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Full Length Research Paper

Development of an indirect method of microalgal lipid quantification using a lysochrome dye, Nile red

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Earlier studies showed that the lipophilic dye, Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one) can be used to measure the lipid content of microalgae by cellular *in vivo* fluorescence. It was observed that a higher amount of lipid present in lipid droplets of microalgal cells would result in higher degree of emitted fluorescent light. In this present study, the feasibility of using Nile red, a fluorescent dye specific for intracellular lipid droplets, as an indirect method of lipid quantifications was investigated. Following cellular staining of different microalgal species with nile red, the *in vivo* fluorescence of the whole cell was visualized by fluorescence microscopy (excitation: 450 to 590 nm and emission: 520 nm). Intensity of the relative *in vivo* fluorescence was measured using a fluorescence spectrophotometer at excitation and emission wavelengths of 485 and 590 nm, respectively. Lipid content was determined gravimetrically and the fluorescence of the extract was measured using the microemulsion method at emission and excitation wavelengths of 540 and 617 nm. The equivalent oil content of the extracted lipid was correlated to the fluorescence of pure olive oil using the microemulsion method. Cellular *in vivo* fluorescence of stained cells (ex: 485 nm and em: 590 nm), fluorescence of extracted lipid (ex: 540 nm and em: 617 nm) and gravimetrically determined lipid were linearly correlated. This suggests that Nile red can serve as a vital stain which allows a relatively rapid method of determining the lipid content of microalgal samples and is as good as the gravimetric method used for lipid determination, eliminating the requirement for the toxic solvents and time-consuming manipulations.

Key words: Nile red, microalgae, lipid, fluorescence.

INTRODUCTION

The realization that the world's supply of crude oil is not everlasting has prompted several researches on the prospects of viable lipid production by microorganisms. Of the numerous candidate microorganisms for lipid production, photoautotrophic organisms showed to have several advantages. The advantages of using microalgae for lipid production are that growth can be done photoautotrophically so that no carbon source is required for growth and any carbon dioxide released from combustion of fossil fuels can be utilized through photo-synthesis so that the energy supply will be carbon dioxide neutral (Scragg et al., 2003). Lipid production by photo-autotrophic organisms plays an important role in aquatic ecosystems. Lipid accumulation serves as storage products of high nutritional value and the production of lipids from microalgae have been regarded as a principal source of energy and a reservoir of essential poly-unsaturated fatty acids for aquaculture industry (Lee et al., 2000).

Moreover, algal lipids have been suggested as a potential diesel fuel substitute with an emphasis on the neutral lipids due to their lower degree of unsaturation and their accumulation in algal cells at the end of the

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growth stage (Casadevall et al., 1985). Algal lipids demonstrated a significant potential value of 75,000 and 60,000 kg ha^{-1} $year^{-1}$ for oil and biodiesel yield, respectively, as compared to plant lipid sources (Moazami et al., 2011). A large-scale of biodiesel production using microalgae in a three raceway ponds of 2000 L capacity each has been successfully illustrated by Moazami et al. (2012). Bulk lipid content of microalgae is usually determined by gravimetric method, that is, by solvent extraction followed by weighing. However, this method requires relatively large sample and often require inexpensive analytical equipment making it unsuitable for rapid and extensive lipid surveys. A simple and relatively sensitive method of measuring total lipids in algae is desirable because the traditional methods require considerable time- consuming manipulations and the biomass available for analysis is often too small in the course of growth, especially when working with limited volumes (Priscu et al., 1990; Lee et al., 1998). This constraint implies that a suitable method could be based on lipid staining with a vital dye that would selectively stain intracellular lipid droplets rather than a procedure dependent on lipid extraction.

Nile red is a phenoxazine lipophilic dye that is present in minute quantities in the commercial preparation of the non-fluorescent stain, Nile blue. Previously, Nile red has been used for the detection and measurement of intracellular lipid droplets by fluorescence microscopy and fluorometry (Greenspan et al., 1984, 1985; Greenspan and Fowler, 1985; Fowler and Greenspan, 1985; Cooksey et al., 1987). Nile red is an uncharged heterocyclic molecule and is quite soluble in organic solvents and lipids but relatively insoluble in water and its fluorescence vary depending on the relative hydrophobicity of the environment. By taking advantage of these properties, Nile red can serve as a vital dye for the detection and measurement of lipid content in microalgal species. In this experiment, the use of Nile red as a vital stain which allows a relatively rapid method of determining the lipid content of microalgal samples and the quantification of the oil content of the cells was investigated.

MATERIALS AND METHODS

Estimation of cell density

The cell density of the culture was estimated by determining the cell number per ml using a haemocytometer and a Sedgewick-Rafter counting cell depending on the size of the microalgal cells. Prior to counting the cells, the aliquots were vigorously vortexed to assure culture homogeneity and to minimize the aggregation of cells.

