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The Vertical Distribution of Sulfate Reducing Bacteria (SRB) by Florescence In Situ Hybridization in Sediments of Lakes in Korea and China

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The vertical distributions of sulfate reducing bacteria (SRB) in sediments of lakes in Korea (Lake Sihwa and Lake Soyang) and China (Lake Aha and Lake Erhai) were investigated by fluorescence in situ hybridization (FISH). SRB from sediment of Lakes of China were located to deeper layer than those in Lakes of Korea. SRB were not detected below 19 cm and 10 cm depth in sediments of Lake Sihwa and Lake Soyang, respectively. SRB numbers were, however, detected at all observed sediments in Lake Aha and Lake Erhai. In case of lakes in Korea, the proportion of SRB ranged from 2.9 to 25.6% (Lake Sihwa) and ranged from 0.6 to 7.1% (Lake Soyang). For lakes in China, the proportions of SRB were from 0.6 to 19.4% and from 2.9 to 11.2% within sediments from Lake Aha and from Lake Erhai, respectively. The high peaks of SRB numbers in sediments of all lakes were appearing at depths between 0 cm and 2 cm.

Key words : FISH, Lake Aha, Lake Erhai, Lake Sihwa, Lake Soyang, sediment, sulfate reducing bacteria

INTRODUCTION

Sulfate reducing bacteria (SRB) play important roles in aquatic sediment for the global cycling of carbon and nutrients (Jørgensen, 1982), which are the main terminal degrader for the mineralization of dead biomass in sulfate rich anoxic habitat (Widdle, 1988). Recently, sulfate (SO_4^{2-}) is more coming from air into lakes and rivers due to acid rain and coal mines, and becoming one of agents for acidification of freshwater. SRB use sulfate as terminal electron acceptor and sulfate is converted into hydrogen sulfide (H_2S), which would be transported into air. By this process, the acidification of lake would be neutralized by microbial activity (Aron *et al.*, 1989). Moreover, sulfides produced by SRB react with dissolved heavy metals in interstitial water, and sulfate elimination by SRB reduces the acidity (Wang *et al.*, 2007). By these processes, the SRB is affecting to the bacterial compositions and activities. Hence, the vertical distribution of SRB is important for understanding of behaviors of metal and organic matters in sediments.

FISH is powerful method for the identification and quantification of complex microbial communities in several environments from domain level to species level (Amann *et al.*, 1995). Recently, 16S rRNA probing method has been applied to assess the diversity of SRB in various ecosystems. The population dynamic of SRB using 16S rRNA-targeted oligonucleotide probes was about 4.1% of total microorganisms in surface

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sediment of Lake Kizaki, Japan (Li *et al.*, 1999). In the community structure analysis of SRB using FISH method, SRB number was detected 5.2 $\times 10^8$ cells mL^{-1} and that was 15% of DAPI-stained cells in marine arctic sediment (Ravenschlag *et al.*, 2000). The annual average of the relative SRB abundance hybridized with 18 probes to DAPI-stained cells was 2.3% in surface layer, and increased to 22.9% between 8 and 14 cm depths of Lake Biwa, Japan (Koizumi *et al.*, 2003).

The Lake Sihwa has been highly polluted due to nearby domestic and industrial waste water including various kinds of metals (Hyun *et al.*, 1999) and the Lake Aha has been polluted by acidic mining drainage and domestic sewages for a long time with iron, manganese, and sulfate excessively enriched in water and sediment (Wang *et al.*, 2003). The other hand, the Lake Soyang was oligotrophic lake in the early 1980s, after then the eutrophication was enhanced (Kim *et al.*, 1989) and recently, the lake is turning into oligo-mesotrophic state (Kim, 2006). The Lake Erhai is one of the typical freshwater at the Yunnan highlands in China (Guodong *et al.*, 2001) and is mesotrophic lake (Sun and Chen, 2000). The environmental conditions of studied lakes are different from each other.

The objective of this study was to analyze the vertical distributions of sulfate reducing bacteria in sediment of four lakes using SRB 385, general probe for SRB (Icgen *et al.*, 2007).

