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Easy and rapid quantification of lipid contents of marine dinoflagellates using the sulpho-phospho-vanillin method

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To develop an easy and rapid method of quantifying lipid contents of marine dinoflagellates, we quantified lipid contents of common dinoflagellate species using a colorimetric method based on the sulpho-phospho-vanillin reaction. In this method, the optical density measured using a spectrophotometer was significantly positively correlated with the known lipid content of a standard oil (Canola oil). When using this method, the lipid content of each of the dinoflagellates *Alexandrium minutum*, *Prorocentrum micans*, *P. minimum*, and *Lingulodinium polyedrum* was also significantly positively correlated with the optical density and equivalent intensity of color. Thus, when comparing the color intensity or the optical density of a sample of a microalgal species with known color intensities or optical density, the lipid content of the target species could be rapidly quantified. Furthermore, the results of the sensitivity tests showed that only 1-3 × 10^5 cells of *P. minimum* and *A. minutum*, 10^4 cells of *P. micans*, and 10^3 cells of *L. polyedrum* (approximately 1-5 mL of dense cultures) were needed to determine the lipid content per cell. When the lipid content per cell of 9 dinoflagellates, a diatom, and a chlorophyte was analyzed using this method, the lipid content per cell of these microalgae, with the exception of the diatom, were significantly positively correlated with cell size, however, volume specific lipid content per cell was negatively correlated with cell size. Thus, this sulpho-phospho-vanillin method is an easy and rapid method of quantifying the lipid content of autotrophic, mixotrophic, and heterotrophic dinoflagellate species.

Key Words: cell size; intensity of color; lipid amount; lipid content per cell; sulfo-phospho-vanilin (SPV); volume specific lipid content

INTRODUCTION

Knowing the lipid content of marine microalgae is important for understanding their metabolism and for the use of their biomass as biofuel. Among them, dinoflagellates contain large amounts of high-quality lipids and dinosterols (Piretti et al. 1997, Mansour et al. 1999).

Dinoflagellates play diverse roles in the marine food web as primary producers, prey, predators, symbionts, and parasites (Muscatine 1990, Coats 1999, Jeong et al. 2010, 2012, Lee et al. 2014*a*, 2014*b*). In the last two decades, many phototrophic dinoflagellates that were previously thought to be exclusively autotrophic have been shown to be mixotrophic (Jacobson and Anderson 1996, Stoecker 1999, Jeong et al. 2005, 2010, Lee et al. 2014*c*). Therefore, these protists not only perform photosynthesis, but also feed on prey as energy sources. Furthermore, many dinoflagellates are important prey for mixotrophic

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and heterotrophic protists and metazoans (e.g., Jeong et al. 2010). Thus, they are a major source of fatty acids, sterols, and other nutrients to these predators. Also, they often dominate the plankton assemblages and sometimes form red tides or harmful algal blooms in the waters of many countries (Hallegraeff 1993, Anderson 1997, Park et al. 2013). Approximately 75% of the harmful algal bloom species are dinoflagellates (Smayda 1997). As a result of their very high concentrations during explosive and extensive blooms, they have been studied as a potential candidate for a source of biofuel (de la Jara et al. 2003, Fuentes-Grünewald et al. 2012, 2016). Thus, it is very important to analyze the lipid content of many dinoflagellates within relatively short time by using reasonable lipid quantification methods.

To analyze the lipid content of a dinoflagellate species, clonal cultures of target species should be established first and then a large amount of the culture should be harvested to obtain a large quantity of its biomass. This process usually takes a long time and is also very laborious. Chloroform and methanol based lipid extraction methods have been widely used for lipid quantification (Folch et al. 1957, Bligh and Dyer 1959). However, the conventional Folch or Bligh and Dyer's method in which lipids are extracted using chloroform and methanol requires a large amount of biomass and thus, usually requires a great deal of time and labor. The process of drying, extraction, weighing, and transesterification of samples for gas chromatography analysis may also take several days and can be laborious work. Thus, development of easier and rapider methods of quantifying the lipid content of microalgae is required.

The sulpho-phospho-vanillin (SPV) method has been used for the determination of the total lipid content in human cerebrospinal fluid (Drevon and Schmit 1964, Vatassery et al. 1981), as well as in serum, food, and ecological samples. The SPV assay produces a distinct pink color when reacting with lipids, and the intensity of the color can be quantified by measuring the absorbance at 530 nm using spectrophotometric methods. The advantages of this technique are that it is able to measure lipid content rapidly and simply and requires only a small amount of target sample. Thus, this method has been employed for rapid quantification of the intracellular lipid contents of some microalgae (Hao et al. 2013, Mishra et al. 2014, Byreddy et al. 2016).

In this study, we applied a modified SPV method for quantifying the lipid content of dinoflagellate species. Furthermore, using this method, we analyzed the lipid content of 9 dinoflagellate species including phototrophic, mixotrophic and heterotrophic species (*Amphidinium carterae*, *Heterocapsa triquetra*, *Prorocentrum minimum*, *Alexandrium minutum*, *Oxyrrhis marina*, *Scrippsiella trochoidea*, *Ostreopsis* cf. *ovata*, *Prorocentrum micans*, and *Lingulodinium polyedrum*), 1 chlorophyta (*Dunaliella tertiolecta*), and 1 diatom (*Thalassiosira* sp., 30 μm). Moreover, the relationships between lipid content and cell sizes or volumes of the dinoflagellates were explored.

