

Analysis of interspecific variation in relative fatty acid composition: use of flow cytometry to estimate unsaturation index and relative polyunsaturated fatty acid content in microalgae

Héctor Mendoza Guzmán · Adelina de la Jara Valido ·
Laura Carmona Duarte · Karen Freijanes Presmanes

Received: 4 November 2009 / Revised and accepted: 5 April 2010 / Published online: 29 April 2010
© Springer Science+Business Media B.V. 2010

Abstract Relative polyunsaturated fatty acid content and unsaturation index are very important composition variables in the use of microalgae both for animal and human nutrition and biofuel production. A readily available technique to rapidly and inexpensively estimate relative fatty acid composition is very important for mass screening of new strains for the production of different types of oil. This study demonstrates the validity of Nile Red staining and flow cytometry for quick estimation of unsaturation index and relative fatty acid content in microalgae. Nile Red staining allows polar and neutral lipid contents to be estimated, and in this study a significant correlation was observed between polar/neutral ratio and fatty acid content in the species studied, corresponding to higher polyunsaturated fatty acid content in the polar lipid fraction of microalgae. This technique enables quick estimation of relative polyunsaturated fatty acid content and interspecific variation, as well as variations caused by culture conditions. In the species studied, most variability in fatty acid composition was due to variation in monounsaturated and

polyunsaturated fatty acids, with less variation observed in saturated fatty acid content.

Keywords Fatty acids · Flow cytometry · Lipids · Nile Red · Microalgae

Introduction

Recent interest in the production of microalgae is due to several factors, most of which are associated with the possibility of using microalgae as a source of fatty acids for aquaculture, human nutrition or biofuel production.

Microalgae play a decisive role in aquaculture. It is estimated that 18% of aquaculture species worldwide depend on microalgae at some stage of their development (Muller-Feuga 2000), as fatty acids are essential in the productivity of aquaculture facilities (Wikfors and Ohno 2001). However, the implications of microalgae go beyond the field of aquaculture. They are now regarded as a prime alternative to fish oils in human nutrition as a source of polyunsaturated fatty acids (PUFAs; Patil et al. 2007). Microalgae are not only rich in PUFAs but also have greater lipid stability due to their high content of antioxidant compounds and bioencapsulation of lipids in membrane structures. Moreover, declining captures in the world's principal fishing grounds and the risk of pollutants have led to a decrease in the use of fish oils in human nutrition, particularly in certain applications subject to high levels of control such as infant formulae preparations (Ward and Singh 2005). This has led to the search for alternatives, in which microalgae have emerged as one of the best options.

H. Mendoza Guzmán (✉) · A. de la Jara Valido ·
L. Carmona Duarte · K. Freijanes Presmanes
Department of Biotechnology, Instituto Tecnológico de Canarias,
Pozo Izquierdo s/n (35119),
Santa Lucía de Tirajana, Las Palmas, Spain
e-mail: hmendoza@itccanarias.org

A. de la Jara Valido
e-mail: adelajara@itccanarias.org

L. Carmona Duarte
e-mail: lcarmona@itccanarias.org

K. Freijanes Presmanes
e-mail: kfreijanes@itccanarias.org

Microalgae have optimum content of fatty acids which are highly important in human nutrition, such as γ -linoleic acid, arachidonic acid, docosahexaenoic acid (DHA) and eicosa-pentanoic acid, whose benefits to human health are widely known. However, the reason why production of oils from microalgae has come to be regarded as a highly strategic activity is found in the numerous studies highlighting microalgae as a promising alternative to traditional biofuel sources, especially biodiesel (Chisti 2007; Li et al. 2008; Hu et al. 2008). Apart from providing a source of high oil content biomass (Gouveia et al. 2009), microalgae culture does not compete with other crops either in terms of fertile land or availability of quality water (unlike traditional energy crops) and is a biomass with very limited use in human nutrition. All of these factors make microalgae an ideal energy crop.

