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Article

What caused the pink coloration of water at the bottom of Lake Shiraishi ?

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Abstract

Pink water was collected from the bottom of Lake Shiraishi, a brackish lake located in Kihoku-cho, Mie Prefecture, in September 2009 and January 2010 for preliminary field investigation. The water quality and bacterial community structure were investigated again in August 2010 to clarify the cause of the pink water; microscopic observation to clarify the morphology and PCR-denaturing gradient gel electrophoresis (DGGE) to characterize bacterial community were performed. The water sampled from near the bottom of the lake at a depth of 8 m was colored pink and smelled of hydrogen sulfide. Microscopic examination revealed that the dominant cells were 5–7 μm in size, spherical or ovoid, pink to purple in color, with gas vesicles, and were not motile. The results of microscopy and phylogenetic analysis using PCR-DGGE suggested that the pink coloration was related to photosynthetic sulfur bacteria of either *Halochromatium roseum* or *Lamprobacter modestohalophilus*.

Key words: Photosynthetic sulfur bacteria, PCR-DGGE, Lake Shiraishi

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Introduction

Lake Shiraishi, located in Kihoku-cho, Mie, Japan, is a brackish lake covering an area of 472,000 m^2 with a maximum depth of 9 m (Tsuchihashi *et al.*, 1996). The lake receives freshwater input from the Funatsu and Choshi rivers, and seawater input from Hikimoto Bay. Brackish lakes have a complex water environment with a mixture of fresh and seawater. In Japan, lakes Suigetsu (Kondo *et al.*, 2009; Mori *et al.*, 2013), Kaiike (Matsuyama 2004), Obuchi (Ueda *et al.*, 2000), Abashiri (Mikami *et al.*, 2002), and others have been reported in studies on brackish lakes. The characteristics of these brackish lakes throughout the year are that density gradients appear and the bottom of the lake is consistently extremely hypoxic.

Microbial communities at layers where the dissolved oxygen gradient changes are interesting from the viewpoint of degradation of organic compounds in the redox system, and analysis of these communities using 16S rRNA genes is advancing. Kondo *et al.* (2009) reported that green sulfur bacteria phylogenetically related to the genera *Prosthecochloris*, *Pelodyctyon*, and *Chlorobium* within the phylum *Chlorobi*, were dominant in the chemocline in Lake Suigetsu; furthermore, Lunina *et al.* (2007) reported that green sulfur bacteria, purple sulfur bacteria, and purple nonsulfur bacteria were detected in Lake Shunet, Khakassia.

In Lake Shiraishi, several studies have been conducted for continuous measurement of water temperature, salinity, dissolved oxygen (Tsuchihashi *et al.* 1996), and bacterial community in the sediment (Santander-De Leon *et al.*

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2013a; 2013b).

Oyster farming is a flourishing industry in this lake and is the main fishing industry present. It was reported that large numbers of oysters died during the summer (Tsuchihashi *et al.* 1996). So, we conducted some basic research into the reason for these deaths in our laboratory (Santander-De Leon *et al.* 2013a). During this investigation, we encountered a pink-colored water. Even those that have been involved in the aquaculture industry for decades do not know about this coloring phenomenon, which suggests that it is not often observed. The purpose of this study was to clarify the cause of the pink water from an ecological viewpoint. To achieve this aim, microorganisms were characterized by the denaturing gradient gel electrophoresis (DGGE) method and microscopic examination.

