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Quantitative Estimation of Phytoplankton Species in Freshwater by Two Step Linear Regression Analysis Using Spectral Absorption Method

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We proposed a passive method to distinguish and to estimate density of the Cyanobacteria (Cyanophyceae) in a mixed population by measuring the spectral absorption of sample waters, based on two step linear regression analysis. Natural freshwater usually contains a few species of algae and dissolved organic carbon (DOC). In the experiment, we picked out four typical algal groups characterized by their own colors, Cyanophycaeae or blue-green alga, Chlorophyceae or green alga, and Bacillariophyceae and Dinophyceae or brown algae. In the first step, for each of the pure sample waters which contained only one of these elemental substances, dependence of spectral characteristic on its density was determined using simple linear regression analysis. Resultant spectral characteristics which we call gradient vectors were used to estimate spectral absorption of mixed sample waters containing the four elementary algae and DOC by multiple linear regression analysis. This method offers new perspectives for identification and estimation of density of blue-green algae and other unialgal species in a mixed population.

Key words: bacillariophyceae, chlorophyceae, cyanophyceae, dinophyceae, gradient vectors, simple linear regression analysis, multiple linear regression analysis, dissolved organic carbon

1. Introduction

Eutrophication or nutrient enrichment of aquatic ecosystems leads to the formation of cyanobacterial floating masses on the surface of the waters. These floating masses are commonly known as water blooms and are one of the major problems in environmental conservation. Surface algal scum has pernicious effects on aquatic organisms. In routine water quality monitoring, Chlorophyll *a* (Chl *a*) is a basic parameter, but the taxonomic composition of phytoplankton is of crucial importance to detect harmful algal blooms.

Phytoplankton in aquatic ecosystems imparts a distinct color to the water which suggests that optical methods provide a better detection methodology and hence predict problematic algal blooms. The individual phytoplankton pigments are characterized by their unique light absorbance features. This property allows detection and identification of algal blooms by color. It has already been proposed that optical properties of algae can be used to distinguish harmful algal blooms from a mixed population.[1] However, studies of the qualitative and quantitative estimation of the distinct phytoplankton species in natural mixed populations have not yet been done in detail.[2] Millie *et al.*[3] proposed a new approach to discriminate red tide dinoflagellates in natural mixed assemblages following absorbance characteristics. Their success in the proposed method was due to there being only one problematic bloom species dominant in the samples.

Beutler *et al.*[4] and Yentsch and Phinney,[5] discussed the possibilities of using fluorescence for taxonomic classification of phytoplankton samples based on their specific excitation spectra of chlorophyll fluorescence. The fluorescence method[4–6] has the potential of estimating quality and quantity of algal species. In this case, sample waters have to be illuminated by a high-energy exciting light such as UV light. However, UV light causes physical damage to the cell structure of target biological components and other living materials.[7] For these reasons, a passive method is preferable to discriminate problematic species from other phytoplankton species. If quantitative estimation of algal species becomes possible using a passive method, it could be widely used in cases for which it would be difficult to apply the fluorescent method such as passive remote sensing of ocean color. Remote-sensed color spectra provide bulk composite signals for a mixed phytoplankton population, and the distinct phytoplankton species are difficult to discriminate by the fluorescence method.

In this paper, we propose a new algorithm to distinguish a specified taxon in a mixed population and to estimate the amount of algae by measuring the spectral absorption of the sample waters, based on two step linear regression analysis. In the first step, spectral absorption characteristics depending on the density of each of pure sample waters containing a unialgal species and dissolved organic carbon (DOC) was determined using simple linear regression analysis. The resultant characteristics represented by gradient vectors are used as basis vectors of the multiple linear regression analysis in the second step.

For this purpose, we prepared two groups of sample waters. The first group, which was prepared to make gradient vectors, consisted of four popular unialgal samples and of pure sample waters of DOC in varying densities. The second group was prepared in order to simulate natural water in which the four algal species and DOC were mixed in different composition ratios.

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Table 1. Spectral Groups of Algae[13] and diluted density series of components of synthesized samples.