In spite of this, microalgae production is not only still at a very early stage but also makes use of a very limited number of species. Just two microalgae species constitute the base of the PUFA production industry for applications in nutraceuticals: *Schizochytrium* and *Cryptocodinium* (Ward and Singh 2005). Little more than 30 species are currently in production in the aquaculture sector (Wikfors and Ohno 2001; Pulz and Cross 2004). This limited number of species in use is also due to the low number of screening studies on new strains with major potential for the production of fatty acids (Griffith and Harrison 2009), despite the high interspecific and intraspecific variability observed in microalgae composition (Patil et al. 2007; Mendoza et al. 2009). Difficulties encountered in screening techniques are in part to blame. The precision required and the number of samples that can be processed are key aspects in screening. In the case of fatty acid composition in microalgae, analysis techniques hinder screening as they are relatively expensive and complicated and require large amounts of biomass to be effective.

Since its development, flow cytometry has been considered the best option for screening studies of single-cell organisms (Davey and Kell 1996). This technique and the equipment it uses not only allow a large number of cell parameters to be checked at one time but also automate cell selection. Flow cytometry has only recently been applied to obtain quick estimates of lipid cell content and select hyperlipogenic species (de la Jara et al. 2003; Gouveia et al. 2009; Mendoza et al. 2009). This type of analysis is performed using the lipid stain Nile Red (NR), which shows varying fluorescence depending on the polarity of the lipids (Alonzo and Mayzaud 1999) and enables neutral and polar lipid cell content in microalgae to be estimated (Mendoza et al. 2009). This study also demonstrates the feasibility of using Nile Red in conjunction with flow cytometry for quick estimation of relative fatty acid content and unsaturation index in microalgae, assessing both intraspecific differences and differences caused by culture conditions. Ability to estimate these variations would be essential in screening studies to select

PUFA-rich species for use in aquaculture and human nutrition and also to identify microalgae for biodiesel production, in which fatty acid composition and unsaturation index are important variables.

Materials and methods

Four microalgae species from different collection were cultured: *Isochrysis galbana* BNA-40-002 and *Chlorella pyrenoidosa* BNA-10-013, from the official collection of algae BNA, *Tetraselmis suecica* 03/0203 from the collection of microalgae of ICMAN-CSIC (Spain) and *Dunaliella salina* ITC-5.003 from the collection of ITC. The first three were cultured in *f*/2 medium (Guillard 1975) (*C. pyrenoidosa* in freshwater). *Dunaliella salina* was cultured in the hypersaline medium of Semenkov and Abdullaev (1980), at a salinity of 1 M NaCl. Cultures were bubbled with 3% CO₂ in air (v/v) at continuous light (200 μ mol photons m⁻² s⁻¹) and grown in 500 mL borosilicate flasks containing 500 mL medium at 25 \pm 2°C in a programmable temperature chamber. Stock was maintained by weekly subculturing. In all cases, the stock culture was used as inoculum.

Two test culture conditions were defined, with three replicates in each condition for each species: (1) control (maintenance conditions of stock culture) and (2) nitrogen starvation (-N), where successive washing by centrifugation (5 min, 5,000 \times g) removed all source of nitrogen. The remaining formula of the culture media was kept constant.

Lipid assessment and chromatography analysis

For the analysis of the lipid composition, samples were taken when they had clearly reached exponential growth in the control and had been exposed to nitrogen starvation in the medium for 48 h. Growth phases were determined from variations in cell density estimated by cytometry analysis. Samples were harvested by centrifugation (5 min, 5,000 \times g, 5°C).

Gravimetric extraction and lipid composition quantification were conducted by the method described by de la Jara et al. (2003). Total lipid content was obtained from a crude extract of each sample. Frozen cells (aliquots of 50–100 μ L) were extracted by adding methanol/chloroform (1:2 v/v). After 2-min centrifugation (5 min, 5,000 \times g, 5°C), the supernatant was collected in a different tube, and a solution of sodium chloride (0.9% w/v) was added in a proportion of 1:5 mL of lipid extract. The mix was vigorously vortexed for approximately 5 min, after which two phases were observed. The supernatant was discarded, as this phase is rich in nonlipid components. The oily phase was recovered as crude lipid extract. Total lipid content was obtained by evaporating the crude lipid extract and oven-

drying it at 85°C for 15 min, after which it was precision-weighed. The polar and neutral lipid fractions were separated by absorption chromatography on silica cartridges as described by Juaneda and Rocquelin (1985), according to the modification made by Yongmanitchai and Ward (1992) for separation of lipids in microalgae.

Fatty acid composition, obtained from fresh samples, was estimated following the method of Mendoza et al. (1999). Biomass aliquots were transmethylated with MeOH acetyl chloride. Gas chromatography analysis was performed in a Varian CP-3800 fitted with an FID detector. Fatty acid methyl esters were identified by comparing retention times with the Supelco standard for FAME Mix C4–C24.