Methods

Sampling station and sample treatments

The sampling station was located at the center of Lake Shiraishi, where the oyster farming was concentrated (Fig. 1). A preliminary field investigation was conducted in September 2009 and January 2010, and the main field sampling commenced in August 2010. Water temperature, salinity, dissolved oxygen and chlorophyll *a* (Chl. *a*) were measured using an automatic water analyzer (HYDROLAB DS5, Kankyo System). Water column samples were collected along a vertical distribution every 1 m from the surface to the bottom (water depth 8 m: 9 layers) to measure nutrient contents and bacterial community composition at the sampling station using a motor pump. Water samples collected for chemical analysis of nutrients were immediately filtered through glass fiber filters (GF-75, Advantec) that had been precombusted at 420℃ . Water samples collected for dissolved silica were immediately filtered through quantitative filter paper (No. 5C, Advantec). Filtrates for determination of nutrients and dissolved silica were stored at -20℃ and 4℃ , respectively, until chemical analysis in the laboratory. Nutrients were measured using an auto analyzer (SWAAT AASU-2000, BLTEC). Dissolved silica concentrations were analyzed spectrophotometrically using the molybdenum blue method of Saijo and Mitamura (1995). Bacterial cells were observed under a microscope without treatment (BZ-9000, Keyence). For PCR-DGGE analysis, the bacterial cells were collected by filtration. We selected a pore size of 3.0 μm to focus on pink-colored bacteria with a large cell size (>3.0 μm) and investigate their distribution. 30 mL water samples (depth of 0 m to 7 m) and 10 mL (depth of 8m) were filtered through a polycarbonate membrane filter (3.0 μm, Advantec) to collect bacterial

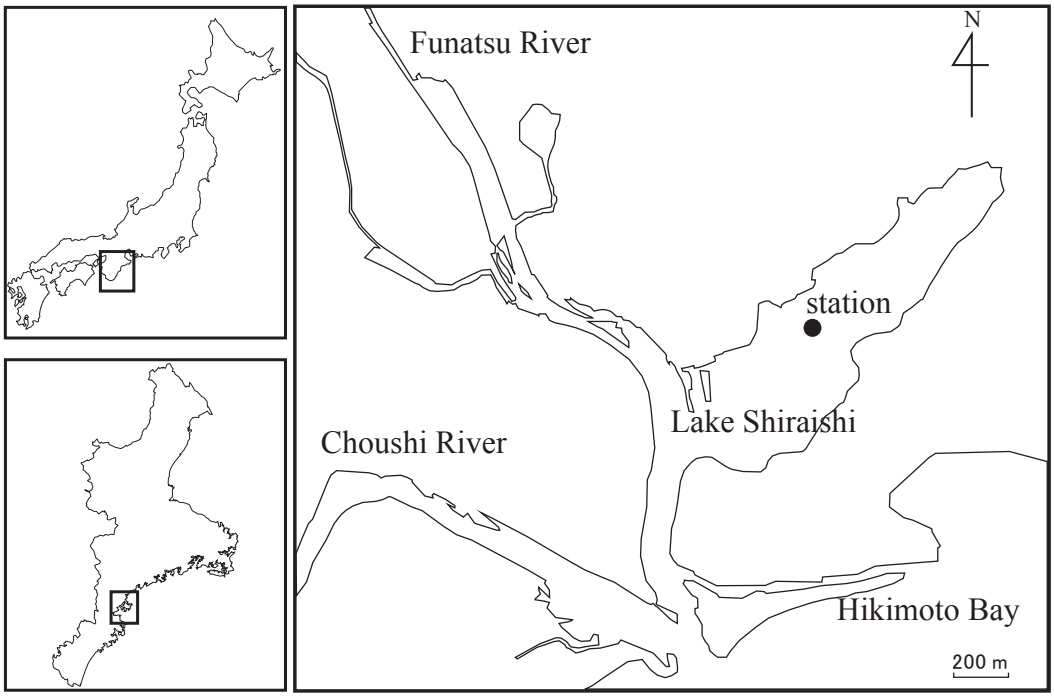

Fig. 1. The location of the sampling station at Lake Shiraishi, Mie, Japan（34°6'47.6"N; 136°14'16.4"E）.

cells. The membrane filter was stored at -20 ℃ until DNA extraction. DNA extraction was carried out according to the manufacturers' instructions (QuickGene DNA tissue kit S, KURABO) using the QG-Mini80 extraction machine. The DNA concentration was measured based on the absorbance value at 260 nm using absorption spectrometer (DU730, BECKMAN COULTER).