MATERIALS AND METHODS

1. Cultivation of bacterial strain

The bacterial strain, *Desulfovibrio* sp. KCTC 2484 was purchased from the Korean Collection for Type Cultures (KCTC, Daejeon, Korea), and cultured on *Desulfovibrio* sp. Medium (NH_4Cl, 1.0 g; Na_2SO_4, 2.0 g; Na-thiosulfate · $5H_2O$, 1.0 g; $NaSO_4$ · $7H_2O$, 1.0 g; $CaCl_2$ · $2H_2O$, 0.1 g; KH_2PO_4, 0.5 g; Yeast extract, 1.0 g; $NaHCO_3$, 2.0 g; Na-pyruvate, 1.0 g; Na-malate, 1.0 g; Resazurin, 0.5 mg; Na_2S · $9H_2O$, 75.0 mg; Trace element solution SL-10, 1.0 mL; Vitamin solution, 10.0 mL; Distilled water, 1 L) in anaerobic chamber at 35 °C for a week.

2. Used probes

The probe SRB385 (5′-CGGCGTCGCTCGCTC AGG-3′) are targeted to most of the known sulfate reducing bacteria in the δ subclass of *Proteobacteria* (Amann *et al.*, 1992). The oligonucleotide probes were purchased from TaKaRa and synthesized with Cy3 fluorochrome at the 5′ end.

3. Probe verification for FISH

For verifying whether the probe SRB385 was specific for the detection of SRB, we tested it with cultured *Desulfovibrio* sp., which has been reported to be the dominant species in sediment of marine Arctic (58-61%), the great Ouse estruary (50-90%), and Lake Biwa (61-86%), repectively (Trimmer *et al.*, 1997; Sahm *et al.*, 1999; Koizumi *et al.*, 2003) before applying the probes for the detection of SRB in environmental sediment. *Desulfovibrio* sp. (strain) was diluted with sterilized distilled water and fixed with 4% paraformaldehyde (sample: PFA=3 : 1) for 4 h. The fixed bacterial strain was filtered onto 0.2 μm pore-sized polycarbonate membrane filters. The filters were washed 3 times with 0.5 mL phosphate buffered saline (1 × PBS) and dehydrated in 50%, 80%, 99.5% ethanol successively. After the final dehydration step, filters were air-dried. Hybridization buffer (0.9 M NaCl, 20 mM tris-HCl, 0.01% SDS, 35% formamide) on filters was added with probe and filters were stored for hybridization at 46°C for 90 min. Following hybridization, filter was washed in preheated washing buffer (40 mM NaCl, 20 mM Tris-HCl, 0.01% SDS, 5 mM EDTA) at 48°C for 30 min.

For counterstaining, total bacterial numbers were stained with 4′, 6′-diamidino-2-phenylindole (DAPI) for 15 min, and were examined by epifluorescence microscope (Hobbie *et al.*, 1977). For statistical confidence, counts for 10 random fields of view were obtained, and triplicate observations were done.

4. Sediment sample collection

The sediment samples were obtained from lakes in Korea (Lake Sihwa and Lake Soyang) and China (Lake Aha and Lake Erhai). The locations (Latitude & Longitude), sampling date and length of core sample are given in Table 1. The core samples in Lake Sihwa and Lake Soyang were collected using a core sampler (5 cm diameter and 50.7 cm length) and those in Lake Aha and Lake Erhai were collected using collector described by Wang *et al.* (1998).

Table 1. The lakes for collecting the sediment.

Station		Latitude (N)	Longitude (E)	Sampling date	Section (cm)
Lake Korea	Sihwa	37° 17′39.5″	126° 48′33.8″	Jun. 2006	0-30
	Soyang	37° 56′43.0″	127° 49′18.8″	Jun. 2006	0-15
Lake China	Aha	26° 32′31.3″	106° 39′0.1″	Dec. 2005	0-30
	Erhai	25° 48′41.1″	100° 12′17.1″	Dec. 2005	0-28

5. Pretreatment of sediment samples

The fresh sediment samples were sliced from surface by 1 cm interval. The collected sediment samples were diluted with autoclaved lake water, sonicated for 5 min, and centrifuged. And then supernatant was transferred to clean conical tube, fixed with 4% paraforaldehyde (sample: PFA=3 : 1) for 4 h and stored under refrigeration for FISH.