Preparation of Nile red and oil standard for fluorescence measurements

Nile red (9-diethylamino-5H-benzo[α]phenoxazine-5-one, $C_{20}H_{18}N_2O_2$) working solution was prepared by dissolving 25 mg of Nile red in 100 ml of acetone (Lee et al., 1998) and stored chilled and protected from light. The oil standard was prepared based on the methodology of Alonzo and Mayzaud (1999) with slight modification. Different concentrations of commercially available 100% olive oil was accomplished as follows: (a) primary stock solution of olive oil was prepared by solubilizing olive oil in absolute isopropanol (to a final concentration of 200 µg of oil/ml of isopropanol); (b) working solution of olive oil was prepared by diluting the primary stock in isopropanol: chloroform (19:1, v/v) to different concentrations ranging from 20 to 200 µg/ml.

Preparation of microalgal samples

Preparation of different microalgal species samples containing different cell numbers was accomplished by diluting the culture with fresh medium. The final volume of the different dilution was kept constant so that these dilutions will represent samples with different cell number, different biomass and hence, different lipid content per sample used.

Nile red staining and fluorescence measurements

Standard and extracted lipid samples were allowed to form microemulsion prior to staining. This was done by taking 50 µl of the sample which was made up to 5 ml with distilled water. It was previously shown that Nile red stain lipids more efficiently in vesicles or micelles (Greenspan and Fowler, 1985). Formation of microemulsion and micelle was achieved by vortexing the resulting solutions vigorously for approximately 1 min, after which 50 µl of Nile red was added immediately to the sample and mixed until the micelles were completely dispersed. The stained solution was measured immediately. 50 µl of isopropanol: chloroform (19:1) in a total volume of 5 ml of distilled water with 40 µl of Nile red was used as blank. The relative intensity of fluorescence was obtained by taking the difference between the fluorescence of the stained sample and the fluorescence of the solvent and Nile red.

Measurement of fluorescence intensity using the microemulsion method was done using 540 and 617 nm as excitation and emission wavelengths, respectively. For *in vivo* fluorescence measurements, 40 µl of Nile red working solution was added to 5 ml of algal suspension of predetermined cell number. The suspension was vigorously agitated on a vortex mixer before measuring the *in vivo* fluorescence. The relative intensity fluorescence intensity of Nile red was obtained after subtraction of both the autofluorescence of algal cells and the fluorescence intensity of Nile red alone in the medium (Lee et al., 1998). Measurement of *in vivo* fluorescence intensity using live microalgal cells was done using 485 nm and 590 nm as excitation and emission wavelengths, respectively. Fluorescence measurements were done with a Hitachi F-2000 Fluorescence Spectrophotometer equipped with concave grating monochromators and sensitive photomultiplier tubes. Fluorescence intensity and spectra were taken at room temperature.

Visualization of Nile red by fluorescence microscopy

Cellular fluorescence of Nile red was visualized using a Nikon Diaphot inverted microscope equipped with epi-fluorescence using an HB-10101AF super high pressure mercury lamp light source. The microscopic observation of cellular Nile red fluorescence was done with the following settings: yellow fluorescence, using 450 to 490 nm short band pass exciter filter, a 455 nm center wavelength chromatic beam splitter; and a 520 nm long band pass emission filter. Microalgal samples were observed using depression slides and photomicrographs were taken before and after addition of Nile red.