Color group	Taxonomic group/Medium	Example	Peripheral antenna pigments	Density (μg/l)
Blue-green	Cyanophyceae	*Microcystis aeruginosa*	Phycobilisomes (Phycocyanin)	20, 50, 200, 400
Green	Chlorophyceae	*Chlorella vulgaris*	Chl *a*, *b*, Carotenoids	50, 100, 200, 400
Brown 1	Bacillariophyceae	*Cyclotella meneghiniana*	Chl *a*, *c*, Carotenoids	20, 40, 80, 150
Brown 2	Dinophyceae	*Peridinium bipes*	(fucoxanthin or peridinin)	
Yellow	Dissolved organic matter	Yellow substances	Carbon	150, 300, 600, 900

2. Preparation of Water Samples

Dominant light absorbing materials in freshwaters are phytoplanktons, detritus (non-living particulate matter), and dissolved organic matter that contains DOC.[8] The color of freshwater is determined by several species of algae and DOC. A few water blooming species of algae live in most lakes and rivers in Japan.[9] We focused on four major algal groups and DOC, as listed in Table 1, exhibiting different photosynthetic pigments. In our present experiments, inorganic particulate matter was not included because its contribution to the spectral absorption is not significant.

The name of the taxonomic group of algae often contains a reference to the color of that organism. In Table 1, the taxonomic group of blue-green algae is referred to as Cyanophyceae. One of the species which belongs to that group is *Microcystis aeruginosa*. The blue-green group possesses phycobilisomes (PB) which function as the peripheral antennae containing mainly phycocyanin (PC). The peripheral antennae mean that pigments can receive light energy efficiently and transfer it to Chl *a* which is the main photosynthetic pigment. The presence of PC determines its typical blue-green color. On the other hand, green algae are referred to as Chlorophyceae. *Chlorella vulgaris* is one such green algae group in which the peripheral antenna contains Chl *a*, Chl *b* and carotenoids. The green color is determined by Chl *a*, and *b*. Members of the brown group are separated into groups of brown 1 and brown 2. Brown 1 is referred to as Bacillariophyceae and brown 2 as Dinophyceae and representative species are *Cyclotella meneghiniana* and *Peridinium bipes*, respectively. Their members contain peripheral antennae pigments of Chl *a*, Chl *c* and carotenoids. Specific carotenoids in brown 1 and brown 2 are fucoxanthin and peridinin which determine brown and dark brown color of the cells, respectively. In addition to algal groups in water, dissolved substances are categorized as the yellow group of DOC, generally referred to as yellow substances (YS) which have been extracted from dried plant materials.[10]

Algal classes can be assigned to spectral color groups and each alga has its own characteristic color or spectral absorption depending on specific photosynthetic pigments in the cells. Therefore, spectral color is a useful taxonomic criterion and algal taxonomic groups differ significantly in their spectral distribution.

We prepared two kinds of water samples: pure sample waters and mixed sample waters. At first, to determine the dependence of spectral characteristic on the density for pure sample waters of each of the four unialgal species (blue-green, green, brown 1 and brown 2) and YS, we prepared them with different densities which are listed in Table 1. The unialgal cultures for laboratory experiments were grown under natural conditions[11] for 14 days under sterile conditions in a growth chamber (Conviron, CMP 3000). Measured amounts of algal densities were based on Chl *a* per liter (μg/l), and that of the YS was based on micrograms of C (Carbon) per liter (μg/l). Chl *a* densities of algal samples were determined using a spectrophotometer (HACH DR/4000) after extracting with 90% aqueous acetone.[12] DOC amounts of YS were measured using a Total Organic Carbon Analyzer (TOC 5000A, Shimadzu, Japan).