PCR amplification of the 16S rRNA gene

PCR amplification of 16S ribosomal RNA genes was carried out using the forward primer 341F (5'-CCTACGGGAGGCAGCAG-3') with a GC-clamp and the reverse primer 907R (5'- CCGTCAATTCCTTTGAGTTT-3') (Muyzer *et al.* 1995) in 25 μL reaction volumes, which contained 0.6 units of Ex Taq (Takara Bio, Otsu, Japan), 2.5 μL of dNTP mixture (2.5 mM each), 2.0 μL of each primer (5 pmol each), 11.0 μL of DNA-free water, and 5 μL of DNA template (containing 1–10 ng of DNA). The thermal cycling conditions were optimized using a touchdown program as follows (Don *et al.*, 1991): after initial denaturation of 5 min at 95℃ , 19 cycles of 95℃ for 1 min, 62℃ for 1 min, and 72℃ for 1 min, decreasing the denaturation temperature after the first cycle by 0.8 ℃ every cycle followed by nine cycles of 95 ℃ for 1 min, 52 ℃ for 1 min, and 72 ℃ for 1 min; a final cycle of 95℃ for 1 min, 55℃ for 1 min, and 72℃ for 10 min for final extension was conducted. The amplicons were confirmed using agarose gel electrophoresis. PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Purified DNA concentrations were measured in the same method as for DNA extracts.

DGGE conditions

DGGE was performed by using a D-Code system (Bio-Rad Laboratories) according to Muyzer *et al.* (1993, 1995). Approximately 700 ng of the purified PCR products were applied to 6% polyacrylamide gel with urea and deionized formamide with a gradient concentration range from 25% to 50% (where 100% denaturing gels contain 7M urea and 40% formamide in 0.5× TAE buffer). Electrophoresis was run at 60V, 60℃ for 16 h. Gels were stained with SYBR Gold (Thermo Fisher, USA) and observed on a blue light transilluminator.

Sequence analysis of DGGE bands

DGGE bands were excised from the gels using Pasteur pipettes. The excised gel pieces were suspended in 200 μL TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediaminetetraacetic acid), and frozen and thawed to elute the amplified DNA. The excised single bands were re-amplified using the same PCR conditions with the reaction volume of 20 μL, and the amplicons were electrophoresed on 1.5% agarose gel at 100 V. The PCR products were purified in the same method as for DGGE analysis. The sequences were determined with ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA), and the BigDye Terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems) was used in accordance with the manufacturer's instructions. The sequencing primer was the 341F primer without a GC clamp.

Phylogenetic analysis

Approximately 500 bp of each DGGE band sequence was used for the phylogenetic analysis. The phylogenetic positions of the excised bands were determined by searching the DDBJ database with the BLAST program (http://blast.ddbj.nig.ac.jp/blastn?lang=ja), and the sequences were aligned using the CLUSTAL X program (version 2.1) (Larkin *et al.*, 2007). A phylogenetic tree was constructed by the neighbor-joining method with 1,000 bootstrap replicates in CLUSTAL X (Saitou and Nei, 1987).

Accession numbers

The partial nucleotide sequences of the 16S rRNA gene fragments are available in the DDBJ database under accession numbers LC543551 to LC543564.

Results

When the water was collected at a depth of 8.5 m, the water was pink in color and smelled of hydrogen sulfide during the preliminary investigation in September 2009. The water samples collected at each depth in September 2009 are shown in Fig. 2. The pink color was evident from a depth of 7.5 m and was the darkest at 8.5 m in September 2009 (Fig. 2-A); even in winter (January 2010) the water was pink at 7.5 m depth (Fig. 2-B). In August 2010, the water taken from 8 m was pink and smelled of hydrogen sulfide. Vertical profiles of the water temperature, salinity, dissolved oxygen, and Chl. *a* were shown in Fig. 3. The water temperature in the 1.5 m layer was slightly higher than that in the surface layer and was almost constant below 1.5 m. A steep salinity gradient was observed between 0 and 1 m, with little change from about 1.5 m down. The vertical profile of dissolved

Fig. 2. Photographs of the water collected during the preliminary investigation. A: vertical sampling of water from 0 to 8.5 m (September 2009), B: comparison of water at depths of 7.5 m (left) and 7 m (right) (January 2010).

oxygen was almost uniform from 0 m to 6 m, and decreased sharply deeper than approximately 6.5 m. The average concentration of Chl. *a* from the surface to 5 m depth was about 1.7 μg L^{-1}, while the value increased with depth and the maximum was about 7.9 μg L^{-1} in the 8 m layer.