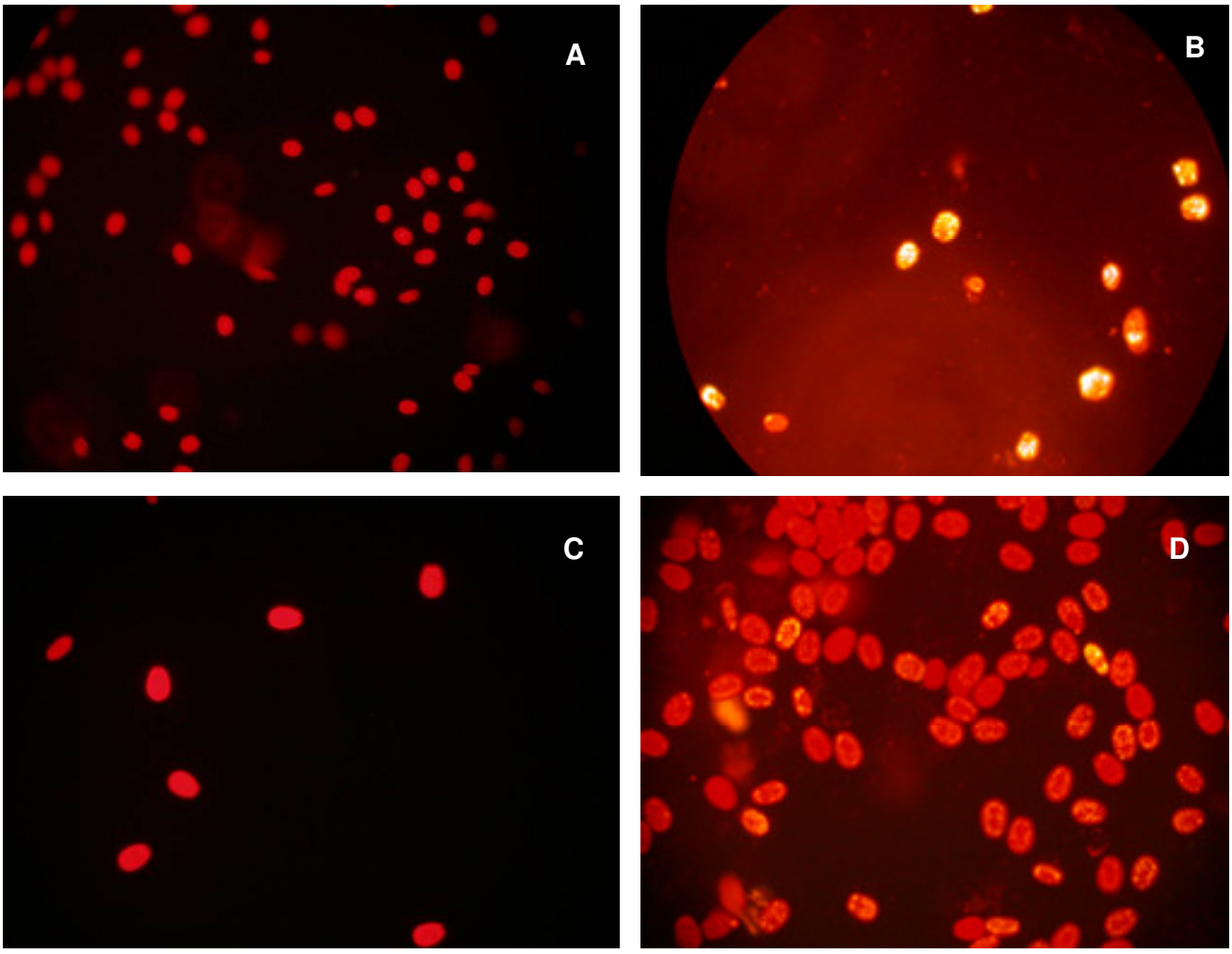

Figure 1. Nile red fluorescence of representative microalgal cells. **A and C,** Fluorescence of unstained Prorocentrum minutum and Prorocentrum lima, repectively. **B and D,** Fluorescence of *P. minutum* and *P. lima* after nile red staining. All cells were viewed for yellow-gold fluorescence with or without nile red stain using excitation band pass filter of 450 to 490 nm and emission band pass filter of 520 nm. The bright yellow to yellow-gold fluorescent are round bodies.

Lipid extraction and gravimetric determination of lipid

Lipid content of algal samples with different biomass was estimated by extracting the crude lipid using dichloromethane: methanol (1:2) solvent system. Extraction was done by adding 5 ml of the solvent to freeze-dried algal sample and extraction was facilitated by sonication for 10 min. To ensure complete extraction, the process was repeated three times. The extract was transferred and filtered (Whatman GF/B) in 50 ml clean centrifuge tubes. 5 ml of dichloromethane and 10 ml of deionized water were added to the filtered solution. The resulting mixture was vortexed vigorously and centrifuged at 3000 rpm for 5 min. After centrifugation, the lower phase (containing the extracted lipid) of the resulting biphasic mixture was gently collected and passed through an anhydrous sodium sulfate filter. The solvent was evaporated to dryness and the samples were transferred to a dessicator to dry overnight. After gravimetric determination of the total lipids, the dried extracted lipid was redissolved in 5 ml of isopropanol: chloroform (19:1) and the fluorescence signal was determined as described in the fluorescence measurement of the methodology.

RESULTS

Fluorescence microscopy of representative Nile red-stained cells

Treatment of cells microalgal cells with dilute solution of Nile red produced an intense fluorescent staining of the cytoplasmic contents. The photomicrographs presented in Figure 1, show the appearance of represented Nile red stained *Prorocentrum* cells viewed for yellow-gold fluorescence (excitation filter of 450 to 490 nm and emission filter of 520 nm). Viewed for yellow-gold fluorescence, the Nile red-stained microalgal cells showed the brightly stained discrete bodies distributed on the periphery of the cells (Figures 1b and d) presumably lipid droplets not readily seen from the same cells without Nile red. In contrast, no significant autofluorescence was

Table 1. Cell density, lipid content *in vivo* and extracted lipid fluorescence of different microalgal species from different dilutions. The volume of the final dilution of each microalgal species was kept constant.