Fatty acid composition was analysed not only for each sample but also for the polar and neutral lipid fractions in the cultures under control conditions. During this analysis, the lipid fractions were concentrated by nitrogen evaporation after separation to avoid degradation of fatty acids.

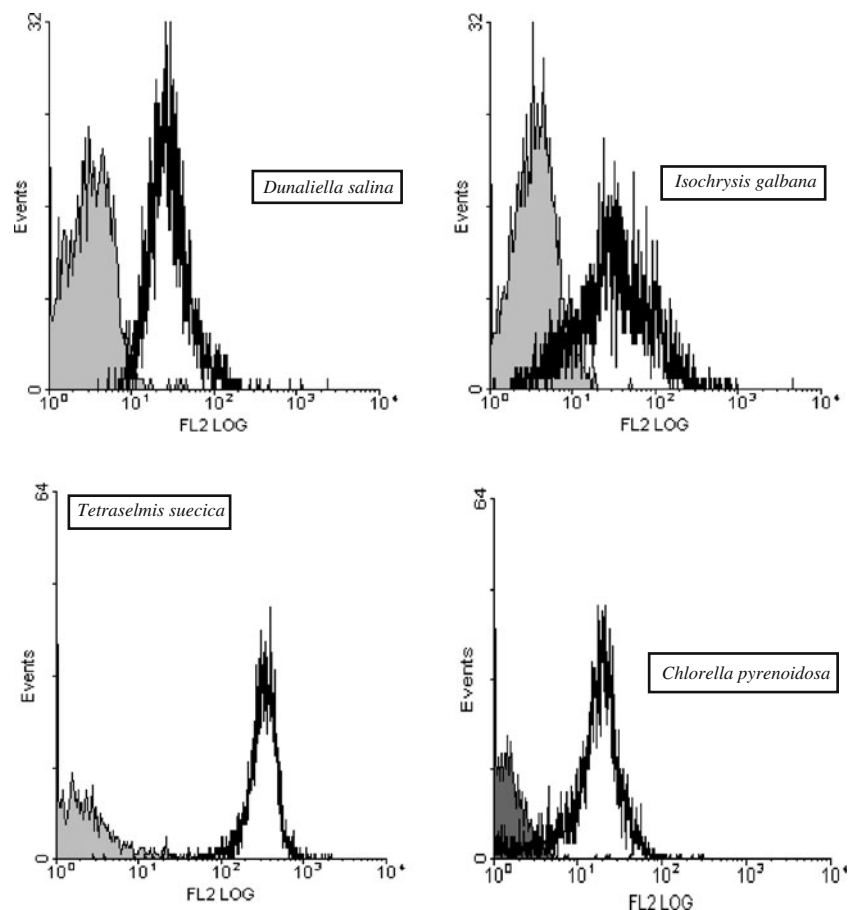
Relative PUFA content is expressed as the ratio between the percentages of the different fatty acids: saturated (SATs), monounsaturated (MUFAs) and PUFAs, using the formula (PUFA/SAT+MUFA) (Guimarães et al. 1991). The unsaturation index was also estimated in accordance with the formula developed by Thi et al. (1987); the percentage

of each fatty acid was multiplied by the number of double bonds presents in the molecule.

Flow cytometry analysis

Cells from the different culture conditions were stained with 15 mM Nile Red (Sigma), as described by de la Jara et al. (2003). Flow cytometry was used to determine yellow and red fluorescence of NR-stained cells, cell size (FSC) and complexity (SSC) and deep red autofluorescence due to chlorophyll, using an EPICS XL flow cytometer (Beckman Coulter Instruments) equipped with an air-cooled 488 nm argon-ion laser. The optical system used in the EPICS XL flow cytometer collects yellow light (575 band pass filter) in the FL2 channel and red light (620 band pass filter) in the FL3 channel. These lengths are similar to the fluorescences of optimum emission described for NR in a neutral lipid fraction (580 nm) and a polar lipid fraction (610 nm), although the excitation length is considerably lower (neutral 530 nm, polar 560 nm; Alonso and Mayzaud 1999). Chlorophyll fluorescence was collected in the FL4 channel (675 band pass filter). Cells were gated according to their chlorophyll fluorescence characteristics to remove nonalgal particles. Approximately 3,000 cells were analysed using a

