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Application of chemical mass balance to determination of phytoplankton composition from pigment profiles in seawater

Yutaka OKUMURA[1)*], Takashi KAMIYAMA[1)] and Toshiyuki SUZUKI[2)]

Abstract: We applied a chemical mass balance (CMB) receptor model to determine the composition of phytoplankton from pigment profiles in seawater collected from Sendai Bay, Japan. In descending order, the relative abundances of the six predominant phytoplankton classes expressed as a proportion of total chlorophyll *a* were Bacillariophyceae > Prasinophyceae ≧ Cryptophyceae ≧ Haptophyceae ≧ Pelagophyceae ≧ Dinophyceae. The results obtained by using CMB model were similar to those obtained by the CHEMTAX software: the Bacillariophyceae accounted for about 65% (CMB) or 64% (CHEMTAX) of the total phytoplankton, and the proportions of the other classes ranged from 5% to 11%. Many DNA sequences obtained from the clone library of the *psbA* gene encoding the D1 protein were highly similar to those of the Bacillariophyceae. The DNA sequencing results were similar to those obtained by CMB. We anticipate that CMB, which is used to investigate sources of pollutants, can be applied as a simple method of evaluating phytoplankton community composition using a pigment ratio approach.

Keywords: *phytoplankton pigment, chemical mass balance analysis, DNA sequencing.*

1. Introduction

Phytoplankton are primary producers in the ocean and use many kinds of pigments for photosynthesis. Pigment profiles vary among phytoplankton classes. Analysis of pigment profiles in seawater has been used to investigate seasonal changes in phytoplankton dominance (METAXATOS and IGNATIADES 2002, RODRIGUEZ *et al.* 2003, HASHIHAMA *et al.* 2008), enabling higher throughput than can be achieved by cell counting under a microscope. Phytoplankton pigments are analyzed by using high-performance liquid chromatography (HPLC) ; the proportional concentration of each phytoplankton class is calculated statistically by, for example, factor analysis using a steepest descent algorithm (CHEMTAX; MACKEY *et al.* 1996) or multiple regression analysis using ordinary least squares (BUSTILLOS-GUZMAN *et al.* 1995, SUZUKI *et al.* 1997, 2002). CHEMTAX software (MACKEY *et al.* 1996, 1997) is frequently used for the calculations (SUZUKI *et al.* 2002, RODRIGUEZ *et al.* 2003, HASHIHAMA *et al.* 2008) and CHEMTAX v. 1 is an excellent free software package, but it functions only within a commercial programming language.

Chemical mass balance (CMB) software is used to solve mass balance equations by an effective variance least-squares method. It was developed by Dr. TOM COULTER of the U.S. Environmental Protection Agency and was

1) Tohoku National Fisheries Research Institute, Fisheries Research Agency, 3－27－5 Shinhama, Shiogama, Miyagi 985－0001, Japan
2) National Research Institute of Fisheries Science, Fisheries Research Agency, Fukuura, Kanazawa, Yokohama, Kanagawa 236－8648, Japan
＊Corresponding author.
TEL: ＋81－22－365－9933
FAX: ＋81－22－367－1250
E-mail: okumura@affrc.go.jp

configured by HAYAKARI and HANAISHI (2001) to operate with add-in Microsoft Excel software (Microsoft, U.S.A.). Because Microsoft Excel is the world's most popular spreadsheet software, CMB has the advantage of ease of use because it does not need a special programming language. Moreover, CMB is different from previous multiple regression analyses of phytoplankton pigments, as it is based on a mass balance equation with a weighted least-squares regression analysis (HOPKE 2002). The CMB receptor model is used in environmental studies to investigate sources of pollutants, such as airborne particulate matter in the atmosphere (HOPKE 2002) and dioxins (KASHIWAGI *et al.* 2006, OKUMURA *et al.* 2008).

If the datasets of mass concentrations of particulate matter components are replaced by pigment profiles, CMB, like CHEMTAX, can be used to estimate phytoplankton composition by a pigment ratio approach. As CMB software (ver. 8J0612) can be used when only few data are available, we expected it to be suitable for monitoring phytoplankton communities in coastal areas in cases where there are only a few sampling points. Here, we used the CMB receptor model to estimate phytoplankton community composition from pigment profiles in seawater. We then compared the results of CMB analysis with those of CHEMTAX and DNA sequencing, both of which have recently been used to investigate plankton diversity (SAVIN *et al.* 2004, ZEIDNER and BEJA 2004, ZHU *et al.* 2005, JING *et al.* 2010).

