RM 528 / DIATOMS

Journal of Microbiological Methods 6 (1987) 333–345
Elsevier

JMM 00216

Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red

Keith E. Cooksey,[a] James B. Guckert,[a] Scott A. Williams[b] and Patrik R. Callis[b]

Departments of [a]Microbiology and [b]Chemistry, Montana State University, Bozeman, MT 59717 (U.S.A.)

(Received 24 November 1986) (Revised version received 6 May 1987) (Accepted 11 May 1987)

Summary

The fluorophore Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) has been used to determine neutral lipid in microalgal cells. Cellular fluorescence of stained cells and gravimetrically or chromatographically determined lipid were linearly correlated when Nile Red was excited at 488–525 nm and the fluorescent emission measured at 570–600 nm. Nile Red is a vital stain which allowed flow cytometric sorting of live microalgal populations based on their lipid content.

Key words: Nile Red; Microalgae; Neutral lipid; Flow cytometry

Introduction

Research concerning the possibility of commercial production of lipid by microorganisms has been stimulated in the last decade by the realization that the world's supply of crude oil is not everlasting. Several types of organisms have been proposed for the purpose of producing lipid, and each has its advantages. The particular advantages in the use of phototrophic microorganisms are that the source of energy and reducing power is the sun, and the carbon requirement of the cell can be met by carbon dioxide. Whatever the kind of microbial cell chosen for the lipid production process, it is probable that no wild-type organism will produce lipid in high enough yield for the process to be cost-effective. It will be necessary therefore to select, or produce by genetic manipulation, high-yielding strains. To do this successfully it is necessary to rapidly screen cellular lipid content in small samples of organisms and to be able to select suitably active cells on the same basis. It would be advantageous if the method discriminated between polar and non-polar lipids since only the non-

Correspondence to: K. E. Cooksey, Department of Microbiology, Montana State University, Bozeman, MT 59717, U.S.A.

polar product is commercially desirable as a fuel fraction [1]. These constraints suggest that a suitable method could be based on lipid staining with a vital dye, rather than a procedure dependent on lipid extraction. Chromatographic techniques, especially when combined with mass spectrometry, provide sensitivity and specificity in lipid analysis but do not overcome the need for an extraction step. Such techniques are also expensive regarding capital equipment and time. At least two staining methods have been proposed previously. Evans et al. [2] have described a semiquantitative procedure for staining, with Sudan Black B, oleaginous yeast colonies on agar plates. Several groups of Russian workers have described lipid analyses based on the use of fluorescent probes. Pomoshnikova et al. [3] have used the fluorescent stains 1,8-naphthoylene-1′-2′-benzimidazole and its methoxy-substituted derivative to determine the lipid content of yeast and mycelial fungi. In later work Pomoshnikova et al. [4] used the fluorescent dye luminor 490PT to investigate the dynamics of lipid formation in fungal mycelia. Neither of these dyes appear to be readily commercially available. More pertinent to our research is the paper by Tertov et al. [5] who used a flow cytometer (Becton-Dickinson FACS II) to determine total intracellular lipid in mammalian tissue-culture cells using a perlenoyl labelled-triglyceride as a lipid probe. A linear relationship between fluorescence and lipid content of cells was found. Disadvantages of the method are (**a**) the probe must be synthesized, (**b**) staining takes several hours, (**c**) not all cells take up triglycerides and (**d**) washing is needed to remove unbound fluorophore.

A series of papers from Greenspan, Mayer and Fowler [6–8] led us to try the commercially available dye Nile Red in our search for a neutral lipid specific fluorophore [9] that could also be used as a vital stain. This paper describes a simple staining method for microalgal cells and its use in lipid determination. An abstract of parts of this paper has been published previously [10].

Materials and Methods

Growth of algal cells

Amphora coffeaeformis, Navicula sp. and *Tropidoneis* sp. were grown on the ASP-2 medium of Provasoli et al. [11] modified to contain 0.25 mM Ca^{2+} [12]. *Chlorella* sp. Chlor-1 (Solar Energy Research Institute, Golden, CO, USA, 80401) was grown on Bold's basal medium [13]. Cell concentrations were determined by using a hemocytometer. Cultures were incubated at 28–30 °C and 100 μE m^{-2} s^{-1}.

Staining of algal cells

Nile Red solution in acetone (250 μg ml^{-1}) and acetone were added to a suspension of cells in growth medium such that the final concentrations were 1 μg ml^{-1} and 0.04% (v/v), respectively. The mixture was vigorously agitated on a vortex mixer. Samples of this suspension were used immediately (or after 30 s) for fluorescence microscopy, fluorometry or flow cytometry. Nile Red (9-diethylamino-5*H*-benzo[α]phenoxazine-5-one) was obtained from Molecular Probes, Junction City, OR 97748 or Kodak, Rochester, NY 14650.