The nutrient concentrations in the water samples are shown in Table 1. The concentrations of ammonia and phosphate were extremely high at the lake bottom. The nitrite concentration was distributed almost uniformly from the surface to the lake bottom. The nitrate concentration in the surface water was markedly high and was distributed uniformly in layers deeper than 1 m. The silica concentration in the surface water was high and was extremely high at the lake bottom.

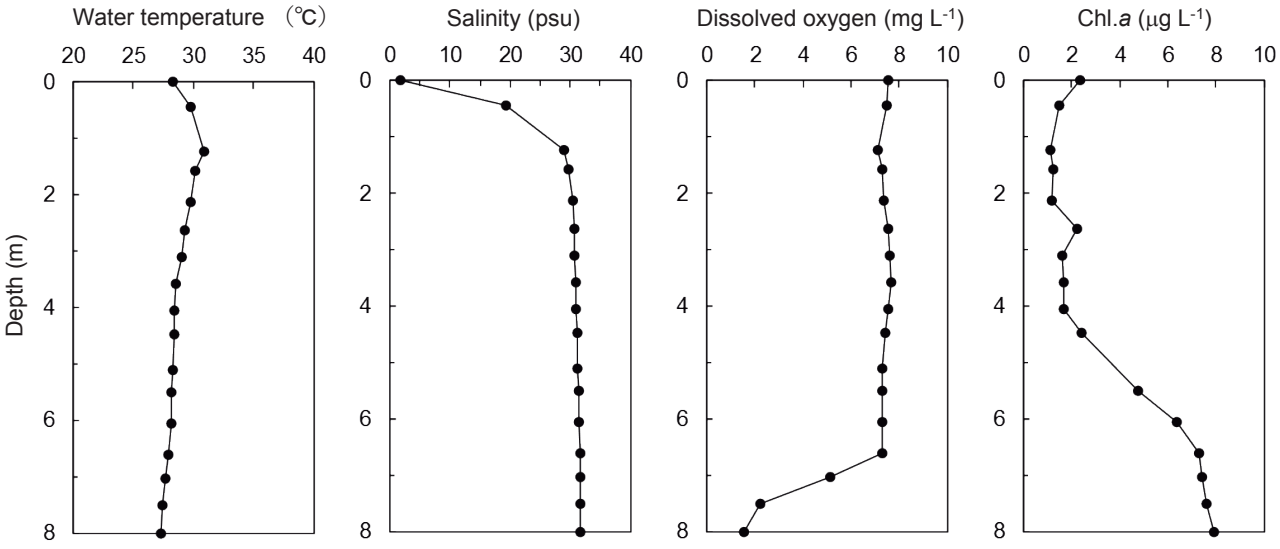

Fig. 3. Vertical distribution of water temperature, salinity, dissolved oxygen and the concentration of chlorophyll *a* (Chl. *a*) in August 2010.

Table 1. Nutrient concentrations in water samples at each sampling depth in August 2010（N.D. = not detected）.

Depth (m)	NH_4^+ - N (mg L^{-1})	NO_2^- - N (mg L^{-1})	NO_3^- - N (mg L^{-1})	PO_4^{3-} - P (mg L^{-1})	Si (mg L^{-1})
0	0.013	0.001	0.026	N.D.	2.16
1	0.012	0.001	0.006	0.002	0.409
2	0.010	N.D.	0.005	0.003	0.481
3	0.018	0.001	0.006	0.004	0.407
4	0.026	N.D.	0.010	0.003	0.270
5	0.015	N.D.	0.005	0.027	0.186
6	0.035	0.002	0.011	0.041	0.315
7	0.051	0.001	0.004	0.126	0.383
8	1.46	0.001	0.005	0.579	4.14

