Application of a Portable Spectrophotometer to Microbial Mat studies: Temperature Dependence of the Distribution of Cyanobacteria and Photosynthetic Bacteria in Hot Spring Water

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We developed a simple method for absorbance spectrophotometry to identify photosynthetic pigments of microbial mats using a portable spectrophotometer in the field. This method was very efficient for the identification of mat-forming phototrophs and estimations of their mixing ratios in the field. It was also applied to describe the structure of hot spring microbial mats developed at the Nakabusa Hot Spring, Nagano Prefecture. The microbial mats consisted of cyanobacteria and Chloroflexus, and their distribution depended on the water temperature. The ratio of these two bacteria determined by absorbance spectra was constant at temperatures ranging from 45 to 60°C, and the spectral mixing ratios of Chloroflexus were about 40%. That ratio increased with temperatures in the range of 60 to 70°C; above 70°C, only Chloroflexus was observed. The spectrophotometry also found a pink bacterial mat which had distinctive absorption peaks at 801 and 878 nm. These peaks strongly suggested that the organism was a novel phototrophic bacterium of a new taxon.

Key words: microbial mat, hot spring, cyanobacteria, Chloroflexus, portable spectrophotometer

The variety of colors found in hot spring microbial mats might be caused by the complexity of microbial components. Microscopic observation has been generally used in microbial mat studies. This method is not sufficient, however, for an identification of their components because bacterial species can not be classified only by their shapes. The PCR method is a powerful one for identification and molecular phylogenetic studies. However, it is not easy to correlate the results with the physical reality of microbial mats in the field. The PCR method is especially poor at describing the structure of microbial mats, because it cannot provide any information on the ratios of bacterial constituents. Moreover, neither microscopic observations nor the PCR method can be performed in the field.

Cyanobacteria-Chloroflexus mats were generally observed at various hot springs. Jørgensen and Nelson described a cyanobacteria-rich layer propagating onto an orange Chloroflexus-dominated layer. Phototrophs consisting of hot-spring mats possess four kinds of pigments: chlorophyll a, phycocyanin, and bacteriochlorophyll a and c. These photosynthetic pigments are useful chemical biomarkers to identify phototrophs. The measurement of absorbance spectra is a very useful and convenient method for determining the structure of microbial mats.

We developed a simple method to measure absorbance spectra with a portable spectrometer in the field. This method enables us to obtain an in situ absorbance spectrum at the sampling site without any degradation of photosynthetic proteins. Using the order of pigment types based on the absorbance spectrum, we classified hot-spring microbial mats and described the constitution of the bacterial community.
Materials and Methods

Absorbance spectrophotometry for field-study

The spectrometric system for field measurements consists of a light source, a spectrometer (MMS VIS, Carl Zeiss) and a computer (Fig. 1). A penlight (Hozan, 3V) was used as a light source. The spectrometer consists of an optical fiber, a concave grating and a photodiode array, and is very small (70×60×40 mm$^3$). The diameter of fiber inlet was 0.4 mm. The 256-channel photodiode array detected dispersed light ranging in wavelength from 300 to 1,100 nm (ultraviolet to near infrared). Wavelength resolution was about 3.3 nm/ch. A hand held computer (Libretto 60, TOSHIBA, with MS-DOS ver.5) was connected to the spectrometer for data acquisition. The electric power of the spectrometer was supplied from the computer battery. Transmitted light was measured by putting samples between the light source and the optical fiber.

Mat samples were placed between two slide glasses. The intensity $I_0$ of the light passing through only slide glasses

![Diagram of the measuring system](image)

Fig. 1. Photograph and schematic figure of the measuring system used in this study. The method for measuring absorbance spectra is schematically shown. $I_0$ is a spectrum of light transmitted hot spring water and slide glasses. $I$ is a spectrum of a light transmitted sample, hot spring water, and slide glasses.
and spring water, and then the light intensity $I$ passing through a sample saturated with spring water were measured (Fig. 1). $I_0$ represented the spectrum of the light source.