2. Materials and methods

2.1. Sampling and HPLC analysis of pigments

In May 2007, a seawater sample was collected from the ocean surface off Oginohama in Sendai Bay, Japan (lat 38°23′N, long 141°24′E). This area contains fertile grounds used for Pacific oyster (*Crassostrea gigas*) aquaculture. The 200-ml sample was filtered through a glass fiber filter (25-mm Whatman GF/F filter, Whatman PLC, Springfield Mill, U.K.). The pigments were extracted from the filter for 24 h in 1 ml methanol, and the extract was centrifuged at 10000×*g* for 10 min. After dilution of the supernatant to 80% with distilled water in an HPLC auto-sampler (Shimadzu, Kyoto, Japan), the pigments were analyzed by the method of ZAPATA *et al.* (2000), with a slight modification whereby a guard column was attached between the injection valve and the analytical column.

Each pigment was identified from the retention times of pigment standards, and the concentration was calculated from the peak area on the chromatograph from the HPLC diode array detector. We used eight pigment standards: 19′-butanoyloxyfucoxanthin (But-fuco), 19′-hexanoyloxyfucoxanthin (Hex-fuco), chlorophyll *a* (Chl-*a*), chlorophyll *b* (Chl-*b*), fucoxanthin (Fuco), neoxanthin (Neo), and peridinin (Perid) were purchased from Wako Chemicals (Osaka, Japan) and DHI Laboratory (Hørsholm, Denmark), and alloxanthin (Allo) was refined by HPLC from pigment extracts of unialgal cultures of *Rhodomonas salina* CS-174 (Cryptophyceae), purchased from CSIRO Marine Research (Hobart, Tasmania, Australia). The concentrations of standard pigments dissolved in acetone or ethanol were calculated from the extinction coefficients ($E_{1\,cm}$; L g^{-1} cm^{-1}), as measured by spectrophotometer (GE Healthcare Ultrospec 3000) over a 1-cm cuvette path length. The $E_{1\,cm}$ and measurement wavelength, and the solvent used for each pigment, were obtained from published data (JGOFS 1994, JEFFREY *et al.* 1997). The concentrations of pigment standards were calculated as follows (JGOFS 1994):

$$C_s = (A_{max} - A_{750\,nm})/E \times 1000\,[mg/g] \qquad (1)$$

where C_s is the pigment concentration (mg L^{-1}), A_{max} is the absorbance maximum, $A_{750\,nm}$ is the absorbance at 750nm to correct for light scattering, and E is the extinction coefficient (L g^{-1} cm^{-1}).

2.2. Analysis by CMB and CHEMTAX

The pigment concentrations determined by HPLC were input into a CMB spreadsheet in Microsoft Excel. Data for the typical pigment ratios of each class of phytoplankton were also input (Table 1). We selected six phytoplankton — *Phaeodactylum tricornutum* (Bacillariophyceae), *Pelagococcus subviridis* (Pelagophyceae), *Pycnococcus provasolii* (Prasino-

Table 1. Typical pigment / chlorophyll a ratios of each class of phytoplankton used in this study

	Perid	But–fuco	Fuco	Neo	Hex–fuco	Allo	Chl *b*	Chl *a*
Prasinophyceae				0.151			0.945	1.000
Bacillariophyceae			0.755					1.000
Dinophyceae	1.063							1.000
Haptophyceae					1.706			1.000
Pelagophyceae		0.368	0.974					1.000
Cryptophyceae						0.229		1.000

phyceae), *Amphidinium carterae* (Dinophyceae), *Emiliania huxleyi* (Haptophyceae), and *Chroomonas salina* (Cryptophyceae) and calculated their pigment ratios from the published data (JEFFREY and WRIGHT 1997) used for the initial ratio matrix in the CHEMTAX user's manual (MACKEY *et al.* 1997). After inputting all data, we analyzed the phytoplankton profiles in the environmental sample by CMB according to the CMB user's manual (HAYAKARI and HANAISHI 2006).