Microscopy

Photographs were taken with a Nikon Optiphot microscope using a 450–490 nm excitation filter, a 510 nm dichroic mirror and a 520 nm barrier filter. Ektachrome film (160 ASA, tungsten) set at ASA 80 was used. Color slide positives were used to make black and white internegatives for Fig. 5.

Flow cytometry

A Becton-Dickinson FACS 440 flow cytometer was used. Diluent sheath fluid contained 308 mM NaCl, 20.2 mM $MgSO_4$, 8.1 mM KCl, 0.25 mM $CaCl_2$ and 8.3 mM Tris-HCl, pH 7.6. The analytical aperture was set at 70 μm. Stained cells were excited at 488 nm and the emission recorded at 575 ± 15 nm in conjunction with a 560 nm dichroic mirror. Results were collected as number of events versus the logarithm of the fluorescence intensity. Instrumental alignments were made daily and were based on the fluorescence of 2.8 μm Nile Red-stained monodisperse latex particles (a gift of Dr. C. J. Wang, Pandex Laboratories, Mundelein, IL 60060). Fluorescence per ml culture was calculated from cells per ml × % fluorescent cells × average fluorescence per cell × 10^{-8}.

Spectrofluorometry

In most experiments, the fluorescent properties of stained and unstained cells were measured using a Spex Fluorolog model F211 spectrofluorometer equipped with a 150 W Xenon lamp. Real time data acquisition and manipulation was performed using a Datamate microprocessor interface. Stained cell (2.5 ml) fluorescence was measured as a function of time for 800 s using a 1 s integration time per point. Wavelengths of excitation and emission were 525 and 580 nm, respectively. Relative fluorescence in arbitrary units was measured 30 s after dye introduction, and this sample was re-mixed at intervals during time course measurements. Emission profiles were generated from 500 to 700 nm using 525 nm excitation and a 0.5 s/nm integration time. In both the time course and emission measurements a bandpass of 4.5 nm on the emission and 2.25 nm on the excitation monochromotor was utilized. In experiments described in Tables 2–4, a Turner model 10 fluorometer with a 480 ± 10 nm excitation filter and a 580 ± 9.8 nm bandpass emission filter was used. This fluorometer was standardized using a suspension of monodisperse latex particles (Pandex Laboratories).

Time-course experiments

A series of 500 ml cultures of *A. coffeaeformis* in 2.8 L Fernbach flasks were examined daily for cell number and Nile Red fluorescence. Based on these parameters, some of the flasks were harvested at early (5 days), mid (9 days) and late stationary phase (12 days). The contents of four flasks were pooled for the 5 day sample, two for the 9 day sample, and for day 12, two flasks were analyzed independently. Cells were collected by centrifugation (3000 × *g*, 15 min, 25 °C) and the supernatant liquid saved for nitrate and silicate analysis. After wet weight determination, cells were lyophilized. Dry weight determinations were made on the lyophilized samples.

Lipid extraction

In the experiments reported in Fig. 4, extractions were made on wet-cell pellets by

the method of Bligh and Dyer [14]. Results were calculated per ml of original culture. For other experiments, dried cell pellets were gently ground to break up large clumps, and dry weight determinations were made. The dry weights of samples for extraction ranged from 42 to 97 mg. The pooled 5 day sample was split into two, approximately 60 mg samples, so that the reproducibility of gravimetric and gas chromatographic lipid analyses could be found. All samples, along with procedural blanks, were randomly assigned to sequentially numbered separatory funnels such that the samples were blindly coded. The samples were then processed in a random order by processing them in this sequence.

A Bligh and Dyer [14] lipid extraction was also used for the lyophilized samples but in these cases as modified by White et al. [15]. The initial single phase was 37.5 ml methanol:18.75 ml chloroform:15.0 ml 50 mM phosphate buffer (pH 7.4). The cells were extracted for 19 h before 18.75 ml each of chloroform and distilled water were added to form 2 phases. The phases were allowed to separate for 24 h before the lower organic phase was removed and filtered through Whatman 2V filter paper into a round-bottom flask for drying.

Total lipid gravimetric determination

The dried total extracted lipid was transferred from round-bottom flasks into previously tared test tubes fitted with teflon-lined screw caps and the solvent evaporated in a water bath ($< 37\,^{\circ}C$) under a stream of dry nitrogen gas. This dried residue was defined as the total extracted lipid for all extraction procedures. The weight of this residue was recorded for a gravimetric estimate of total lipid yield.