MATERIALS AND METHODS

Reagents and standard curve preparation

Concentrated sulfuric acid, *o*-phosphoric acid (85%), chloroform, and methanol, all American Chemical Society (ACS) grade, were purchased from Thermo Fisher Scientific Inc. (Waltham, MA, USA) and Junsei Chemical Co., Ltd. (Tokyo, Japan). Vanillin (≥98%) was purchased from Sigma-Aldrich (St. Louis, MO, USA). The phospho-vanillin reagent was prepared by dissolving 0.75 g vanillin in 0.125 L distilled water and mixed with 500 mL of 85% phosphoric acid solution. The final concentration of reagent was 1.2 mg vanillin per mL of 68% phosphoric acid (Inouye and Lotufo 2006). For standard lipid stock, commercial Canola oil was obtained from the local market.

For preparation of the standard curve, commercial Canola oil was dissolved in chloroform (10 mg in 10 mL for a final concentration of 1 mg mL^{-1}), and different concentrations (10-150 μg) of standard lipid samples were prepared in clean glass vials. The vials were incubated at 90°C for 10 min to evaporate the chloroform. Concentrated sulfuric acid (0.1 mL) was added to each vial, and then heated at 90°C for 10-20 min. After cooling on ice about 5 min, 2.4 mL of phospho-vanillin reagent was added and allowed to develop for 10 min, until the color of the sample turned pink. Absorbance was measured at 530 nm using a spectrophotometer (Jenway-7300; Bibby Scientific, Stone, UK).

Color reaction of four dinoflagellate species in different cell numbers

To observe the color reaction of dinoflagellate lipids at different cell concentrations, we analyzed lipid contents of four dinoflagellate species: *Prorocentrum minimum*, *Alexandrium minutum*, *P. micans*, and *Lingulodinium polyedrum*.

Each species was grown at 20°C in enriched f/2 seawater media (Guillard and Ryther 1962) under a continuous

illumination of 20 μE m^{-2} s^{-1} provided by cool white fluorescent lights. Cultures were grown for 5-7 days, until the cell number was high enough for analysis (over 1.5×10^7 cells), at which point, the cells were collected. Four dinoflagellate species were prepared depending on cell concentration from 1,000 to 2,000,000 cells mL^{-1} (10^3, 10^4, 10^5, 3×10^5, 5×10^5, 7×10^5, 10^6, and 2×10^6). Triplicated samples of each cell number were prepared for lipid analysis. Pellets of each cell number were collected into a 15-mL glass vial, and then freeze dried for analysis.

After analyzing lipid content using the SPV method, the lipid content data analyzed for each cell number was calculated using the standard curve, and the lipid content of individual cells was calculated. A t-test and ANOVA were performed to determine if the lipid content of individual cells has any significant difference with the lipid content of the individual cells calculated for each different cell number.

Preparation of 11 experimental organisms

We prepared nine dinoflagellates species including a benthic dinoflagellate (*Ostreopsis* cf. *ovata*) and a heterotrophic dinoflagellate (*Oxyrrhis marina*), and a chlorophyte, and a diatom species for comparison (Table 1). All cultures were grown at 20°C in enriched f/2-Si seawater media (Guillard and Ryther 1962) under a continuous illumination of 20 μE m^{-2} s^{-1} provided by cool white fluorescent lights except *O. marina* and diatom (f/2 media).

The phototrophic and mixotrophic species were grown in 2-L PC bottles containing 1 L of f/2-Si or f/2 media per a bottle. The cultures of each species were grown over a week until they ended exponential growth and entered stationary phase. The cell concentrations of these cultures were more than 3,000 cells mL^{-1}. Aliquots of 10 mL were taken from each bottle and then fixed with Lugol's solution in order to count their cell concentrations. Additionally, triplicate 50-mL aliquots were prepared for lipid content analysis using the SPV method.

O. marina was grown on dried yeast (*Saccharomyces cerevisiae*, Red Star; Lesaffre Yeast Corporation, St. Milwaukee, WI, USA) at 20°C. Yeast (0.1 g L^{-1}) was supplied to the predator cells every day for 5 days, but not on the last day, in order to eliminate all yeast from the *O. marina* culture medium. After yeast removal, the samples were prepared for lipid quantification in the same manner as the other species.

The mean equivalent spherical diameter (ESD) was measured by an electronic particle counter (Coulter Multisizer II; Coulter Corporation, Miami, FL, USA).

Micro-colorimetric method using the SPV reaction

The direct measurement of lipid content using the SPV reaction in this study was modified from the Inouye and Lotufo protocol (Inouye and Lotufo 2006); in this method, 0.2 mL sample, 0.1 mL sulfuric acid, and 2.4 mL vanillin reagent (1.2 mg vanillin per milliliter of 68% phosphoric acid) were used.

