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# A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae

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ABSTRACT

Isolation of high neutral lipid-containing microalgae is key to the commercial success of microalgae-based biofuel production. The Nile red fluorescence method has been successfully applied to the determination of lipids in certain microalgae, but has been unsuccessful in many others, particularly those with thick, rigid cell walls that prevent the penetration of the fluorescence dye. The conventional "one sample at a time" method was also time-consuming. In this study, the solvent dimethyl sulfoxide (DMSO) was introduced to microalgal samples as the stain carrier at an elevated temperature. The cellular neutral lipids were determined and quantified using a 96-well plate on a fluorescence spectrophotometer with an excitation wavelength of 530 nm and an emission wavelength of 575 nm. An optimized procedure yielded a high correlation coefficient ($R^2 = 0.998$) with the lipid standard triolein and repeated measurements of replicates. Application of the improved method to several green algal strains gave very reproducible results with relative standard errors of 8.5%, 3.9% and 8.6%, 4.5% for repeatability and reproducibility at two concentration levels (2.0 μg/mL and 20 μg/mL), respectively. Moreover, the detection and quantification limits of the improved Nile red staining method were 0.8 μg/mL and 2.0 μg/mL for the neutral lipid standard triolein, respectively. The modified method and a conventional gravimetric determination method provided similar results on replicate samples. The 96-well plate-based Nile red method can be used as a high throughput technique for rapid screening of a broader spectrum of naturally-occurring and genetically-modified algal strains and mutants for high neutral lipid/oil production.



## 1. Introduction

Due to the limited stocks of fossil fuels and the increasing emission of greenhouse gas carbon dioxide into the atmosphere from the combustion of fossil fuels, research has begun to focus on alternative biomass-derived fuels (Scurlock et al., 1993; Kosaric and Velikonja, 1995). One promising source of biomass for alternative fuel production is microalgae that have the ability to grow rapidly, and synthesize and accumulate large amounts (20–50% of dry weight) of neutral lipid (mainly in the form of triacylglycerol, TAG) stored in cytosolic lipid bodies (Day et al., 1999; Hu et al., 2008). Some species of diatoms (e.g., *Chaetoceros muelleri*) and green algae (e.g., *Chlorella vulgaris*) have been considered to be ideal sources of neutral lipids suitable for biodiesel production (McGinnis et al., 1997; Illman et al., 2000). However, there is still much to be done to move the effort from laboratory research to commercial production of biofuel feedstock. The critical starting point for this process is identification of suitable algal strains that possess high constituent amounts of total lipids in general, and neutral lipids, in particular, and are capable of rapid accumulation of large quantities of neutral lipids under various culture conditions. Identification of desirable algal species/strains will require screening of the lipid contents in both naturally-occurring and genetically-modified algae.

The conventional method used for lipid determination involves solvent extraction and gravimetric determination (Bligh and Dyer, 1959). Further quantification of neutral lipids requires the separation of the crude extractions and quantification of the lipid fractions by thin-layer chromatography (TLC), HPLC or gas chromatography (GC) (Eltgroth et al., 2005). The procedure used for lipid analysis must ensure complete extraction and at the same time avoid decomposition and/or oxidation of the lipid constituents. A major drawback of the conventional method is that it is time- and labor-intensive, making it difficult to screen large numbers of algae. As a result, increasing attention has focused on *in situ* measurements of the lipid contents (Cooksey et al., 1987; Izard and Limberger, 2003).

Nile red, a lipid-soluble fluorescent dye, has been frequently employed to evaluate the lipid content of animal cells and microorganisms, such as mammalian cells (Genicot et al., 2005), bacteria (Izard and Limberger, 2003), yeasts (Evans et al., 1985; Kimura et al., 2004), zooplankton (Kamisaka et al., 1999), and microalgae (McGinnis et al., 1997; Eltgroth et al., 2005; Elsey et al., 2007). However, most of the studies have provided only a qualitative or semi-quantitative analysis of

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the lipids. An *in situ* quantitative determination of cellular neutral lipid content using a Nile red method has yet to be established. Furthermore, the Nile red assay was usually carried out in individual cuvettes, using a UV spectrophotometer or flow cytometer one sample at a time, resulting in a time-consuming process.

Of the algal species and strains analyzed by the Nile red method, most have belonged to the Bacillariophyceae (Cooksey et al., 1987; McGinnis et al., 1997), Xanthophyceae (Eltgroth et al., 2005), Chrysophyceae (de la Jara et al., 2003) Phaeophyceae (Brzezinski et al., 1993) with only a few species from the Chlorophyceae (Lee et al., 1998; Elsey et al., 2007). On the other hand, over the past several decades, numerous algal species and strains belonging to the Chlorophyceae have been reported to contain, based on the conventional solvent extraction and gravimetric determination analysis, high levels (25–50% of dry weight) of neutral lipid, suggesting that this class of algae may represents a large pool of organisms from which desirable candidates could be obtained for lipid/oil feedstock production (Hu et al., 2006, 2008). During our preliminary screening of algae for high neutral lipid producers, we realized that the high neutral lipid content measured in a number of green algal species resulted from the solvent extraction and gravimetric method (Bligh and Dyer, 1959), but failed to be detected by the commonly used Nile red method (Cooksey et al., 1987). We speculated that the failure of the Nile red method to determine the neutral lipid content in the green algae in our studies and perhaps also in previous efforts was due to the composition and structure of the thick and rigid cell walls common in many green algae which may prevent the Nile red dye from penetrating the cell wall and cytoplasmic membrane and subsequently binding with the intracellular neutral lipid and polar lipids to give the desired fluorescence.

