples were also obtained in October 2010 from site A and site C (Fig. 1). For the

investigation of the horizontal distribution, samples were obtained from 16 sites, including sites A and C, in the period from November to December in 2009 (Fig. 1, Table S1). All sediment samples were obtained using a core sampler equipped with an acrylic tube (inside diameter 110 mm) and subcored (diameter 41mm) immediately after collection. The subcore samples were kept at 4°C in the dark until further processing in the laboratory.

Chemical analyses

Interstitial water samples were obtained by centrifugation of the sediment samples sectioned in to five layers (the top 2 cm and at 3-cm intervals below that). The concentrations of chloride, nitrate, and sulfate were measured with an ion chromatograph (DX-120; Dionex, Sunnyvale, CA) equipped with a column for anion analyses (IonPac AS4ASC; Dionex). The concentrations of ammonium, nitrite, and ferrous iron were determined colorimetrically by the indophenol method, naphthyl ethylene diamine method, and ferrozine method, respectively.

To extract methane, portions of sediment (1.2–1.8 mL) were thoroughly mixed with 0.4 g of NaCl and 1mL distilled water in gas-tight vials. The resulting slurries were heated at 60°C for 30 min to enhance release of methane and suppress microbial activities. The concentration of methane released into the head space was measured by using a gas

chromatograph (model 2014; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector. The stable carbon isotope ratio of methane was determined with a gas-chromatograph-combustion isotope ratio mass spectrometer (GC/C/IRMS, Finnigan MAT-252).

To examine oxygen penetration into the sediment, microprofile of oxygen was investigated using intact sediment sample of site A obtained in 2010, stored for two days at 4°C. During the measurement, the subcore sample was set in a water bath at 7°C, *in situ* temperature of sediment surface. To compensate for decrease in oxygen during the storage, overlying lake water (ca. 5 cm depth) was briefly aerated by bubbling air prior to the measurement. Concentration of oxygen was measured with a Clark-type oxygen microsensor (OX-25; Unisense, Århus, Denmark) connected to a picoammeter (PA 2000, Unisense).

Clone library analysis

The diversity of NC10 bacteria in the profundal site was analyzed by constructing clone libraries of 16S rRNA and *pmoA* genes (Table 2). From the surface layer of the sediment obtained from site A, genomic DNA was extracted using the Ultra Clean Soil DNA kit (MoBio Laboratories, Solana Beach, CA). From the extracted DNA, fragments of 16S rRNA genes were

amplified using primers designed for the specific amplification of 16S rRNA genes from NC10 bacteria [3]. To maximize the recovery of a diversity of NC10 bacteria, two specific pairs, 202F and 1043R were used in combination with universal bacterial primers, 1492R and 27F, respectively (Table 2). Fragments of the 16S rRNA gene were also amplified with the universal primer pair 27F/1492R. All PCR amplifications initiated with 2 min of denaturation at 94°C. Each thermal cycle consisted of 30 s of denaturation at 94°C, 30 s of annealing, and elongation at 72°C. The annealing temperature, extension time, and total cycle number for each primer pair are shown in Table 2. Additional extension was carried out for 10 min at 72°C. The amplified fragments were ligated into the pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA), and the resulting vectors were transformed into competent TOP10 cells (Invitrogen).

From the resulting three libraries of 16S rRNA genes, clones of NC10 were selected for further analysis. Based on the alignment of the region shared by all clones (822 sites), pairwise genetic distances were calculated using the Tajima-Nei model. Subsequently, the clones were grouped into operational taxonomic units (OTUs) in such a way that the distances between the clones of the same OTU did not exceed 0.01 in any combination.

A clone library was also constructed from PCR-amplified fragments of the genes for particulate methane monooxygenase (*pmo*A). For specific amplification of the *pmoA* gene

related to "*M. oxyfera*", a new primer, 682_NC10 (5′-AAATCCGGCGAAGAACGA-3′) was designed at the position of the primer A682 [4]. This primer, perfectly matching the gene sequence of "*M. oxyfera*", was used in combination with A189 [4]. The PCR amplification and subsequent cloning steps were carried as described above.

PCR- denaturing gradient gel electrophoresis

The vertical and horizontal distributions of the NC10 bacteria were investigated by denaturing gradient gel electrophoresis (DGGE). Depth-related changes within the sediment were investigated for the samples from site A and site Sh obtained in 2004. With the templates of genomic DNA extracted from five sediment layers of two sites, NC10 specific PCR amplifications were performed using the primer pair 202F/1492R. The products obtained from the specific amplifications were used as templates for a second amplification with the primer pair GC341f and 907r [12, 13]. The amplicons obtained were subjected to DGGE as described previously [15].

The distribution of NC10 bacteria within the whole lake area was investigated with the samples of the surface layer of sediment (0–2 cm) obtained from the 16 sites in 2009. The first round of the nested PCR was performed with the primer pair 27F/1043R, and the following

procedures were carried out as described above, but range of denaturant gradient was changed to 20-50% and electrophoresis was conducted at 200 V for 4 h in DGGE.