For analysis of the lipid content of the 11 experimental species, triplicates of 50 mL samples were harvested using centrifugation at 3,500 rpm for 10 min. Pellets were rinsed with distilled water 2-3 times, and transferred into 15-mL glass centrifuge tubes. The pellets were freeze dried, and then 2 mL of a chloroform : methanol mixture (2 : 1 [v:v]) was added into the tubes, vortexing for 1 min for extrac-

Table 1. List of the experimental organisms and their cell size (ESD, μm), trophic modes, and origins

Class	Species name	ESD (μm)	Trophic mode	Origin
Dinoflagellate	*Amphidinium carterae*	9.8	Mixotrophic	USA
	Heterocapsa triquetra	15	Mixotrophic	Masan bay, Korea
	Prorocentrum minimum	12.1	Mixotrophic	Kunsan, Korea
	Alexandrium minutum	19	Mixotrophic	CCMP 113
	Oxyrrhis marina	20	Heterotrophic	Kunsan, Korea
	Scrippsiella trochoidea	22.8	Mixotrophic	Jeju, Korea
	Ostreopsis cf. *ovata*	26	Autotrophic	Jeju, Korea
	Prorocentrum micans	26.6	Mixotrophic	Shiwha, Korea
	Lingulodinium polyedrum	38.2	Mixotrophic	USA
Chlorophyte	*Dunaliella tertiolecta*	5	Autotrophic	UTEX
Diatom	*Thalassiosira* sp.	30	Autotrophic	Kunsan, Korea

ESD, equivalent spherical diameter.

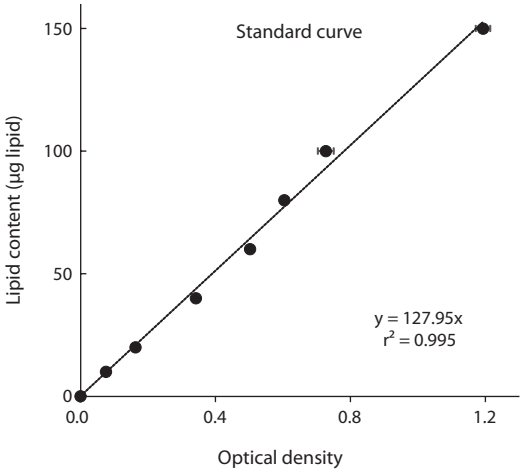

Fig. 1. The standard curve obtained by plotting lipid content (LC, µg lipid) of Canola oil as a function of optical density (OD). The optical density was measured using a spectrophotometer. Symbols represent treatment means ± standard deviation (n = 3). The equation of the curve was y (LC) = 127.95x (OD), $r^2 = 0.995$.

tion. Subsequently, 1.5 mL of a 0.9% NaCl solution was added and the samples were centrifuged at 3,500 rpm for 15 s. The supernatant (methanol + 0.9% NaCl solution) was carefully removed, and 0.2-1 mL of the remaining materials (chloroform + extract) was transferred into a 4-mL glass vial. The vial was placed on the hot plate set at 90°C to allow the solvent to evaporate. Once the solvent was removed, 0.1 mL of concentrated sulfuric acid was added to each vial, and then heated at 90°C for 10 min until the color of sample changed to light brown. The vial was then removed from the hot plate and allowed to cool on ice for 5 min. Phospho-vanillin reagent (2.4 mL) was added and allowed to develop for 10 min, until the color of samples turned to pink. The absorbance was measured at 530 nm using a spectrophotometer (Jenway-7300; Bibby Scientific).

RESULTS AND DISCUSSION

The optical density measured using a spectrophotometer (and equivalent intensity of color) was significantly positively correlated with the known lipid content of the standard oil (i.e., Canola oil) ($p < 0.01$, linear regression ANOVA) (Fig. 1). Furthermore, the r^2 value of the standard curve, which was obtained by plotting the optical density as a function of the lipid quantity, was 0.995.

When the lipid contents of the dinoflagellates *A. minutum*, *P. micans*, *P. minimum*, and *L. polyedrum* were calculated using the standard curve, the lipid amount of each species was also significantly positively correlated with optical density and equivalent intensity of color ($p < 0.01$ for each of all 4 species, linear regression ANOVA) (Fig. 2). Thus, when comparing color intensity or optical density of samples with these known color intensities or optical densities of each dinoflagellates species, the lipid content of the target species can be easily and rapidly quantified.

The lipid content per cell of *P. minimum* which was measured using 1×10^3 or 1×10^4 cells was significantly higher than that measured using the other 6 concentrations of cells (1×10^5 to 2×10^6; $p < 0.01$, one tailed t-test). However, the lipid content per cell for these 6 concentrations (1×10^5 to 2×10^6) were not significantly different from one another ($p > 0.1$, ANOVA) (Fig. 3A).

The lipid content per cell of *A. minutum* which was measured using 1×10^3, 1×10^4, or 1×10^5 cells was significantly higher than those measured using the other 5 concentrations of cells (3×10^5 to 2×10^6; $p < 0.01$, one tailed t-test), however, the lipid content per cell for these 5 concentrations of cells (3×10^5 to 2×10^6) were not significantly different from one another ($p > 0.1$, ANOVA) (Fig. 3B).