Cell density (cells/ml)	Lipid content (ug/ml)	*in vivo* fluorescence (ave.) exλ = 485: emλ = 590	Extract fluorescence (ave.) exλ = 570: emλ = 617
***Isochrysis galbana* (Ig 01)**			
12500000	97.8	372.42	1.443
20600000	166.7	562.28	1.8467
35600000	228.9	648.75	2.0887
41100000	275.6	709.06	2.4833
46000000	342.2	752.35	2.6257
***Tetraselmis sp.* (Ts 01)**			
2040000	40	39.103	1.2583
3790000	53.3	41.381	1.2983
6060000	82.2	47.183	1.449
8150000	128.9	48.348	1.4863
11320000	148.9	58.459	1.5647
***Alexandrium minutum* (Ammk 02)**			
101390	31.1	53.236	1.955
172220	68.9	58.138	2.1013
281480	88.9	77.166	2.2743
341670	102.2	85.651	2.586
***Prorocentrum lima* (Pl 12)**			
30700	62.2	43.086	1.5997
55900	75.6	45.306	1.6653
89375	100	58.999	1.7593
139580	144.4	66.168	1.892
163670	177.8	89.383	1.9273

observed when unstained microalgal cells were viewed for yellow-gold fluorescence (Figures 1a and 1c). This staining presents the native state of the cell before and after the addition of the stain. The relative position of the lipid droplets represents their actual location, since no mechanical or chemical treatments were imposed on the cells.

Relationships of cell number, gravimetrically determined lipid content, *in vivo* fluorescence and fluorescence of extracted lipid

Table 1 presents the cell density, lipid content and the *in vivo* and extracted lipid fluorescence of different microalgal species from the different dilutions. In all of the microalgal species assayed, there was a direct correlation of all the measured parameters. As evident in Figure 2, the *in vivo* fluorescence and the lipid content of the different microalgal species correlates directly to the cell number, suggestive that the increase in the *in vivo* fluorescence showed a corresponding increase in the lipid content of increasing cell number. In addition, the *in vivo* fluorescence of the cells parallels the fluorescence intensity of the extracted lipid at varying lipid concentrations which was determined gravimetrically which shows that the relative *in vivo* fluorescence approximates the total lipid content although relative intensity of the fluorescence of the extracted lipid are much lower than the *in vivo* fluorescence (Figure 3).

In order to determine the oil equivalent of the total lipid content (determined gravimetrically) of different microalgal species, the fluorescence of the extracted lipid was extrapolated from the relative fluorescence of calibration curve of commercial oil (Figure 5) using the regression equation. The oil equivalent of the fluorescence of the extracted lipid was calculated as follows: relative fluorescence of the extracted lipid = - 1.1941 + (0.0408 × µg oil equivalent ml^{-1}); r^2 = 0.9305. As seen in Figure 4, there was a significant relationship between the oil equivalent of the different microalgal species and the lipid content of the cells determined gravimetrically. This relationship was linear for all the tested microalgal species. However, the computed equivalence of the total