6. FISH

After confirming that the probe SRB385 was effective for determining *Desulfovibrio* sp. (strian), the probe SRB385 was applied to assess SRB in environmental sediments with same FISH method above.

RESULTS AND DISCUSSTION

1. Probe verification for FISH

The average SRB proportion hybridized with the probe SRB385 to DAPI-stained cells was 92.8% (Table 2). This result showed that the probe SRB385 was able to detect effectively *Desulfovibrio* sp. (strain) which has been reported to be the dominant species in marine and freshwater sediment (Trimmer *et al.*, 1997; Sahm *et al.*, 1999; Koizumi *et al.*, 2003).

Table 2. The efficiencies of probe for detection of *Desulfovibrio* sp.

Probe name	Experiment number	DAPI ($\times 10^6$ cells mL^{-1})	FISH ($\times 10^6$ cells mL^{-1})	FISH/DAPI (%)
SRB385	1	4.0±0.7	3.7±0.7	92.6±3.0
	2	4.5±0.6	4.2±0.6	93.8±3.9
	3	4.8±0.8	4.4±0.7	91.9±2.9

The formamide concentration and hybridization temperature are important conditions in hybridization step. For detection of the family *Desulfovibrionaceae* by FISH, Okabe *et al.* (1999) used 30% formamide concentration and 46°C hybridization temperature while Tonolla *et al.* (2000) used 20% and 53°C. The result showed that hybridized *Desulfovibrio* sp. (KCTC 2484) was visualized with strong fluorescence signal (Fig. 1.) when we used 35% formamide concentration and 46°C hybridization temperature (data not shown).

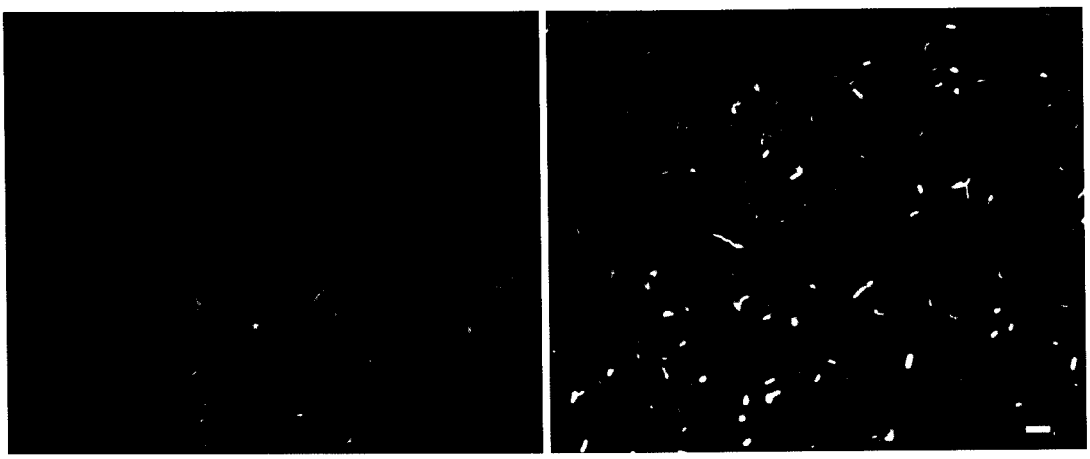

Fig. 1. Microphotographs of FISH (left) and DAPI (right) of cultured *Desulfovibrio* sp. (KCTC 2484). FISH image is showing bacteria hybridized with SRB385 probe (Cy3 labeled, red). Bar=5 μm.