To simulate natural freshwater samples, we prepared mixed sample waters with the known densities listed in Table 1. Four different densities (20, 50, 200, and 400 μg/l) of blue-green (50, 100, 200, and 400 μg/l), of green and (150, 300, 600, and 900 μg/l) of YS, and three different densities (20, 80, and 150 μg/l) of both brown 1 and brown 2 were used for these mixed sample waters. These fourteen samples of unialgal species and four samples of YS were mixed with each other and subsequently 56 sample waters were prepared. In total, 77 samples (56 mixed algal samples, one distilled water sample and twenty pure sample waters) were used for the laboratory experiment. Total volume of each mixture was 15 ml in a cuvette, containing 3 ml from each of the four algal samples and 3 ml from one of the YS samples (thereby, four sets of mixtures were prepared for the four YS densities), whereas the pure sample waters contained only unialgal samples and YS only. The absorbance of each sample in a cuvette of 15 ml (cell width 10 mm) was measured using a spectral colorimeter (Minolta CM-3600d) in the band of 360–740 nm at 10 nm intervals. The measurements were repeated three times for sixteen unialgal samples and four YS samples and for 56 mixed sample waters and then averaged. Some of the resultant spectral absorption curves of the pure and mixed sample waters are shown in Figs. 1 and 2, respectively. Curves in Fig. 1 have their own spectral characteristics. Each of them was 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. All unialgal species

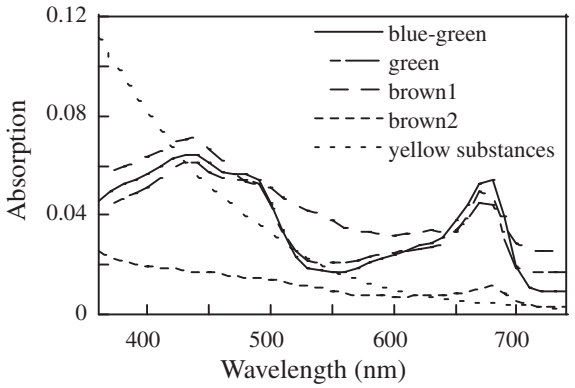

Fig. 1. Spectral absorption characteristics of five pure sample waters. *Microcystis aeruginosa* (solid line, blue-green), *Chlorella vulgaris* (long-dash short-dash line, green), *Cyclotella meneghiniana* (dash space line, brown 1), *Peridinium bipes* (dash line, brown 2) and yellow substances (dotted line).

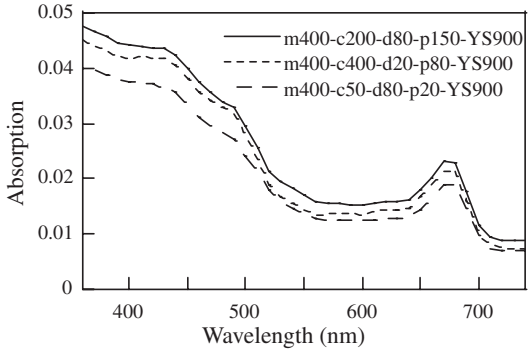

Fig. 2. Spectral absorption characteristics of three different mixed sample waters. Blue-green (m), and YS densities were kept constant (blue-green 400 µg/l, YS 900 µg/l) while green (c), brown 1 (d) and brown 2 (p) densities were changed.

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. In the blue-green group, the phycocyanin peak at 630 nm is characteristic of them. However, its intensity relative to 680 nm of Chl *a* is much less and overlaps with Chl *a*. The green group is characterized by a Chl *b* peak at 460 nm which overlaps with the carotenoids. Brown 1 and brown 2 show small absorption peaks at 630 nm due to Chl *c*. An overlap of carotenoids and Chl *a* within the spectral region 440 to 525 nm caused a broad absorption peak in brown 2. Brown 1 and brown 2 are characterized by the specific carotenoids fucoxanthin and peridinin, respectively.[13] In contrast to Fig. 1, curves of mixed sample waters as shown in Fig. 2 seem to have very similar spectral shapes.

3. Two Step Linear Regression Analysis

It is difficult to identify contained algal species and YS and to estimate their densities in mixed sample waters from the curves in Fig. 2 alone. But if we have *a priori* knowledge of the algal species contained and YS together with the density dependence of their spectral absorption, we can distinguish the contained elemental species quantitatively just from the spectral absorption curves. We assume that each of elements is thin enough that multiple scattering does not happen. Spectral absorption of sample waters is directly proportional to the density of each element they contain, based on Lambert–Beer law.