The mass environmental concentration of pigment i (C_i) is shown in Equation (2) : (in the original equation, C_i is the mass concentration of component i of particulate matter) ;

$$C_i = \sum_{j=1}^{p} a_{ij} S_j \quad i=1,.....,n \qquad (2)$$

where a_{ij} is the fractional concentration of pigment i of class j (in the original equation, a_{ij} is the density of particulate matter containing component i in emissions from source j to the receptor) ; S_j is the total mass concentration contributed by class j (originally the total mass concentration contributed by source j) ; p is the number of classes and n is the number of pigments, with $n \geqq p$ (originally, p was the number of sources and n was the number of components). C_i and a_{ij} are known, and S_j is found from the effective variance least-squares solution of an over-determined system of equations.

The calculations are accompanied by an error in the fractional concentration of the pigment from each class (or, in the Air Pollution Control Technology Manual [ENVIRONMENT AGENCY, GOVERNMENT of JAPAN 1997], the emission source profile data) and by an error in the established data on the environmental concentration of the pigment (or, in the abovementioned manual, the concentration of the component on environmental particles). Both of these errors must be considered. The effective variance least-squares method is solved in Equation (2) by multiplying the concentration of each pigment (or, in the manual, the concentration of each chemical) by a percentage weight that is proportional to the fractional concentration of the pigment from each class (or, in the manual, the precision of the emission source profile data) and the measured environmental concentration of each pigment (or, in the manual, the environmental concentration of each chemical component). Repeat computations are conducted to seek S_j, which minimizes the function x^2 in Equation (3) :

$$x^2 = \sum_{j=1}^{n} \frac{(C_i - \sum_{j=1}^{p} a_{ij} S_j)^2}{V_i} \qquad (3)$$

In Equation (4) V_i is the effective variance :

$$V_i = \sigma c_i^2 + \sum_{j=1}^{p} \sigma_{aij}^2 S_j^2 \qquad (4)$$

where σ_{Ci} is the error that accompanies component i, and σa_{ij} is the error that accompanies the measurement of a_{ij}.

To evaluate whether the calculated values were appropriate, the χ^2, R^2, and percent mass values were also calculated by CMB. In the CMB manual, the χ^2 value is the weighted sum-of-squares of the differences between the calculated and measured fitting species concentrations; a value less than 1 indicates a very good fit to the data, and values between 1 and 2 are acceptable. The R^2 value is the fraction of the variance in the measured concentrations

that is explained by the variance in the calculated species concentrations. R^2 ranges from 0 to 1.0; the closer the value is to 1.0, the better the source contribution estimates explain the measured concentrations. Percent mass is the ratio of the model-calculated source contribution to the measured mass concentrations, expressed as a percentage. This ratio should equal 100%, although values ranging from 80% to 120% are acceptable (COULTER 2004, HAYAKARI and HANAISHI 2006).

After the data for the typical pigment ratios of each phytoplankton class were input (Table 1), we also used CHEMTAX to analyze the phytoplankton community profile in the same environmental sample according to the CHEMTAX user's manual (MACKEY *et al.* 1997).

2.3. DNA sequencing and BLAST similarity search

For DNA sequencing, a 500-ml seawater sample was filtered through a Durapore membrane filter (diameter, 47 mm; pore size, 0.45 μm; Millipore, Merck KGaA, Darmstadt, Germany). The filter containing the sample was suspended in 1 ml of lysis buffer (20 mM Tris • HCl [pH8.0], 5 mM EDTA, 0.3% (wt : vol) SDS, 200 μg/ml proteinase K). The suspension was vortexed for 30s and then incubated at 55℃ for 1 h and at 95℃ for 5 min. The filter was then removed from the lysis buffer and the liquid phase containing nucleic acids was separated by centrifugation at 10,000×g for 10 min. The supernatant diluted with distilled water was used as a template for polymerase chain reaction (PCR) analysis.

The *psbA* gene, which encodes polypeptide D1 of the photosystem II reaction center complex, was amplified by PCR with the degenerate primers PsbAF (5′-TTC GGT CAA GAA GAA GAG ACT TA−3′) and PsbAMR (5′-YTC RTG CAT HAC YTC VAW RCC−3′), which have specificity for many algae. Primers were selected by using Primer3 software (ROZEN and SKALETSKY 2000). PCR was performed under the following conditions: 94℃ for 10 min; 40 cycles of 94℃ for 30s, 55℃ for 1 min, and 72℃ for 1 min; and a final step at 72℃ for 7 min.