The lipid content per cell of *P. micans* which was measured using 1×10^3 cells was significantly higher than those measured using the other 7 concentrations of cells (1×10^4 to 2×10^6; $p < 0.01$, one tailed t-test), but the lipid contents per cell for these 7 concentrations of cells (1×10^4 to 2×10^6) were not significantly different from one another ($p > 0.1$, ANOVA) (Fig. 3C).

The lipid content per cell of *L. polyedrum* which was measured using 1×10^3 cells was not significantly different from those measured using the other 7 concentrations of cells (1×10^4 to 2×10^6; $p > 0.05$, one tailed t-test), and the lipid contents per cell, which were measured using the 8 different concentrations cells (1×10^5 to 2×10^6), were not significantly different from one another ($p > 0.1$, ANOVA) (Fig. 3D). Therefore, when no more than 10^4 cells of *A. minutum* and *P. minimum* which are <20 µm in ESD cell size are analyzed, the lipid content per cell can be overestimated. Similarly, when no more than 10^3 cells of *P. micans*, which is 26 µm in ESD cell size, are analyzed, the lipid content per cell can be overestimated. However, when no less than 10^3 cells of *L. polyedrum* which is 38 µm in ESD cell size are analyzed, the lipid content per cell may not be overestimated.

When the lipid content per cell of the 9 dinoflagellate species, a diatom, and a chlorophyte was analyzed using this method, the lipid concentration of *L. polyedrum* was highest (0.460 ± 0.012 ng lipid $cell^{-1}$), while that of *D. ter-*