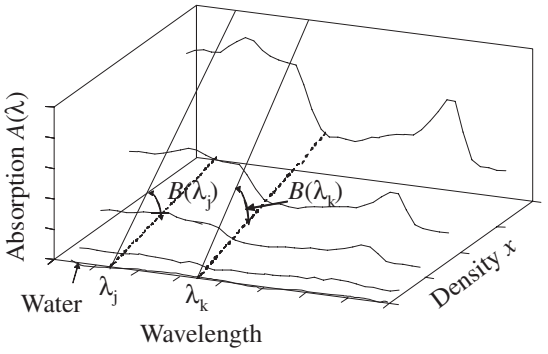

Fig. 3. Schematic diagram of making gradient vectors $B(\lambda)$.

At the first step, a linear regression model can be made for each pure sample water between logarithmic absorption, $A(\lambda)$, and density x,

$$A(\lambda) = B_0 + B(\lambda)x, \tag{1}$$

where λ is the wavelength and x, is the density of Chl *a* in µg/l. $B(\lambda)$ is the regression coefficient which is the gradient of a regression line of a spectral absorption to density at a particular wavelength schematically shown in Fig. 3. A regression coefficient $B(\lambda)$ for a specific value of wavelength λ is calculated by least squares method with units of $(\mu g/l)^{-1}$. The regression coefficients for an array of sampling wavelengths describe the sequence of $B(\lambda)$ in a spectral wavelength and construct a n-dimensional vector for one element. The gradient $B(\lambda)$ is the function of wavelength λ and can be described by a column vector form as

$$B = [B(\lambda_1), B(\lambda_2), \ldots, B(\lambda_n)]^T, \tag{2}$$

where T is the vector transpose and $n = 39$ is the sampling number along the wavelength axis ranging from 360 to 740 at 10 nm intervals. We call B the gradient vector. The gradient vectors were determined for five pure sample waters. The coefficient B_0 is a constant vector, explaining the contribution of water which does not contain algae or YS (Fig. 3).

In the second step, multiple linear regression analysis was applied to the models of mixed sample waters to estimate the density of each of the five elements. Five gradient vectors, $B_m(\lambda)$, $B_c(\lambda)$, $B_d(\lambda)$, $B_p(\lambda)$ and $B_{YS}(\lambda)$ for the blue-green group, green group, brown 1 group, brown 2 group and YS respectively, are known by the simple linear regression as described previously. Spectral absorption, $A_{mix}(\lambda)$ is a known value that can be described simply by a linear combination of the five gradient vectors by

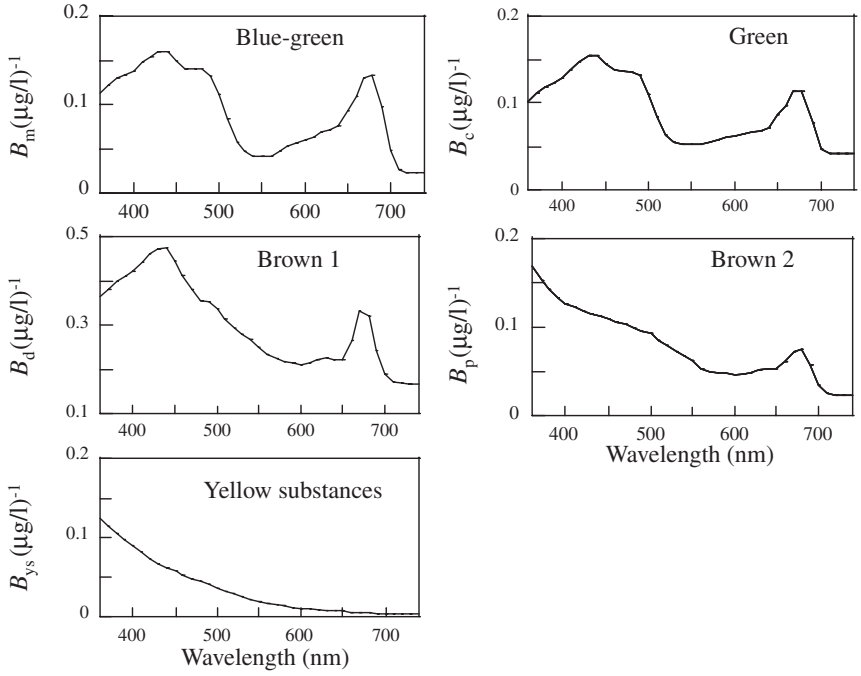

Fig. 4. Gradient vectors for five spectral elements.