Quantitative real-time PCR

The copy number of the 16S rRNA gene of the NC10 bacteria was assessed by quantitative real-time PCR as described previously [3]. The reaction mixture consisted of iQ SYBR Green Supermix (Bio-Rad), the primers NC10qP1F and NC10qP1R [3], and 4.9–14.3 ng of the genomic DNA samples. The conditions for the reactions were the same as described previously, and fluorescence acquisition during the amplification was carried out using the Mini-Option real-time PCR system (Bio-Rad). A standard DNA solution was prepared from the plasmid obtained during the cloning analysis and quantified using the Quant-iT dsDNA Assay Kit, Broad Range (Invitrogen, Carlsbad, CA). By diluting the solution, a linear standard curve ranging from 10^1 to 10^9 copies per reaction was generated. Specificity of the amplification was confirmed by direct sequencing of the amplicon obtained from surface layer of site A, resulting in successful acquisition of the target sequence.

Catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH)

CARD-FISH was performed as described previously [7], to visualize cells of the NC10 bacteria in the sediment. The sediment sample from site A obtained in October 2010 was fixed with 4% paraformaldehyde. Sediment particles were trapped on membrane filter and then embedded in low-melting-point agarose. The agarose-embedded sample was treated with lysozyme for 25 min for permeabilization, and then hybridized with the probe DBACT-1027 [3] labeled with peroxidases for 15 h at 40% formamide.

Nucleotide sequence accession numbers.

The nucleotide sequences determined in this study have been assigned the DDBJ/EMBL/GenBank accession numbers AB661465-AB661625.

RESULTS

Physicochemical characteristics of the sediments

The characteristics of the sediments are summarized in Table 1. The bottom water of site A contained 294 μM chloride, 97.3 μM sulfate, 20.7 μM nitrate, and 178 μM dissolved oxygen. As to vertical profiles of methane, increasing concentration and decreasing $\delta^{13}C$ value with increasing sediment depth were observed at both sites, but these trends were more apparent at site

A. Nitrate could not be detected in the interstitial water samples of either site, but trace amounts of nitrite were detected.

A microprofile of oxygen in sediment of site A, measured in the laboratory is shown in Fig. S1. Under the applied conditions, overlying water contained more dissolved oxygen (243 μM) than the water *in situ*, but it depleted within the top 3 mm of sediment.

16S rRNA diversity of NC10 bacteria at the profundal site

Using the specific primers, non-target organisms were detected in the two NC10-specific clone libraries only in low frequencies (Table 2). From the three (specific and general) 16S rRNA gene libraries, 39 clones of NC10 were obtained and grouped into 6 OTUs. A single OTU was dominant in the two libraries constructed using specific primers, and it was also detected in the general 16S rRNA gene library. This dominant phylotype was closely related to "*M. oxyfera*" (represented by B84 in Fig. 2) and corresponded to LB13, a DGGE band reported in a previous study [8]. The other 5 OTUs were all members of group *b*.

In the general 16S rRNA gene clone library, 11 clones were closely related to diatom chloroplasts. Besides the one clone of NC10 bacteria, two clones of aerobic methane-oxidizing bacteria were detected in this library. On the basis of analysis with the RDP Classifier [16], they

were inferred to belong to the genus *Methylobacter* and the genus *Methylosarcina*, respectively.

Phylogenetic analysis of the *pmoA* genes

In the clone library of the *pmoA* genes constructed with a NC10-specific primer, all clones sequenced were closely related to each other (Table 2). In a pairwise comparison of the deduced protein sequences, the differences observed were restricted to 3 amino acids or fewer out of 166 amino acid positions. These sequences were related to the *pmoA* gene sequences of "*M. oxyfera*" and the clones from Lake Constance (Fig. 3). No PCR product was obtained from sites B, C, D, and E. As to these four sites, two other primer sets specific for *pmoA* gene of NC10 bacteria, A189_b/cmo682 and cmo182/cmo682 [11] also did not give a PCR product of the expected size.

Vertical related distribution of NC10 in sediments

The vertical distribution of the NC10 bacteria in the sediments was investigated by DGGE for the samples obtained from two sites. A single dominant band was observed in the sample of the surface layer of site A, and its relative intensity decreased with increasing sediment depth. This band corresponded to the dominant phylotype identified in the cloning

analysis. Another band, detected only in the sample of surface layer of site A, was very closely related to the dominant band (99.5% sequence identity). All other bands sequenced were affiliated with group *b*. At site Sh, no dominant phylotype was observed, and only members of group *b* were detected (Fig. 4, Fig. S2).

Horizontal distribution of NC10 bacteria in Lake Biwa sedimemts

PCR products were obtained from all sites, but DGGE and sequencing revealed that gene fragments from non-target organisms were also amplified with the method applied. The major DGGE bands were all sequenced but NC10 could not be detected at two of the investigated sites (Fig. 1, Table S1). From the 11 sites in the northern area, a band corresponding to the phylotype dominant in the clone libraries was obtained, and it was only a single band of NC10 detected in each site (Fig. 1). Two DGGE bands of NC10 were obtained from site B and one band each was detected in sites D and E. These bands were distinct from each other and all belonged to group *b* (Fig. S2).

Quantification by real time-PCR

The copy number of the 16S rRNA genes of the NC10 bacteria was estimated by

real-time PCR, as shown in Fig. 5. Depth-related changes were observed only at site A. Differences between the two sites were apparent in the shallow layers, indicating a higher relative abundance of NC10 at site A.

CARD-FISH

In the sediment sample of the 0–2 cm layer obtained from site A in October 2010, CARD-FISH-positive cells were detected with a low frequency; they were less than 1% of all 4′,6-diamidino-2-phenylindole (DAPI)-stained cells. They appeared as short rods (0.8–1.2 μm in length) and occurred single or in clusters with a few cells (Fig. 6). The surface sediment sample contained a large amount of diatom debris, and large amount of this rather fluffy sample could not be stably retained on a membrane filter. As a result, it was not possible to accomplish exact counting of the rare CARD-FISH-positive cells in limited volume of the sediment.