Silicic acid column chromatography

The total extracted lipid was separated into three general classes by silicic acid column chromatography (SAC) [16]. Columns were disposable, large volume, glass pasteur pipets prepared with the approximate ratios of 50:1 stationary phase to lipid (dry weight) and 6.5:1 stationary phase column bed height to cross-sectional area. The stationary phase was Biosil A (100–200 mesh, Bio-Rad Labs) activated at 120 °C for at least 18 h. The sample was loaded onto the column in a minimal volume of chloroform and a series of mobile phases of increasing polarity was used to separate the lipid classes: neutral lipids in 10 ml chloroform, glycolipids in 10 ml acetone, and polar lipids in 10 ml methanol. The fractions were collected in previously tared test tubes fitted with teflon-lined screw caps and dried under a stream of nitrogen, as before. Gravimetric estimates of lipid class yields were made as described for total lipid yield.

Mild alkaline methanolysis

This procedure will not methylate free fatty acids, but transesterification of fatty acids esterified to glycerol backbones is complete in just 15 min at 37 °C [17]. The procedure (detailed in Ref. 18) results in fatty acid methyl esters (FAME) from each of the lipid classes separated by SAC.

Separation and quantification of FAME by gas chromatography (GC)

Dry FAME were dissolved in appropriate volumes of iso-octane which contained

approximately 500 pmol/μl of FAME (either 19:0 or 20:0, Sigma Chemical Co.) as internal standards. Samples (1.0 μl) were injected for separation on a Varian 3700 GC (Sunnyvale, CA) using a 30 m, polar, DB-225 fused silica capillary column (J&W Scientific, Folsom, CA). A splitless injection system with injector temperature at 200 °C was used. Helium was the carrier gas (linear velocity, 35 cm/s). The temperature program used began at 130 °C and rose at 2° /min to 200 °C with a final isothermal period of 10 min (total analysis time, 45 min). The GC was equipped with a flame ionization detector (flame:hydrogen/air at 30/300 ml/min, make-up gas:helium at 30 ml/min, detector temperature, 210 °C). An equimolar response was assumed for all FAME in the range analyzed (14:0 to 24:0). Peak areas were quantified by a programmable Waters Data Module M730 (Milford, MA). The area of each peak was compared to the internal injection standard area to determine the quantity of each separated FAME.

Data reported in this study are the total molar amount of FAME for each lipid class (gas chromatographic method, e.g., Table 2). GC peaks were identified by two methods. First, by co-elution or identical relative retention times with standards obtained from Supelco, Inc. (Bellefont, PA); Applied Science Laboratories, Inc. (State College, PA); Nu-Chek Prep (Elysian, MN); or previously identified laboratory standards. Second, GC/mass spectrometry (MS) was utilized to verify the FAME as previously described [18]. Samples were analyzed on a VG MM-16 GC/MS (Manchester, UK) fitted with a direct capillary inlet. The separation conditions were identical to those described above for GC. Mass spectrometer operating conditions were: electron multiplier voltage, 4000 volts; transfer line temperature, 200 °C; source and analyzer temperature, 200 °C; calibration file tuned to per-fluorokerosene-H (PFK); and electron impact energy, 70 eV.

Analytical reproducibility of the Nile Red method

So that the variability of the methods could be compared, five replicate 6 ml samples of an *Amphora* culture in stationary phase were analyzed for cell concentration and Nile Red fluorescence. The coefficient of variation of this method was compared with those for gravimetric and GC lipid estimation.

Analysis of silicate and nitrate

The concentrations of nitrate (mg N l^{-1}) and silicon (total SiO_2 mg l^{-1}) were estimated by the cadmium reduction and silicomolybdate methods, respectively, using commercially available test kits (Hach Inc., Loveland, CO).

Statistical analyses

These were performed using a Honeywell level 66/DPS computer with a CP-6 operating system utilizing programs available in the MiniTab Software package. Correlations were calculated as Pearson's product-moment correlation coefficients [19].

Results and Discussion

Table 1 indicates that the Nile Red concentration needed to achieve maximal

TABLE 1

STAINING OF *A. COFFEAEFORMIS* WITH NILE RED

Concentration of Nile Red ($\mu l\ ml^{-1}$)	Average fluorescence per cell (arbitrary units)	Cells fluorescent (%)
0.01	38.4	13.9
0.10	44.8	28.3
1.00	61.0	95.4
10.00	62.9	92.8

fluorescence in the flow cytometer was not critical. In fluorometric measurements it is sometimes necessary to wash stained cells by centrifugation to lower background fluorescence. With Nile Red, the fluorescence yield in polar environments is so low [7] that this step is not necessary. Fluorescence which developed rapidly in *Amphora* at 585 nm (Fig. 1) when stained cells were excited at 525 nm, was stable for between 2 and 7 min. After that time, fading, but not settling of the stained cells, took place (Fig. 1). With other algae (data not shown here), the shape of this curve was different. It is necessary, therefore, to investigate the development of fluorescence with time for individual species. The time course of fluorescence development is probably related to the cellular permeability for Nile Red and whether the lipid is in small (*Chlorella*) or large (*Amphora*) droplets within the cell. If sample numbers were kept within a

