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Possible Underestimation of Chlorophyll *a* Measurements for Subtropical Phytoplankton Community by the Pigment Extraction and the Fluorometric Determination

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Abstract

Chlorophyll *a* concentrations of subtropical phytoplankton community extracting with 90% acetone and *N, N*-dimethylformamide (DMF) were determined with the acidified and non-acidified fluorometric methods. Chlorophyll *a* concentrations extracted with 90% acetone and determined with the acidified fluorometric method showed a considerably underestimation than those extracted with DMF and determined with the non-acidified fluorometric measurements. The underestimation was not size-specific but observed for pico-, nano- and micro-phytoplankton. Based on variance analysis (two-way ANOVA), the extraction with 90% acetone and the acidified fluorometric determination are both significant as the underestimating sources although the former was more important. From these results, we recommend combination of pigment extraction with DMF and the non-acidified fluorometric measurement in chlorophyll *a* determinations for subtropical phytoplankton community.
Key words: chlorophyll *a*, fluorometric measurement, pigment extraction, phytoplankton, subtropical

Introduction

Since chlorophyll *a* is a major photosynthetic pigment observed for various phytoplankton taxonomic groups (CHIBARA 1999), the pigment has been extensively used as an important indicator to estimate phytoplankton biomass in various aquatic environments. In general, spectrophotometric (PARSONS and STRICKLANDS 1963), fluorometric (YENTSCH and MENZEL 1963), or high-performance liquid chromatography methods (HPLC: FURUYA *et al.* 1998) are used for chlorophyll *a* determination. Because of the sensitive measurement and simple procedure, fluorometric method has been widely used for chlorophyll *a* determination in oceanography.

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Currently, two fluorometric methods are proposed by HOLM-HANSEN *et al*. (1965) and WELSCHMEYER (1994) for chlorophyll *a* measurements. In the method of HOLM-HANSEN *et al*. (1965), chlorophyll *a* concentrations can be determined with the difference of fluorescence before and after acidification of pigment extracts. Because of the simple procedures, it has been accepted as a worldwide standard method for chlorophyll measurement. However, there are many reports that chlorophyll *b* interferes with the precise measurement of chlorophyll *a* in the acidified fluorometric method (GIBBS 1979, TREES *et al*. 1985) and can be quite common in marine systems (BIDIGARE *et al*. 1986, GIESKES and KRAAY 1986). Thereby, some underestimation is considered for chlorophyll measurements with the acidified fluorometric method. WELSCHMEYER (1994) proposed the improved method for chlorophyll measurements. He mentioned that chlorophyll *a* concentration can be precisely determined without acidification of pigment extracts because chlorophyll *b* and its degradation products are highly eliminated by the specific excited (436 nm) and emitted wavelengths (680 nm).

In the fluorometric chlorophyll measurements, pigment extraction from phytoplankton has been performed with aqueous 90% acetone (GIESKES and KRAAY 1986, WRIGHT *et al*. 1997). However, chlorophyll *a* is decomposed to chlorophyllide *a* or pheopigments during the extractions (SUZUKI and FUJITA 1986). Moreover, further procedures such as grinding and centrifuge are needed due to a low extraction efficiency of the solvents (HOLM-HANSEN *et al*. 1965). Thereby, SUZUKI and ISHIMARU (1990) recommended *N, N*-dimethylformamide (DMF) as an organic solvent for the pigment extraction because of the higher extraction efficiency, rapid extraction time, simple extraction procedure (only soaking) and a long stable life of chlorophyll *a*. Recently, the high extraction efficiency was confirmed for the coastal phytoplankton community (TADA *et al*. 2004).

It is well known that *Prochlorococcus* cells contain divinyl chlorophyll *b* as a major accessory pigment (CHISHOLM *et al*. 1992) and numerically dominates phytoplankton community in tropical to subtropical waters (CAMPBELL *et al*. 1997, DURAND *et al*. 2001). In addition to the low chlorophyll *a* concentrations in these waters, considerable underestimation could be resulted from the interference of divinyl chlorophyll *b* in the commonly used method of the pigment extraction with aqueous 90% acetone and the acidified fluorometric determination.

In the present study, we report some underestimation of chlorophyll *a* concentrations in the 90% acetone extraction and the acidified fluorometric determination, compared with and DMF extraction and the non-acidified fluorometric determination. From these results, we recommend the appropriate method of chlorophyll *a* determination in tropical to subtropical phytoplankton community.