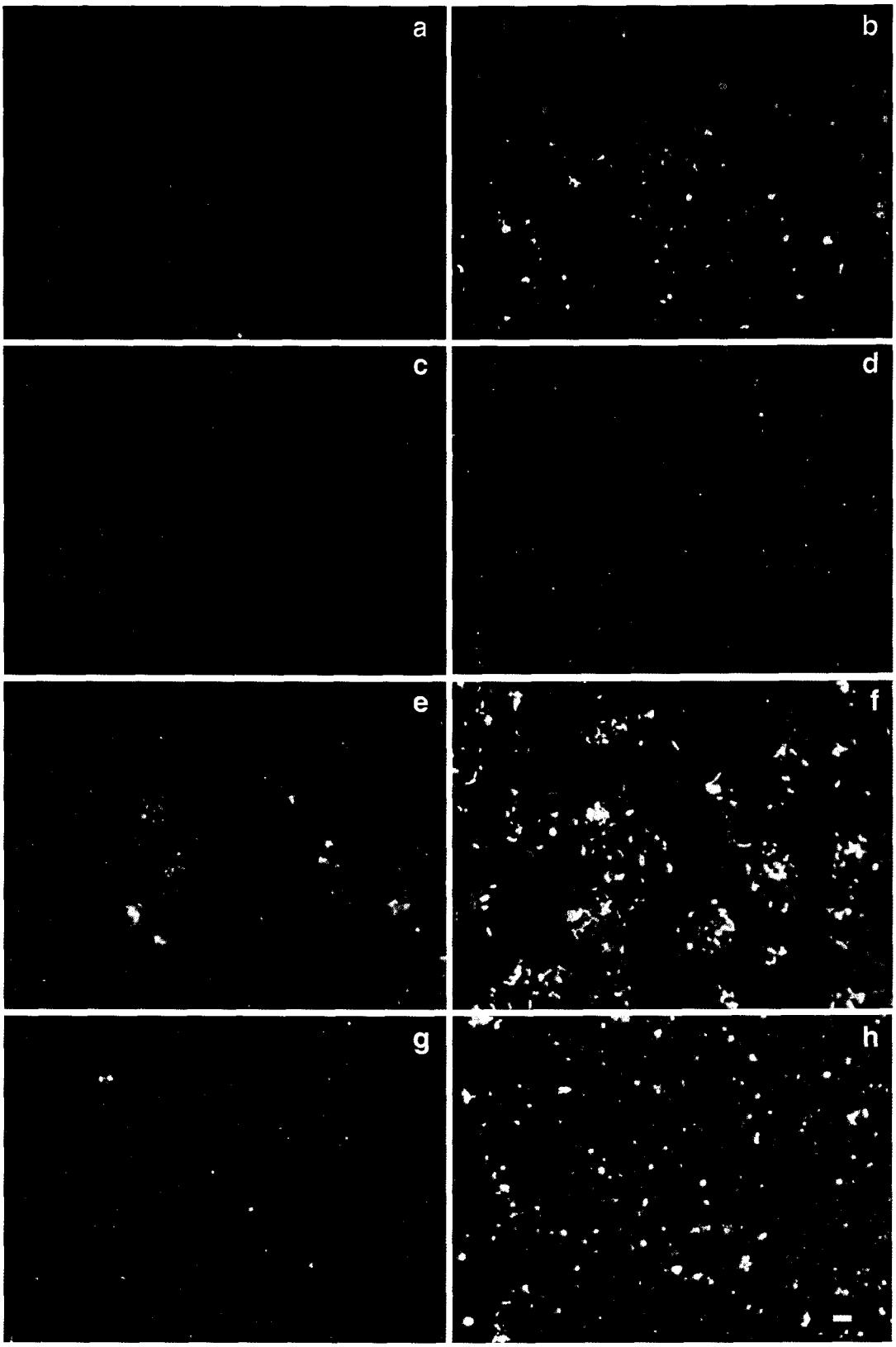

Fig. 2. Microphotographs of FISH (left) and DAPI (right) of SRB in sediments of Lake Sihwa (a, b), Lake Soyang (c, d), Lake Aha (e, f) and Lake Erhai (g, h). Bar=5 μm.