DISCUSSION

The microprofile of oxygen shown in Fig. S1 was obtained in the laboratory, and therefore, cannot be regarded as direct representation of oxygen availability *in situ*. Considering oxygen concentration lower than that in the experimental conditions, however, oxygen

penetration into the sediment would not exceed 3 mm under the actual conditions at the lake bottom in site A. On the other hand, the profiles of methane concentration and its stable isotope ratio suggest biological methane oxidation in much deeper sediment. Although the isotopic fractionation factor in nitrite-dependant methane oxidation has not been revealed yet, a value similar to those observed for aerobic methanotrophs can be expected since they share the same enzyme system for oxidizing methane. The activities of denitrifying methanotrophs should result in the enrichment of ^{13}C in the residual methane, and therefore, can be a cause of the profiles of methane observed in this study. In the sediment of the profundal site, accordingly, the 16S rRNA gene sequences related to "*M. oxyfera*" were detected. The phylotype, henceforth referred to as putative methanotroph, was consistently detected by other methods. Through CARD-FISH, the presence of the putative methanotrophs in the sediment was visualized directly.

The results of the DGGE suggested that the putative methanotroph preferred upper layers of sediment. This result was also supported by the results of the quantitative real-time PCR. The primer pair used in the real-time PCR was designed for specific quantification of group *a* in the phylum NC10 [3], and the putative methanotroph was the only member of this phylogenetic group detected in this study. In a previous study, it was shown that real-time PCR

was not sufficient in absolute quantification, but its estimation was correlated to activity and biomass of the denitrifying methanotrophic enrichment culture [3]. The higher abundance of the putative methanotroph in shallow layers may be related to the supply of electron acceptor. Although no clear depth-related trend was observed in the vertical profile of nitrite concentration, abundance does not necessarily correspond to the availability since nitrite is concurrently produced and consumed in the sediments. As major sources of nitrite, partial reduction of nitrate and ammonium oxidation active at the sediment surface may support growth of putative methanotrophs.

The putative methanotroph was detected only in the northern area of the lake, which is characterized by deep water (Fig. 1). In the case of Lake Constance, the putative methanotroph of NC10 was dominant in the profundal sediments but not detected in the littoral sediments [1]. Slow-growing denitrifying methanotrophs might be enriched only in profundal sediments characterized by stable environmental conditions.

By using the specific primer which perfectly matches to "*M. oxyfera*", sequences of the *pmoA* gene were obtained from site A. It is likely that these sequences originated from the putative methanotroph detected as 16S rRNA gene sequences, but the coverage of the primer pair should be taken into account. Besides the primer used in this study, several specific primers

have been reported to detect the *pmoA* gene of NC10 bacteria [1, 11]. All these primers were designed on the basis of a very limited number of sequences obtained from the metagenomic analysis of enrichment cultures. In fact, there are mismatches between these primers and the sequences obtained in this study. The reverse primer used in this study was also designed solely on the basis of the sequence of "*M. oxyfera*", and there might have been sequences missed because of the poor primer affinity. Further improvement of the specific primer might be required to explore the diversity of the denitrifying methanotrophs by the *pmoA* gene-targeted PCR-based methods.

On the whole, the results obtained in this study were good agreement with those obtained in the study of Lake Constance. This consistency implies the universality of the phenomena observed in these lakes. In addition, the DGGE and real-time PCR analyses performed in this study indicated that denitrifying methanotrophs prefer sediment surface to deeper sediment. Therefore, the upper layer of lake sediments in areas characterized by deep water is suggested to be a major habitat for denitrifying methane-oxidizing bacteria in the natural environment.

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REFERENCES

[1] Deutzmann, J.S., Schink, B. (2011) Anaerobic oxidation of methane in sediments of Lake Constance, an oligotrophic freshwater lake. Appl. Environ. Microbiol. 77, 4429–4436.

[2] Ettwig, K.F., Butler, M.K., Le Paslier. D., Pelletier, E., Mangenot, S., Kuypers, M.M., Schreiber, F., Dutilh, B.E., Zedelius, J., de Beer, D., Gloerich, J., Wessels, H.J., van Alen, T., Luesken, F., Wu, M.L., van de Pas-Schoonen, K.T., Op den Camp, H.J., Janssen-Megens, E.M., Francoijs, K.J., Stunnenberg, H., Weissenbach, J., Jetten, M.S., Strous, M. (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature 464, 543–548.

[3] Ettwig, K.F., van Alen, T., van de Pas-Schoonen, K.T., Jetten, M.S., Strous, M. (2009). Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl. Environ. Microbiol. 75, 3656–3662.

[4] Holmes, A., Costello, A., Lidstrom, M., Murrell J. (1996) Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily. FEMS Microbiol. Letters. 132, 203–208.

[5] Hu, S., Zeng, R.J., Burow, L.C., Lant, P., Keller, J., Yuan, Z. (2009) Enrichment of denitrifying anaerobic methane oxidizing microorganisms. Environ. Microbiol. Rep. 1, 377–384.

[6] Hu, S., Zeng, R.J., Keller, J., Lant, P.A., Yuan, Z. (2011) Effect of nitrate and nitrite on the selection of microorganisms in the denitrifying anaerobic methane oxidation process. Environ. Microbiol. Rep. 3, 315–319

[7] Ishii, K., Mußmann, M., MacGregor, B.M., Amann, R. (2004) An improved fluorescence in situ hybridization protocol for the identification of Bacteria and Archaea in marine sediments. FEMS Microbiol. Ecol. 50, 203–212.