Fig. 1 NR-stained cells (3,000 cells analysed). NR staining causes an increase in the FL2 and FL3 signals, corresponding to fluorescence in a neutral lipid matrix and a polar lipid matrix. In the figure is reflected the variation of FL2 signal in the cells stained with NR



log amplification of the fluorescent signals. Equipment was calibrated daily using Flow-Check™ fluorospheres (Beckman Coulter). Unstained cells were used as autofluorescence control. Data were expressed as fluorescence (arbitrary units). The reference data used were the arithmetic mean of the cytometric data of all parameters for all cells analysed (3,000) for each test condition, species and replicate.

Data analysis

In all cases, data represent the mean of the three replicates. Data were analysed for statistical significance using linear regression analysis. The *t* test was used to establish statistically significant differences for the culture conditions. The statistical significance values chosen were $p < 0.05$ for the comparison of test conditions and species and $p < 0.01$ for correlations.

Results

All species responded positively to staining, as there was effective incorporation of Nile Red in the cells (Fig. 1). The

coefficient of variation (CV) of FL2 and FL3 signal values in Nile-red-stained cells was low in all species studied (Table 1); in general, the coefficient of variation was not higher on average than 0.097 or 0.060 in FL2 and FL3, respectively (Table 1), and therefore the mean of these parameters was taken as a reference value for all events (3,000 cells analysed) for each cytometric analysis.

A significant correlation was observed ($p < 0.01$) between variations in the polar/neutral ratio estimated by flow cytometry for Nile-red-stained cells (FL3/FL2) and relative PUFA content and the unsaturation index for all species, culture conditions and replicates (Figs. 2 and 3). The correlation between the polar/neutral ratio and relative fatty acid composition corresponds to the differences observed between the fatty acid content of the polar and neutral lipid fractions in all species, with relative PUFA content and unsaturation index significantly higher in the polar fraction (Fig. 4).

The highest unsaturation index was seen in *I. galbana*, in both polar and neutral lipids (Fig. 4). Similarly, this species showed greatest variation in the unsaturation index between the polar and neutral lipid fractions (Fig. 4). The highest

Table 1 Mean and standard deviation of cytometry variables for each species, test condition and replicate

Species	Conditions	Replicates	FL2			FL3		
			Mean	Standard deviation	Coefficient of variation	Mean	Standard deviation	Coefficient of variation
<i>Isochrysis galbana</i>	Exponential growth	1	6.5	0.3	0.046	15.2	0.7	0.046
		2	6.4	0.6	0.094	14.3	0.7	0.049
		3	5.2	0.4	0.077	12.4	0.6	0.048
	-N	1	11.5	0.4	0.035	15.1	0.2	0.013
		2	16.3	0.9	0.055	20.3	1.8	0.089
		3	9.1	0.2	0.022	11.6	0.8	0.069
<i>Tetraselmis suecica</i>	Exponential growth	1	38.0	3.0	0.079	67.0	2.1	0.031
		2	22.1	1.8	0.081	43.1	1.5	0.035
		3	34.7	1.2	0.035	62.8	1.8	0.029
	-N	1	35.1	1.5	0.043	40.7	0.6	0.015
		2	33.9	1.1	0.032	38.7	1.1	0.028
		3	31.7	0.4	0.013	35.9	1.1	0.031
<i>Dunaliella salina</i>	Exponential growth	1	3.5	0.6	0.171	6.7	0.3	0.045
		2	3.5	0.9	0.257	6.9	1.3	0.188
		3	3.6	0.9	0.250	6.9	0.5	0.072
	-N	1	35.3	0.1	0.003	43.8	0.4	0.009
		2	32.9	0.3	0.009	43.4	0.4	0.009
		3	40.7	0.3	0.007	50.2	0.1	0.002
<i>Chlorella pyrenoidosa</i>	Exponential growth	1	1.1	0.1	0.091	2.9	0.5	0.172
		2	1.2	0.2	0.167	2.6	0.3	0.115
		3	1.7	0.1	0.059	5.3	0.2	0.038
	-N	1	1.5	0.2	0.133	3.8	0.3	0.080
		2	2.0	0.3	0.150	5.1	0.5	0.098
		3	2.1	0.3	0.143	4.4	0.6	0.136

Cytometry measurements were taken on 3,000 events for each test condition and replicate