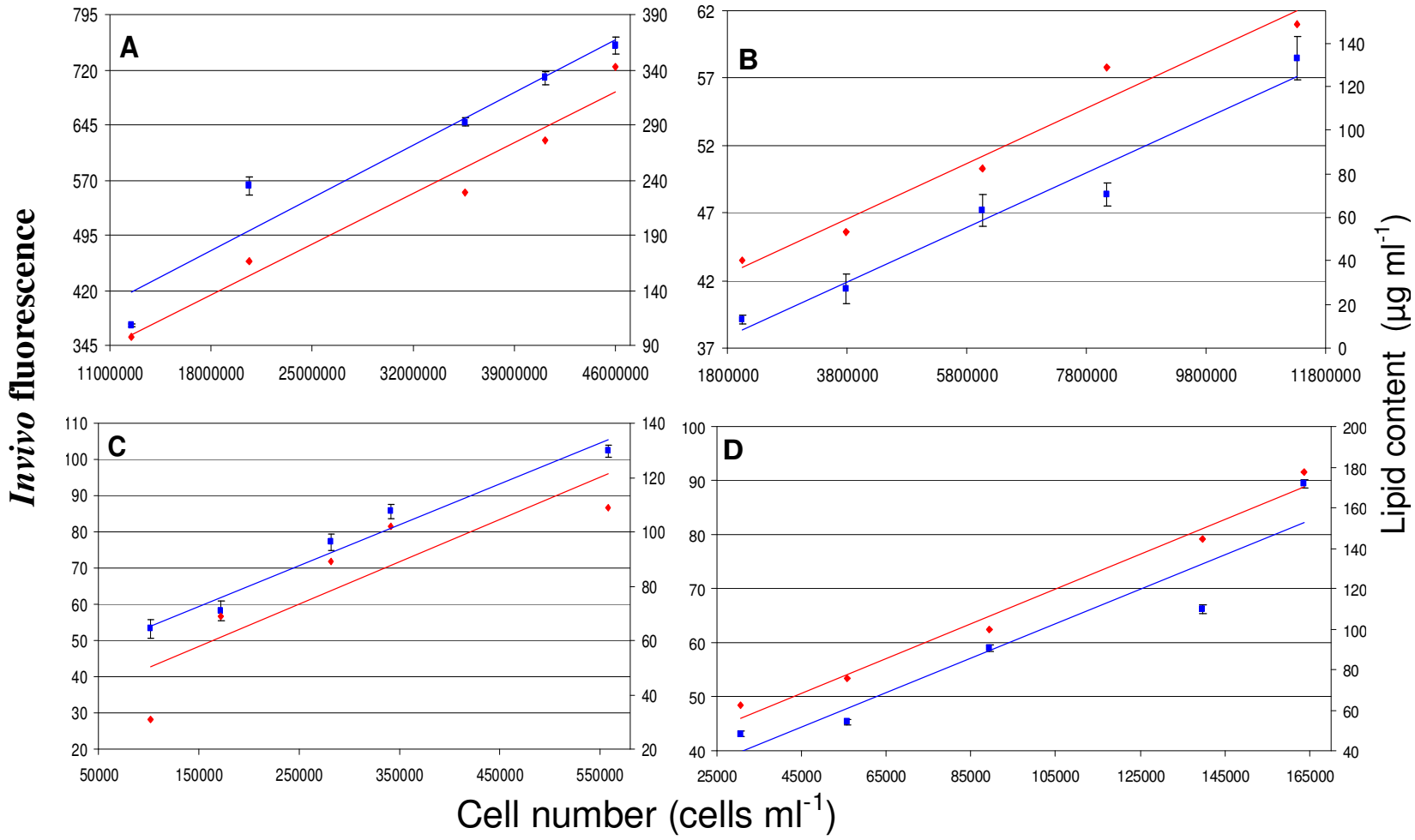

Figure 2. Relationship of *in vivo* fluorescence (♦) and lipid contents of the same cell counts by gravimetric methods (■) of (A) *Isochrysis galbana*, (B) *Tetraselmis* sp., (C) *Alexandrium minutum* and (D) *Prorocentrum lima* after Nile red staining.

lipid content for all the microalgal species tested showed an inverse relationship with the lipid content (Table 2).

DISCUSSION

Most of the common way of determining bulk lipid content from microalgae employs the use of ample samples, toxic extracting solvents, expensive equipment and time- consuming manipulations,