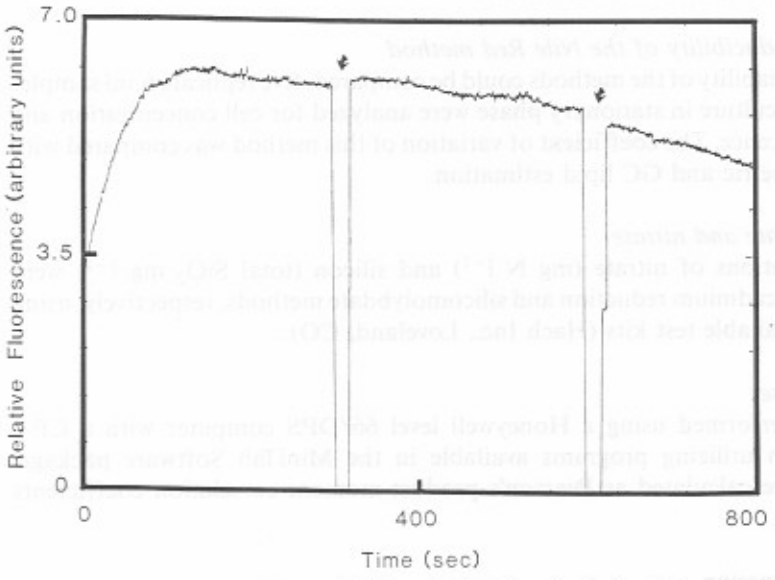

Fig. 1. Time-course of staining and fading of Nile Red-stained cells of *A. coffeaeformis* (8.8×10^5 cells ml^{-1}). Arrow indicates cuvette contents re-mixed. Zero time represents 30 s since dye was added to cell suspension. Excitation 525 nm, emission 585 nm.

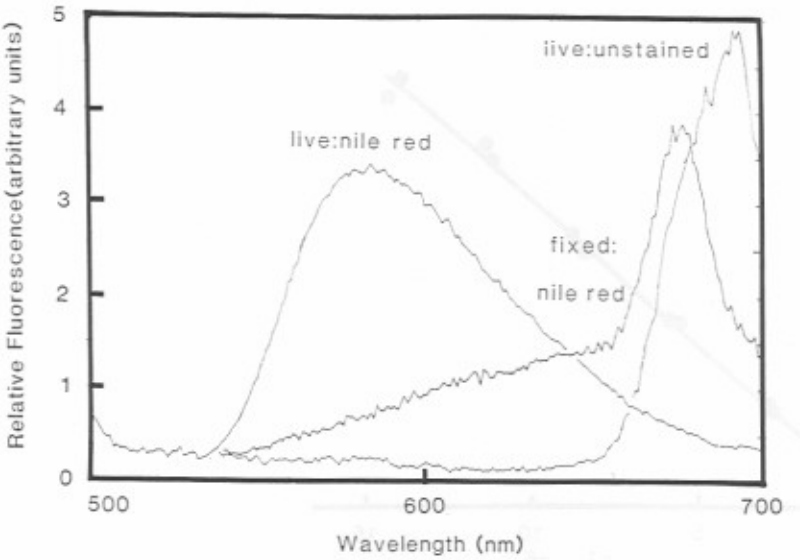

Fig. 2. Relative fluorescence emission of Nile Red-stained, unstained and fixed cells of *A. coffeaeformis* (8.3×10^5 cells ml^{-1}). Excitation at 525 nm.

manageable size or measurements were made individually, the length of this time window was not inconvenient. Formaldehyde-fixed cells did not stain (Fig. 2), however, cells could be fixed in such a solution (5%) or ethanol (70% v/v) after staining. The fluorescence of stained, fixed and washed cells was stable for about 2 h (data not shown), however the inclusion of a washing step in a quantitative procedure was undesirable from the point of view of cell recovery. The fluorescence generated by intracellular lipid was linear (Figs. 3 and 4). In Fig. 3, cells of a *Navicula* sp. were grown until NO_3^- in the medium was depleted and lipid synthesis in the cell was advanced. The fluorescence of various culture dilutions was measured using the spectrofluorometer. The relationship was linear with a correlation coefficient of $r = 1.00$. In the experiments shown in Fig. 4, samples of a growing culture of *A. coffeaeformis* were taken over a period of 1–11 days. During this time cells accumulated lipid which was determined gravimetrically as total lipid. Parallel determination of the fluorescence of Nile Red-stained cells gave a linear relationship ($r = 0.95$). This data differs from that in Fig. 3 in that here fluorescence determinations were made by flow cytometer and the lipid content of the cells varied, rather than their concentration.