In this study, we re-examined the Nile red method with nine algal species of various classes. Various physical and chemical treatments were applied to the existing Nile red method to improve the effectiveness and efficiency. A modified, improved Nile red method was developed primarily using the green alga *C. vulgaris* as a model system. An optimal protocol was successfully applied to six other green algal species and strains varying in cell size and morphology. The modified, improved Nile red method can be used as a sensitive, quantitative, and high throughput method for screening of cellular neutral lipid content in green algae as well as other classes of algae.

## 2. Materials and methods

### 2.1. Organisms and culture conditions

Species of algae were obtained from the Culture Collection of Algae at the University of Texas at Austin (*Ankistrodesmus pseudobraunii* UTEX LB1380, *Nannochloris* sp. UTEX LB2291 the Provasoli-Guillard National Center for Culture of Marine Phytoplankton, U.S.A..(*Chaetoceros lauderi CCMP 193*; *Emiliania huxleyi CCMP 372*; *Crypthecodinium cohnii CCMP 316*) the CSIRO Marine Microalgae Research Center, Australia. (*Rhodomonas salina CS 24*; *Nannochloropsis* sp. CS 246; *Pavlova pinguis CS 375*), and from our laboratory isolations (*Chlorella zofingiensis. C. vulgaris LARB#2*, *Palmelococcus miniatus*, *Desmodesmus quadricauda*, *Pseudochlorococcum* sp.). *C. vulgaris*; and *C. zofingiensis,* and *Pseudochlorococcum* sp. were maintained in BG-11 growth medium, *Nannochloropsis* sp, *R. salina*, *P. pinguis*, and *E. huxleyi* were grown in f/2 growth medium, and *C. cohnii* was grown in f/2+NPM culture medium. *C. lauderi* was grown in f/2 + Si medium. All the algal strains were maintained in a glass column photobioreactor at 25 °C, and exposed to a continuous illumination at a light intensity of 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$. Culture mixing was provided by bubbling air containing 1.5% $CO_2$ (v/v).

### 2.2. Chemicals and reagents

Nile red and lipid standards were purchased from Sigma (USA). Silicon gel plates used for separation of neutral lipids was from Merck (Germany). All other chemicals and solvents of analytical grade were purchased from Sigma or other commercial suppliers.

### 2.3. Lipid analysis

#### 2.3.1. Gravimetric determination of neutral lipids

Lipids were extracted in a chloroform–methanol–water system according to Bligh and Dyer (1959). The extract was evaporated in a water bath (30 °C) using a rotary evaporator (Büchi, Switzerland) to remove solvents. Crude lipids were further separated by column chromatography using silicon gel (60–200 mesh) (Merck Corp., Germany) according to Alonzo and Mayzaud (1999) using the following solvent sequence: 6 volumes of chloroform to collect the neutral lipid class and 6 volumes of methanol to collect the polar lipids. Each lipid fraction was transferred into a pre-weighed vial, initially evaporated in a water bath (30 °C) using a rotary evaporator and then dried under high vacuum. The dried residuals were placed under nitrogen and then weighed.

#### 2.3.2. Nile red fluorescence determination of neutral lipid

After the algal cell suspensions were stained with Nile red, fluorescence was measured on a Varian 96-well plate spectrofluorometer using medium scan control and high PMT detector voltage mode. According to the pre-scan of excitation and emission characteristics of neutral lipid standards, the excitation and emission wavelengths of 530 nm and 575 nm were selected.

### 2.4. Optimization of the modified Nile red fluorescence method

#### 2.4.1. DMSO concentration

5 μL algal samples of known cell concentration were introduced into individual wells of a 96-microplate containing 3 μL of a 50 μg $Ml^{-1}$ Nile red solution. Then, 292 μL DMSO aqueous solutions with the concentrations ranging from 1% (v/v) to 40% were added. The 96-well plate was vortexed (120 rpm) and incubated at 40 °C for 10 min. After algal cells were stained, fluorescence emissions were recorded with a Varian spectrophotometer equipped with a 96-well plate reading mode. Unless stated otherwise, six replicates of each treatment were analyzed.

#### 2.4.2. Nile red dye concentration

Nile red dye, at different concentrations, ranging from 0.1 to 10 μg/mL was used following the experimental procedure detailed above to optimize the dye concentration. In this experiment, 25% DMSO (v/v) was used in the staining solutions.

#### 2.4.3. Staining time

For the optimization of staining time, algal suspensions of defined cell concentrations were stained with Nile red in 25% DMSO aqueous solutions. Staining times of 5 min, 10 min, 20 min, 30 min, 60 min and 100 min were evaluated.

#### 2.4.4. Staining temperature

Staining temperatures, ranging from 20 °C to 80 °C were investigated following the staining procedure described above.