$$A_{\mathrm{mix}}(\lambda) = B_{\mathrm{m}}(\lambda)x_{\mathrm{m}} + B_{\mathrm{c}}(\lambda)x_{\mathrm{c}} + B_{\mathrm{d}}(\lambda)x_{\mathrm{d}} + B_{\mathrm{p}}(\lambda)x_{\mathrm{p}} + B_{\mathrm{YS}}(\lambda)x_{\mathrm{YS}}, \quad (3)$$

where x_{m}, x_{c}, x_{d}, x_{p} and x_{YS} are the unknown densities of the respective elements in mixed sample waters. Gradient vectors are important parameters for the evaluation to distinguish and to determine the density of each element in mixed sample waters. Provided that a matrix $\|B(\lambda)\|$, the gradient vectors in eq. (3) are simply written as **B**. Equation (3) can be represented by,

$$\mathbf{A} = \mathbf{BX}, \quad (4)$$

where matrix **X** is the vector format of densities of x_{m}, x_{c}, x_{d}, x_{p} and x_{YS}. It is known from the least squares theory that density **X** is estimated as a minimum residual sum of squares. The simple solution is:

$$\mathbf{X} = [\mathbf{B}^T\mathbf{B}]^{-1}\mathbf{B}^T\mathbf{A}, \quad (5)$$

where $[\mathbf{B}^T\mathbf{B}]^{-1}$ is the inversion matrix of the matrix $[\mathbf{B}^T\mathbf{B}]$. The strength of the relationship between estimated densities and known densities is measured by the square of correlation coefficient.

4. Experimental Results

4.1 Determination of gradient vectors and estimation of mixed sample waters

Calculations of the linear regression written in eq. (1) were done for each data set of spectral absorption of five pure sample waters for four different values of density. We determined the gradient vector, $B(\lambda)$, for every known element of these waters. Figure 4 shows the resultant gradient vectors $B_{\mathrm{m}}(\lambda)$, $B_{\mathrm{c}}(\lambda)$, $B_{\mathrm{d}}(\lambda)$, $B_{\mathrm{p}}(\lambda)$ and $B_{\mathrm{YS}}(\lambda)$ for each group of the five elements, blue-green alga, green alga, brown 1 alga, brown 2 alga and YS, respectively. Each group is characterized by a specific gradient vector $B(\lambda)$, obtained for five elements of pure sample waters in 360–740 nm wavelength bands. In Fig. 4, the differences in the shapes of gradient vectors between brown 1, brown 2, and YS are more obvious than those for blue-green or green. In the blue-green group, there is a low intense monotonous increment of spectral shape from the 570 to 640 nm range. However, a specific pigment, phycocyanin can be observed around the 630 nm area slightly. The green group does not show a peak in this region, and the blue-green and green group spectral shapes do not provide a discernable difference between them. In the brown 1 and brown 2, 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 fact, in the brown 2 group, the intensity of peridinin is much higher than that of fucoxanthin in the brown 1 group spectra. In both the brown 1 and brown 2 groups, a small peak of Chl *c* appeared near 630 nm, however, its intensity relative to 680 nm of Chl *a* is much lower than that of phycocyanin in the blue-green. In contrast to the algal groups, the YS group has a smooth spectral curve and the maximum gradient is observed near the blue region of the spectrum. Resultant gradient vectors were used to identify and to estimate the elements in mixed sample waters.