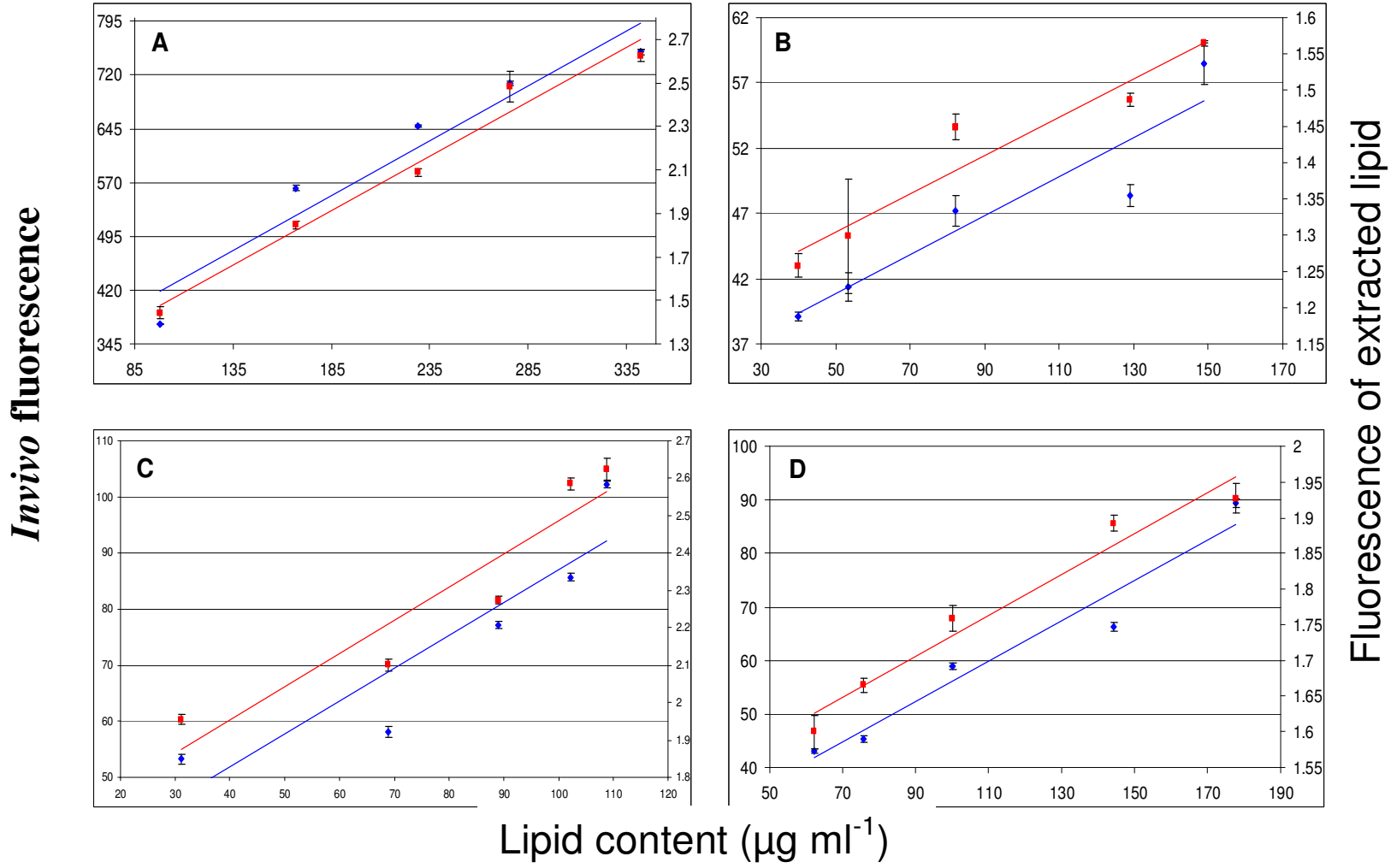

Figure 3. Relationship of *in vivo* fluorescence (♦) and fluorescence intensity of the extracted lipid (■) to the gravimetrically determined lipid content of (A) *Isochrysis galbana*, (B) *Tetraselmis* sp., (C) *Alexandrium minutum* and (D) *Prorocentrum lima* after Nile red staining.

thus, working with numerous samples become tedious and cumbersome. Previously, it was shown that a relatively rapid way of estimating the lipid content for microalgal strain selection can be accomplished using lipophilic dyes, which will preferentially stain intracellular lipid droplets.

Results of the experiment presented here describe the utilization of Nile red to demonstrate intracellular lipid droplets by fluorescence