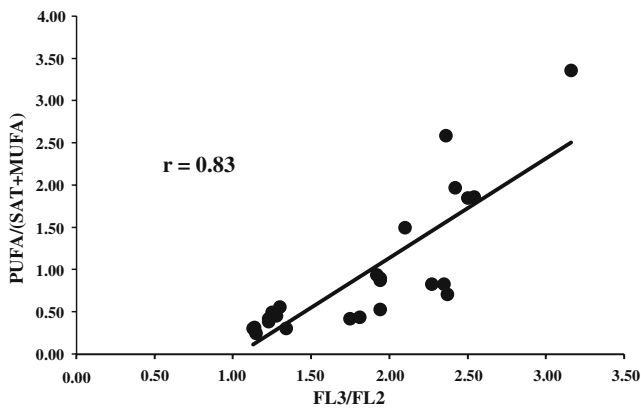


Fig. 2 Correlation between relative PUFA content and polar/neutral lipid ratio estimated by cytometry (FL3/FL2) in NR-stained cells for all species, culture conditions and replicates

relative PUFA content (PUFA/SAT+MUFA) in both lipid fractions was found in *C. pyrenoidosa*.

In general, in the four species, the main differences between polar and neutral fraction were observed in the PUFA contents whereas the SATs showed less variation between polar and neutral lipids (Table 2); variation in SATs between polar and neutral lipids in *D. salina* and *T. suecica* was not detected, while a significant variation in the content of PUFAs and MUFAs in all species was observed. The greatest variations in the fatty acid profile between both fractions of lipids were observed in *D. salina* and *C. pyrenoidosa*, with significant differences in the content of most fatty acids. In *T. suecica*, variation between the two fractions was lower.

16:0 was one of the predominant fatty acids in the composition of all species (Tables 2 and 3), with similar relative content in the neutral and polar lipid fractions; only in *C. pyrenoidosa* was variation detected in the content of 16:0 (Table 2). In contrast, high variability was observed in the

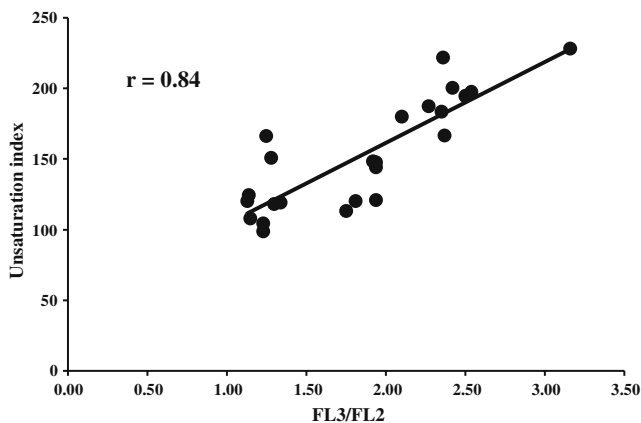


Fig. 3 Correlation between fatty acid unsaturation index and polar/neutral lipid ratio estimated on NR-stained cells by flow cytometry for all species, culture conditions and replicates

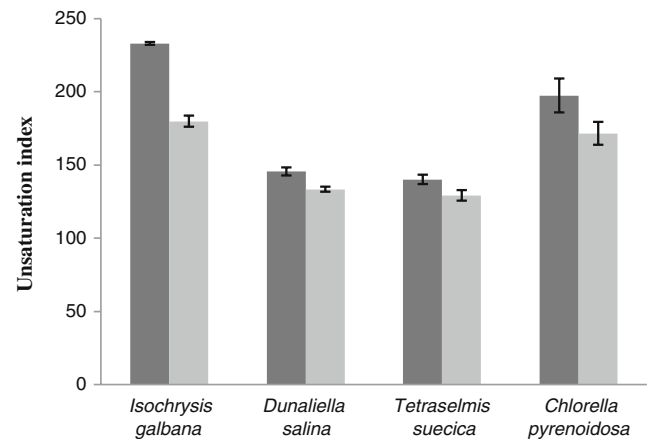
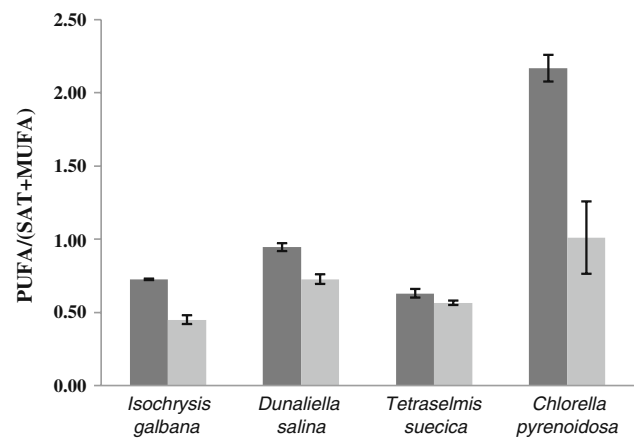


