Two cyanobacterial strains can be distinguished from each other and other eukaryotic algae by spectral absorption method
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ABSTRACT

Spectral absorption method based on two step linear regression analyses (TSLR) was applied for detection of two strains of cyanobacterium, Microcystis (blue-green algae) from eukaryotic algae. Both blue-green algae, algae and dissolved organic carbon (DOC) were considered from freshwater bodies in Kanto region, Japan. The results show that blue-green species can be detected from other algal species using absorption spectra of water samples. In this study statistical analysis was done by TSLR method, which determined the gradient vectors of single algal species and DOC. We believe that this method might be useful in environmental monitoring of freshwater algae.

Key words | absorption, cyanobacteria, dissolved organic carbon, eukaryotic algae, linear regression, spectral absorption

INTRODUCTION

Cyanobacteria are most interesting organisms in ecological and physiological studies. The occurrence of cyanobacterial blooms in eutrophied freshwater bodies has become a worldwide concern over the past few decades. Microcystis aeruginosa is one of the most common species found during cyanobacterial blooms (Wu et al. 2007). Some species of M. aeruginosa produce microcystin (MC) which poses potential hazard to aquatic and terrestrial organisms as well as humans, while other species are non-toxic. In eutrophied waters, MC-containing strains and MC-free strains usually coexist and both impact the aquatic ecosystems (Lrling 2003). Correct identification of microalgae and cyanobacteria species in natural waters is essential for environmental management and research.

Consequently, various techniques such as high performance liquid chromatography (Wong & Wong 2003) and fluorometric analysis (Beutler et al. 2002, 2003) have been developed to discriminate problematic cyanobacterial species in fresh water. Among these methods, fluorometric analysis become more and more common for detection and quantification of phytoplankton (Poryvkina et al. 2000), but the consistency of this method is lower for cyanobacteria (Beutler et al. 2002). Like all algae, cyanobacteria possess the ability to acclimate their photosynthetic apparatus to environmental conditions. When cyanobacteria become limited by iron or due to lack of isiA gene, phycobilisomes (PBSs) especially PC may be degraded. Those kinds of cyanobacterial species are difficult to differentiate from their common companions i.e., PC containing cyanobacteria and can affect fluorometric method (Beutler et al. 2003; Lewis et al. 2010). However, fluorescence method does not have enough information for species/strains level identification of cyanobacteria. Then they are overlooked. Hence it is crucial importance to develop a reliable tool to detect cyanobacteria species in fresh water. To overcome this problem, passive method is preferable to discriminate problematic species of cyanobacteria. Recently proposed spectral absorption method can facilitate the discrimination of blue-green (Cyanophyceae) and eukaryotic algae (Chlorophyceae, Bacillariophyceae, and Dinophyceae) and of dissolved organic

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carbon (DOC) in fresh water samples, based on two step linear regression (TSLR) (Lokuhewage et al. 2005). The specific spectral characteristics of each algal species may lead to differentiate them by TSLR method. However, species or strain level identification of cyanobacteria was not considered.

In the present study, we applied the spectral absorption method to analyze the cyanobacterium Microcystis sp. in fresh water. Two strains of Microcystis sp. were observed in mixed phytoplankton populations in some freshwater samples collected from wetlands, ponds, rivers, and lakes in Saitama, Japan. Both strains were detected from each other by the TSLR method, which determined the gradient vectors of single algal species and DOC. The other eukaryotic algal species were also distinguished from those strains. We used the method TSLR for detection of Microcystis strains, which are responsible for local degeneration of water quality in freshwater samples.

**MATERIALS AND METHODS**

**Field and laboratory experiment**

The survey (including microscopic counting) started, after two strains of Microcystis had been detected at some sites of freshwater samples (three wetlands, two ponds, one river, one inlet, and three sampling sites in one lake) in Saitama at 35° 51’ N, 139° 39’ E, Japan. Fresh water samples were collected from surface waters at 10 sites. Each water sample was immediately preserved with 2% formaldehyde solution and kept for further taxonomic analysis. Phytoplankton species were identified using Bellinger (1992) and the number of cells was determined using an optical microscope (OLYMPUS BX40). In the experiment identified cyanobacterial species named as blue-green 1 and 2 and the others are green (Chlorophyceae), brown 1 (Bacillariophyceae), brown 2 (Dinophyceae). Total Chl. a concentration was determined for freshwater samples (APHA 1995).