Although previous work by Greenspan, Mayer and Fowler [5–9] led us to believe that the increase in fluorescence of lipid-accumulating algal cells was due to the increased synthesis of neutral lipid, we had no proof that this was so in algae. Accordingly, a time-course experiment was performed wherein the fluorescence of Nile Red-stained *A. coffeaeformis* was compared to the major lipid components of the culture during the time period that Nile Red-staining droplets began to be observed microscopically within the cell. The results of this experiment appear in Tables 2 and

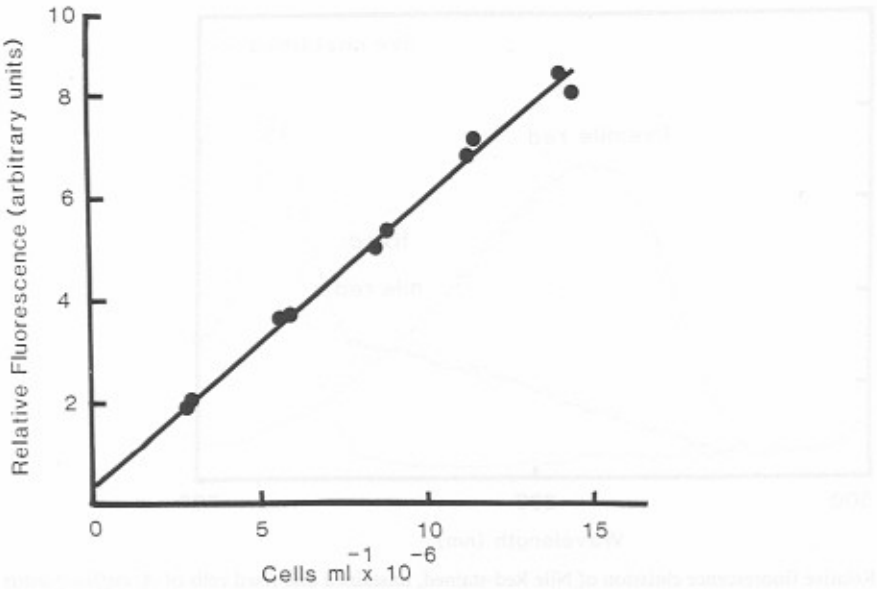

Fig. 3. Fluorescence of *Navicula* sp. 9D stained with Nile Red: spectrofluorometric data.

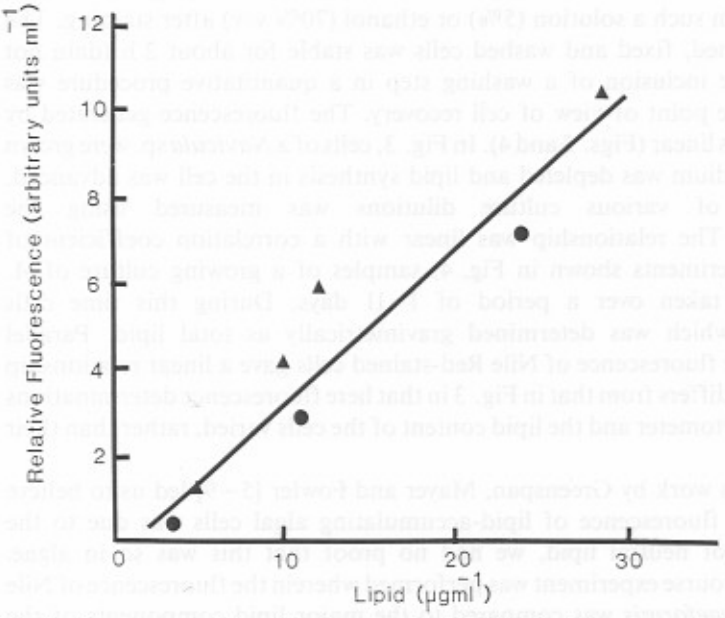

Fig. 4. Fluorescence of *A. coffeaeformis* stained with Nile Red: flow cytometer data. For details of calculation of fluorescence per ml, see text. Total lipid determined gravimetrically. Circles and triangles represent independent experiments.