A calibration line for each element was determined to four points of known densities of pure sample waters by least squares method as shown in Figs. 5(a)–5(e). Known densities of pure sample waters used in calibration of the data are shown as diamond marks in the graphs. The unknown density of each element in mixed sample waters was estimated by solving eq. (5). The estimated densities were

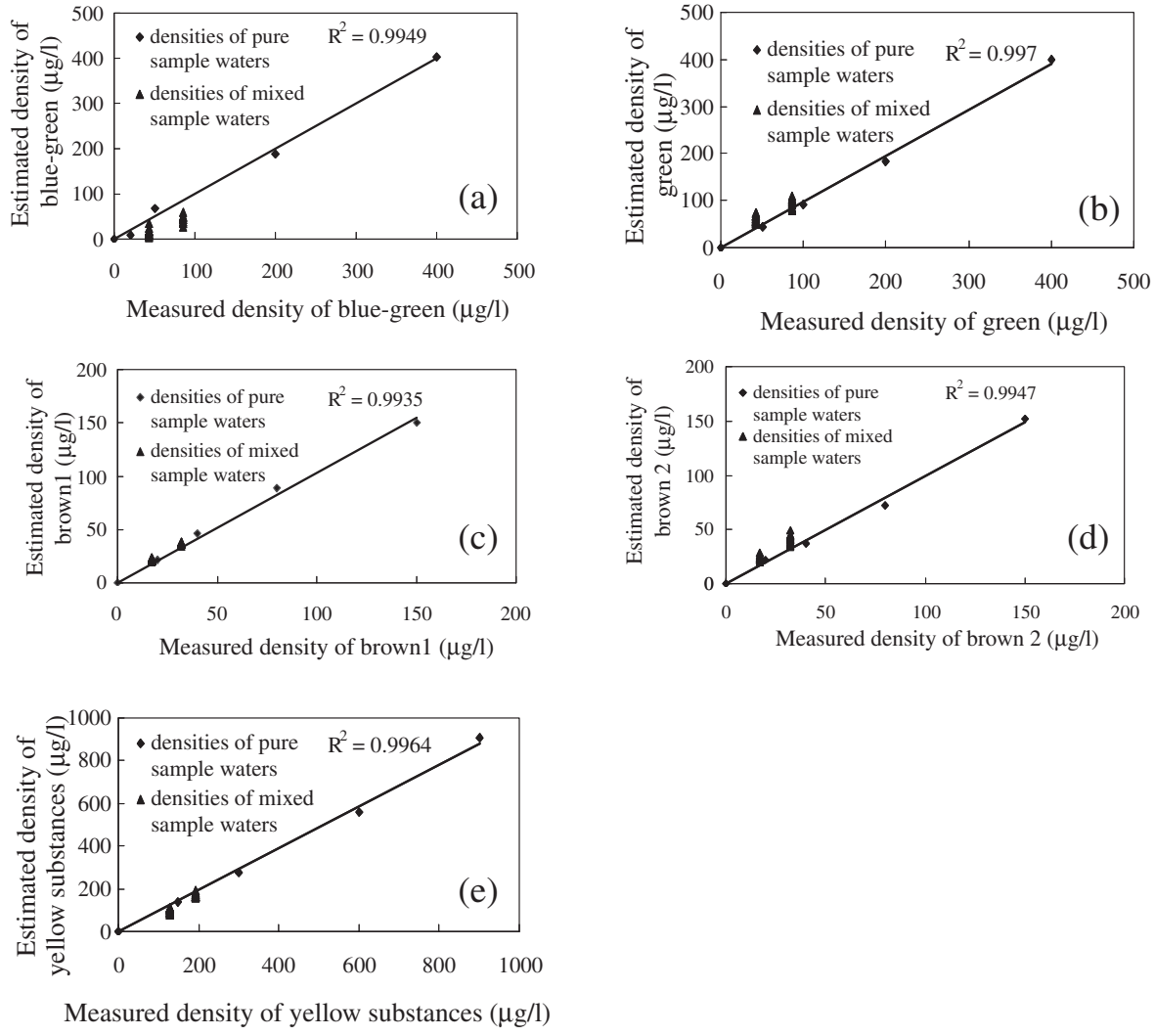

Fig. 5. Calculated results versus measured densities of the amount of individual elementary substance in mixed sample waters. The solid line represents the 1 : 1 ratio. Correlation coefficient indicates pure sample waters (a) blue-green, (b) green, (c) brown group 1, (d) brown group 2, and (e) yellow substances.

plotted on the calibration line *versus* the measured (results of Chl *a* obtained spectrophotometrically) amount of each element as shown in Figs. 5(a)–5(e).