[8] Koizumi, Y., Kojima, H., Fukui, M. (2003) Characterization of depth-related microbial community structure in lake sediment by denaturing gradient gel electrophoresis of amplified 16S rDNA and reversely transcribed 16S rRNA fragments. FEMS Microbiol. Ecol. 46, 147–157.

[9] Kojima, H., Nakajima, T., Fukui, M. (2007) Carbon source utilization and accumulation of respiration-related substances by freshwater *Thioploca* species. FEMS Microbiol. Ecol. 59, 23–31.

[10] Luesken, F.A., van Alen, T.A., van der Biezen, E., Frijters, C., Toonen, G., Kampman, C., Hendrickx, T.L., Zeeman, G., Temmink, H., Strous, M., Op den Camp, H.J,. Jetten, M.S. (2011a) Diversity and enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from wastewater sludge. Appl. Microbiol. Biotechnol. 92, 845–854.

[11] Luesken, F.A., Zhu, B., van Alen, T.A., Butler, M.K., Diaz, M.R., Song, B., Op den Camp, H.J.M, Jetten, M.S.M, Ettwig, K.F. (2011b) pmoA primers for detection of anaerobic methanotrophs. Appl. Environ. Microbiol. 77, 3877–3880.

[12] Muyzer, G., de Waal, E.C., Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl. Environ. Microbiol. 59, 695–700.

[13] Muyzer, G., Teske, A., Wirsen, C.O., Jannasch, H.W. (1995) Phylogenetic relationships of Thiomicrospira species and their identification in deep-sea hydrothermal vent samples by denaturing gradient gel electrophoresis of 16S rDNA fragmen. Arch. Microbiol. 164, 165–172.

[14] Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J., Ettwig, K.F., Rijpstra, W.I., Schouten, S., Damsté, J.S., Op den Camp, H.J., Jetten, M.S., Strous, M. (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. Nature 440, 918–921.

[15] Tsutsumi, M., Kojima, H., Fukui, M. (2012) Vertical profiles of abundance and potential activity of methane-oxidizing bacteria in sediment of Lake Biwa, Japan. Microbes. Environ. *In press*

[16] Wang, Q., Garrity, G.M., Tiedje, J.M., Cole, J.R. (2007) Naïve Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Appl. Environ. Microbiol. 73, 5261–5267.

Figure legends

Fig. 1. Map showing the sampling sites and distribution of NC10 bacteria in Lake Biwa, based on the survey performed in 2009. Contour lines represent water depth in meters. Symbols correspond to detected NC 10 bacteria; closed circle, group *a*; triangle, group *b*; open circle, not detected.

Fig. 2. Phylogenetic relationships of the 16S rRNA gene clones within the phylum NC10. The sequence of *Geothrix fermentans* was included as an outgroup. The clones obtained in this study are shown in bold (only representative clones of 6 OTUs are shown). Name of clone prefixed B, F, and R indicate the clones from the respective libraries shown in Table 2. The tree was constructed with minimum evolution method, based on alignment of 762 nucleotide positions. Phylogenetic distances were calculated with the Kimura 2-parameter model. Numbers on nodes are percentage values of 1000 bootstrap resampling (values larger than 50 are shown). Four phylogenetic groups, *a*, *b*, *c*, and *d* were defined in reference 3.

Fig. 3. Phylogenetic position of the predominant PmoA sequence deduced from 10 of the 21

pmoA gene clones obtained in this study (from the library P, Table 2). This minimum evolution tree was constructed with 148 amino acid sites, and genetic distances were calculated with the Poisson correction. Numbers on nodes are percentage values of 1000 bootstrap resamplings (values larger than 50 are shown).

Fig. 4. DGGE band patterns of the 16S rRNA gene fragments amplified with a primer pair specific for NC10 bacteria, showing depth-related changes in the sediments of two sampling sites obtained in 2004. Bands labeled with an arrow correspond to the dominant phylotype of group *a*, identified in the cloning analysis. Bands labeled with a triangle are members of group *b* in the phylum NC10.

Fig. 5. Abundance of 16S rRNA gene copy number of NC10 bacteria belonging to the group *a* in sediments obtained in 2004, assessed by real-time PCR.

Fig. 6. Cells of NC10 bacteria in sediment from site A obtained in 2010, visualized by CARD-FISH. A cluster of CARD-FISH-positive cells are indicated by arrow in upper panel, and DAPI staining of the same field is shown in lower panel. Bar indicates 5 μm.