#### 2.4.5. Algal cell concentration

To optimize cell concentrations for determination of cellular neutral lipid, several cell concentrations, ranging from 10 to $8 \times 10^5$ cells $mL^{-1}$ were evaluated using the above procedures for cell staining.

### 2.5. Comparison of lipid content by Nile red fluorescence method and conventional gravimetric method

To verify that the modified Nile red fluorescence method was effective in determining neutral lipid content, the modified method and conventional gravimetric method were compared for the green alga *C. vulgaris*. The cells used were from a 12-day liquid culture. For

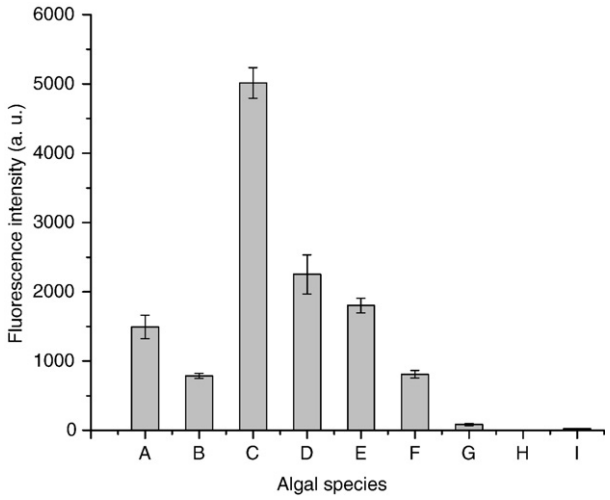


**Fig. 1.** Fluorescence emission of Nile red-stained microalgae A) *Chaetoceros lauderi*; B) *Emiliania huxleyi*; C) *Rhodomonas salina*; D) *Nannochloropsis* sp.; E) *Pavlova pinguis*; F) *Crypthecodinium cohnii*; G) *C. vulgaris*; H) *C. zofingiensis*; I) *Pseudochlorococcum* sp. The excitation and emission wavelengths for fluorescence measurement were at 480 nm and 585 nm, respectively. The optical density of the cell suspensions used for analysis was 0.3 ($OD_{750\ nm}$). Nile red staining followed the procedures described by Cooksey et al. (1987), Lee et al. (1998) and Elsey et al. (2007). Data were the mean values and standard deviations of four replicates.

fluorescence determination, six replicates were used and for gravimetric determination, 3 replicates were used.

### 2.6. Growth and neutral lipid accumulation in the green alga, C. vulgaris, over a 12-day cultivation period

The initial cell concentration of *C. vulgaris* was 0.5 ($OD_{750}$). The growth and neutral lipid accumulation in *C. vulgaris* was determined on days 0, 3, 5, 8, 10 and 12.

To measure growth, 10 mL of the algal culture was collected and filtered using a pre-weighed GF-C filter paper (Whatman, U.S.A.) and dried in a 105 °C oven overnight. Algal growth was expressed as the increase in dry algal biomass as a function of time (days) on a volumetric basis. For the determination of neutral lipid content, 1 mL of the algal samples were collected and diluted. The final cell concentration for staining was 0.06 ($OD_{750}$). For both growth and neutral lipid accumulation measurements, eight replicates were used.

### 2.7. Statistical analysis

Comparisons of means were conducted by one-way analysis of variance (ANOVA), followed by Bonferroni tests to identify the sources of detected significance. In all cases, comparisons that showed a $p$ value $<0.05$ were considered significant.

## 3. Results

### 3.1. Limitations of the existing Nile red fluorescence method on neutral lipid analysis in green algae

A Nile red fluorescence method which has been previously successfully applied to a number of microalgae (Cooksey et al., 1987; Lee et al., 1998 and Elsey et al., 2007) was evaluated for nine species of microalgae representing various classes, including *C. lauderi*, *E. huxleyi*, *R. salina*, *Nannochloropsis* sp., *P. pinguis*, *C. cohnii*, *C. vulgaris*, *C. zofingiensis,* and *Pseudochlorococcum* sp. After staining with Nile red, strong fluorescence signals were detected in all strains except for the three green algae strains (Fig. 1). In particular, *R. salina* and *Nannochloropsis* sp. yielded very strong fluorescence emissions, with the highest intensity 5013 a. u. In contrast, *C. vulgaris* and *Pseudochlorococcum* sp. samples gave very week fluorescence (i.e., 37–80 a. u.) and no fluorescence emissions were detected in the third green algal species. High fluorescence intensities measured in the other four species were indicative of the high neutral lipid content in these organisms, confirming the effectiveness of this method for algae belonging to certain taxonomic classes.

### 3.2. Modification of the existing Nile red method

In order to determine if these green algae contained neutral lipids, the conventional gravimetric method was applied. It revealed that the neutral lipid contents of *C. vulgaris*, *C. zofingiensis* and *Pseudochlorococcum* sp. were 42.9%, 30.9% and 51.5% of dry biomass, respectively. This verified that the existing Nile red method, while working well on many other types of microalgae, is inefficient for some green algae species. Based on the gravimetric analysis, we hypothesized that the failure of the existing Nile red method to effectively detect cytosolic neutral lipids in the green algae cells was due to the inability of the Nile red dye solution to enter the cell. If this was true, then any treatment that would affect the integrity of the cell wall and/or cytoplasmic membrane could facilitate the penetration of the Nile red dye into the cell and to bind to neutral lipid, and therefore provide maximum fluorescence emission.