Fig. 4 Variations in relative PUFAs content (expressed as PUFA/SAT+MUFA) and fatty acid unsaturation index between polar lipid fractions (dark grey bars) and neutral lipid fractions (light grey bars) of the species studied

oleic acid content (18:1 ω 9), in both the *cis* and *trans* forms, *C. pyrenoidosa* was also the exception in this case, presenting similar content of 18:1 ω 9 in polar and in neutral lipids. This variability was also high in the content of the principal PUFAs between the two lipid fractions of the four species (Table 2).

Higher variability between MUFAs and PUFAs compared to the relative stability of the principal SAT contents also occurred in the variation in the composition of fatty acids in the culture conditions studied. In *I. galbana*, *T. suecica* and *D. salina*, a significant decrease was observed in relative PUFA content and unsaturation index between cultures that had clearly reached exponential growth (control cultures) and cultures exposed to nitrogen starvation (Fig. 5). This variation occurred primarily in relation to variation in MUFA content (which increased in the nitrogen-starved (-N) cultures) and PUFA content, with higher relative content in cultures in the exponential growth phase (control conditions). In contrast, SAT content showed little variation or remained practically stable, particularly in the case of *T. suecica*, in which a decrease in SAT content was seen in the control, although this was not significant (Fig. 5). By contrast,

Table 2 Fatty acid composition of polar and neutral lipid fractions in the species studied