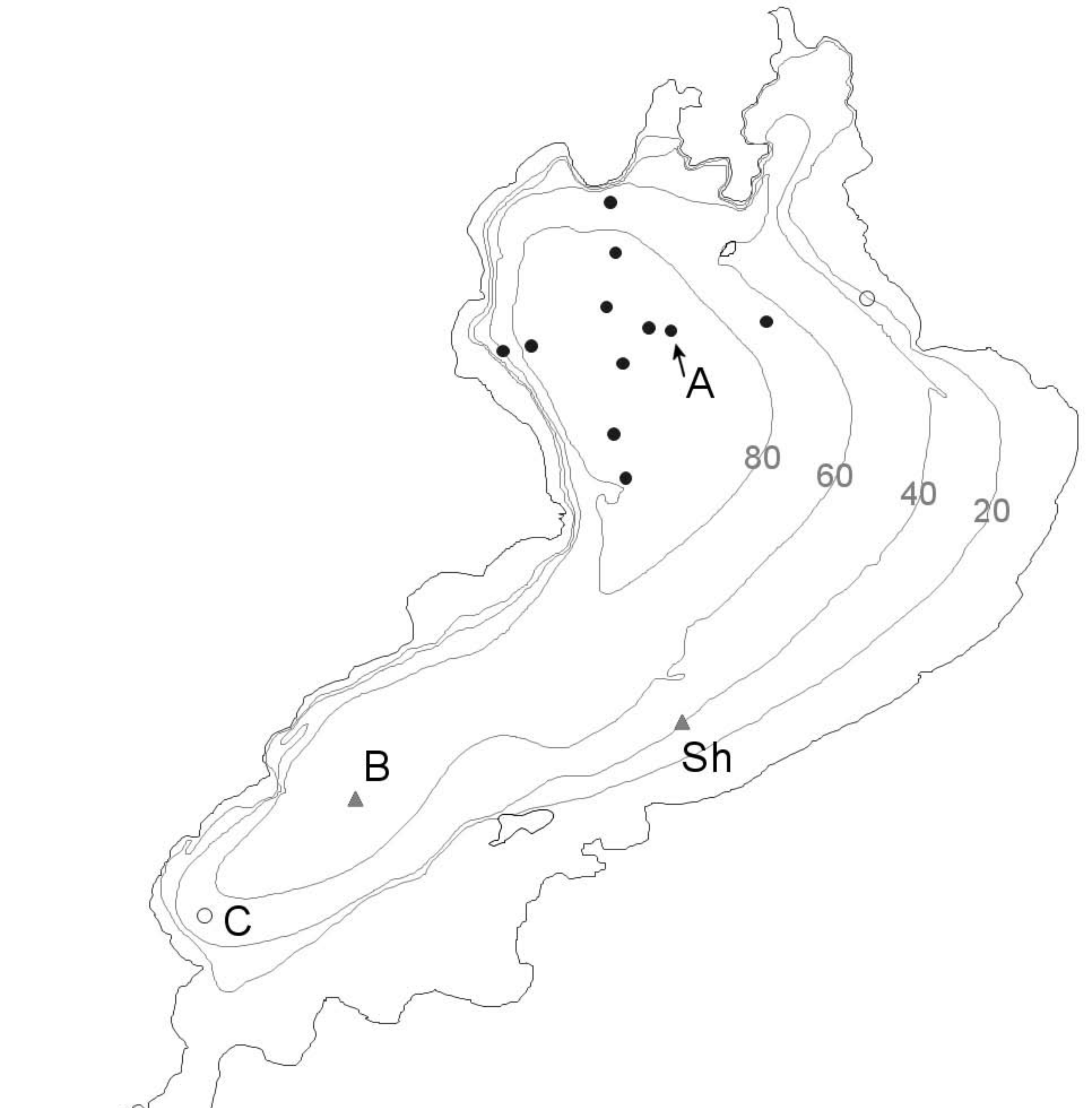
Table 1. Characteristics of sediment samples obtained in October 2010.