In order to test our hypothesis, several physical and chemical approaches were tested, using the green alga, *C. vulgaris*, as a model system. Prior to addition of Nile red, algal samples were pretreated, e.g., algal cells were 1) ground in liquid nitrogen; 2) treated with 20% methanol (v/v) for 10 min at 25 °C; 3) treated with 20% DMSO (v/v) for 10 min at 25 °C; and 4) treated with 20% ethanol (v/v) for 10 min at 25 °C. Fig. 2 shows fluorescence emissions of *C. vulgaris* cells treated with the different methods. Clearly, either grinding or the solvent treatments improved the penetrative characteristics of the cell wall and cytoplasmic membranes and thus resulted in enhanced Nile red derived neutral lipid-specific fluorescence. Because the algal sample treated with 20% DMSO (v/v) resulted in the highest fluorescence

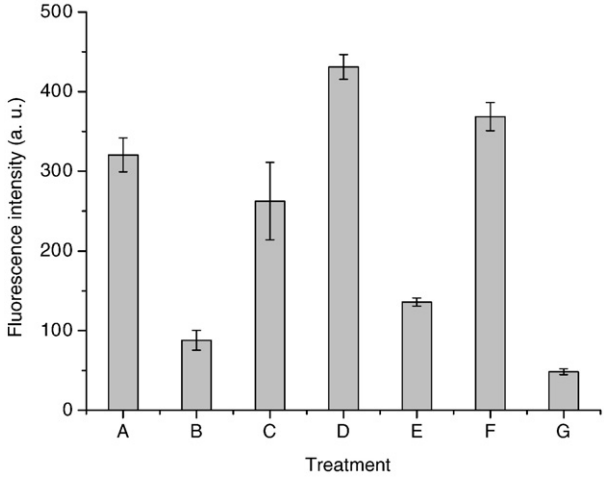


**Fig. 2.** Fluorescence emission of the green alga *C. vulgaris* cells subjected to different treatments prior to Nile red staining: A) cells were ground in liquid nitrogen; B) cell suspensions were heated to 80 °C for 10 min; C) cell suspensions were treated with 20% methanol (v/v) for 10 min at 25 C; D) cell suspensions were treated with 20% DMSO (v/v) for 10 min at 25 °C; E) cell suspensions were treated with 20% acetone (v/v) for 10 min at 25 °C; F) cell suspensions were treated with 20% ethanol (v/v) for 10 min at 25 °C; G) cell suspensions were treated with 20% isopropanol (v/v) for 10 min at 25 °C. Fluorescence measurements were carried out at peak excitation and emission wavelengths of 530 nm and 575 nm, respectively. Cell concentration for analysis was 0.06 ($OD_{750\ nm}$). Data were the mean values and standard deviations of six replicates.

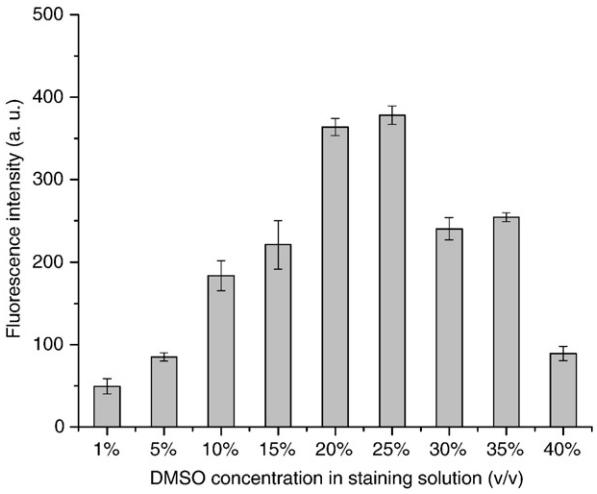


**Fig. 3.** The effect of DMSO concentrations in Nile red staining solution on fluorescence intensity of the green alga *C. vulgaris*. The excitation and emission wavelengths for fluorescence determination were 530 nm and 575 nm, respectively. Optical density of cell suspension used for analysis was 0.06 ($OD_{750\ nm}$). Treatments and analyses were performed at 40 °C. Data were expressed as the means with standard deviations of six replicates.