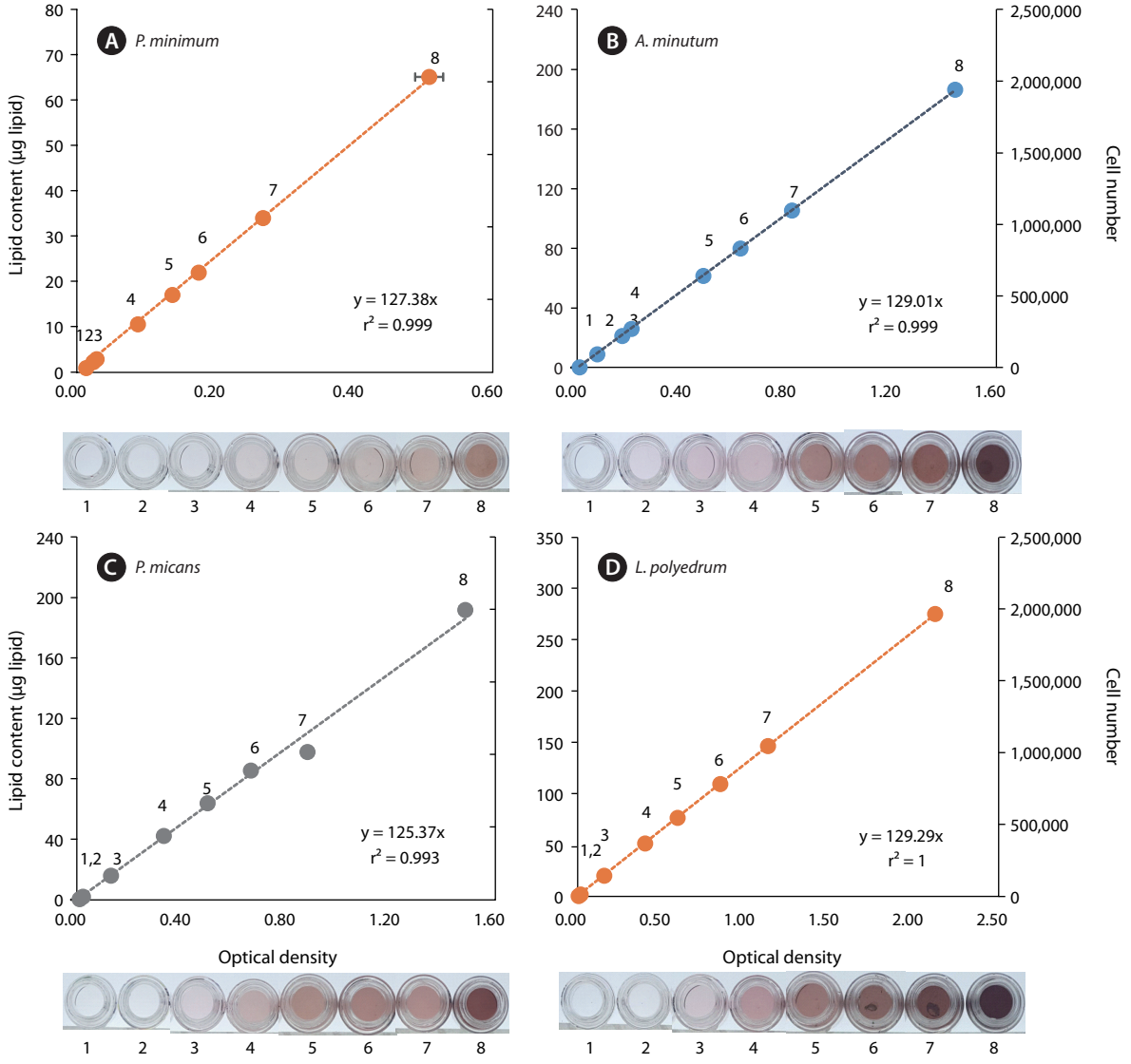

Fig. 2. Lipid content (LC, μg lipid) and cell numbers of *Prorocentrum minimum* (A), *Alexandrium minutum* (B), *P. micans* (C), and *Lingulodinium polyedrum* (D) as a function of the optical density (OD) obtained from samples when measured using the sulpho-phospho-vanillin method. Symbols represent treatment means ± standard deviation (n = 3). The equation of the curve was y (LC) = 127.38x (OD), r^2 = 0.999 for *P. minimum* (A); LC = 129.01x, r^2 = 0.999 for *A. minutum* (B); LC = 125.37x, r^2 = 0.993 for *P. micans* (C); LC = 129.29x, r^2 = 1 for *L. polyedrum* (D). Note different scales on axes.

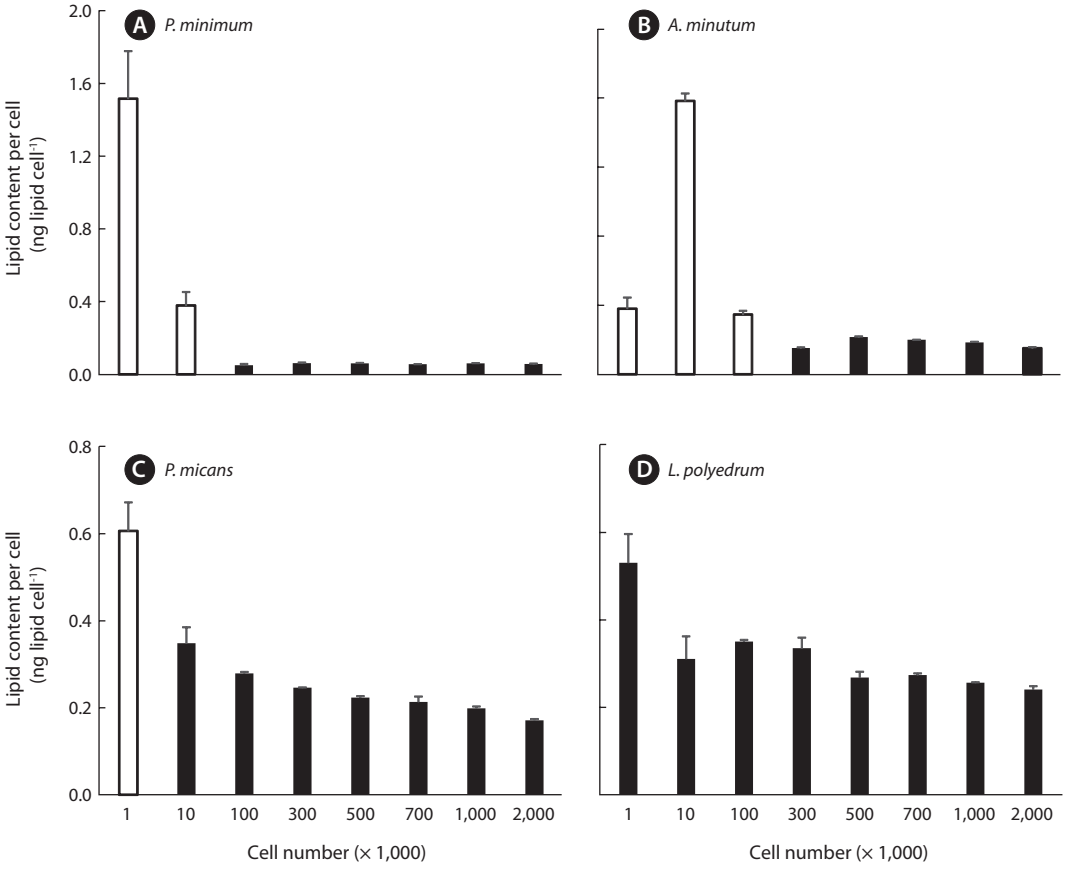

Fig. 3. Lipid content per cell (ng lipid cell^{-1}) of *Prorocentrum minimum* (A), *Alexandrium minutum* (B), *P. micans* (C), and *Lingulodinium polyedrum* (D) measured using the 8 different cell numbers ranging from 1×10^3 to 2×10^6 in the sulpho-phospho-vanillin method. Bars represent treatment means ± standard deviation (n = 3). The open bars indicate that the lipid content per cell of each species at one cell number or lesser cell numbers was significantly higher than those at the greater cell numbers ($p < 0.05$, one tailed t-test). The closed bars indicate that the lipid content per cell of each species at these cell numbers was not significantly different from one another ($p > 0.05$, ANOVA test).