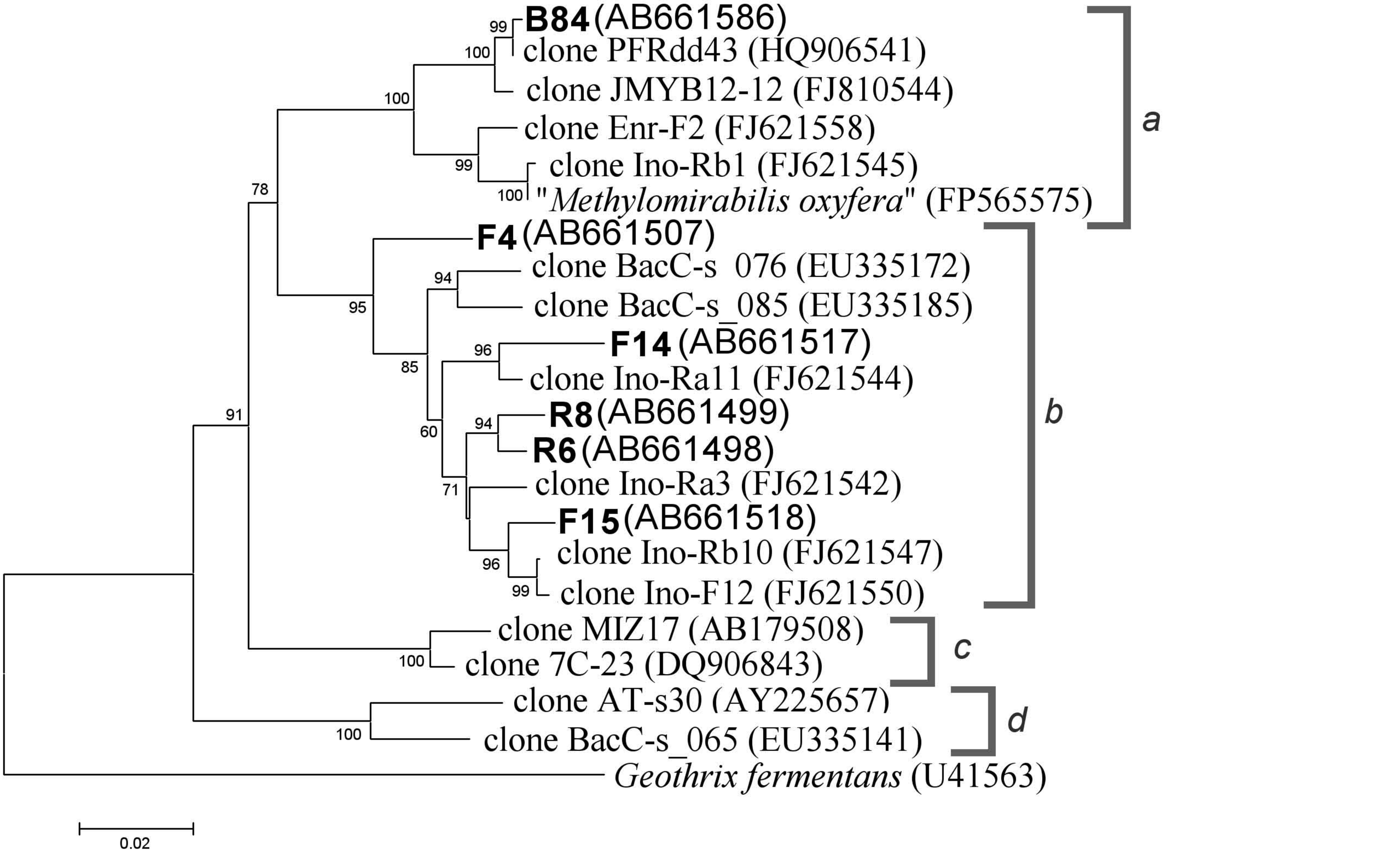
		Water content (%, w/w)	Ignition loss (%)	Methane concentration (nmol/ml-sediment)	$\delta^{13}C$-CH_4	Dissolved ions in interstitial water (μM)			
						Sulfate	Ammonium	Ferrous iron	Nitrite
site A	0-2 cm	91.5	20.0	283.8	-62.5	33.7	68.0	50.7	0.5
	2-5 cm	87.5	8.3	885.5	-68.7	12.3	41.0	133.4	0.5
	5-8 cm	83.7	9.4	1700	-71.7	9.6	49.0	117.4	0.7
	8-11 cm	80.6	8.1	2523	-73.0	12.7	18.5	221.4	0.9
	11-14 cm	79.9	7.5	2785	-73.4	9.0	42.5	272.1	0.7
site C	0-2 cm	91.8	20.0	193.3	-70.9	40.2	76.5	238.7	1.1
	2-5 cm	85.6	10.7	386.8	-72.6	11.2	94.0	256.7	0.9
	5-8 cm	87.1	12.5	334.9	-72.6	12.0	80.5	234.1	0.7
	8-11 cm	80.9	10.5	355.6	-72.5	19.3	77.5	192.7	0.8
	11-14 cm	79.9	10.0	599.4	-73.9	13.6	111.5	164.1	0.9

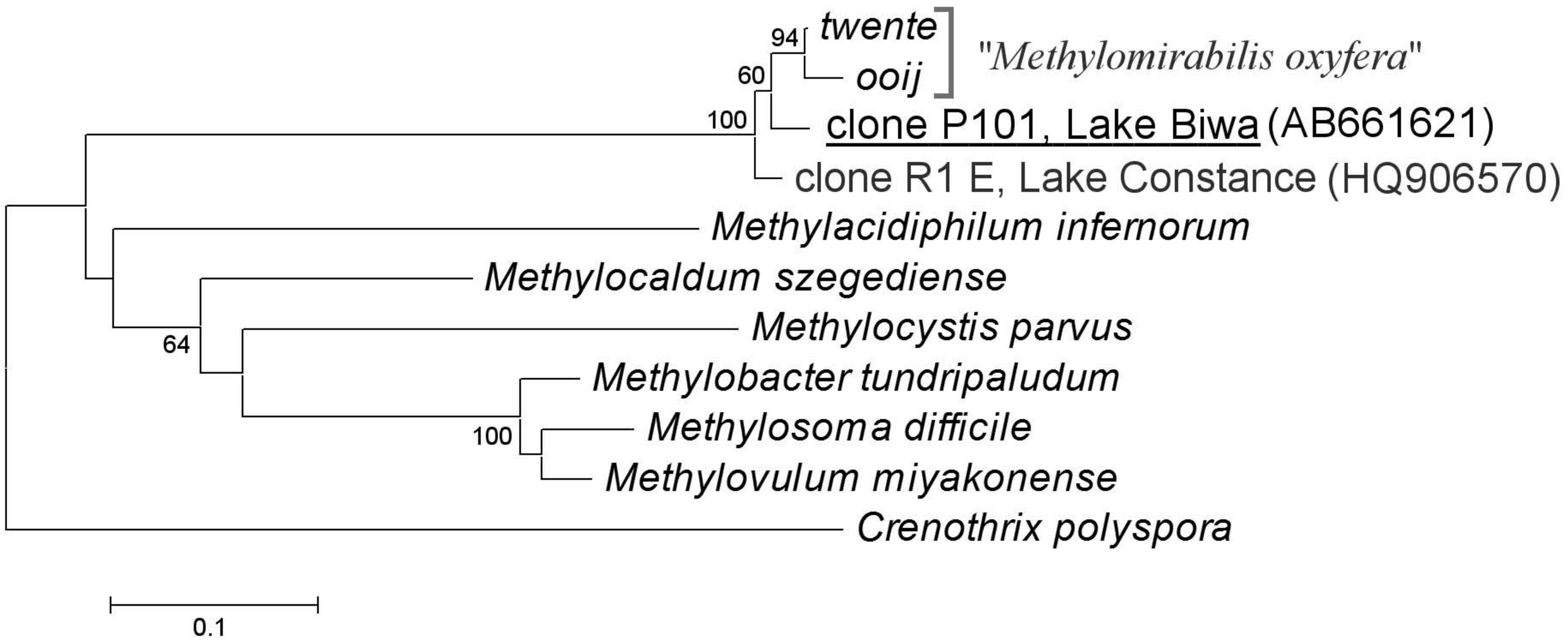
Table 2. Clone libraries established from the sediment samples (0–2 cm layer) of site A