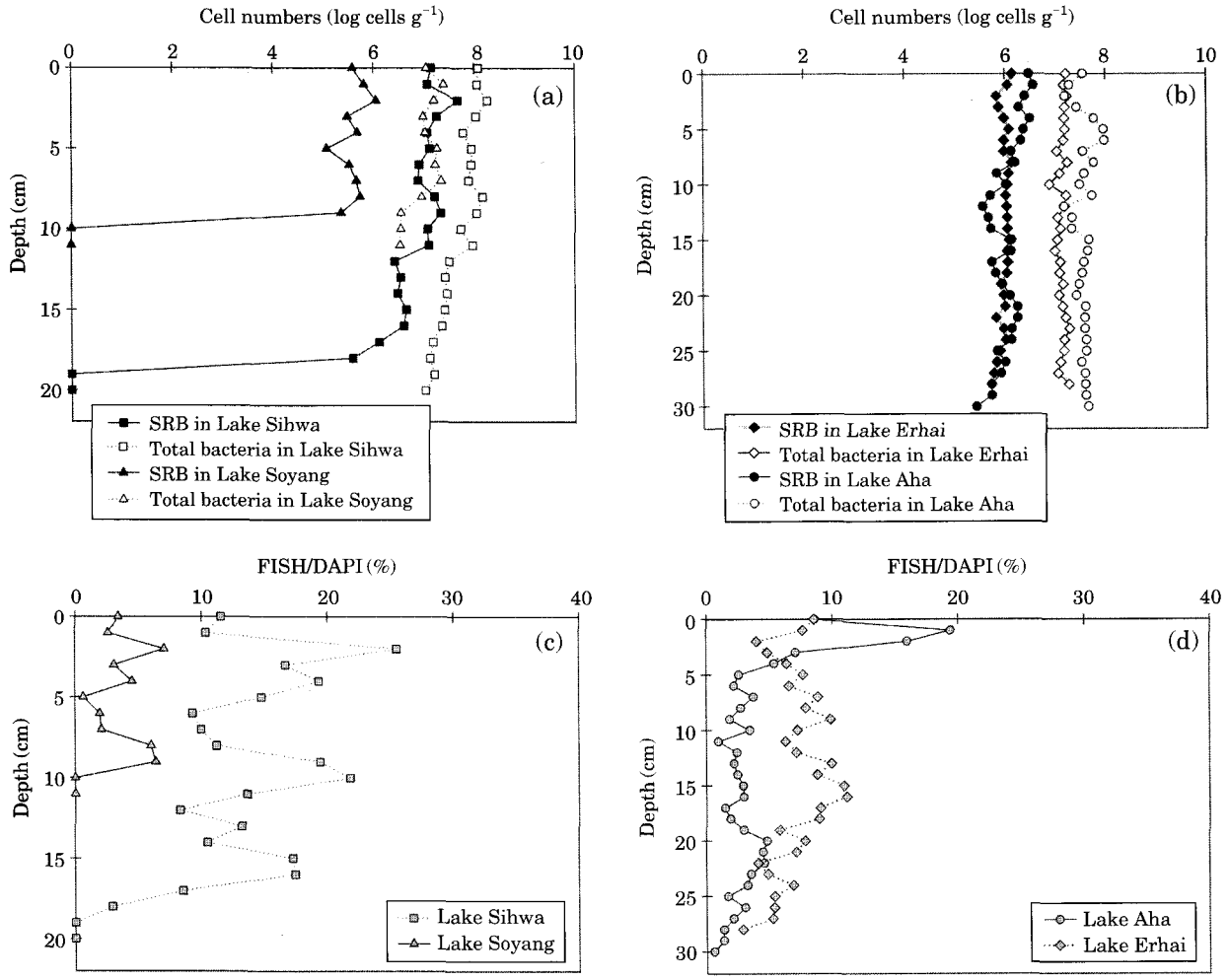

Fig. 3. The vertical distribution of total bacterial numbers and SRB numbers (a, b) and the proportion of SRB to DAPI-stained cells (c, d) in sediments of Korean and Chinese lakes.