We analysed field and pure samples from which we derived gradient vectors, were isolated from field samples using an optical microscope and micropipette, and transferred to pure water to clean the cells. Isolated algal samples were also washed with culture media containing a single species. This process was repeated several times to reduce bacterial contamination (Tomar 1999). Each algal species for laboratory experiment was cultured under natural conditions (Watanabe et al. 2000) for 14 days in reference medium WC (Guillard & Lorenzen 1972) in a controlled incubator (Conviron, CMP 3000). A fluorescence light (VHO-SYLVA-NIA) was used as the source of light. The photon flux density of each light source was maintained at c. 28 μE m⁻² s⁻¹ of photosynthetically available light (PAR), because phycobiliproteins decrease dramatically at higher photon flux density (Wyman & Fay 1986). The light:dark cycle was 12:12 h. Chlorophyll a (Chl. a) concentrations of algal species were determined using a spectrophotometer (HACH DR/4000) after extraction with 90% aqueous acetone (Jeffrey & Humphrey 1975). In addition, we studied dissolved organic substances extracted from dried plant materials, categorised into the yellow group of DOC, and referred to as yellow substances (YS) (Kirk 1985), which used as a reference in the experiments. Concentrations of YS were measured using a total organic carbon analyser (TOC 5000A, Shimadzu, Japan). The measured amounts of algal species and YS are described as values of Chl. a per litre (μg/litre), and micrograms C (Carbon) per litre (μg/litre), respectively.

The absorbance of each pure and field sample in a cuvette of 15 ml (cell width 10 mm) was measured in the band of 360–740 nm at 10 nm intervals using a spectral colorimeter (Minolta CM-3600d). The measurements were repeated three times for each laboratory and field samples and subsequently averaged. Spectral absorption measurements were taken for field samples after non-living coarse particulate matter was removed by filtration (mesh size 50 μm) to minimize unexpected light scattering (Becker et al. 2002; Beutler et al. 2002). To determine the dependence of spectral characteristics on the concentration of each algal groups and YS, we prepared pure samples at four different concentrations similar to Lokuhewage et al. (2005). Four different concentrations (20, 100, 200, and 400 μg/l) of blue green 1 and 2, (50, 100, 200, and 400 μg/l) of green (20, 40, 80, and 150 μg/l) of brown-1 and 2 (150, 300, 600, and 900 μg/l) of YS were used for laboratory experiment.

**Two step linear regression analysis**

Spectral absorptions of freshwater samples containing phytoplankton and YS can be considered as the weighted sum of the characteristic spectrum of each constituent in proportion to its concentrations (Falkowski & Raven 1997). With the priori knowledge of unialgal species and YS together spectral absorption characteristics on concentration, we can distinguish contained unialgal species and YS only from spectral absorption curves (Lokuhewage et al. 2005).

In the present study, we assume that concentration of algae and YS are low enough to satisfy Lambert-Beer Law (Falkowski & Raven 1997). At the first step, a linear
regression model can be made for each of pure water samples between logarithm absorption $A(\lambda)$, and concentration $x$, is the Chl. $a$ $\mu$g/litre.