TABLE 2

TIME-COURSE EXPERIMENT WITH *A. COFFEAEFORMIS:* ANALYSIS

Parameter	Units	Incubation Time (days)			
		5	9	12	12
Cell density	10^3 cells ml^{-1}	379	638	570	771
Dry wt. of cells analyzed	mg	119.2	96.8	52.0	64.7
Nile Red fluorescence	relative fluorescence[a]	1	21	43	30
Silicate	% used[b]	28	70	100	100
Nitrate	% used[b]	16	33	61	74
Gravimetric analysis					
Total lipid	mg	9.3	14.0	13.4	14.1
Neutral lipid		3.3	8.6	9.5	9.6
Glycolipid		5.0	4.1	2.9	3.6
Polar lipid		1.5	1.1	0.8	1.0
GC analysis					
Neutral lipid	μmol	1.8	16.0	24.3	22.9
Glycolipid		6.1	5.4	3.7	5.0
Polar lipid		1.8	1.4	1.2	1.2

[a] Scale reading corrected for dilution of sample.
[b] Initial silicate and nitrate concentrations, 0.53 and 0.59 mM, respectively.

3. During the 4 day period from day 5 to day 9, Nile Red fluorescence rose 21-fold, however, cell density rose less than 2-fold. Also over this period the neutral lipid fraction of the cell was the only lipid fraction to change materially. From day 9 to 12, there was no increase in cell number, but the Nile Red fluorescence rose approximately 2-fold. Once more the only lipid fraction to increase was the neutral lipid one. These

TABLE 3

CORRELATION OF NILE RED FLUORESCENCE AND LIPID CLASSES ESTIMATED GRAVIMETRICALLY AND BY GAS CHROMATOGRAPHIC ANALYSES

Lipid Estimate	Coefficient[a] (r)
Gravimetric Analysis (mg)	
Total lipid	0.79
Neutral lipid	0.91
Glycolipid	−1.00
Polar lipid	−1.00
Gas Chromatographic Analysis (μmol)	
Neutral lipid	0.97
Glycolipid	−0.96
Polar lipid	−0.95

[a] Pearson's product-moment correlation.

relationships are correlated statistically in Table 3. Cellular Nile Red fluorescence is correlated most positively to the neutral lipid fraction of the cell. The neutral lipid fraction, as determined by GC analysis, represents the esterified fatty acid fraction of the cell, i.e., mostly triglycerides.

This method was developed, in part, for use in a screening program to discover and select high lipid-producing algae. However, another use in our laboratory is to define the point in time when neutral lipid accumulation is triggered. It is well accepted that the increase in total lipid of microalgal cultures becomes noticeable when cells can no longer divide because of nitrogen or, for diatoms, silicate-limitation (reviewed in Refs. 20, 21). Although the work of Shifrin and Chisholm [20] and Ben-Amotz et al. [21] confirmed earlier reports that these limitations did result in lipid accumulation, their experiments are not strictly comparable to those reported in Table 2. Whereas these workers measured total lipid [20] or neutral lipid [21] only after cells had experienced nutrient limitation for some time, we, because of the sensitivity of the Nile Red method, were able to measure cellular lipid during growth, when the cells were not obviously nutrient limited. Thus, it appears, at least in *A. coffeaeformis*, the onset of lipid accumulation takes place well before NO_3^- or Si limitation is evident (Table 2, lines 4, 5). Our confidence in such a conclusion is supported by the reproducibility of the Nile Red method. An experiment to compare the reproducibility of the three lipid determinations used here showed that the Nile Red procedure was as good as the gravimetric method commonly used for lipid determination (e.g., Ref. 20) and, as one would expect, was somewhat better than the GC analytical method, which required a considerably greater number of experimental manipulations.

The general applicability of the method, at least for algae, is shown in Fig. 5 and Table 5. All organisms showed an increased fluorescence and thus lipid content as they entered the stationary phase of growth. The method allowed comparisons of relative lipid contents of small quantities of cell ($< 5 \times 10^6$). Since Nile Red does not kill algal cells, it was possible, using a flow cytometer in the 'sort' mode, to select cells of above average lipid content for further study (data not shown). Where absolute lipid content of a population was required, it was necessary first to prepare a standard such as that depicted in Fig. 4. We see no reason why this method could not be applied to determine neutral lipid in other types of cells.

TABLE 4

COMPARISON OF METHODS

Expt. No.	Nile Red fluorescence/10^5 cells (relative units)	Gravimetric analysis (mg)	GC analysis (μmol)
1	0.52 ± 0.03[a]	–	–
2	–	1.6 ± 0.10[b]	0.92 ± 0.10[b]
Coefficient of variation (%)	5.6	6.2	9.2

[a] $n = 5$, mean $\pm$ one standard deviation.
[b] $n = 2$, mean $\pm$ one standard deviation.