The spectrometric measurement conditions were as follows. Transmitted light was integrated for 6.10 ms and stacked for 100 times, and the averaged value was then recorded as the voltage proportional to the light intensity. Thus, each measurement took about 0.6 sec. Circles at a about 1 mm in diameter on the slide glasses were measured at one at a time. Since the samples sandwiched between two glasses were not always uniform in color, 2–20 spots were measured on each set of slide glasses.

**Data analysis**

The optical density of the sample represents an absorbance spectrum and is written as follows.

$$\text{Optical Density} = OD(\lambda) = -\log(I(\lambda)/I_0(\lambda)),$$

where $I(\lambda)$, $I_0(\lambda)$ are the photo intensity of the light source and the transmitted light of a sample, respectively. According to Lambert-Beer’s law, the optical density can also be written in the following form,

$$\text{Optical Density} = c(\lambda)d\log_{10}e,$$

where $c(\lambda)$ is the molar absorption coefficient, $c$ is the molarity of the absorbent, and $d$ is the thickness of the sample. The optical density depends on wavelength because the molar absorption coefficient $c(\lambda)$ is a function of wavelength.

As mentioned below, the spectra obtained were classified into five groups. Three appeared to be the absorbance spectra of mainly one species of phototroph. Here these spectra were regarded as identical spectra, which denotes the spectra of a pure bacterial component, and each was named a “pure spectrum” of the species. The rest of the data were grouped into the “mixed spectrum” and “null spectrum.” The mixed spectra appeared to represent the composite spectra of identical spectra with different mixing ratios. The null spectra showed no absorbance maxima and were regarded as microbial mats composed of chemotrophs, ranging in color from transparent to white. The mats having null spectra in this study generally consisted of so-called “sulfur-turf”\(^\text{12}\). “Null spectrum” is not discussed here.

Here, we modeled a “mixed spectrum” as the sum of identical spectra (Fig. 2). The model spectrum $OD_m(\lambda)$ was assumed to be written as a linear combination of each pure spectrum,

$$OD_m(\lambda) = \sum_k^n (a_k \times OD_k(\lambda)),$$

where $OD_k(\lambda)$ is the optical density for $k$-th identical spectra, and $n$ is the number of identical spectrum components considered. Coefficients $a_k$ were determined by the least squares method as model parameters were selected for fitting the synthetic spectrum with observed one.

The residual of the fitting was calculated by equation (4), and the data with residuals over 0.006 were excluded from the results because they were considered to be a poor fit with the observations.

$$\text{residual} = \frac{\sum_N ((OD_d(\lambda) - OD_m(\lambda))/OD_d(\lambda))^2}{N},$$

where $OD_d$ is data spectrum and $OD_m$ is model spectrum. $N$ is channel number. The spectral mixing ratio was defined using the coefficients as follows,

$$\text{Spectral Mixing Ratio of Type B} = \frac{a_k}{\sum_k a_k}.$$
ing ratio would be perfectly equal to the ratio of pigment concentration. However, since the sample thickness could not be controlled in this study, the spectral mixing ratio is an proxy of the concentration mixing ratio with some nonlinearity.

Site description and treatment of samples

Microbial mats occurring at the Nakabusa Hot Spring, Nagano Prefecture, were studied on August 9, 1998 and May 14 and 15, 1999. Nakabusa Hot Spring (137°45'E in Longitude, and 36°24'N in Latitude) is located beside the Nakabusa River which runs along a valley in the Chubu Mountains. Our study site was a debris dam across the river. The height of the dam wall was about 3.6 m, and there was a horizontal crack in the wall 1.8 m from ground level. Hot water flowed from the crack and down the wall surface, then ran into the Nakabusa River.

Microbial mats developed on the wall. We took samples from two sites (Fig. 3). Mat samples of different temperatures were taken along a horizontal line 1.2 m above ground level (“Site 1” on Fig. 3) on May 15, 1999.

At Site 2, the thickness of the mat was about 15 mm and the mat was composed of five layers. Each layer was removed sequentially from the surface to the bottom, and was first described in terms of colors visible to the naked eye and then quantified by measuring their absorbance spectra. The sampling and observation were carried out on August 9, 1998.