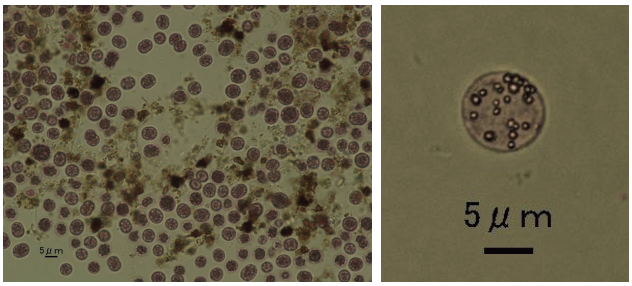

Fig. 4. Observation of microorganisms in samples taken from pink-colored water（8 m layer）of Lake Shiraishi in August 2010 using a microscope.

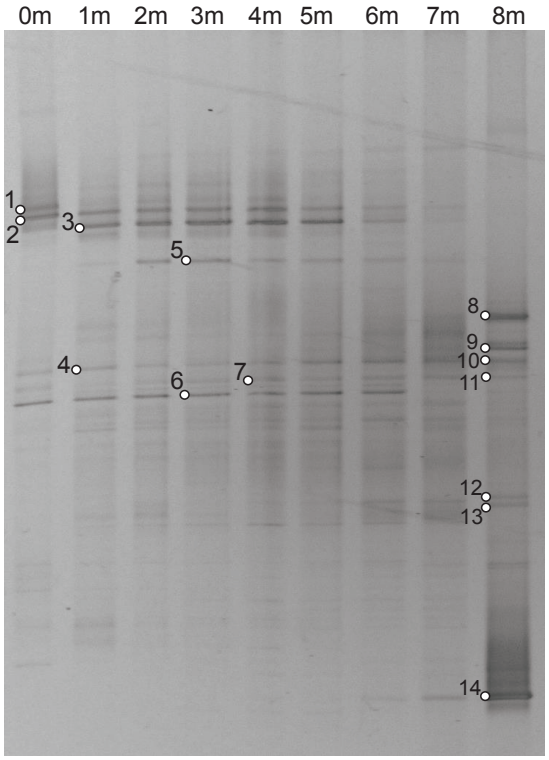

Fig. 5. Denaturing gradient gel electrophoresis profiles of universal 16S rRNA gene amplicons of each layer of Lake Shiraishi. Open circles with numbers indicate excised bands. These bands were analyzed by sequencing, with the results of sequencing shown in Table 2.

Microscopic observation showed that the dominant cells in the pink colored lake bottom water were coccoid or ovoid, approximately 5–7 μm in length with some vesicles in the enteric cytoplasm. Cells were either purple or pink in color (Fig. 4). No flagellum was found, and no motility was observed.

The DGGE banding patterns of the microbial populations at different depths are shown in Fig. 5. The band pattern was very similar among samples from 0–6 m in depth. The pattern below 7 m was different when compared with the upper layers; in particular, the band pattern of 8 m depth was greatly different from that of the other layers. Four bands (bands 2, 3, 4 and 6) showed dominance in the upper layer from 6 m. Bands that were unique at the depth of 8 m were observed from the DGGE profile. DNA sequences were obtained from a total of 14 bands on the DGGE gels. The closest strain and phylogenetic group of each sequence are listed in Table 2. Bands 1–3 and 5 were derived from the chloroplast of phytoplankton. Bands 4 and 6 that dominated from 0 to 7 m depth were uncultured bacteria belonging to *Bacterioidetes* and *Ralstonia insidiosa*, respectively, of the β-*proteobacteria*. Bands 8–11, which appeared only at 8 m depth, were closely homologous to the green sulfur bacterium, *Chlorobium* spp. Band 14 was very similar to both *Halochromatium roseum* and *Lamprobacter modestohalophilus* belonging to the purple sulfur bacteria, with the same similarity to each strain in the BLAST search. The phylogenetic tree containing sequencing results of the DGGE bands and the closest strains is shown in Fig. 6. The microorganisms detected by DGGE were chloroplast derived from phytoplankton, *Chlorobi*, *Bacteroidetes*, *Chloroflexi*, β-*proteobacteria*, and γ-*proteobacteria*. Band 14 showed the same homology value (99.4%) for both *L. modestohalophilus* and *H. roseum*, but the phylogenetic tree indicated that band 14 was more closely related to *L. modestohalophilus*.