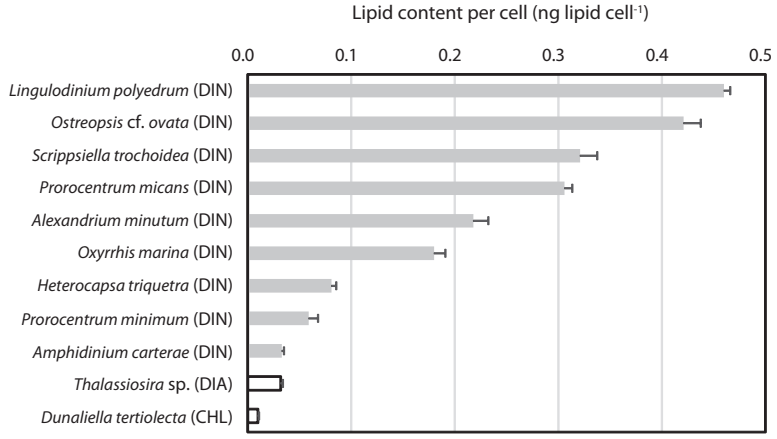

Fig. 4. Lipid content per cell (ng lipid cell^{-1}) of the 11 experimental species tested when measured using the sulpho-phospho-vanillin method. Bars represent treatment means ± standard deviation (n = 3).

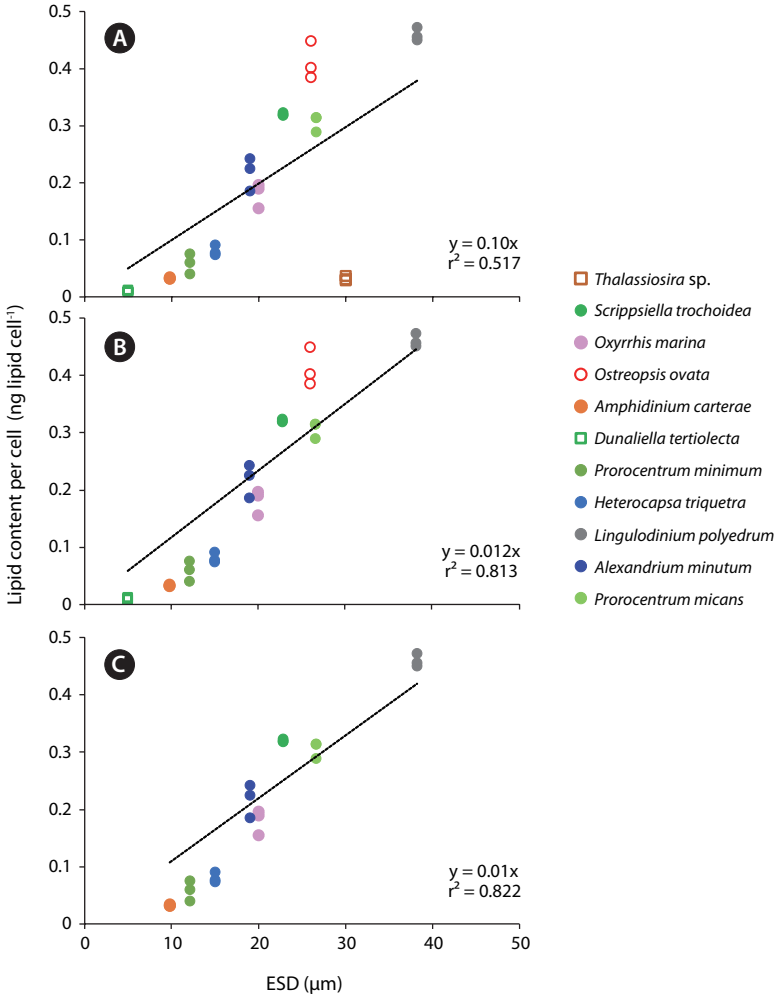

Fig. 5. Lipid content per cell (LCPC, ng lipid cell^{-1}) of all 11 species (A), the dinoflagellates only (B), and planktonic dinoflagellates only (C) as a function of cell size (equivalent spherical diameter, ESD, μm) when measured using the sulpho-phospho-vanillin method. Among the 11 species, the diatom *Thalassiosira* sp. in (B) and (C) and the benthic dinoflagellate *Ostreopsis* cf. *ovata* in (C) were deleted from (A) and (B), respectively. The equation of the curve was y (LCPC) = 0.10x (ESD), r^2 = 0.517 (A); LCPC = 0.012x, r^2 = 0.813 (B); LCPC = 0.01x, r^2 = 0.822 (C).