Hot spring water also gushed out of a holding tank and flowed about 50 meters from the debris dam. A massive microbial mat occurred in the stream. The color of the mat varied according to temperature from transparent (76–70°C), to orange (70–65°C) to green (65–62°C) with decreasing water temperature along the stream. Samples were taken from the mat in the stream. The temperature was measured using a thermoelectric thermometer (IM245506, YOKOGAWA).

Half of the samples were fixed in formalin (ca. 20%), and brought back to the laboratory for microscopic observation. The samples were stained with ca. 5 μg/mL 4′,6-diamidino-2-phenylindole (DAPI) and observed under a fluorescent microscope (BX50, Olympus with Color Child 3CCD Camera, Hamamatsu Photonics).

Results and Discussion

Spectral types and identification of bacteria

Fig. 4 showed three types of “pure spectra” observed at Nakabusa Hot Spring. The left-ascending pattern appearing on each of them was due to Rayleigh scattering, and the steeply ascending pattern toward 500 nm was due to the existence of carotenoids, which have absorbance maxima in the blue spectral region.

The maxima of 630 and 680 nm in absorbance spectrum type A can be attributed to phycocyanin and chlorophyll a, respectively (see Brock et al., p. 587). Therefore, type A was thought to be composed of cyanobacteria. The microbial mat with spectrum type A occurred below 65°C and usually showed green. Rod-shaped bacteria with red autofluorescence were found in the mat under the fluorescent

![Fig. 3](image-url)  
Fig. 3. Microbial mats at Nakabusa Hot Spring on August 9, 1998. Absorbance spectra and temperatures were measured along a horizontal line (site 1) on the mat surface. The distribution of phototrophs from the mat surface to the bottom was examined at site 2.

![Fig. 4](image-url)  
Fig. 4. Absorption spectra of cyanobacteria, Chloroflexus and type C mats. Type A spectrum is a typical absorption spectrum of Cyanobacteria which has chlorophyll a (680 nm) and phycocyanin (630 nm). Type B is a typical spectrum of Chloroflexus which has bacteriochlorophyll c (740 nm) and bacteriochlorophyll a (808 and 868 nm). Type C spectrum has distinctive absorption peaks of bacteriochlorophyll a (801 and 878 nm).
microscopy, and their morphology resembled that of the genus *Synechococcus*.

Type B had absorbance maxima at 740 nm corresponding to bacteriochlorophyll *c*, and 808 and 868 nm to bacteriochlorophyll *a* (see the list of the absorption maxima wavelengths for various bacteriochlorophylls compiled by Brock et al.)*3, p. 720*. This type of spectrum was thought to be typical of *Chloroflexus*.*7*. The mats with this type appeared more yellowish than those with type A spectra. The bundles of bacterial filaments were visible to the naked eye. They showed fine filaments several \( \mu m \) in width and dozens of \( \mu m \) in length under fluorescent microscopy, and morphologically resembled to *Chloroflexus aurantiacus*.*1*.

Type C had absorbance maxima at 801 and 878 nm due to bacteriochlorophyll *a* (see also Brock et al.*3, p. 720*). Its unique spectrum can be distinguished from the typical spectrum of *Chloroflexus* (Fig. 5), strongly suggesting that the organism forming the mat might be a novel phototrophic bacterium.*5*.

**Temperature dependency of the bacterial distribution**

White, green and yellow-green microbial mats occurred on the surface of the wall (Fig. 3). The thickness of the mats differed from 2–3 mm at site 1 to about 15 mm at site 2 where mat colors changed vertically, transparent, green, pink and gray from the surface to the bottom. The highest water temperatures at the source of the flowing hot water on the wall was 72°C and pH was about 8.5 on August 9, 1998, and 71°C on May 15, 1999.

Mats collected along a horizontal line were green to yellowish green, corresponding to different degrees of mixing of type A (cyanobacteria) and type B (*Chloroflexus*) spectra. The relationship between temperature and the distribution of microorganisms on the wall surface is shown in Fig. 6. Their mixing ratios seemed to be almost constant with some scattering in the temperature range of 45–60°C. The fraction of *Chloroflexus* increased with temperatures over 60°C, and the pure spectrum of *Chloroflexus* was observed at about 70°C. This suggests that temperature was a major factor restricting the locations of cyanobacteria and *Chloroflexus*. In contrast, bacteria with type C spectrum were found in the mats over broad temperature range of 45.5–68.5°C.