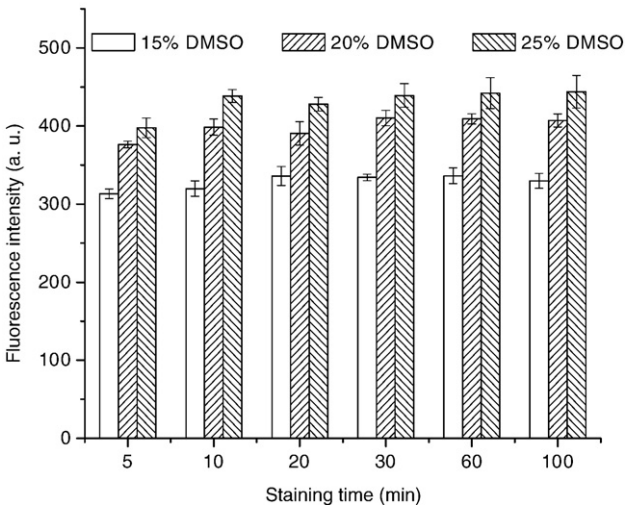


**Fig. 5.** The effect of Nile red staining time on fluorescence intensity of the green alga *C. vulgaris*. The excitation and emission wavelengths used for the fluorescence determination were 530 nm and 575 nm, respectively. Optical density of cell suspensions used for analyses was 0.06 ($OD_{750\ nm}$). Treatments and analyses were performed at 40 °C. Data were the means with standard deviations of six replicates.

intensity, the DMSO-enhanced Nile red method was selected for further investigation.

### 3.3. Optimization of DMSO-assisted/enhanced Nile red fluorescence method

The DMSO-enhanced Nile red straining method was further optimized with regard to DMSO concentration, Nile red dye concentration, staining temperature and time, and algal cell concentration, using *C. vulgaris* as the model system.

#### 3.3.1. Effect of DMSO concentrations

In Fig. 3, the Nile red staining efficiency of *C. vulgaris* increased with increasing DMSO concentrations in the staining solution and reached maximum efficiency (with the highest fluorescence intensity of 380 a. u.) at a DMSO concentration of 25% (v/v) in the staining solution. Thereafter, the fluorescence intensity decreased with the further increase of DMSO concentration.

#### 3.3.2. Effect of Nile red dye concentrations

Fig. 4 shows that Nile red concentrations ranging from 0.25 to 2 μg $mL^{-1}$ resulted in similar fluorescence intensities for *C. vulgaris* cell suspensions (cell concentration ca. $4\times10^5$ cells $mL^{-1}$). A significant decrease ($p<0.05$) in fluorescence intensities was observed when Nile red concentration was below 0.1 μg $mL^{-1}$ or higher than 5 μg $mL^{-1}$. The optimal concentration of Nile red dye was 0.5 μg $mL^{-1}$ under our experimental condition. This concentration was similar to that used for analysis of *Navicula* sp. and *Tropidoneis* sp. (Cooksey et al., 1987), but considerably lower than that used for lipid analysis in ciliates (Cole, 1990).

#### 3.3.3. Effect of staining time

The changes in staining efficiency of *C. vulgaris* as a function of staining time are shown in Fig. 5. Two other DMSO concentrations (15% and 20%, v/v) in addition to the optimum DMSO concentration (25%, v/v) were also evaluated with the increase in staining time. Results indicated that the staining efficiency did not increase after the

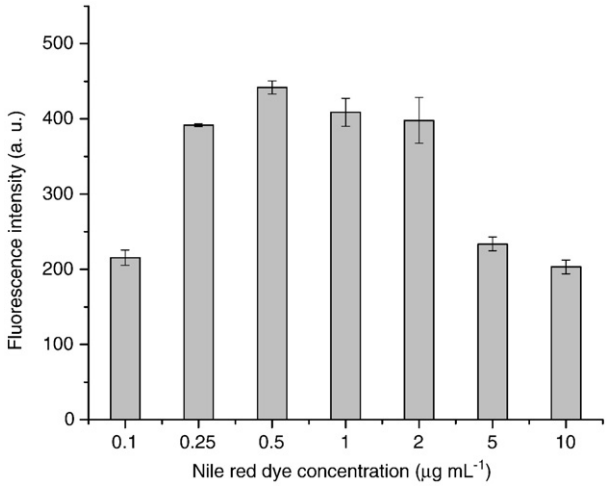


**Fig. 4.** The affect of Nile red dye concentrations on fluorescence intensity of the stained green alga *C. vulgaris*. The excitation and emission wavelengths used were 530 nm and 575 nm, respectively. Optical density of cell suspensions was 0.06 ($OD_{750\ nm}$). Treatments and analyses were performed at 40 °C. Data were expressed as the means and standard deviations of six replicates.

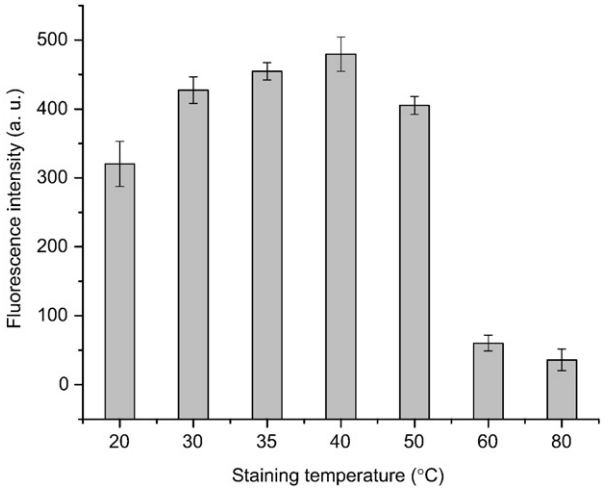


**Fig. 6.** The effect of staining temperature on the fluorescence intensity of the green alga *C. vulgaris* stained with Nile red dye. The excitation and emission wavelengths used for fluorescence determinations were 530 nm and 575 nm, respectively. Optical density of cell suspensions used for analysis was 0.06 ($OD_{750\ nm}$). Data were expressed as the means and standard deviations of six replicates.