The Figures illustrate the results of variation of the density of each element in mixed sample waters by a triangular mark. Estimated densities of the blue-green algae were at an accepted level with measured densities as shown in Fig. 5(a). The correlation between the estimated and measured densities of the mixed sample waters was ($r^2 = +0.7620$) with an experimental error of less than 17%. All other groups were identified well as shown in Figs. 5(b)–5(e). According to the figures, the brown group 1 and YS were identified properly by regression method with the strong correlation ($r^2 = +0.968$ and $r^2 = +0.9166$) and an error less than 4% and 6% respectively. Green and brown group 2 were estimated with the correlation ($r^2 = +0.7493$ and $r^2 = +0.8182$) and an average error less than 13 and 25%, respectively. The calculated error between the estimated densities and measured densities of brown 2 was higher than other groups. This method thus seems to be a promising approach as indicated by the experiments shown in Figs. 5(a)–5(e).

5. Discussion

The present study was an attempt to distinguish and to estimate the quantity of phytoplankton species in mixed sample waters using two step linear regression analysis. Densities of each element in mixed sample waters were estimated by using gradient vectors, and the proposed method depends significantly on the reliability of these gradient vectors. Laboratory work suggested that the identification and estimation of densities of the blue-green group and other groups of fresh water was possible.

The spectral similarities could be assessed by computing the angle between the gradient vectors comprising regression for the five elements in the multidimensional space (39-D). A crucial requirement for the spectral similarity analysis is linearity between the gradient vectors B_k and B_l of any

Table 2. Angle, $\theta(^\circ)$ between gradient vectors on original space and 3-D subspace for pure sample waters.

Group	Angle, θ (39-D)	Angle, θ (3-D)
Blue-green to green (m–c)	5	4
Blue-green to brown 1 (m–d)	12	12
Blue-green to brown 2 (m–p)	15	15
Green to brown 1(c–d)	8	8
Green to brown 2 (c–p)	15	13
Brown 1 to brown 2 (d–p)	12	11
Blue-green to YS (m–YS)	32	32
Green to YS (c–YS)	33	33
Brown 1 to YS (d–YS)	32	32
Brown 2 to YS (p–YS)	21	21

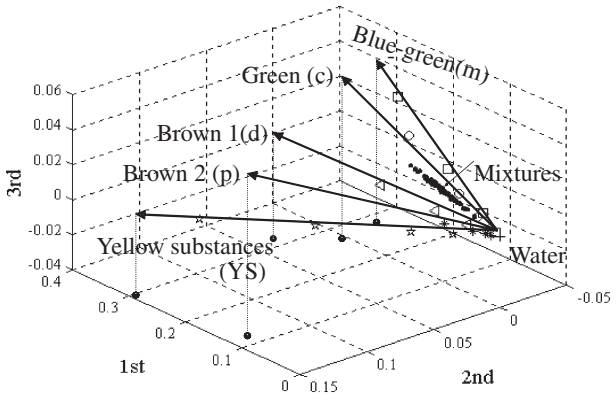

Fig. 7. Projection of spectral absorption characteristic of each sample waters on a three dimensional subspace.

species. If this requirement is fulfilled, angles between B_k and B_l can be defined in the multidimensional space as follows,

$$\theta_{kl} = \cos^{-1} \frac{(B_k B_l)}{|B_k| \times |B_l|}, \tag{6}$$

where θ_{kl} is the angle between B_k and B_l in radians. The angle between the two vectors is very small, which means that two spectra of the elements approach each other similarly. The calculated results are listed in Table 2, as degree of the angles.