Materials and Methods

Samplings were done in the southwestern Japan (30° N, 131° E) on 8 November 2004. Water samples for chlorophyll measurements were collected from depths of 10, 30, and 50 m with a CTD-RMS at intervals of 4 hours on the day. Surface water samples were collected with a plastic bucket. Water samples from each depth (1000 ml) were filtered through a nylon plankton net (NYTAL HD20: 20- μ m mesh opening), a polycarbonate membrane filter (Millipore TTTP: 2- μ m pore size) and a glass fiber filter (Whatman GF/F) under lower vacuum pressure than 150 mmHg. Thereafter, chlorophyll pigments on the filter were immediately extracted by direct immersion into aqueous 90% acetone (SATO *et al*. 1981) and *N, N*-dimethylformamide (DMF: SUZUKI and ISHIMARU 1990). The pigment extraction in both organic solvents was done at 5℃ under dark condition more than 24 hours. Filters without the filtration (controls) were soaked into the two extracting solvents under same conditions. After sonication of the pigment extracts for 30 seconds at 30W using a UCHIDA P1 sonicator (WRIGHT *et al*. 1997), chlorophyll *a* concentrations were measured with a Turner Designs fluorometer (TD-700) based on the acidified (HOLM-HANSEN *et al*. 1965) or non-acidified fluorometric method (WELSCHMEYER 1994). The fluorometer was calibrated with a standard chlorophyll *a*; a chlorophyll *a* reagent (SIGMA) was dissolved in aqueous 90% acetone and DMF, and the concentrations of the standard chlorophyll *a* solutions were determined spectrophotometrically using the equation:

$$CHL_{ST} = A_{CHL} \,/\, (\text{specific absorption coefficient} \times L) \quad (1)$$

where CHL_{ST} is standard chlorophyll *a* concentration (g l^{-1}) and A_{CHL} is the difference between absorbance at 663.8 nm and 750 nm. The specific absorption coefficient is 87.67 l g^{-1} cm^{-1} and 88.74 l g^{-1} cm^{-1} in the case of aqueous 90% acetone and DMF, respectively (JEFFREY and HUMPHREY 1975, PORRA *et al*. 1989). L means path-length (1 cm). Chlorophyll *a* determined with the acidified fluorometric method (CHL_H : μg l^{-1}) was estimated from the following equations:

$$CHL_H = K \times (Fo - Fa) \times (v / V) \quad (2)$$

where *v* is the volume of the pigment-extracting solvents (ml), *V* is the volume of water samples (ml), and *Fo* and *Fa* is the fluorescence before and after acidification, respectively. A factor of the equation (*K*) was determined from the standard chlorophyll *a*. Chlorophyll *a* determined with the non-acidified fluorometric method (CHL_W : μg l^{-1}) was estimated from the following equations:

$$CHLw = CHLo \times (v / V) \quad (3)$$

where *CHLo* is the chlorophyll *a* concentration of pigment extracts in organic solvents (μg l^{-1}). Since controls showed a little fluorescence, CHL_H and CHL_W were calibrated with them.

Result and Discussion

First, we have investigated vertical distributions and temporal changes in chlorophyll *a* concentrations determined with the two extracting solvents and the two different fluorometric methods. On Figure 1, subscripts at CHL indicate chlorophyll determination (*H* : acidified method, *W* : non-acidified method) and extracting solvent (*A*: aqueous 90% acetone, *D*: DMF). $CHL_{W\text{-}D}$ showed the highest chlorophyll among the methods and $CHL_{H\text{-}D}$ was second (Fig. 1). $CHL_{W\text{-}A}$ and $CHL_{H\text{-}A}$ revealed the low-