$$A(\lambda) = B_0 + B(\lambda)x$$

where $\lambda$ is the wavelength of light. The regression coefficient $B(\lambda)$ is the gradient at a particular wavelength. Calculations of the linear regression (Equation (1)) were made to determine $B(\lambda)$, for each spectral absorption data set of six pure samples at four different concentrations by least square method (Weisberg 1985). The gradient $B(\lambda)$ is the function of wavelength $\lambda$, described as vector form,

$$B = [B(\lambda_1), B(\lambda_2), \ldots, B(\lambda_n)]^T$$

where $T$ is the vector transpose. Then $n$-dimensional gradient vector $B$ was constructed. In the present experiment, $n = 39$ was the sample number along the wavelength axis ranging from 360 to 740 nm at 10 nm intervals and the unit of the gradient vector $B(\lambda)$ is $(\mu$g/litre)$^{-1}$. $B_0$ is a constant vector for the contribution of pure water which does not contain algae or YS. The gradient vectors $B_m(\lambda)$, $B_{m2}(\lambda)$, $B_c(\lambda)$, $B_d(\lambda)$, $B_p(\lambda)$, and $B_{YS}(\lambda)$ were determined for six pure samples for the blue-green 1, blue-green 2 green, brown 1, brown 2, and YS, respectively.

In the second step, multiple linear regression analysis was applied to field water samples to estimate concentrations of each of the six elements. In field water samples, total bulk spectral absorption $M(\lambda)$ is defined as the linear summation by all absorbing six gradient vectors and their unknown concentrations $x_{m1}$, $x_{m2}$, $x_c$, $x_d$, $x_p$, and $x_{YS}$ of respective single algal species and YS in the mixed sample:

$$M(\lambda) = B_{m1}(\lambda)x_{m1} + B_{m2}(\lambda)x_{m2} + B_c(\lambda)x_c + B_d(\lambda)x_d + B_p(\lambda)x_p + B_{YS}(\lambda)x_{YS}$$

where absorption spectra of mixed sample $M(\lambda)$ obtained smooth curves which include spectral characteristics of six components (five algal species and YS). Gradient vectors are important for the evaluation to distinguish and to estimate the concentration of each algal species and YS in mixed samples.

From least square theory values of $x_{m1}$, $x_{m2}$, $x_c$, $x_d$, $x_p$, and $x_{YS}$ were estimated as a minimum sum of the residuals:

$$S = \sum_{i=1}^{n}(M(\lambda_i) - B_{m1}(\lambda_i)x_{m1} - B_{m2}(\lambda_i)x_{m2} - B_c(\lambda_i)x_c - B_d(\lambda_i)x_d - B_p(\lambda_i)x_p - B_{YS}(\lambda_i)x_{YS})^2$$

where $\lambda_i$ indicates wavelength $i$ in the $n$-dimensional measured spectrum, $M(\lambda)$ and six gradient vectors become variables in the multiple linear regression analysis. The estimates $x_{m1}$, $x_{m2}$, $x_c$, $x_d$, $x_p$, and $x_{YS}$ are determined by minimizing $S$ from Equation (4) using the Gaussian normal equation (Weisberg 1985).

Similarity Index between the normalized spectra

Similarity Index (SI) is useful to understand the spectral similarities and differences between the algal species (Mayer 2000). The resulting absorption spectra were normalized to the mean absorption between 360–740 nm wavelengths. Spectral similarities and differences can be assessed by computing the SI between the normalized spectra comprising TSLR for the five algal species and YS in the $n$-dimensional space (39-D) similar to Lokuhewage et al. (2005). Absorption spectra of algal species were compared using SI described previously by Millie et al. 1997. A crucial requirement for the analysis of spectral similarities and differences analysis is linearity between the normalized spectra $A_m$ and $A_n$ of any algal species. If this requirement is fulfilled, the SI between the normalized spectra $A_m$ and $A_n$ can be defined in 39-D space. The degree of similarity between spectra was computed using an index (SI) algorithm:

$$SI = 1 - \frac{2 \times \arccos \left( \frac{A_m \cdot A_n}{\|A_m\| \cdot \|A_n\|} \right)}{\pi}$$

where SI is the similarity index between $A_m$ and $A_n$. The arccosine transformation and division by $\pi/2$ converts SI values from zero to one. The SI value is very close to one which means that two spectra of algal species approach each other similarly.

For visualization of differences among the spectra of algal species and YS, contour plots were constructed in low dimensional space using principal component analysis (PCA) (Jaaskelainen et al. 1990).