Table 2. Cell volume (CV), lipid content per cell (LCPC), and volume specific lipid content per cell (VLCPC) of the 11 experimental species when measured using the sulpho-phospho-vanillin method

Species name	CV (μm^3)	LCPC (ng lipid cell^{-1})	VLCPC (pg lipid cell^{-1})
Amphidinium carterae	493	0.033 ± 0.004	0.064-0.070
Heterocapsa triquetra	1,766	0.081 ± 0.009	0.042-0.052
Prorocentrum minimum	927	0.059 ± 0.018	0.043-0.081
Alexandrium minutum	3,590	0.218 ± 0.029	0.052-0.068
Oxyrrhis marina	4,187	0.180 ± 0.022	0.037-0.047
Scrippsiella trochoidea	6,203	0.321 ± 0.002	0.051-0.052
Ostreopsis cf. *ovata*	9,198	0.421 ± 0.033	0.042-0.049
Prorocentrum micans	9,850	0.306 ± 0.015	0.029-0.032
Lingulodinium polyedrum	29,172	0.460 ± 0.012	0.015-0.016
Dunaliella tertiolecta	65	0.010 ± 0.002	0.131-0.181
Thalassiosira sp.	14,130	0.032 ± 0.004	0.002-0.003

tiolecta was lowest (0.010 ± 0.002 ng lipid cell^{-1}) (Table 2, Fig. 4). The lipid content per cell of *A. minutum* measured using the SPV method in this study (0.186-0.345 ng lipid cell^{-1}) was comparable to those measured using other methods (0.288-0.400 ng lipid cell^{-1}) (Table 3). Thus, the SPV method is valid.

The lipid content per cell of the 11 microalgal species measured using SPV method increased with increasing the cell size (ESD, μm) with the exception of the diatom *Thalassiosira* sp. (Fig. 5A). This *Thalassiosira* sp. had a lipid content much lower than that of the dinoflagellates having similar sizes. The lipid content of all 11 microalgae species, 9 dinoflagellate species, or 8 planktonic species were significantly positively correlated with cell size with different values of r^2 (Fig. 5B-D). The benthic dinoflagellate *Ostreopsis* cf. *ovata* had a lipid content approximately 25% higher than that of *P. micans* having a similar size (Fig. 5C).

Among the tested species, the calculated volume of specific lipid content was highest for *D. tertiolecta* and lowest for *Thalassiosira* sp. (Fig. 6A). Among the dinoflagellate species tested, *A. carterae* had the highest volume specific lipid content, while *L. polyedrum* had the lowest. Furthermore, the volume specific lipid content of all dinoflagellates was significantly negatively correlated with cell size (Fig. 6B).

The conventional methods of quantifying the lipid contents of microalgae require large amounts of biomass and thus, usually requires a great deal of time and labor; the processing of samples for gas chromatography analysis may also take several days and be laborious work. The results of this study clearly show that both optical density and intensity of color had strong correlations with the lipid content of each of the 4 dinoflagellates tested and thus, the lipid content of a sample containing each species can be measured using spectrophotometry or even the naked eye. Thus, the SPV method applied in this study can be used as an easy and rapid method of quantifying the lipid content of microalgal species. The dry weight biomass of 10^5 cells of *A. minutum* and *P. minimum* are approximately 0.9 mg and 0.2 mg, respectively. These biomasses are much smaller than the minimum detectable biomasses

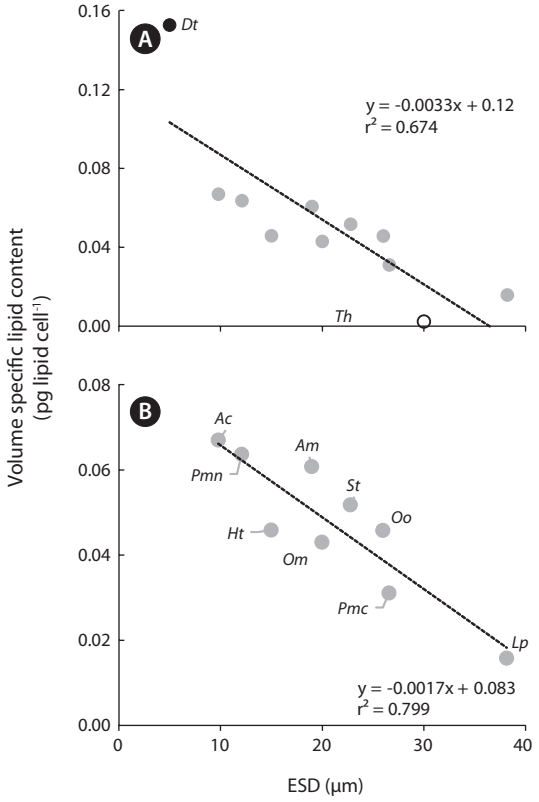

Fig. 6. Volume specific lipid content per cell (VLCPC, pg lipid cell^{-1}) of all 11 species (A) and the dinoflagellates only (B) as a function of cell size (ESD, μm) when measured using the sulpho-phospho-vanillin method. The equation of the curve was y (VLCPC) = -0.0033x (ESD) + 0.12, r^2 = 0.674 (A); VLCPC = -0.0017x + 0.083, r^2 = 0.799 (B). *Lp*, *Lingulodinium polyedrum*; *Oo*, *Ostreopsis* cf. *ovata*; *St*, *Scrippsiella trochoidea*; *Pmc*, *Prorocentrum micans*; *Am*, *Alexandrium minutum*; *Om*, *Oxyrrhis marina*; *Ht*, *Heterocapsa triquetra*; *Pmn*, *Prorocentrum minimum*; *Ac*, *Amphidinium carterae*; *Th*, *Thalassiosira* sp.; *Dt*, *Dunaliella tertiolecta*.