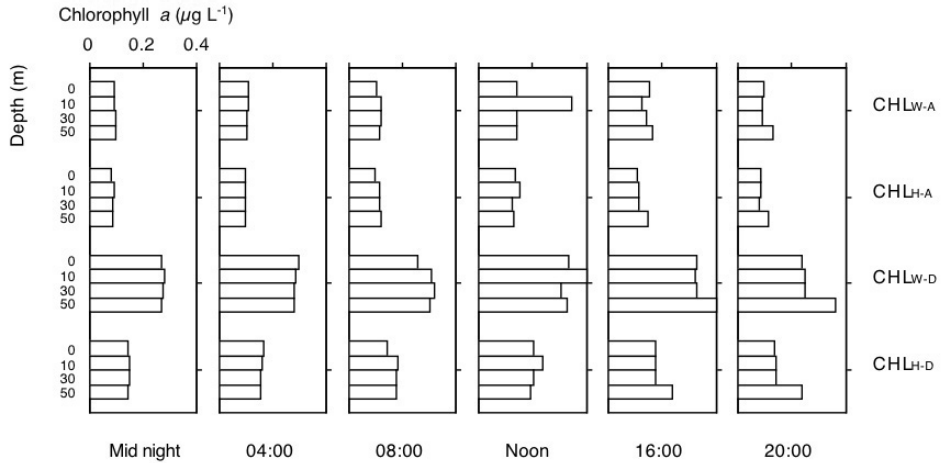

Fig. 1. Vertical distribution of chlorophyll *a* concentrations in acetone (A) and DMF extracts (D) determined with the acidified (H) and non-acidified fluorometric methods (W).

est value and was 40.7% and 35.3% of $CHL_{W\text{-}D}$, respectively. Similar results were observed for the mean chlorophyll *a* in the water column above 50 m, as $CHL_{W\text{-}A}$ and $CHL_{H\text{-}A}$ were less than half of $CHL_{W\text{-}D}$ (Fig. 2). Although the absolute chlorophyll was different, vertical and temporal patterns were similar among the chlorophyll *a* con-

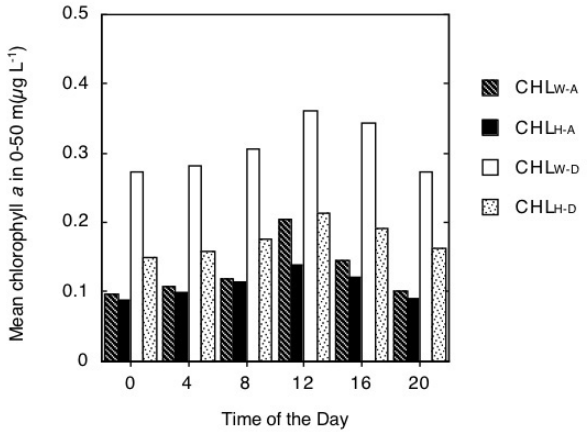

Fig. 2. Diel changes in mean chlorophyll *a* concentrations above 50 m in acetone (A) and DMF extracts (D) determined with the acidified (H) and non-acidified fluorometric methods (W).

centrations determined with these methods.

Second, we evaluated which size group of phytoplankton was contributed to the difference (Fig. 3). Relative contribution to *CHLW-D* (the highest chlorophyll observed in this study) showed that the difference was observed for all size groups of the phytoplankton community.

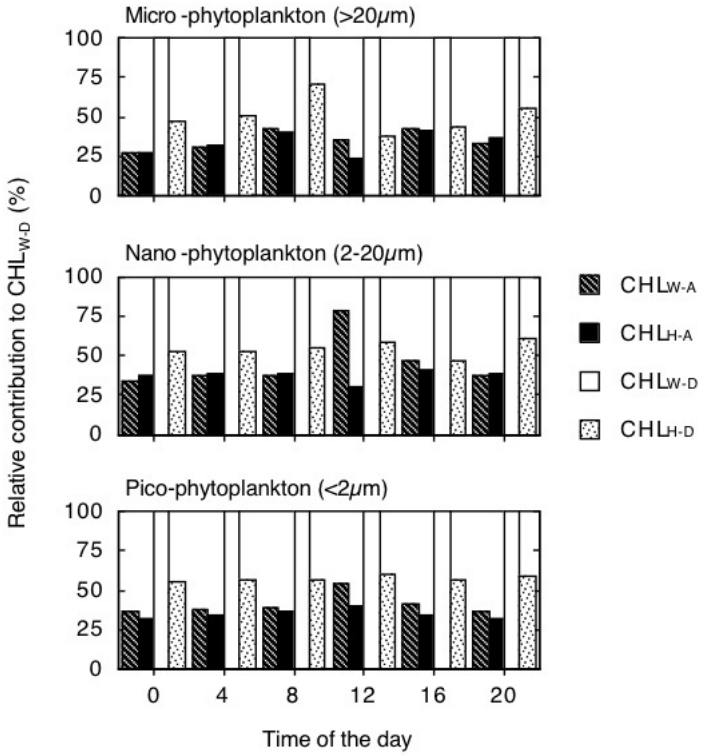

Fig. 3. Relative contribution of chlorophyll *a* concentrations in DMF extracts determined with the acidified fluorometric methods. Upper: micro-phytoplankton, Middle: nano-phytoplankton, Bottom: pico-phytoplankton. Abbreviations are the same in Figure 1.