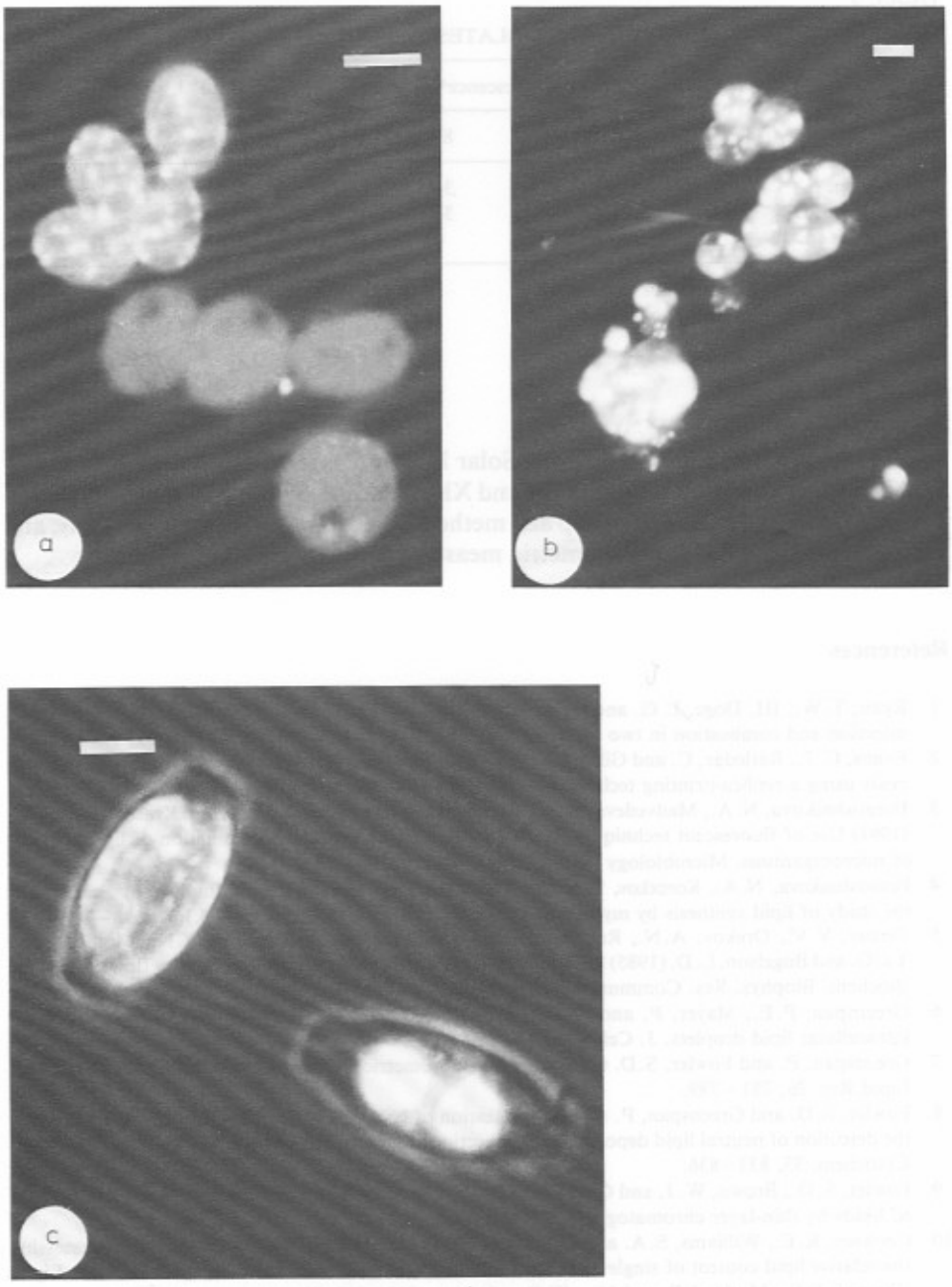

Fig. 5. Cells of *Chlorella* sp. Chlor-1 (Solar Energy Research Institute Culture Collection) in nitrogen sufficient (a) and nitrogen depleted (b) media. (c) *Amphora* sp. in nitrogen depleted media. Note that lipid accumulates on one side of the chloroplast only in this organism.

TABLE 5

FLUORESCENCE OF THREE DIATOM ISOLATES

Organism	Relative fluorescence[a] after		Cell ml^{-1} $\times 10^{-6}$	NO_3^- used[b]
	4 days	8 days	8 days	(%)
Navicula sp. 6	179.1	340.7	2.66	99.4
Navicula sp. 9D	159.4	387.7	2.55	99.0
Tropidoneis sp. 17	29.8	67.7	0.42	99.6

[a] See Materials and Methods for calculation.
[b] Initial nitrate concentration = 0.59 mM.