Discussion

One of the major characteristics of brackish water lakes is that seawater and freshwater are intricately mixed, which greatly affects the distribution of chemical water quality and the biology of microorganisms (Clark, 1977; Barnes, 1980). The water temperature and salinity profiles indicated that a halocline was formed as a result of freshwater flowing from the river during the investigation (Fig. 3). The high concentrations of nitrate and silica in the surface water

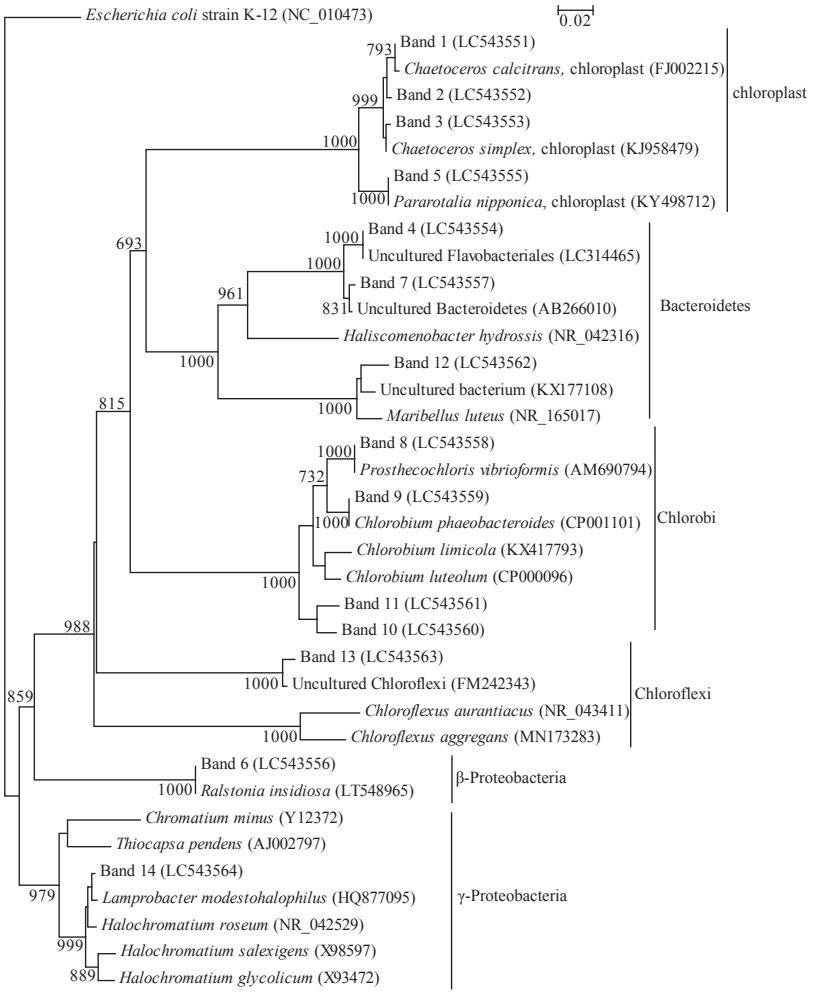

Fig. 6. Neighbor-joining tree of partial 16S rRNA gene sequences recovered from denaturing gradient gel electrophoresis bands. Accession number appears after the genus name. The scale bar indicates 0.05 changes per nucleotide. The numbers on the branches refer to bootstrap values for 1,000 replicates; only those above 650 are shown. *Escherichia coli* was used as an outgroup.

Table 2. Basic local alignment search tool（BLAST）homology searches of denaturing gradient gel electrophoresis bands obtained from Lake Shiraishi.