2. The vertical distribution of total bacterial numbers and SRB in sediments

Fig. 3 shows the vertical distributions of SRB in core sediments of Lake Sihwa, Lake Soyang, Lake Aha and Lake Erhai. Microphotographs of hybridized cells with the probe SRB385 and DAPI staining are presented in Fig. 2. Here, total bacterial numbers are lower than those obtained in marine sediments (Llobet-Brossa *et al.*, 1998).

In the case of lakes in Korea, DAPI-stained cells ranged from 5.8×10^6 to 1.9×10^8 cells g^{-1} and from 3.4×10^6 to 2.5×10^7 cells g^{-1} within sediments of Lake Sihwa and Lake Soyang, respectively (Fig. 3a). The numbers of SRB ranged from 3.8×10^5 to 4.7×10^7 cells g^{-1} in Lake Sihwa and those in Lake Soyang were from 1.2×10^5 to 1.1×10^6 cells g^{-1} (Fig. 3a). The number of SRB in Lake Sihwa was 24 times higher than of Lake Soyang. These differences probably caused by organic matter content of the sediments (Hyllerberg *et al.*, 1984). Comparing both lakes, Lake Sihwa is more polluted than Lake Soyang. The concentrations of COD were 1.5 and 10 mg L^{-1} in Lake Soyang (Kim *et al.*, 2007) and Lake Sihwa (Choi, 2007) on June of 2006, respectively. The number of SRB is closely related with the concentration of organic materials and sulfate is not affecting the abundance of SRB (Hyun *et al.*, 1999). The higher number of SRB in Lake Sihwa would be related to the higher concentration of available organic matter. In Lake Sihwa and Lake Soyang, SRB were not detected below 19 and 10 cm depths, respectively. This means that SRB in Lake Sihwa

is located to deeper layer than that in Lake Soyang. Although SRB were not detected under 19 cm and 10 cm depths of both lakes, total bacterial numbers were still abundant at all observed sediment. Generally, sediment may contain oxygen-rich, oxygen-poor, and oxygen depletion zones, and the bacteria found within its different zones will vary according to the chemical reactions they use for energy. The order of aerobic and anaerobic respiration in soil and sediment are as follows; O_2 - MnO_2 - Fe^{3+} - SO_4^{2-} - CO_2 (Fredrickson and Onstott, 1996). Therefore, methanogenesis is processing below 19 and 10 cm depths at Lake Soyang and Lake Sihwa, respectively.

The high peaks were appearing at depth of 2 cm in both lakes (Fig. 3a). This is accordance with results obtained in Wadden Sea sediments of the German North Sea coast by Llobet-Brossa *et al.* (1998) and marine Arctic sediment of Smeerenburgfjorden, Svalbard by Ravenschlag *et al.* (2000).

For the lakes in China, total bacterial numbers were from 1.5×10^7 to 9.5×10^7 cells g^{-1} and from 1.0×10^7 to 1.9×10^7 cells g^{-1} in Lake Aha and Lake Erhai, respectively. Each depth of sediment showed similar total bacterial numbers in Lake Erhai but in Lake Aha, from surface to 15 cm depth, total bacterial numbers showed different values. The pattern of total bacterial numbers in here was similar with the vertical distribution of SRB. Irregular SRB distribution in Lake Aha than Lake Erhai might be caused by sulfate, iron, manganese and organic matters inflow from coal mine around Lake Aha. In Lake Aha, SRB ranged from 2.7×10^5 cells g^{-1} to 3.7×10^6 cells g^{-1}, which is lower range than that of Lake Sihwa. Maximum density of SRB was shown at the 1 cm depth. In Lake Erhai, that ranged from 0.5×10^6 to 1.4×10^6 cells g^{-1}, with maximum at the surface layer (Fig. 3b). The numbers of SRB were similar in both Lake Aha and Lake Erhai although sulfate becomes known to be excessively enriched in water and sediment of Lake Aha (Wang *et al.*, 2003). As described above, this result suggests that the abundance of SRB is not related with sulfate concentration.