Third, we quantitatively compared the difference of chlorophyll *a* concentrations determined with the pigment extraction and the fluorometric determination (Fig. 4). A significant regression line was observed for chlorophyll determined with acidified and non-acidified methods ($p<0.001$). Based on the regression equations, the decrease by the acidified determination was 28% in acetone extracts and 42% in DMF extracts. However, chlorophyll extracted with 90% acetone was comparable between the acidified and non-acidified methods if the higher data points were excluded. These results indicate that the disparity between the two fluorometric methods is more pronounced in DMF extracts.

Finally, we specified the underestimating source for the chlorophyll determination. According to the results of variance analysis (two-way ANOVA), the extracting solvents and the fluorometric determinations are both important as the un-

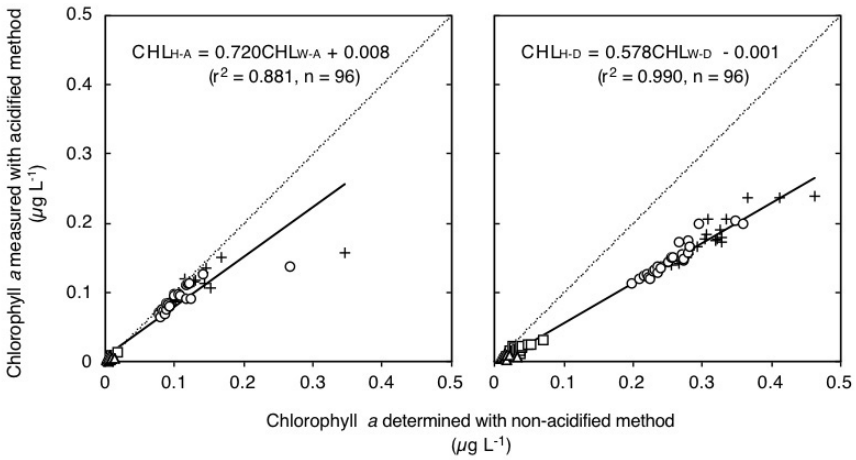

Fig. 4. Comparison between chlorophyll *a* concentrations determined with the acidified and non-acidified fluorometric methods. Dotted lines mean same between the two fluorometric methods. Triangles: pico-phytoplankton, Squares: nano-phytoplankton, Circles: micro-phytoplankton, Crosses: all size groups. Abbreviations are the same in Figure 1.

derestimating sources, but the formers were more significant (Table 1).

Table1. The summery results of variance analysis (two-way ANOVA) of the mean chlorophyll *a* concentrations in the water column(0-50m). df: degree of freedom. SS: sum of squares. MS: mean squares.

Underestimating sources	df	SS	MS	F-value	P-value
Extracting solvents	1	0.089	0.089	48.321	<0.001
Fluorometric determination	1	0.035	0.035	18.789	<0.001
Residual	21	0.039	0.020		

The large difference of chlorophyll extracted in 90% acetone was in good agreement with the previous comparisons of the extracting solvents (SUZUKI and ISHIMARU 1990, WRIGHT *et al*. 1997, TADA *et al*. 2004); i.e. chlorophyll *a* could not be completely extracted with direct immersion in 90% acetone. We also observed some difference of chlorophyll determined with acidified and non-acidified fluorometric methods, suggested by WELSCHMEYER (1994). However, this disparity between the two methods was more pronounced for DMF extracts. The interference by chlorophyll *b* and pheopigments could be a important cause for DMF extracts, whereas such effects might be of minor importance in acetone extracts. Sometimes, either DMF extraction or the non-acidified fluorometric determination has been applied for chlorophyll *a* measurement. However, the present results indicate that chlorophyll *a* extracted with DMF should be determined with the non-acidified fluorometric, at least for subtropical phytoplankton. In conclusion, we recommend combinations of the pigment extraction with DMF and the non-acidified fluorometric measurement in chlorophyll *a* measurements for subtropical phytoplankton community.

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