DGGE band	Most homologus relatives	Sequence Identity（%）			Taxonomic group	Accession no.
1	chloroplast	505/507	（	99.6% ）	chloroplast	FJ002215
2	chloroplast	494/495	（	99.8% ）	chloroplast	JN207230
3	chloroplast	493/506	（	97.4% ）	chloroplast	KJ958479
4	Uncultured bacterium	517/517	（	100% ）	Bacteroidetes	JQ198577
5	chloroplast	503/505	（	99.6% ）	chloroplast	KY498712
6	*Ralstonia insidiosa*	517/517	（	100% ）	β -Proteobacteria	LT548965
7	Uncultured bacterium	510/510	（	100% ）	Bacteroidetes	GQ916124
8	*Prosthecochloris vibrioformis*	513/513	（	100% ）	Chlorobi	AM690794
9	*Chlorobium phaeobacteroides*	508/508	（	100% ）	Chlorobi	CP001101
10	*Chlorobium limicola*	484/504	（	96.0% ）	Chlorobi	KX417793
11	*Chlorobium luteolum*	493/504	（	97.8% ）	Chlorobi	CP000096
12	Uncultured bacterium	500/512	（	97.7% ）	Bacteroidetes	KX177108
13	Uncultured Chloroflexi bacterium	488/495	（	98.6% ）	Chloroflexi	FM242343
14	*Halochromatium roseum*	521/524	（	99.4% ）	γ -Proteobacteria	NR042529
	Lamprobacter modestohalophilus	521/524	（	99.4% ）		HQ877095

also supported this fact. Conversely, the concentrations of ammonia and phosphate were high at the lake bottom (Table 1). The tendency of these vertical distributions was similar in various brackish water lakes, such as Lake Nakaumi (Kondo *et al.*, 1994) and Lake Abashiri (Mikami *et al.*, 2002). In addition, the dissolved oxygen was consumed in the bottom layer and the oxygen concentration was greatly reduced near the bottom of the lake.

Similar to the distribution of photosynthetic bacteria in the chemocline in Lake Suigetsu (Mori *et al.*, 2013) and in the upper part of the anoxic layer in Lake Abashiri (Mikami *et al.*, 2002), the gradient in oxygen concentration affected the microbial community composition in Lake Shiraishi. The microbial community in the 8 m layer where dissolved oxygen was low was very different from that of the other layers, which was consistent with the other features of water from this layer (pink colored and smelled of hydrogen sulfide). Pimenov *et al.* (2003) reported a pale pink layer of water because of the development of purple bacteria (6×10^5 cells mL^{-1}), which were assigned by their morphological and spectral characteristics to *L. purpurea* in Lake Shira (Khakassia). In the present study, the number of cells that caused the pink color shown in Figure 4 could not be accurately counted but the total bacterial count by DAPI (4',6-diamidino-2-phnylindole) staining was on the order of 10^6 cells mL^{-1}, and many of the cells were larger than 3 μm (data not shown). Considering the water quality environment at a depth of 8 m, a high percentage of the bacteria in the lake bottom water are perhaps photosynthetic bacteria. Therefore, photosynthetic bacteria are the most likely candidates responsible for the pink color. In fact, the Chl. *a* concentration was elevated near the bottom layer, but this might have been because of photosynthetic bacteria capable of producing bacteriochlorophyll *a* rather than by phytoplankton.

Although a halocline was formed near the surface and the salinity changed significantly, the banding pattern was almost the same from the surface to a depth of 7 m except for band 5 (Fig. 5). In other words, the flow of water from the river had no significant effect on the microbial community composition collected by the 3 μm pore size filter. In contrast, the community composition was drastically altered in the lake bottom water with low oxygen. This implies that the gradient of dissolved oxygen concentration had a significant effect on the microbial community composition. The results of the DGGE sequencing showed the presence of bacteria of purple sulfur bacteria of the γ-*Proteobacteria* and green sulfur bacteria of the phylum *Chlorobi*, in the 8 m layer (Fig. 5, Table 2), indicating that the gradient of oxygen concentration greatly affected the microbial community composition. Normally, the cells of *Chlorobi* group, which have a small cell size, would pass through the 3μm filter. However, the filter was clogged with pink bacteria and suspended solid when the cells were collected. It is possible that the cells of *Chlorobi* were trapped with other bacteria.