Table 3. Comparison of the lipid content per cell (LCPC) of the dinoflagellate *Alexandrium minutum* measured using different methods

LCPC (ng lipid cell^{-1})	Analyzing method	Reference	Culture condition
0.308	Solid phase extraction, GC	Fuentes-Grüneward et al. (2009)	L2 media, 12 : 12 LD cycle, 21°C
0.288	Microwave assisted extraction, GC	Fuentes-Grüneward et al. (2012)	L1 media, 12 : 12 LD cycle, 20°C
0.299-0.400	Gravimetric determination	Malapascua et al. (2012)	
0.186-0.243	Sulpho-phospho-vanillin method	This study (cell conc. = ca 10^5)	f/2 media, 24 h light, 20°C
0.200-0.345	Sulpho-phospho-vanillin method	This study (cell conc. 10^3-2 × 10^6)	f/2 media, 24 h light, 20°C

Gravimetric determination is the Bligh & Dyer method using chloroform-methanol-DDW extraction.
GC, gas chromatography.

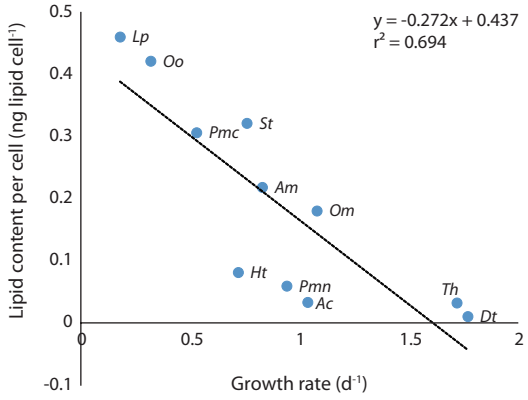

Fig. 7. Lipid content per cell (LCPC, ng lipid cell^{-1}) of all 11 species as a function of the specific growth rate (GR, d^{-1}) when measured using the sulpho-phospho-vanillin method. The equation of the curve was y (LCPC) = -0.272x (GR) + 0.437, r^2 = 0.694. *Lp*, *Lingulodinium polyedrum*; *Oo*, *Ostreopsis* cf. *ovata*; *St*, *Scrippsiella trochoidea*; *Pmc*, *Prorocentrum micans*; *Am*, *Alexandrium minutum*; *Om*, *Oxyrrhis marina*; *Ht*, *Heterocapsa triquetra*; *Pmn*, *Prorocentrum minimum*; *Ac*, *Amphidinium carterae*; *Th*, *Thalassiosira* sp.; *Dt*, *Dunaliella tertiolecta*. Specific growth rate data refer to data from Jeong et al. (2005) (*Lp*), Guerrini et al. (2010) (*Oo*), Costas (1990) (*St*), Jeong et al. (2010) (*Pmc*), Grzebyk et al. (2003) (*Am*), unpublished data (*Om*), Hansen (1992) (*Ht*), Kondo et al. (1990) (*Pmn*), Furnas (1990) (*Ac*), and Eppley and Sloan (1966) (*Th*, *Dt*).

in the Inouye and Lotufo method (2006), which were 10-50 mg. Therefore, the modified SPV method requires much less biomass than the method described by Inouye and Lotufo (2006).

The lipid content of the diatom *Thalassiosira* sp. was much lower than that of *P. micans* which has a similar size, while that of the benthic dinoflagellate *O.* cf. *ovata* was much greater. The lipid content of the microalgae tested in this study is significantly negatively correlated with their growth rates (Fig. 7). Therefore, a high growth rate is likely to lower lipid content because lipids are a reserved material (Kagami and Urabe 2001, Converti et al. 2009). In addition, reserving lipids is likely to cause a low growth rate.

There have been many studies on the utilization of *A. carterae* as a source of raw materials for biofuel (Doan et al. 2011, Fuentes-Grünewald et al. 2016). Among the dinoflagellates tested in this study, *A. carterae* had the highest volume specific lipid content even though its absolute lipid content was lower than several other dinoflagellates. Thus, for the use of raw materials as biofuel, determining volume specific lipid content of a microalgae is important.

CONCLUSION

We applied a method of quantifying lipid content using SPV. In this method, the lipid content of a sample containing each species of dinoflagellates can be measured using spectrophotometry or the naked eye. Furthermore, when this method was used, only 1-3 × 10^5 cells of *P. minimum* and *A. minutum*, 10^4 cells of *P. micans*, and 10^3 cells of *L. polyedrum* (approximately 1-5 mL of dense cultures) were needed to quantify lipid content per cell. The lipid content per cell of the 9 dinoflagellates tested was significantly positive correlated with cell size, but the volume specific lipid content per cell was negatively correlated with the cell size. Thus, this SPV method is an easy and rapid method of quantifying the lipid content of autotrophic, mixotrophic, and heterotrophic species of dinoflagellates.

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