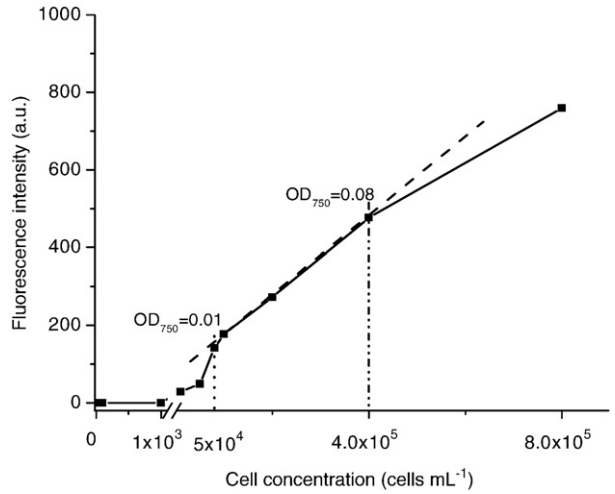


**Fig. 7.** The effect of cell concentration on the fluorescence intensity of the green alga *C. vulgaris* stained with Nile red dye. The excitation and emission wavelengths used for fluorescence determination were 530 nm and 575 nm, respectively. Data were expressed as the mean value of triplicate determinations.

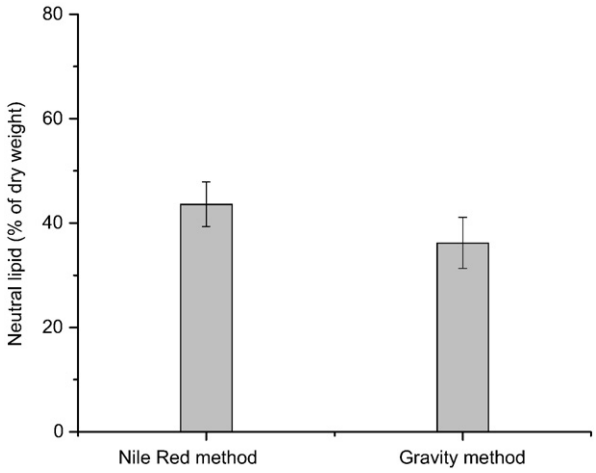


**Fig. 8.** Comparison of lipid content in *C. vulgaris* determined by the Nile red fluorescence method and the conventional gravimetric method. The excitation and emission wavelengths used for fluorescence determination were 530 nm and 575 nm, respectively. The gravimetric method used was according to Bligh and Dyer (1959). Data were expressed as the means and standard deviations obtained from triplicate determinations.

cell suspensions were stained for longer than 10 min (Fig. 5), and thus 10 min was selected as the optimum staining time.

*3.3.4. Effect of temperature*

As indicated in Fig. 6, the fluorescence intensities of the green algae *C. vulgaris* stained with Nile red dye were affected by the temperature used during the staining process. The optimum temperature for staining was 35–40 °C; however, when the temperature exceeded 60 °C, a significant decrease in fluorescence intensity ($p<0.05$ vs. 40 °C) was observed.

*3.3.5. Effect of algal cell concentration*

Effect of cell concentration on the fluorescence of neutral lipids in *C. vulgaris* is shown in Fig. 7. A range of cell concentrations that resulted in a linear relationship between the cell concentration and fluorescence intensity was between $5\times10^4$ cells $mL^{-1}$ (equivalent to ~2.0 μg $mL^{-1}$ triolein) and $4\times10^5$ cells $mL^{-1}$ (equivalent to ~20 μg $mL^{-1}$ triolein) for *C. vulgaris*. Thus, in the present study, the concentration of $3\times10^5$ cells $mL^{-1}$ (equal to $OD_{750\ nm}=0.06$) was used for optimization of the staining method.

*3.4. Correlation coefficient, repeatability, level of detection (LOD) and level of quantification (LOQ) of the modified Nile red fluorescence method*

A correlation coefficient of $R^2=0.998$ (Table 1) between the Nile red derived fluorescence intensity and the neutral lipid content was obtained using the improved Nile red method. Based on the standard curve generated, the linearity ranged from 2.0 μg $mL^{-1}$ to 20.0 μg $mL^{-1}$ for triolein. Repeated measurements for different green algal species or the same algal species at different stages of the life cycle gave very reproducible results, with relative standard errors of 8.5, 3.9 and 8.6, 4.5% for repeatability and reproducibility at two concentration levels (2.0 μg/mL and 20 μg/mL), respectively. Moreover, LOD and LOQ of the improved Nile red fluorescence method were 0.8 μg $mL^{-1}$ and 2.0 μg $mL^{-1}$ for the neutral lipid standard, triolein, respectively.