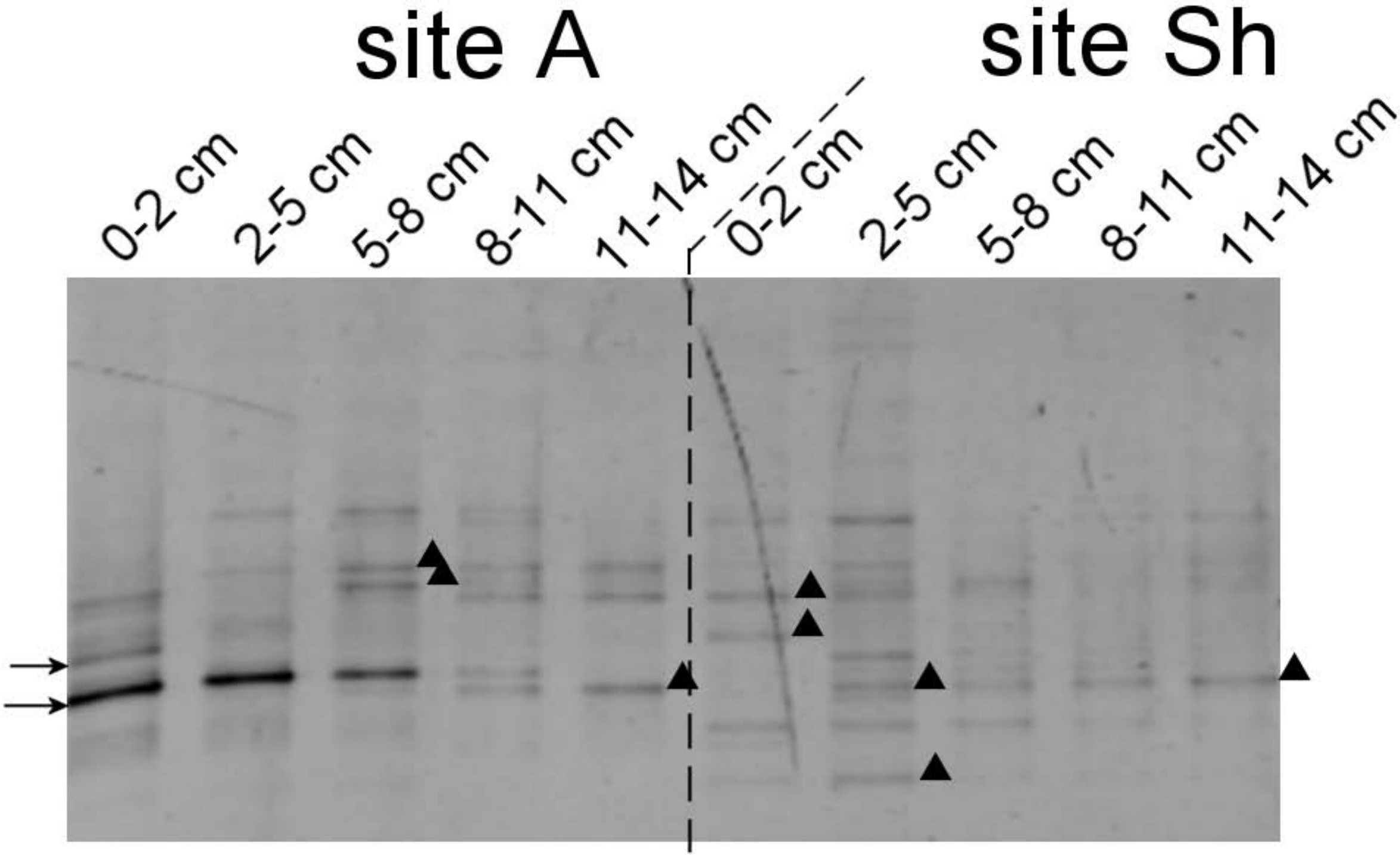
Library	Date of sediment sampling	Primer pair	PCR conditions			No. of clone			Accession No.
			Annealing temperature (°C)	Extension time (sec)	Cycle number	Sequenced	NC10	Group *a*	
F	Sep, 2004	27F/1043R	56	60	34	17	15	12	AB661505-521
R	Sep, 2004	202F/1492R	56	60	34	26	23	20	AB661479-504
B	Oct, 2009	27F/1492R	45	90	25	83	1	1	AB661522-604
P	Sep, 2004	A189/682_NC10	55	45	34	21	21	NA*	AB661605-625