3. The ratio of sulfate-reducing bacteria (SRB) to total bacterial numbers in sediments

The proportion of SRB to DAPI-stained cells ranged from 2.9 to 25.6% (Lake Sihwa) and from 0.6 to 7.1% (Lake Soyang) (Fig. 3c). The sediment of Lake Sihwa contained mud, whereas that of Lake Soyang contained thick-gained sand. The SRB proportion in muddy sediment was much higher than that in sandy sediment. This result was same with the research by Llobet-Brossa (Llobet-Brossa *et al.*, 1998), which compared the proportion of SRB between sediments with different content.

In case of lakes in China, the proportions of SRB to DAPI-stained cells were from 0.6 to 19.4% in Lake Aha, and from 2.9 to 11.2% in Lake Erhai, respectively (Fig. 3d). In case of an intertidal mud flat of the German Wadden Sea, SRB ranged from 1.7 to 12.3% (Llobet-Brossa *et al.*, 2002) which is similar with the result of Lakes in China. Averagely, the proportion of SRB in Lake Sihwa, Lake Soyang, Lake Aha, and in Lake Erhai were 13.8%, 3.8%, 4.0%, 7.2%, respectively.

Through this study, we confirmed that the vertical distribution patterns of SRB within sediment were different with sample sites. Especially, the SRB existing layer of sediments in China was deeper than that in Korea. The highest SRB numbers showed, however, at surface layer (0-2 cm) in all samples. This may be related to the higher concentration of available organic matter, and heavy metals at surface layer.

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LITERATURE CITED

Amann, R.I., J. Stromley, R. Devereux, R. Key and D.A. Stahl. 1992. Molecular and microscopic identification of sulfate-reducing bacteria in multispecies biofilms. *Appl. Environ. Microbiol.* **58**: 614-623.

Amann, R.I., W. Ludwig and K.H. Schleifer. 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**: 143-169.

Aron, L.M., E.B. Pamela and T.H. Alan. 1989. Acid stress and aquatic microbial interactions, p. 1-19. *In*: Salem, S.R. and P.D. Rao (eds.), CRC Press. Inc., Florida, USA.

Choi, K.S. 2007. Management of brackish lake-case

of Lake Shiwa. Proceeding of Symposium for management of brackish lakes in korea. *Wonju Regional Enrironmental Office*. 120-137.

Fredrickson, J.K. and T.C. Onstott. 1996. Microbes deep inside the earth. *Sci. Americans*. **275**: 68-73.

Guodong, Z., B. Takano, A. Kuno and M. Matsuo. 2001. Iron speciation in modern sediment from Erhai Lake, southwestern China Redox conditions in an ancient environment. *Appl. Geochem*. **10**: 1201-1213.

Hobbie, J.E., R.J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol*. **33**: 1225-1228.

Hyllerberg, J. and H. Riis-Vestergaard. 1984. Marine environments; the fate of detritus. Akademisk Forlag, Copenhagen, Denmark.

Hyun, M.S., I.S. Chang, H.S. Park, B.H. Kim, H.J. Kim, H.K. Lee and K.K. Kwon. 1999. Relationship between the organic content, heavy metal concentration and anaerobic respiration bacteria in the sediment of Shihwa-ho. *Kor. J. Appl. Microbiol. Biotechnol*. **3**: 252-259.

Icgen, B., S. Moosa and S.T.L. Harrison. 2007. A study of the relative dominance of selected anaerobic sulfate-reducing bacteria in a continuous bioreactor by fluorescence in situ hybridization. *Microbiol Ecol*. **53**: 43-52.

Jørgensen, B.B. 1982. Mineralization of organic matter in the sea bed-the role of sulphate reduction. *Nature*. **296**: 643-645.

Kim, B., K.S. Cho, W.M. Heo and D.S. Kim. 1989. The eutrophication of Lake Soyang, *Korean J Limnol*. **31**: 17-24.

Kim, B.C. 2006. Survey of ecosystem structure of Lakes in the North-Han River system. *National Institude of Environmental Research* 161.