**Table 1**
Correlation coefficient, repeatability, detection (LOD) and quantification limit (LOQ) of the improved Nile red staining method

| Correlation coefficient ($R^2$) | Repeatability (RSD %) | | Reproducibility (RSD %) | | LOD (s/n = 3) | LOQ (s/n = 10) |
|---|---|---|---|---|---|---|
| | 2 μg/mL | 20 μg/mL | 2 μg/mL | 20 μg/mL | | |
| 0.998 | 8.5 | 3.9 | 8.6 | 4.5 | 0.8 μg/mL | 2.0 μg/mL |

*3.5. Comparison of the modified Nile red fluorescence method with the conventional gravimetric method*

To verify that the improved Nile red fluorescence method for neutral lipid determination provides similar results to the conventional gravimetric method, culture samples of *C. vulgaris* were collected and measured using both methods. The average neutral lipid contents of 43% and 36% were obtained by the modified Nile red fluorescence method and the gravimetric method, respectively (Fig. 8). No significant difference was detected between the results from the two methods ($p>0.05$).

*3.6. Growth and neutral lipid accumulation in the green alga C. vulgaris over a 12-day cultivation period*

During a 12-day cultivation of *C. vulgaris*, growth and cellular lipid accumulation were determined and the data are shown in Fig. 9.

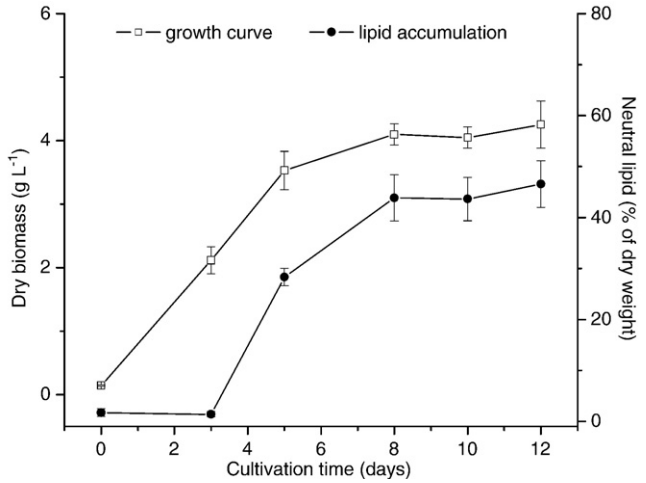


**Fig. 9.** Growth and cellular accumulation of neutral lipid in *C. vulgaris* over a 12-day cultivation. The accumulation of neutral lipids was measured using the improved Nile red fluorescence method. Data were expressed as the means and standard deviations of eight replicates.

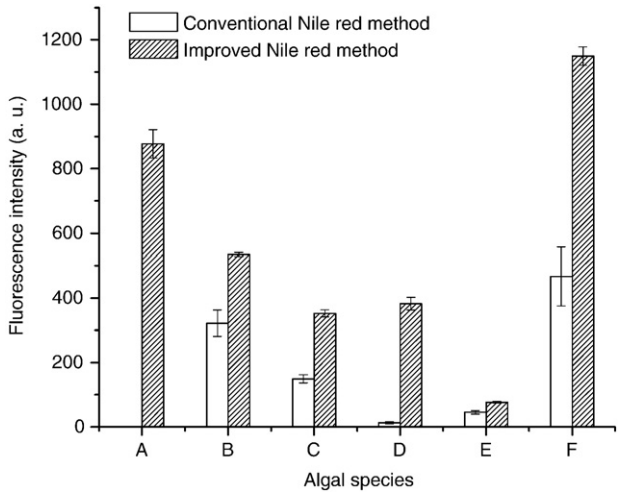


**Fig. 10.** Fluorescence emission of Nile red-stained microalgae using the conventional Nile red method and the improved Nile red method as follows: A) *C. vulgaris*; B) *Ankistrodemus pseudobraunii*; C) *Nannochloris* sp.; D) *C. zofingiensis*; E) *Desmodesmus quadricauda*; F) *Palmellococcus miniatus*. The excitation and emission wavelengths used for fluorescence measurements were 530 nm and 575 nm, respectively. The optical density of cell suspensions used for analysis was 0.30 ($OD_{750\ nm}$). The conventional Nile red staining procedure was described by Cooksey et al. (1987), Lee et al. (1998) and Elsey et al. (2007). Data were expressed as the means and standard deviations of four replicates.

During a 12-day cultivation of *C. vulgaris*, growth and cellular lipid accumulation were determined and the data are shown in Fig. 9. The biomass increased from the initial value of 0.146 g $L^{-1}$ at day 1 to 4.3 g $L^{-1}$ at day 12. The neutral lipid content was below 2% of dry weight for the first three days of cultivation, then started to accumulate at day 5 and reached the highest neutral lipid content of 56% at day 12.

### *3.7. Applications of the modified Nile red fluorescence method to other green algal species*

To verify that the improved Nile red method can be applicable to other green algae, six green algal species i.e., *C. vulgaris*, *A. pseudobraunii*, *Nannochloris* sp., *C. zofingiensis*, *D. quadricauda* and *P. miniatus*, were compared using the conventional Nile red staining procedure and the modified Nile red fluorescence method. As shown in Fig. 10, the fluorescence intensity dramatically increased in the samples treated by the modified Nile red method compared to the conventional Nile red method, indicating the greater effectiveness of the modified, Nile red method for selected green algae.