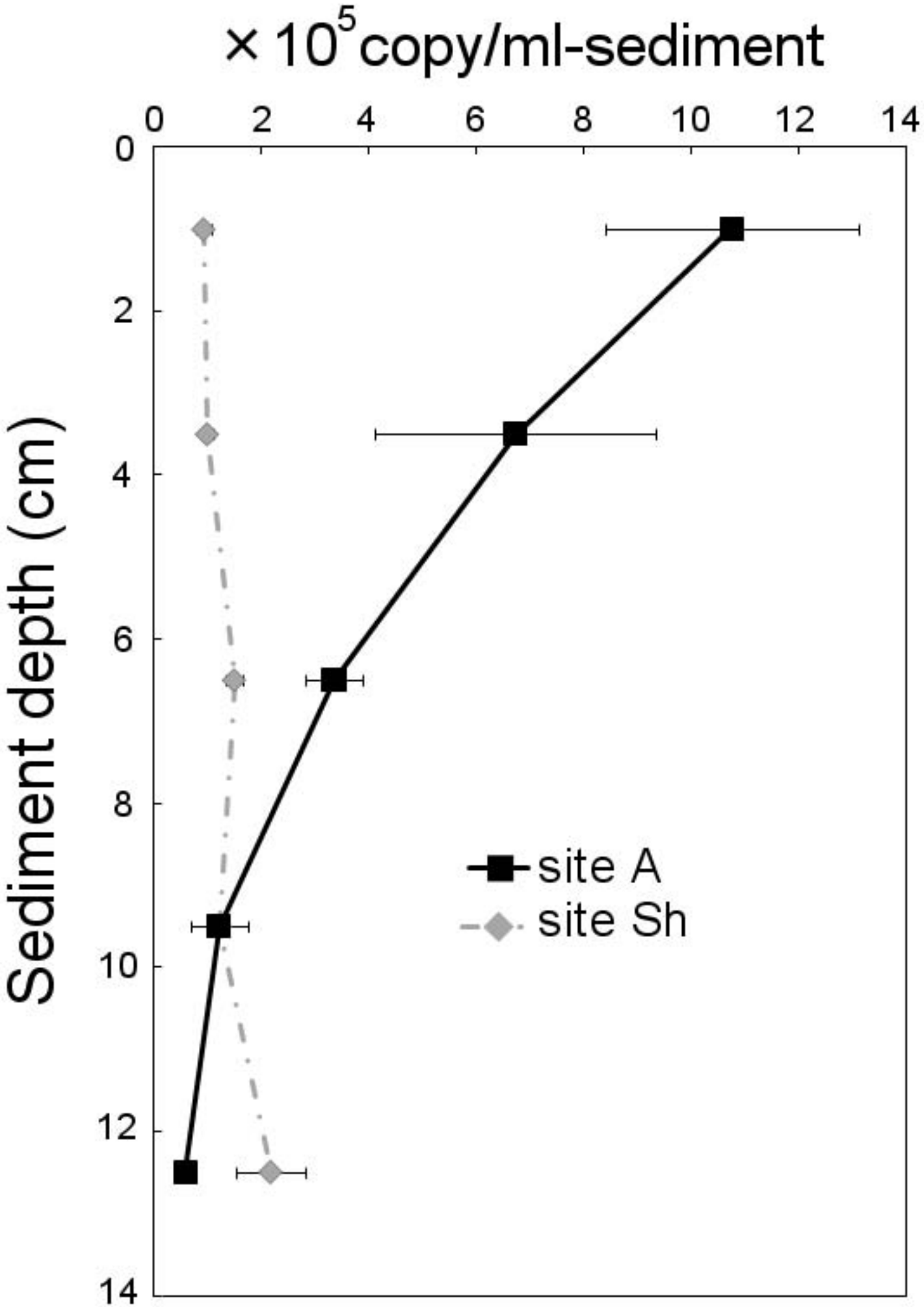
*Not applicable


A
80
60
40
20
B
Sh
C
D
E
10 km


B84 (AB661586)
clone PFRdd43 (HQ906541)
clone JMYB12-12 (FJ810544)
clone Enr-F2 (FJ621558)
clone Ino-Rb1 (FJ621545)
"Methylomirabilis oxyfera" (FP565575)
F4 (AB661507)
clone BacC-s 076 (EU335172)
clone BacC-s_085 (EU335185)
F14 (AB661517)
clone Ino-Ra11 (FJ621544)
R8 (AB661499)
R6 (AB661498)
clone Ino-Ra3 (FJ621542)
F15 (AB661518)
clone Ino-Rb10 (FJ621547)
clone Ino-F12 (FJ621550)
clone MIZ17 (AB179508)
clone 7C-23 (DQ906843)
clone AT-s30 (AY225657)
clone BacC-s_065 (EU335141)
Geothrix fermentans (U41563)
99
100
100
99
100
78
95
94
85
96
91
60
94
71
96
99
100
100
a
b
c
d
0.02


twente
ooij
"Methylomirabilis oxyfera"
clone P101, Lake Biwa (AB661621)
clone R1 E, Lake Constance (HQ906570)
Methylacidiphilum infernorum
Methylocaldum szegediense
Methylocystis parvus
Methylobacter tundripaludum
Methylosoma difficile
Methylovulum miyakonense
Crenothrix polyspora
94
60
100
64
100
0.1


site A
site Sh
0-2 cm
2-5 cm
5-8 cm
8-11 cm
11-14 cm
0-2 cm
2-5 cm
5-8 cm
8-11 cm
11-14 cm


× 10^5 copy/ml-sediment
0
2
4
6
8
10
12
14
Sediment depth (cm)
0
2
4
6
8
10
12
14
site A
site Sh