Acknowledgements

This research was supported by the Solar Energy Research Institute, Golden, CO, USA, contract number XK-4-04136-04 and XK-5-05073-1. We thank David Davies and Barbara Cooksey for technical help and methodological suggestions, respectively, and Deborah Bergland for flow cytometric measurements.

References

1 Ryan, T.W., III, Doge, L.G. and Callahan, T.J. (1984) The effects of vegetable oil properties on injection and combustion in two different diesel engines. J. Am. Oil Chem. Soc. 61, 1610–1619.
2 Evans, C.T., Ratledge, C. and Gilbert, S.C. (1985) A rapid screening method for lipid-accumulating yeast using a replica-printing technique. J. Microbiol. Methods 4, 203–210.
3 Pomoshnikova, N.A., Medvedeva, G.A., Levchenko, M.N., Meisel, M.N. and Krasovitskii, B.M. (1981) Use of fluorescent techniques for detection and quantitative determination of lipids in cells of microorganisms. Microbiology (USSR Intern. Ed.) 50, 176–182.
4 Pomoshnikova, N.A., Korotkov, S.A. and Galanina, L.A. (1983) Use of fluorescent analysis for the study of lipid synthesis by mycelial fungi. Microbiology (USSR Intern. Ed.) 52, 698–701.
5 Tertov, V.V., Orekov, A.N., Rudchenko, S.A., Mukhin, D.N., Smirnov, V.N., Molotkovsky, Yu, G. and Bugelson, L.D. (1985) Determination of total intracellular lipid content by flow cytometry. Biochem. Biophys. Res. Commun. 128, 1196–1202.
6 Greenspan, P.E., Mayer, P. and Fowler, S.D. (1985) Nile Red: a selective fluorescent stain for intracellular lipid droplets. J. Cell Biol. 100, 965–973.
7 Greenspan, P. and Fowler, S.D. (1985) Spectrofluorometric studies of the lipid probe, Nile Red. J. Lipid Res. 26, 781–789.
8 Fowler, S.D. and Greenspan, P. (1985) Application of Nile Red fluorescent hydrophobic probe for the detection of neutral lipid deposits in tissue sections: Comparisons with Oil Red O. J. Histochem. Cytochem. 33, 833–836.
9 Fowler, S.D., Brown, W.J. and Greenspan, P. (1986) Use of Nile Red for rapid qualitative analysis of lipids by thin-layer chromatography. Fed. Proc. 45, 1669.
10 Cooksey, K.E., Williams, S.A. and Calis, P.R. (1986) Nile Red: A fluorophore useful in assessing the relative lipid content of single cells. In: The Metabolism, Structure and Function of Plant Lipids (Stumpf, P.K., Mudd, J.B. and Nes, W.D., eds.), pp. 645–647, Plenum Press, New York.
11 Provasoli, L., McLaughlin, J.J.A. and Droop, M.R. (1957) The development of artificial media for marine algae. Arch. Mikrobiol. 25, 392–428.
12 Cooksey, K.E. and Chansang, H. (1976) Isolation and physiological studies on three isolates of *Amphora* (Bacillariophyceae). J. Phycol. 12, 455–460.

13 Nichols, H.W. (1973) Growth media – freshwater. In: Handbook of Phycological Methods: Culture Methods and Growth Measurements (Stein, J.R., ed.) pp. 7–24, Cambridge University Press, London.
14 Bligh, E.G. and Dyer, W.J. (1959) A rapid method of lipid extraction and purification. Can. J. Biochem. Physiol. 35, 911–917.
15 White, D.C., Davis, W.M., Nickels, J.S., King, J.D. and Robbie, R.J. (1979) Determination of sedimentary microbial biomass by extractable lipid phosphate. Oecologia (Berl.) 40, 51–62.
16 Gehron, M.J. and White, D.C. (1983) Sensitive assay for phospholipid glycerol in environmental samples. J. Microbiol. Methods 1, 23–32.
17 Christie, W.M. (1973) Lipid Analysis: Isolation, Separation, Identification and Structural Analysis of Lipids. Pergamon Press, Oxford. 338 pp.
18 Guckert, J.B., Antworth, C.P., Nichols, P.D. and White, D.C. (1985) Phospholipid, ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. FEMS Microbiol. Ecol. 31, 147–158.
19 Sokal, R.R. and Rohlf, F.J. (1981) Biometry, 2nd Edn., W.H. Freeman, San Francisco. 859 pp.
20 Shifrin, N.S. and Chisholm, S.W. (1981) Phytoplankton lipids: interspecific differences and effects of nitrate, silicate and light-dark cycles. J. Phycol. 17, 374–384.
21 Ben-Amotz, A., Tornabene, T.G. and Thomas, W.H. (1985) Chemical profiles of selected species of microalgae with emphasis on lipids. J. Phycol. 21, 72–81.