RESULTS

Field experiment

Spectral absorption curves of algal species collected from field samples were characterized by a specific composition of photosynthetic antennae pigments. The significant absorption peaks of Chl $a$ are observed at 440 and 680 nm for the four algal groups and a broad absorption peak from 480 to 525 nm is caused by carotenoids. Allunalgal species contained Chl $a$ and carotenoid pigments which allow them to be
distinguished from samples containing only YS where absorption is high in the blue region and exponentially decreases with the wavelength. All unialgal species contained Chl $a$ and carotenoid pigments which allow them to be distinguished from samples containing only YS where absorption is high in the blue region and exponentially decreases with the wavelength. Spectral absorption curves of field samples seem to have very similar spectral shapes for wetlands, pond, inlet, river, and lakes (data not shown). The absorption peaks around 440 nm and 680 nm corresponding to Chl $a$ were observed in field samples. To determine the concentration of blue-green algae in a mixture of different algal species, it is necessary to account for the presence of other algal species in field samples. This can be achieved by including gradient vectors for other phytoplankton species in a TSLR method.

**Determination of gradient vectors from field samples**

Calculations were made for each data set of spectral absorption of six pure samples at four different concentrations, using simple linear regression of Equation (1). We determined the gradient vectors $B_{m1}(\lambda)$, $B_{m2}(\lambda)$, $B_{c}(\lambda)$, $B_{d}(\lambda)$, $B_{p}(\lambda)$ and $B_{YS}(\lambda)$ for each algal group; blue-green 1, blue-green 2, green, brown 1, and brown 2 and YS, respectively (Figure 1). Each group has characterized by a specific gradient vector $B(\lambda)$, obtained for six components (five algal species and YS) of pure samples at 360–740 nm wavelength bands, with differences in the shapes of the gradient vectors evident. The differences in the shapes of gradient vectors between blue-green 2, brown 1, brown 2, and YS are more obvious than those of blue-green 1 or green. In the blue-green group 1, there was a slow intense monotonous increment of the spectral shape from the 560 to 640 nm wavelength range, caused by phycocyanin. However, a specific peak, phycocyanin at 630 nm for the blue-green algae 1 was hidden and was observed in the spectra of blue-green algae 2. The green group does not show a peak in this region (560 to 640 nm) and the spectral shapes of blue-green 1 and green group are nearly similar. In the brown 1 group, a peak of Chl $a$ at 440 nm was observed with high intensity and that of peak was not recognized in the spectrum of brown 2 group. In the brown 1 and brown 2 groups, the respective carotenoids of fucoxanthin and peridinin are overlapped on the peak of Chl $a$ at 440 nm, thereby significantly broadening the overall spectral peak range from 440 to 550 nm. In the brown 1 and

![Figure 1](https://iwaponline.com/wst/article-pdf/63/6/1203/445595/1203.pdf)
brown 2 groups, a small peak of Chl. c appeared near 630 nm, but its intensity relative to 680 nm of Chl. a is much lower. The variations of spectral characteristics of blue-green 1, blue-green 2, green, brown 1 and brown 2 were observed (Figure 1). On the other hand, the intensities of the phycocyanin peaks at 630 nm, compared to the Chl a peak, were not similar in the blue-green 1 and blue-green 2. In contrast to the algae groups, the YS group spectrum has a smooth curve and the maximum gradient is observed near the blue region of the spectrum.

Gradient vectors for five algal groups and YS were determined for the evaluation of cyanobacteria from other algal species and YS in field samples (Figure 1). We assumed that gradient vectors were obtained under the same conditions as the investigated samples. The crucial premise of the evaluation is based on the assumption that the characteristic spectra (gradient vectors) of investigated algal groups are constant. This was valid for green, brown 1, and brown 2 groups. The spectral characteristics of gradient vectors of the blue-green group were not identical.