After PCR amplification, the PCR products were purified with a High Pure PCR Cleanup Micro Kit (ROCHE DIAGNOSTICS, Germany). The purified products were cloned by DYNAEXPRESS TA PCR Cloning Kit (BIODYNAMICS LABORATORY INC., Tokyo, Japan). After colony-direct PCR (30 cycles of 98℃ for 10 s, then annealing and extension at 68℃ for 1 min) with the universal primers M13P7 (5′-CGC CAG GGT TTT CCC AGT CAC GAC−3′) and M13P8 (5′-AGC GGA TAA CAA TTT CAC ACA GGA AAC−3′), colonies containing inserts were identified by agarose gel electrophoresis. After purification of the PCR products of the insert colonies using ExoSAP-IT (GE HEALTHCARE, England), DNA sequencing was performed with a DYEnamic ET Terminator Cycle Sequencing Kit (GE HEALTHCARE) by using the primer M13P8 on an automated ABI 3100 DNA sequencer (APPLIED BIOSYSTEMS USA). Sequences were then aligned by using phred/phrap/consed software (EWING *et al.* 1998, GORDON *et al.* 1998, SAITO 2009) and phytoplankton species were identified from sequences aligned by using BLAST.

3. Results and discussion

3.1. Measured pigment concentrations and concentrations calculated by the CMB model

We compared measured and calculated pigment concentrations (Fig. 1). Measured pigment concentrations were in the order Chl-a >

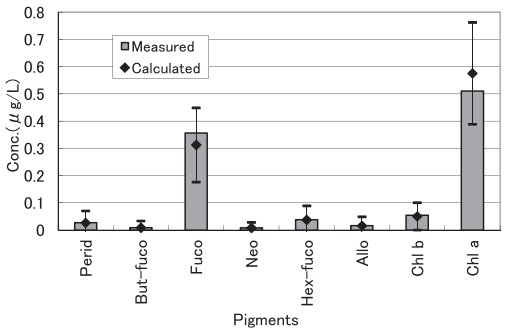

Fig. 1. Comparison of measured and calculated pigment concentrations in a seawater sample from Sendai Bay, Japan. Error bars were calculated by assuming a 5% error in the measured concentrations.

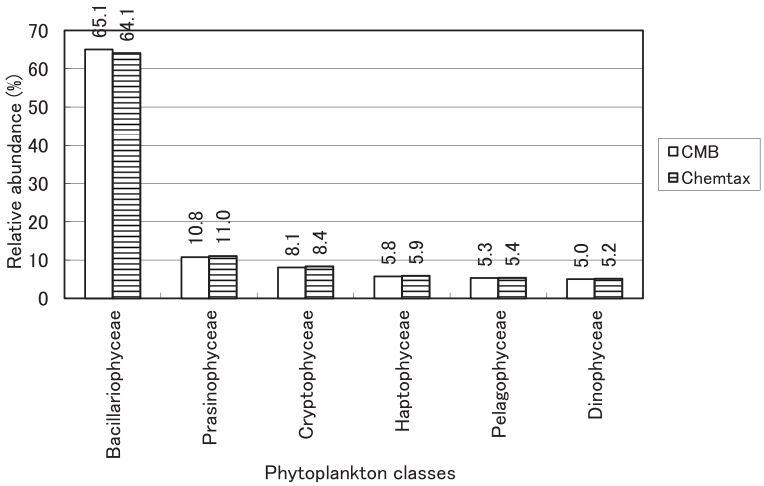

Fig. 2. Relative contributions of six phytoplankton classes to total chlorophyll a in a sample of seawater from Sendai Bay, Japan, as calculated by CMB and CHEMTAX.

Fuco > Chl-*b*. Hex-fuco, Perid, Allo, But-fuco, and Neo were at the same low level as Chl-*b*. The calculated concentration of each pigment was consistent with the measured concentration. The χ^2, R^2, and percent mass values calculated by CMB were 0.62, 0.99, and 101.6%, respectively. We consider that these three values indicate the validity of the class contribution estimates.