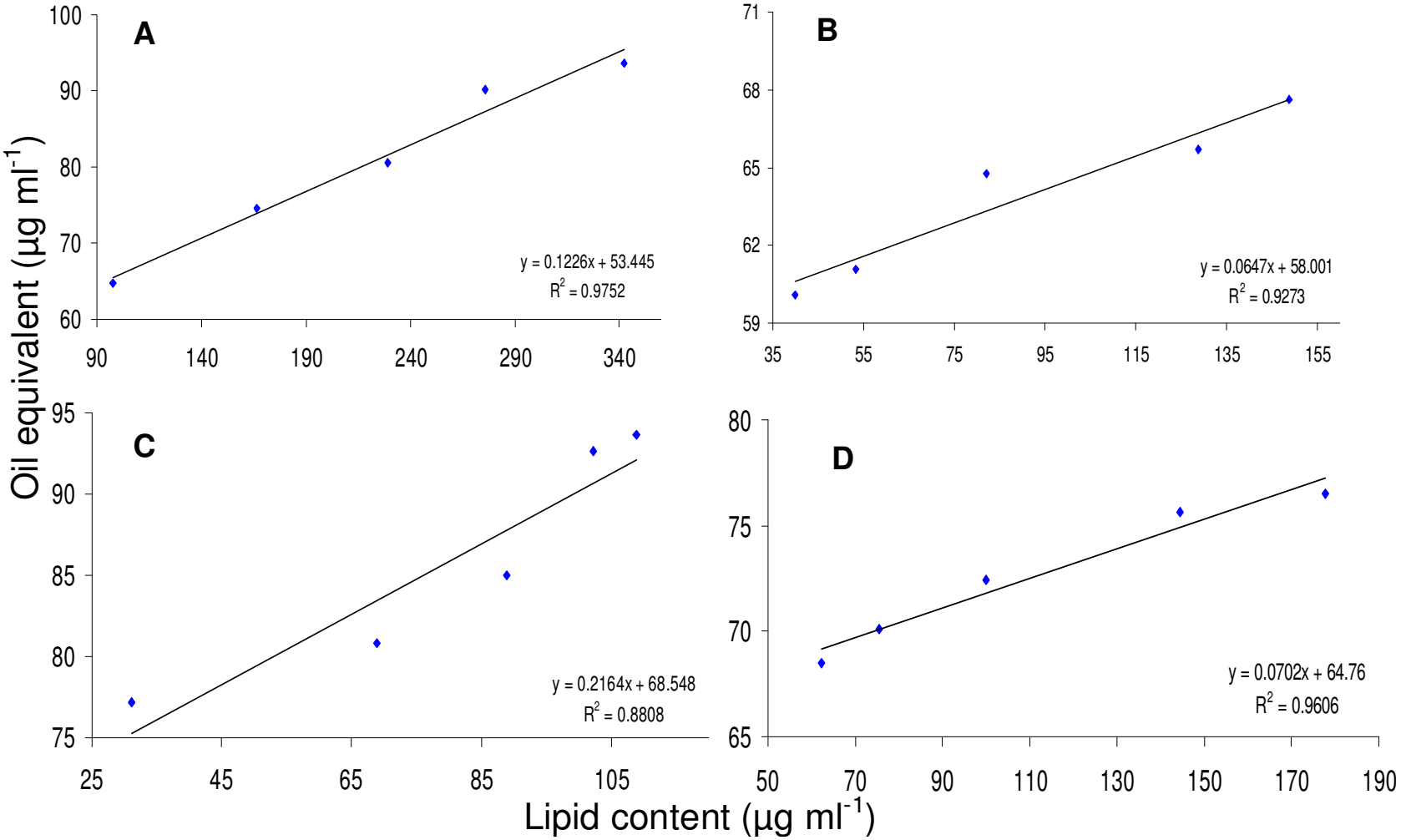

Figure 4. Oil equivalent (♦) and the gravimetrically determined lipid content of (A) *Isochrysis galbana,* (B) *Tetraselmis sp.,* (C) *Alexandrium minutum* and (D) *Prorocentrum lima*. The oil equivalent of the total lipid content was obtained from calibration curve of commercial oil after nile red staining.

microscopy and an attempt to quantify the oil content of different microalgal species using the fluorescence of the extracted lipid.

As mentioned earlier, Nile red possesses properties that can be used to selectively stain intracellular lipid droplets. Nile red is a dye that exhibits solvatochromism and the spectral posi-tion, shape and intensity of the absorption and emission band may vary depending on the nature of the solvent

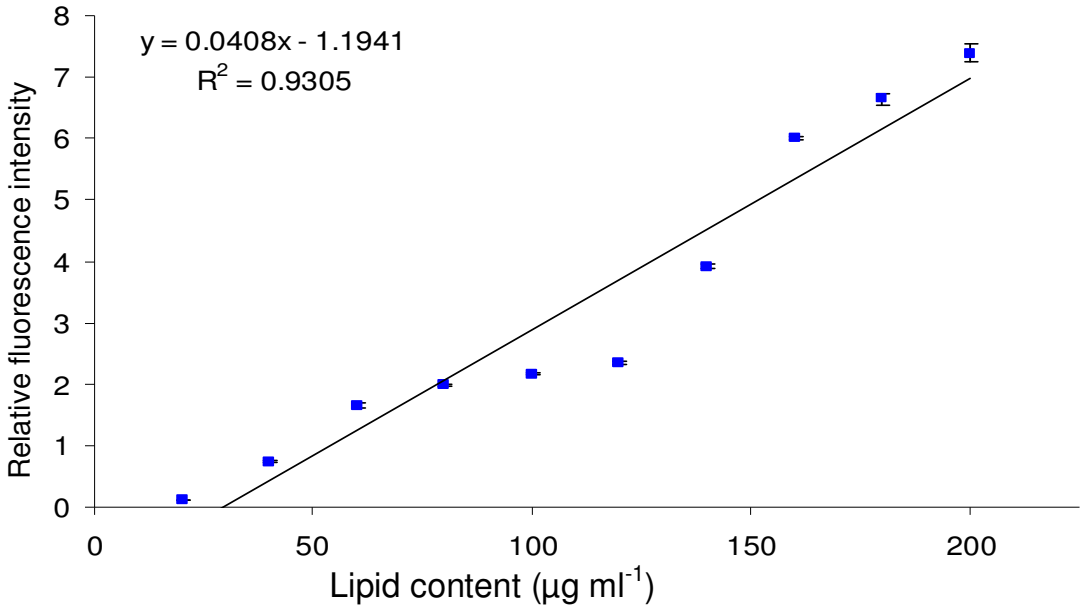

Figure 5. Relative fluorescence intensity (♦) of different concentrations of commercial oil after Nile red staining. The fluorescence intensity was obtained from the difference between the fluorescence of the sample and the fluorescence of Nile red alone.