In the 8 m layer, the intensities of bands 8 and 14 were stronger than those of the other bands. Erroneous results can occur when PCR-DGGE analysis is used to quantify the microbial community in the environment (Nakagawa and Fukui, 2002). However, it has also been reported that when a high percentage of cells of a particular microorganism are present in a microbial community, the strength of the DGGE band corresponding to that microorganism was also strong (Casamayor *et al.*, 2000). In addition, Okunishi *et al.* (2012) showed that the abundance of microorganisms was reflected in the strength of the band. Therefore, the abundance of *Prosthecochloris vibrioformis* (band 8), a green sulfur bacterium, and *L. modestohalophilus* or *H. roseum* (band 14), a purple sulfur bacterium, are likely to be higher than those of other microorganisms inhabiting the lake bottom

Table 3. Comparison of the characteristics of *Halochromatium roseum*, *Lamprobacter modestohalophilus* and the cells of this study. "+" and "−" stand for the presence or absence of a feature. Data for reference species were taken from Kumar *et al.*（2007）（*H. roseum* strain JA134^T）and Gorlenko and Imhoff（2005）（*L. modestohalophilus*）.

Characteristic	*H. roseum*	*L. modestohalophilus*	This study
Cell shape	Rod	Ovoid to rod	Spheriacl or Ovoid
Motility	−	+	−
Cell size（μm）	2.0–3.0×3.0-5.0	2.0 – 2.5	5 – 7
Gas vesicles	+	+	+
Colour of cell suspension	Purple-pink	Purple-red	Purple-pink
NaCl optimum（%）	1.5 – 2.5	1 – 4	

water, based on the density of bands 8 and 14. Among them, the pink color of the lake bottom water suggested that the bacteria that appeared as band 14, which is closely related to purple sulfur bacteria, made a significant contribution.

Sequence analysis of band 14 showed that it was closely related to two bacteria, *L. modestohalophilus* and *H. roseum*, with the same homology (Table 2). However, the results of the phylogenetic tree showed that band 14 was more closely related to *L. modestohalophilus* (Fig. 6). Since the results of microscopic observation reflected the results of the nucleotide sequence and phylogenetic tree, band 14 was considered to be originated from the cells observed in Fig. 4. Table 3 summarizes the morphological characteristics of *L. modestohalophilus* (Gorlenko and Imhoff, 2005), *H. roseum* (Kumar *et al.*, 2007) and the characteristics of the bacteria collected from Lake Shiraishi as determined from microscopic observations. The cells in Lake Shiraishi were oval-shaped with a diameter of 5–7 μm and were larger than those of both *H. roseum* and *L. modestohalophilus*. Additionally, the cells were growing at salinity of about 30 psu, and motility was not observed. Consequently, it was morphologically difficult to identify them as either *H. roseum* or *L. modestohalophilus*.

In this study, it was revealed that the pink water taken from the bottom layer of Lake Shiraishi in August 2010 was caused by photosynthetic sulfur bacteria, *H. roseum* or *L. modestohalophilus*. However, neither *H. roseum* nor *L. modestohalophilus* could be confirmed genetically or morphologically. To confirm the identity of this photosynthetic bacterium, it is necessary to isolate and characterize this photosynthetic bacterium from the field and examine it in more detail using primers specific to sulfur photosynthetic bacteria.

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摘　要

白石湖におけるピンク色の湖底水の要因は何か？

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三重県紀北町にある汽水湖の白石湖において2009年9月および2010年1月の予備調査で，湖底付近からピンク色の水が採水された。このピンク色の水を解明するために，2010年8月に再度調査を行い，同時に顕微鏡観察，PCR-DGGE 解析を行った。その結果，水深8m の湖底付近から採水した水はピンク色で硫化水素のにおいがした。また顕微鏡観察では，細胞大きさは5-7 μm で球形または卵形をしており，色はピンクから紫色，ガス胞を持ち，運動性はなかった。顕微鏡観察の結果と PCR-DGGE の解析結果から，ピンク色の要因は *Halochromatium roseum* または *Lamprobacter modestohalophilus* のどちらかの光合成硫黄細菌のよるものだと考えられた。

キーワード：光合成硫黄細菌，PCR-DGGE，白石湖

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