Fatty acids	<i>Isochrysis galbana</i>		<i>Tetraselmis suecica</i>		<i>Dunaliella salina</i>		<i>Chlorella pyrenoidosa</i>	
	Polar fraction	Neutral fraction	Polar fraction	Neutral fraction	Polar fraction	Neutral fraction	Polar fraction	Neutral fraction
13:0	<i>0.04±0.01</i>	<i>0.08±0.02</i>			<i>0.47±0.12</i>	<i>4.27±0.54</i>	<i>0.15±0.05</i>	<i>1.48±0.59</i>
14:0	19.90±0.52	18.62±1.81		<i>0.45±0.22</i>	<i>0.23±0.03</i>	<i>1.48±0.46</i>	<i>0.84±0.62</i>	<i>4.73±2.83</i>
14:1	0.13±0.07	0.24±0.17				<i>0.52±0.26</i>	<i>0.05±0.02</i>	<i>0.09±0.07</i>
16:0	16.37±0.50	19.89±2.56	28.14±1.82	24.66±5.65	23.55±0.51	24.12±1.14	<i>20.65±1.29</i>	<i>30.88±4.13</i>
16:1	3.74±0.56	2.02±0.71	7.76±0.33	7.08±1.94	<i>1.45±0.03</i>	<i>5.73±0.58</i>	<i>2.40±0.66</i>	<i>1.85±0.34</i>
16:2	0.73±0.11	0.60±0.32	1.58±0.40	1.12±0.10	<i>1.45±0.09</i>	<i>0.86±0.14</i>	<i>10.05±1.54</i>	<i>5.69±1.57</i>
16:3	0.59±0.35	0.56±0.11	2.29±0.90	1.63±0.45	<i>0.94±0.06</i>	<i>0.30±0.22</i>	<i>0.04±0.02</i>	<i>1.91±0.42</i>
16:4	<i>0.14±0.04</i>	<i>0.65±0.19</i>	<i>1.73±0.11</i>		<i>3.41±0.03</i>	<i>2.25±0.15</i>	<i>13.39±1.65</i>	<i>6.61±0.73</i>
17:1						<i>1.11±0.88</i>		
18:0	<i>0.30±0.09</i>	<i>0.06±0.04</i>	10.41±0.93	8.46±0.77	<i>20.60±0.78</i>	<i>13.26±1.10</i>	<i>0.24±0.11</i>	<i>1.87±0.72</i>
18:1ω9c	<i>17.28±1.89</i>	<i>24.48±1.06</i>	<i>20.74±0.25</i>	<i>32.80±3.45</i>	<i>2.43±0.08</i>	<i>3.76±0.12</i>	6.91±2.66	9.36±.68
18:1ω9t	1.94±0.62	0.74±0.05	7.55±2.76	8.29±0.36	<i>2.32±0.30</i>	3.41±0.28	0.35±0.08	0.22±0.16
18:2ω6	3.40±0.46	4.05±0.54	4.44±0.32	4.08±0.08	8.13±0.39	8.60±0.30	22.39±2.71	19.10±4.02
18:3ω6	0.05±0.01	0.03±0.01			1.97±0.05	1.45±0.81	<i>0.11±0.02</i>	
18:3ω3	4.06±0.30	2.95±0.50	8.55±1.62	10.46±2.49	<i>33.05±0.23</i>	<i>28.88±0.46</i>	<i>22.14±2.84</i>	<i>16.21±1.34</i>
18:4ω3	<i>13.04±0.61</i>	<i>10.17±0.94</i>	1.85±0.32	0.43±0.05			<i>0.29±0.14</i>	
20:3ω6	<i>0.82±0.08</i>	<i>0.30±0.21</i>						
20:5ω3	<i>0.29±0.04</i>	<i>0.54±0.06</i>	<i>4.96±2.00</i>	<i>0.54±0.11</i>				
22:2	0.05±0.04							
24:0	<i>0.06±0.02</i>	<i>4.28±0.47</i>						
24:4ω6	2.77±0.31	1.60±0.79						
22:6ω3	<i>14.30±1.30</i>	<i>8.10±0.70</i>						
SATs	<i>36.53±0.27</i>	<i>42.95±3.85</i>	<i>38.55±0.89</i>	<i>35.23±4.50</i>	<i>46.58±0.74</i>	<i>43.14±1.92</i>	<i>21.89±0.89</i>	<i>38.96±7.71</i>
MUFAs	<i>23.03±1.92</i>	<i>27.49±0.39</i>	<i>36.05±3.33</i>	<i>47.63±2.60</i>	<i>4.86±0.98</i>	<i>14.86±0.82</i>	<i>9.71±1.21</i>	<i>11.51±3.14</i>
PUFAs	<i>40.44±1.86</i>	<i>29.56±3.47</i>	<i>25.39±2.46</i>	<i>17.14±1.91</i>	<i>48.56±0.30</i>	<i>42.00±1.10</i>	<i>68.40±0.94</i>	<i>49.53±5.81</i>

Cultures analysed were kept in control conditions and harvested when they had clearly reached exponential growth. Results correspond to three replicates per species in the control condition (data as percentage of total fatty acids ± SD; $n=3$). Significant differences were detected ($p<0.05$) in the fatty acids in italics

there were no great differences in the fatty acid composition between the two experimental growth conditions.

Discussion

Earlier studies demonstrated the possibility of using flow cytometry and Nile Red staining for quick estimation of lipid content in microalgae (Cooksey et al. 1987; de la Jara et al. 2003; Mendoza et al. 2009; da Silva et al. 2009). This method makes it possible not only to detect total lipid content but also to quickly determine the ratio of polar/neutral lipids, which earlier studies had associated with relative fatty acid content in microalgae (Roessler 1990; de la Jara et al. 2003). In general, polar lipids, which are associated with membrane structures, have higher PUFA content than other lipids (Roessler 1990), which means that the polar/neutral ratio could be used as an effective

indicator of relative PUFA content in microalgae for screening studies, as shown in the present study.

The polar/neutral ratio estimated by cytometry and Nile Red staining has been used to quickly determine variations in relative fatty acid content in microalgae in response to culture conditions (Mendoza et al. 2009). The present study, at least in the case of the species studied, demonstrates the validity of using this indicator to study interspecific differences in fatty acid composition and even to quickly analyse variations in response to culture conditions.