## 4. Discussion

In previous studies, Nile red has been successfully used as a fluorescence probe for the detection of both neutral and polar lipids in algae from several classes (Cooksey et al., 1987; Lee et al., 1998; Elsey et al., 2007). However, this method failed to adequately detect neutral lipid content in several green algae (Fig. 1). This might explain why over the past two decades numerous high lipid containing green algae have been identified through the use of the conventional solvent extraction and gravimetric methods, but few by the Nile red fluorescence method. The improvement in staining effectiveness and efficiency with the aid of physical grinding or a hydrophobic solvent as a stain carrier confirmed our hypothesis that the failure of the original Nile red fluorescence method in determining the neutral lipid content in green algal taxa is due to the presence of thick, rigid cell walls associated with these algae that prevent Nile red dye from effectively entering the cell and thus binding lipids to give rise to distinctive fluorescence. Previously, Castell and Mann (1994) suggested that the addition of solvents such as acetone or ethylene glycol might improve the partition of the dye into lipids. However, DMSO has not been previously used as a stain carrier for Nile red staining of algal samples.

In this study, the excitation and emission wavelengths for neutral lipid determination were 530 nm and 575 nm, respectively, using Triolein or extracted neutral lipids from *C. vulgaris* as the standard. These wavelengths were different from the wavelengths 480 nm/580 nm used by several investigators (Genicot et al., 2005; Elsey et al., 2007), but similar to that reported by Alonzo and Mayzaud (1999), in which 530/580 nm was selected for the neutral lipid determination in zooplankton. The excitation and emission wavelengths for total lipid measurement ranged from 470 to 549 nm and from 540 to 628 nm, respectively, depending on the individual organisms tested, and the composition and content of intracellular lipids. The excitation wavelengths selected for neutral lipid analysis were also different, ranging from 470 nm to 530 nm. Therefore, prior to fluorescence measurement, optimal excitation and emission wavelengths for maximum fluorescence yield must be identified. Given that many classes of algae, particularly the green algae taxa (Chlorophyceae) contain large amounts of chlorophyll (1–4% of dry weight) which can considerably increase the fluorescence background, making it difficult, if not impossible, to use the Nile red method to quantify cellular polar lipids using the wavelengths of 560/620 nm, which were previously used to determine neutral and polar lipids in non- or low chlorophyll-containing cells, tissues, and organisms (e.g., mammalian cells, zooplankton, yeast, diatoms and chrysophycean species) (Cole, 1990; Alonzo and Mayzaud, 1999; de la Jara et al., 2003).

The concentration of Nile red used for cell staining varied considerably (e.g., 0.01–100 μg $mL^{-1}$) when the fluorescence was detected by flow cytometry or fluorescence microscopy (de la Jara et al., 2003). However, for quantitative analysis of neutral lipid using a spectrofluorometer, the background fluorescence associated with the Nile red concentration should be taken into consideration. In some cases, it was necessary to wash stained cells by centrifugation or filtration to remove residual Nile red and thus lower the background fluorescence level (Cooksey et al., 1987). In this study, 0.5 μg $mL^{-1}$ of Nile red was found to be the optimal concentration at cell densities ranging from $5\times10^4$ to $4\times10^5$ cells $mL^{-1}$.

Nile red fluorescence was also strongly influenced by temperature and duration of staining with high temperatures or lengthy staining periods leading to the quenching of the Nile red fluorescence (Deye et al., 1990). Under our experimental conditions, staining algal cells with Nile red at 40 °C for 10 min was found to yield optimal neutral lipid-derived fluorescence.

Little information is available regarding determination of the absolute content of neutral lipids in algae using the conventional gravimetric method calibrated with a lipid standard (Alonzo and Mayzaud, 1999). Our results indicated no significant difference between the two methods based on the mean values, whereas, an absolute error (7%) and a relative standard error (15%) between the two methods in neutral lipid content from the same algal biomass was observed. One plausible reason for the noticeable errors was that the conventional gravimetric method involves several complicated steps, such as lipid extraction, separation, and concentration, which might result in loss of some neutral lipid, as observed by other investigators (Lee et al., 1998; Elsey et al., 2007). The Nile red fluorescence method, on the other hand, may, or may not, either slightly overestimate or underestimate the neutral lipid content, depending on characteristics of individual algal strains, particularly the cell wall composition, as well as concentrations of chlorophyll, polar membrane lipids, and various other lipophilic compounds in the cell that may affect the amount of fluorescence background. Therefore, further investigations and refinements on the modified Nile red method using additional green and other types of algae may be useful.

In summary, the typical analysis of neutral lipids requires the use of a two-phase solvent separation that utilizes considerable amounts of biological material and organic solvent and a series of manipulations that precede the quantification. This makes it difficult for wide-scale screening of naturally-occurring and genetically-modified algal strains and mutants for high neutral lipid/oil producers, which is a prerequisite for the success of algae-based biofuel production technology and commercialization. The modified Nile red fluorescence method described in this study provides a rapid, easily manipulated and reliable method for *in vivo* quantification of neutral lipids in various algal taxa, particularly those belonging to the Chlorophyceae, that were previously found difficult to stain with the original Nile red method. The introduction of 96-well plate technology to the Nile red method can further increase its high throughput capability and robustness.

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