A calibration line for each algal group and YS was made in advanced by solving Equation (1) using simple linear regression for four points of known concentrations of pure samples, sampled from field. Absorption spectra of field samples (data not shown) were analyzed by Equation (3) using gradient vectors (Figure 1). The concentrations of each algal group and YS in field samples were estimated using multiple linear regression analyses and plotted on the calibration line against the observed cell densities of each algal group and the measured amounts of C of YS (Figure 2). There was agreement between both methods (measured cell densities from microscopic observation and estimated Chl. a concentrations from TSLR) for blue-green algae 1 (y = 0.001x, r = +0.9506 at P < 0.0001) and blue-green algae 2 (y = 0.0012x, r = +0.9944 at P < 0.0001) as well as for the other groups (data not shown). The discrimination of algal species and YS in mixed phytoplankton population indicates that it is feasible to use spectral absorption based on TSLR method.

**TSLR method comparison with spectrophotometric determinations**

The reliability of the analysis by TSLR was assessed by comparing the results with those of spectrophotometric determinations. The results of the TSLR determined and of spectrophotometrically analyses for the total Chl content are compared. The results illustrated close relationship between the values from the TSLR method and the spectrophotometric method with an error of less than 5% (data not shown) (y = 0.9419x, r = +0.9948, r significant at P < 0.0001).

**Spectral variability of cyanobacteria and other algal species**

Cyanobacteria were the main focus of this investigation. We determined the variability of the spectral characteristics of blue-green algae using the similarity index in 39-D space. SI values between YS and algal groups consistently were greater than SI values between algal groups (Table 1). Consequently, YS was the most dissimilar from other absorption spectra. Blue-green algae 1 and green groups displayed limited close relationship to spectra with respect the SI values between them. In order to incorporate the variable spectra of algal species and YS, contour plots can be constructed to expand the spectral absorption data of them to visualize the differences between the spectra of the blue-green group in low dimensional space.

According to the PCA, a small number of eigenvectors were determined from correlation matrix of the spectral data $A(\lambda)$. Resultant eigenvectors are orthogonal to each other. The resultant first three eigen vectors where fidelity value was 0.998, and most statistically important characteristics of the original data are included in these few upper eigenvectors (data not shown). Then contour plots were constructed on subspace in two dimensional spaces. Constructed contour plots illustrated clear differences and similarities of algal
species and YS each other. Blue-green 1 and blue-green 2 were dissimilar with respect to the SI values but contour plot shows that they are belong to one spectral group. Consequently, as for algal groups, absorbance spectra alone did not provide adequate discrimination among the spectra.

**DISCUSSION**

The aim of this work was to apply TSLR method (Lokuhewage et al. 2005) for detection of two blue-green species from other algal species in freshwater. These two cyanobacterial species were different in their spectral characteristics (Figure 1). This kind of analysis depends entirely on the reliability of the gradient vectors. TSLR method is suitable, valuable and practical tool for the detection of two blue-green species from eukaryotic algae in freshwater samples (Lokuhewage et al. 2005).

Spectral characteristics of phytoplankton depend not only on the taxonomic position, but also on the photo adaptation state. A crucial premise is the constancy of the gradient vectors of each algal group of phytoplankton in a specific living environment (Lokuhewage et al. 2005; Lokuhewage & Toyooka 2007). Further, the two different blue-green species described above may not hold unique spectral characteristics. The characteristics spectra of cyanobacterial species are helpful in advance to monitoring problematic species and to predict the water quality based on the amount of algae as a bio-indicator. We need to account for variable spectral properties of cyanoabacteria in different fresh water fields together with their inherent gradient vectors, to reveal the water quality correctly. The technology of the method applied here, could be used to discriminate the causative species in *vivo* and in *situ*, when harmful algal specie occur. The approach presented here should be adequate for fresh water systems.

An important consideration in the application of TSLR method for identification of algal groups in field samples is an accurate deduction of their gradient vectors. Lokuhewage et al. (2005) proposed that TSLR analysis can be applied to identify algal species and YS, and to estimate their concentrations in mixed samples. It is highly desirable to determine the gradient vectors under the same experimental condition as those used for the field samples. The present results suggested that quantitative estimation of algae groups can be achieved by TSLR with sufficient precision (error below 15%).