Table 2. Oil equivalent and % equivalence of the total lipid content determined gravimetrically from different microalgal species.

Lipid content (ug/ml)	Oil equivalent (ug/ml)	% equivalence (oil equivalent/lipid content)
***Isochrysis galbana* (Ig 01)**		
97.8	64.6411	0.6610
166.7	74.5359	0.4471
228.9	80.4679	0.3515
275.6	90.142	0.3271
342.2	93.6309	0.2736
***Tetraselmis sp.* (Ts 01)**		
40	60.1146	1.5029
53.3	61.095	1.1462
82.2	64.7882	0.7882
128.9	65.7033	0.5097
148.9	67.6235	0.4542
***Alexandrium minutum* (Ammk 02)**		
31.1	77.1914	2.4820
68.9	80.7783	1.1724
88.9	85.019	0.9563
102.2	92.6586	0.9066
108.8	93.6227	0.8605
***Prorocentrum lima* (Pl 12)**		
62.2	68.4814	1.1010
75.6	70.091	0.9271
100	72.3952	0.7240
144.4	75.6471	0.5239
177.8	76.5132	0.4303

(Davis and Hetzer, 1966; Greenspan and Fowler, 1985). The emission spectra of fluorescent compounds, such as Nile red, containing polar substituents on the aromatic rings have been shown to be highly sensitive to the chemical and physical properties of the solvents. As a consequence, the spectral band may exhibit blue or red shift depending on the relative polarity and hydrophobicity of the solvent (McClure and Edelman, 1966). Such is observed in the cellular fluorescence of the different microalgal samples, the fluorescence of the extracted lipid and the oil standard. As noted in Table 1, the pair of excitation and emission wavelengths of cellular (*in vivo*) fluorescence was different from that of the fluorescence of the extracted lipid.

The excitation and emission wavelengths used for *in vivo* fluorescence represent a blue shift and the fluorescence of Nile red-stained cells falls on the yellow to yellow-gold region of the spectrum. Indeed, when the cellular fluorescence of the cells were visualized using fluorescence microscopy, the nile red-stained microalgal cells showed the brightly stained discrete bodies distributed on the periphery of the cells (Figures 1b and d) presumably lipid droplets not readily seen from the same cells without nile red (Figure 1a and c). The apparent intense yellow to yellow-gold fluorescence observed in the cells after staining the cells with Nile red could suggest the nature of the lipid droplet. Presumably, the stained lipid body contained neutral lipids such as triacylglycerols, since the observed fluorescence is seen when Nile red is solubilized in relatively hydrophobic and nonpolar solvents. On the other hand, the excitation and emission wavelengths used for measuring the fluorescence intensity of the extracted lipid and the standard commercial oil represent a red shift. The shift in the excitation and emission wavelengths is mainly due the nature of the solvent used in solubilizing the oil and the extracted lipid.

The chloroform-isopropanol solvent used in the fluorescence measurements exhibited maximum emission within 592 to 630 nm, which is in accordance with the red shift in the fluorescence intensity in relation to the polarity and hydrophobicity of the solvent (Greenspan and Fowler, 1985). It can therefore be surmised that the relative hydrophobicity and polarity of the solvent does indeed, exert a considerable effect on the maximum excitation and emission wavelengths. Based on these observations, the maximum excitation and emission wavelengths as well as the shifts in the spectral band of the fluorescence of Nile red could be an indication of the type of lipid that Nile red is staining. The preference of Nile red to stain lipid bodies is also seen in the significant correlation of all the parameters measured. Both the *in vivo* and fluorescence of the extracted lipid showed to linearly correlate well with the cell number and gravimetrically determined lipid content (Figures 2 and 3). Similar correlation was also observed in the extrapolated oil equivalent of the fluorescence of the extracted lipid. What is interesting to note is that the computed oil equivalence of the total lipid content for all microalgal species showed an inverse relationship with the lipid content (Table 2). In an examination of the fluorescence spectra of Nile red in organic solvents, Greenspan and Fowler (1985) observed that alterations appeared on the spectral curves and they hypothesized that specific solvent effect such as hydrogen bonding and some other interaction could have been exerted by the solvent. It is noteworthy that the extracted crude lipid used in this experiment is pigmented and that a combination of chloroform and isopropanol was used to re-dissolve the dried lipid for fluorescence measurement and the decreasing oil equivalence in the estimation of the extracted lipid could have arisen from the interaction of the components of the extract lipid with the solvent system used. Compared to the commercial oil used, its solubilization yields a relatively clear and colorless solution, and therefore the interference of possible sources of interfering or interacting compounds is minimized.

Conclusion

The parallelism observed between the *in vivo* fluorescence and the fluorescence intensity of the extracted lipid at varying lipid concentrations could imply that the Nile red staining is as good as the gravimetric method of determining lipid content.

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