We carried out a similar study at Nakanoyu hot spring on August 8, 1998. A cyanobacteria-*Chloroflexus* mats also developed there, and a similar trend of changes in mixing ratio determined by temperature was observed.

The upper temperature limit for cyanobacteria was 71°C, and 77°C for *Chloroflexus* at the study areas so far examined. The previously reported temperature limits were 73°C for cyanobacteria and 68°C for *Chloroflexus*, respectively, at Buffalo Spring, White Creek Drainage, Yellowstone National Park.*4* The temperature limit for cyanobacteria at Nakabusa Hot Spring almost reached the highest value reported*4* and was even higher than for *Chloroflexus*.

**Layered structure**

The surface temperature of the Nakabusa microbial mats
at site 2 was 60°C, and the bottom layer was slightly lower; 59°C. The mat thickness was ca. 15 mm, and we identified five layers with different colors from the surface to the bottom: 1) transparent-green, 2) green, 3) dark green, 4) dark pink, and 5) gray. Spectral mixing ratios of each layer are shown in Fig. 7. At the surface, the bacterium with type C spectrum was predominant and cyanobacteria was subordinate in the transparent-green layer. In the second green layer, the dominant component was cyanobacteria. In the third dark-green layer, three spectra types occurred in almost equal proportions. Chloroflexus existed widely from surface to deeper layers in the mat, especially concentrated in the middle layer. In the bottom layer, dark pink and gray parts consisted mainly of bacteria with a type C spectrum. The mat with type C spectrum was found in the surface layer as well as in the lower thick pink layer.

**On-site spectrophotometry for describing microbial mats**

Pierson et al.\textsuperscript{10} and Nicholson et al.\textsuperscript{8} measured absorbance spectra in a laboratory by extracting pigments with a solvent or by disrupting the cells to obtain in vivo spectra. Although Pierson et al.\textsuperscript{11} measured absorbance spectra in the field, their instruments appeared to be too expensive for general use in microbial mat studies.

In contrast, the portable spectrometer we employed in this study was inexpensive, handy, and able to show in vivo absorbance spectra instantly in the field. Decomposition of pigment can be avoided because samples are measured immediately after sampling. This spectrophotometric system is very useful also in determining the appropriate samples for further studies such as cultures, gene analyses, and organic or geochemical analyses.

![Fig. 7. Spectral mixing ratios of cyanobacteria (no pattern), Chloroflexus (vertical lines), and type C (slanted lines) in the laminated structure. Layers were numbered 1 to 5 from the surface to the bottom.](image-url)
A newly discovered bacterium

The portable spectrophotometric system contributed to the discovery of a novel phototroph by revealing that a pink-colored bacterial mat in Nakabusa hot spring had distinctive absorption peaks at 801 and 878 nm. The absorption spectrum of the mat (type C spectrum) lacked a peak at 740 nm. Such a peak at 740 nm is due to the light-harvesting organs called chlorosomes. These spectroscopic findings strongly suggested that the organism forming the pink-colored mat is a new phototrophic bacterium.

After vigorous efforts to isolate novel phototroph from the pink-colored mat a new strain was obtained\(^5\). The isolate was a filamentous anoxygenic phototroph that resembled Chloroflexus in morphological and physiological respects but that lacked the chlorosomes that were a notable feature in Chloroflexus. The absence of chlorosomes was indicated by the following findings: the in vivo spectrum of phototrophically grown cells lacked an absorption peak around 740 nm that is typical of chlorosomes; the pigment analysis of chloroform/methanol extract showed that the bacterium had no bacteriochlorophyll \(c\), the main pigment in chlorosomes\(^5\).

We expect that this initial success will promote widespread use of the spectrometer in microbial mat studies in situ. The portable spectrometric system we described in this paper is a simple and useful tool for on-site spectrophotometry and the widespread investigations of microbial mats.

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