### Cyanobacteria and spectral variations

The present investigation illustrated that physiological differences has a profound influence on spectral characteristics of blue-green algae compared with other algal groups (Figure 2). The variability of pigment composition affects the spectral absorption characteristics of cyanobacterial photosynthetic apparatus (Wyman & Fay 1986; Tandeau de

![Table 1](https://iwaponline.com/wst/article-pdf/63/6/1203/445595/1203.pdf)

**Table 1 | Similarity index for algal species and YS in 3-D subspace**

<table>
<thead>
<tr>
<th>Species</th>
<th>Similarity Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue-green 1 to green</td>
<td>0.93</td>
</tr>
<tr>
<td>Blue-green 1 to brown 1</td>
<td>0.86</td>
</tr>
<tr>
<td>Blue-green 1 to brown 2</td>
<td>0.82</td>
</tr>
<tr>
<td>Green to brown 1</td>
<td>0.90</td>
</tr>
<tr>
<td>Green to brown 2</td>
<td>0.89</td>
</tr>
<tr>
<td>Brown 1 to brown 2</td>
<td>0.86</td>
</tr>
<tr>
<td>Blue-green 2 to brown 1</td>
<td>0.86</td>
</tr>
<tr>
<td>Blue-green 2 to green</td>
<td>0.90</td>
</tr>
<tr>
<td>Blue-green 2 to brown 1</td>
<td>0.92</td>
</tr>
<tr>
<td>Blue-green 2 to brown 2</td>
<td>0.82</td>
</tr>
<tr>
<td>Blue-green 1 to DOC</td>
<td>0.52</td>
</tr>
<tr>
<td>Blue-green 2 to DOC</td>
<td>0.54</td>
</tr>
<tr>
<td>Green to DOC</td>
<td>0.54</td>
</tr>
<tr>
<td>Brown 1 to DOC</td>
<td>0.57</td>
</tr>
<tr>
<td>Brown 2 to DOC</td>
<td>0.68</td>
</tr>
</tbody>
</table>
Marsac & Houmard 1993). The spectral similarity and differences were observed using similarity index of them in the 39-D (Table 1). Consequently, spectral characteristics of blue-green 1 and blue-green 2 were closely related to green algae compared to other spectra of algae and YS (Table 1). The reason for the spectral variability of the blue-green originates from large changes in the ratios of phycocyanin to Chl a, which reported previously (Schubert et al. 1993; Bryant 1995; Beutler et al. 2005). Variability of blue-green algae may be induced by an increase of phycocyanin within the phycobilisomes (PBSs), and/or changes in size and number of PS I reaction centers, with the ratio of PBSs to Photosystem II being accepted as constant (Myers et al. 1980). The variation of spectral characteristics of blue-green algae owing to PBS reduces the reliability of estimates of algal concentrations in fresh water. We overcome the problem with blue-green to increase the accuracy of the TSLR method and to yield reliable results by obtaining appropriate gradient vectors under the same environmental conditions as investigated samples from field.

The study presented here demonstrated that presence of two species of cyanobacteria in freshwater can be monitored by TSLR method. Measured values were in good correlation with the other parameters of cyanobacterial biomass (chlorophyll a, cell counts). It may be possible to use this method to provide a warning system and to detect the toxin-producing cyanobacteria in a mixed population. This method is versatile and easy to use in different fresh water studies as long as there is prior knowledge of inherent gradient vectors for the occurrence of algae species. Specially, the pigment composition and spectral characteristics of cyanobacterial species are specific to living sites in fresh water. As we have demonstrated, the site-specific spectral characteristics of algal species in each sampling site in fresh water bodies are important for algal differentiation. Furthermore, this study can be extended to gain knowledge of the spectral properties of potentially toxic species to monitoring harmful algal blooms and help in interpreting quantity, physiology and ecology of phytoplankton in fresh water bodies.

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