It is impossible to visualize the differences between angles for comparison of gradient vectors in the 39-D space. The vector subspace method can be used to describe discernable differences and similarities of gradient vectors by using principal component analysis.[14] In fact, a small number of eigen vectors were determined from correlation matrix of the spectral data $A(\lambda)$. Figure 6 shows the resultant first three eigen vectors where fidelity value was 0.998, and then three-dimensional subspace was constructed. Every sample (blue-green, green, brown 1, brown 2, YS, mixtures and pure water) was projected on the subspace as shown in Fig. 7. The same figure illustrates the differences between angle values of gradient vectors and clear understanding of the distribution of mixed sample waters on the subspace. Angles

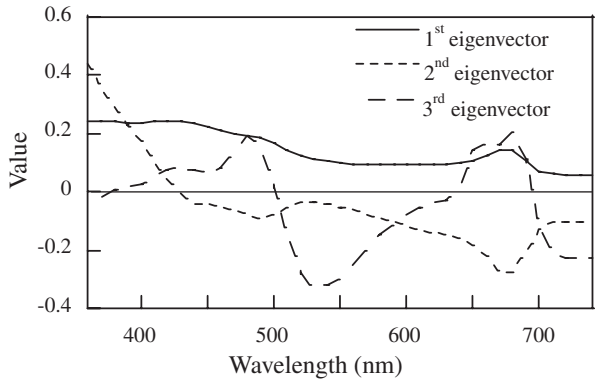

Fig. 6. First three eigen vectors.

between gradient vectors were calculated on 3-D subspace using eq. (6), as shown in Table 2.

According to the results in Table 2 and as shown in Fig. 7, angles between YS and unialgal groups consistently were greater than angles between unialgal elements. Consequently, YS was the most dissimilar from other absorption spectra. Blue-green and green groups displayed a limited close relationship to spectra with respect to the angle between them, not merely their spectral shape. Figure 7 provides a clear interpretation of differences of gradient vectors with respect to the angles.

In the figure, points denoted by squares (□), circles (○), triangles (△), asterisks (∗) and stars (☆) are the density points of blue-green, green, brown 1, brown 2, and YS respectively. The density of pure sample waters was proportional to the distance between a point on a relevant elementary substance line and the point of water (+). When we consider the mixed sample waters consisting of blue-green, green and brown 1 should be located on a plane which is parallel to the plane made by the green and brown 1 line. Then the point on the blue-green line intersected by the plane gives the density of blue-green in these sample waters. Using the calibration line of blue-green, the unknown density in mixed sample waters can be calculated. In our case, its similar implementation allows us to regard the high dimensional subspace relevant to the number of elementary substances in mixed sample waters. It is clear that dimensionality of the subspace should be determined first by considering the number of elementary substances in these waters. If we are going to apply the proposed method to a natural field to estimate of blue-green, we must observe other living elements in natural water samples.

Before the cyanobacterial bloom formation, natural fresh waters are usually dominated by green or brown algae. It is therefore important to detect blue-green and other unialgal groups selectively in a mixed population. The results for the unialgal groups and YS suggest that the demand for the selectivity can be satisfied by using two step linear regression analysis. The results of Fig. 5(a) are very encouraging and indicate the blue-green density could be monitored from the early stage of waterbloom.

6. Conclusions

We proposed a passive method based on a new algorithm for qualitative and quantitative estimation of phytoplankton species in a freshwater ecosystem. Mathematical evaluation was done by two step linear regression analysis based on least squares method. Resultant gradient vectors are significant parameters for quantitative estimation of phytoplankton in mixed sample waters using spectral absorption curves. The present studies have provided a simple classification pathway for differentiation of unialgal species and YS in a mixed population. The correlation between measured Chl *a* density and estimated Chl *a* density of a blue-green group in the laboratory experiment illustrates the usefulness of this quantitative analysis. This study shows that the spectral analysis is suitable for algal classification, while such a method of analysis provides certain advantages in identifying different algal types and estimated densities of an algal species.

We believe that it may be possible to use this proposed two step linear regression analysis in the remote determination of problematic species in vast marine ecosystems, in some cases where a more active method cannot be applied. Furthermore, this study can be extended to monitoring problematic species and to predict the water quality of freshwater bodies based on the amount of algae as a bio-indicator.

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