Kim, J.K., M.S. Shin, C.W. Jang, S.M. Jung and B.C. Kim. 2007. Comparison of TOC and DOC Distribution and the Oxidation Efficiency of BOD and COD in Several Reservoirs and Rivers in the Han River System. *Journal of Korean Society on Water Quality* **23**: 72-80.

Koizumi, Y., S. Takii, M. Nishino and T. Nakajima. 2003. Vertical distributions of sulfate-reducing bacteria and methane-producing archaea quantified by oligonucleotide probe hybridization in the profundal sediment of a mesotrophic lake. *FEMS Microbiol. Ecol*. **44**: 101-108.

Li, J.H., K.J. Purdy, S. Takii and H. Hayashi. 1999. Seasonal changes in ribosomal RNA of sulfate-reducing bacteria and sulfate reducing activity in a freshwater lake sediment. *FEMS Microbiol. Ecol*. **24**: 221-234.

Llobet-Brossa, E., R. Rossello-Mora and R. Amann. 1998. Microbial community composition of Wadden sea sediment as revealed by fluorescence in situ hybridization. *Appl. Environ. Microbiol*. **64**: 2691-2696.

Llobet-Brossa, E., R. Rabus, M.E. Bottcher, M. Konneke, N. Finke, A. Schramm, R.L. Meyer, S. Grotzchel, R. Rossello-Mora and R. Amann. 2002. Community structure and activity of sulfate-reducing bacteria in an intertidal surface sediment: a multi-method approach. *Aquat. Microb. Ecol*. **29**: 211-226.

Okabe, S., T. Itoh, H. Satoh and Y. Watanabe. 1999. Analyses of spatial distributions of sulfate-reducing bacteria and their activity in aerobic wastewater biofilms. *Appl. Environ. Microbiol*. **65**: 5107 -5116.

Ravenschlag, K., K. Sahm, C. Knoblauch, B.B. Jørgensen and R. Amann. 2000. Community structure, cellular rRNA content, and activity of sulfate-reducing bacteria in marine arctic sediments. *Appl. Environ. Microbiol*. **66**: 3592-3602.

Sahm, K., C. Knoblauch and R. Amann. 1999. Phylogenetic affiliation and quantification of psychrophilic sulfate-reducing isolates in marine arctic sediments. *Appl. Environ. Microbiol*. **65**: 3976-3981.

Sun, S. and Z. Chen. 2000. Nitrogen distribution in the lakes and lacustrine of China. *Nutrient Cycling in Agroecosystems* **57**: 23-31.

Trimmer, M., K.J. Purdy and D.B. Nedwell. 1997. Process measurement and phylogenic analysis of the sulfate reducing bacterial communities of two contrasting benthic sites in the upper estuary of the Great Ouse, Norfolk, UK. *FEMS Microbiol. Ecol*. **24**: 333-342.

Tonolla, M., A. Demarta, S. Peduzzi and D. Hahn. 2000. In situ analysis of sulfate-reducing bacteria related to *Desulfocapsa thiozymogenes* in the chemocline of meromictic (Switzerland). *Appl. Environ. Microbiol*. **66**: 820-824.

Wang, Y.C., R.G. Huang, and G.J. Wan. 1998. The newly developed sample for collecting samples near the lacstrine sediment-water interface. Geol. Geochem. **1**: 94-96.

Wang, F., C. Liu, X. Liang and Z. Wei. 2003. Remobilization of trace metals induced by microbiological activities near sediment-water interface, Aha Lake, Guiyang. *Chinese Science Bulletin* **48**: 2352-2356.

Wang, M.Y., X.B. Liang, X.Y. Yuan, W. Zhang and J. Zeng. 2007. Analyses of the vertical and temporal distribution of sulfate-reducing bacteria in Lake Aha (China). *Envrion. Geol*. dio: 10.1007/s 00254-007-0787-6.

Widdle, F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria, p. 469-585. *In*: A. J. B. Zebnder (ed.), Biology of anaerobic microorganisms. John Wiley and Sons, Inc., New York, N.Y.

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