Chl-*a*, which is in all phytoplankton, usually has the highest concentration of all pigments detected in environmental samples (WRIGHT *et al.* 1991, ZAPATA *et al.* 2000), as it did in this study. Fucoxanthin, which is found in the Bacillariophyceae and Pelagophyceae (JEFFREY and WRIGHT 1997), was also detected at high concentrations. Our pigment profiles are therefore similar to those reported elsewhere (WRIGHT *et al.* 1991, ZAPATA *et al.* 2000).

3.2. Comparison between CMB and CHEMTAX

We compared the relative contributions of six phytoplankton classes to total chlorophyll *a* calculated by CMB and CHEMTAX (Fig. 2). Both approaches indicated that the relative abundance of the Bacillariophyceae was highest, followed in descending order by Prasinophyceae ≧ Cryptophyceae ≧ Haptophyceae ≧ Pelagophyceae ≧ Dinophyceae. The results of the CMB and CHEMTAX analyses were similar. The Bacillariophyceae accounted for about 65% (CMB) or 64% (CHEMTAX) of all phytoplankton (Fig. 2). The proportions of Prasinophyceae, Cryptophyceae, Haptophyceae, Pelagophyceae, and Dinophyceae were about 11%, 8%, 6%, 5%, and 5% (both CMB and CHEMTAX), respectively. In Sendai Bay, the Bacillariophyceae outnumber the Dinophyceae in all seasons (IIJIMA *et al.* 2004) ; therefore, we believe that the phytoplankton community composition determined in this study is characteristic of that in Sendai Bay.

The pigment concentrations in phytoplankton are known to vary with environmental conditions such as light and nutrient conditions, and among species or strains within the same class (MACKEY *et al.* 1998, SCHLUTER *et al.* 2000, LIONARD *et al.* 2008). As the change of the pigment : chlorophyll a ratios of phytoplankton accompanying environmental conditions are reflected in the CMB analyses, we anticipate that CMB is a useful tool for investigating phytoplankton composition in the environment.

3.3. DNA sequencing and BLAST similarity search

The BLAST search showed that many of the *psbA* gene DNA sequences were highly similar to those of the Bacillariophyceae (Table 2).

Table 2. Results of a BLAST search of DNA partial sequences from water samples from Sendai Bay

Phylogenetic group	Sequences producing significant alignment (GenBank accession no.)	Identity (%)	Number of aligned reads	Ratio of each phylogenetic group (%)
Bacillariophytceae	*Odontella sinensis* (Z67753)	268/285 (94%)	12	63.6
	Thalassiosira pseudonana (EF067921)	256/260 (98%)	2	
Haptophyceae	*Isochrysis* sp. (AY119753)	271/282 (96%)	1	13.6
	Emiliania huxleyi (AY741371)	243/255 (95%)	1	
	Phaeocystis antarctica (AY119756)	232/241 (96%)	1	
Prasinophyceae	*Ostreococcus* sp. (EU851961)	272/278 (97%)	1	9.1
	Micromonas pusilla (FJ858269)	278/285 (97%)	1	
Rhizaria	*Paulinella chromatophora* (DQ789030)	184/215 (85%)	3	13.6

Bacillariophyceae sequences accounted for about 64% of all phytoplankton. This proportion is close to the values obtained by CMB (65%) and CHEMTAX (64%). Within the Bacillariophyceae, *Odontella sinensis* was significantly aligned by BLAST. *Odontella* spp. are frequently observed in the coastal zones of the Tohoku region of Honshu, Japan (Takano 1990) ; our results are consistent with these observations. The results of the BLAST search for similar DNA sequences suggest that the Haptophyte species were *Isochrysis* sp., *Emiliania huxleyi* and *Phaeocystis antarctica*, and the Prasinophytes were *Ostreococcus* sp., *Micromonas pusilla* and Rhizaria was *Paulinella chromatophora*. Although we did not detect any Cryptophyceae, Pelagophyceae or Dinophyceae DNA sequences, the pigments characteristic of these phylogenetic groups indicate that they accounted for 5.0% to 8.4% of the total phytoplankton, as determined by CMB and CHEMTAX. If more DNA sequences are available, it should be possible to detect the DNA sequences of these classes. Although the bias of PCR frequently adds uncertainty to the quantification of organisms (Diez 2001), our DNA sequencing results were roughly similar to those of CMB and CHEMTAX. As more results of CMB analysis accumulate, CMB will become an easy-to-use tool for investigating natural phytoplankton community composition.

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