The polar/neutral ratio is closely related to the two basic indicators of relative fatty acid content (unsaturation index and PUFA/MUFA+SAT ratio), which further supports the validity of this ratio as a quick indicator of relative fatty acid content. At present, it is not possible to specifically stain fatty acids to analyse their composition using fluorometry techniques, although analogous fluorescent lipids can be used in studies on metabolism (Bai and Pagano 1997). Although the

Table 3 Fatty acid content in the species studied for the two experimental conditions: control (cultures clearly at exponential growth phase) and -N (nitrogen-starved)

Fatty acids	<i>Isochrysis galbana</i>		<i>Tetraselmis suecica</i>		<i>Dunaliella salina</i>		<i>Chlorella Pyrenoidosa</i>	
	Control exponential growth	-N	Control exponential growth	-N	Control exponential growth	-N	Control exponential growth	-N
13:0		<i>1.10±0.61</i>	0.65±0.30	0.29±0.12	0.68±0.14	0.68±0.24		2.95±1.62
14:0	23.92±0.45	24.38±0.88	0.57±0.27	0.76±0.13	0.92±0.11	1.08±0.09	0.85±0.05	0.85±0.01
14:1		<i>0.64±0.36</i>		<i>0.10±0.02</i>	0.06±0.02	0.06±0.05	0.05±0.02	0.13±0.10
16:0	<i>16.67±0.63</i>	<i>20.06±1.92</i>	27.41±4.15	27.5±0.95	<i>24.99±0.82</i>	<i>34.33±2.45</i>	20.54±1.73	21.13±0.95
16:1	2.83±0.19	3.64±0.47	<i>3.00±0.33</i>	<i>7.82±0.37</i>	3.03±0.23	2.62±0.21	3.29±1.14	2.48±0.54
16:2	0.83±0.59	1.15±0.26	2.00±0.52	1.84±0.13	0.08±0.02	1.20±0.32	<i>7.59±0.59</i>	<i>10.39±0.44</i>
16:3	0.44±0.22	0.50±0.12	<i>2.66±1.11</i>	<i>0.56±0.09</i>	0.52±0.18	0.62±0.17		
16:4		<i>0.38±0.16</i>	1.06±0.37	1.13±0.08	<i>2.17±0.10</i>	<i>1.21±0.18</i>	19.36±1.73	13.00±0.95
17:1								
18:0		<i>0.48±0.12</i>	<i>12.43±1.01</i>	<i>6.27±0.03</i>	<i>15.92±0.31</i>	<i>12.60±0.15</i>	0.24±0.19	0.38±0.16
18:1ω9c	<i>12.47±0.37</i>	<i>17.54±1.74</i>	<i>16.44±2.80</i>	<i>29.10±0.48</i>	<i>3.59±0.31</i>	<i>15.52±1.86</i>	<i>2.30±0.42</i>	<i>3.45±0.26</i>
18:1ω9t	<i>0.98±0.23</i>	<i>2.09±0.24</i>	6.14±2.02	5.57±0.30	<i>3.22±0.41</i>	<i>1.95±0.08</i>	0.90±0.66	0.80±0.45
18:2ω6	3.17±0.66	3.19±0.27	<i>5.4±0.99</i>	<i>2.30±0.32</i>	<i>8.59±0.52</i>	<i>6.64±0.52</i>	17.75±1.18	18.10±1.05
18:3ω6	<i>1.78±0.79</i>	<i>0.48±0.19</i>		<i>0.04±0.05</i>	1.37±0.40	0.68±0.57		
18:3ω3	<i>5.91±0.49</i>	<i>2.31±0.48</i>	15.65±2.44	10.34±0.30	<i>34.86±0.73</i>	<i>20.81±3.01</i>	26.36±2.55	24.91±0.05
18:4ω3	<i>15.10±3.01</i>	<i>6.61±0.92</i>		<i>1.98±0.40</i>				
20:3ω6	<i>5.14±0.54</i>	<i>0.62±0.48</i>		<i>0.09±0.03</i>				
20:5ω3	0.48±0.35	0.95±0.45	4.39±0.68	4.16±0.31				
22:2	1.12±0.40	0.83±0.30	<i>2.20±1.02</i>				<i>0.27±0.19</i>	<i>1.43±0.23</i>
24:0								
24:4ω6	<i>1.94±0.69</i>	<i>5.36±0.55</i>		<i>0.15±0.06</i>				
22:6ω3	7.22±0.79	7.69±2.67						

Results correspond to three replicates per species and experimental condition (data as percentage of total fatty acids ± SD; n=3). Significant differences ($p < 0.05$) were detected in the fatty acids in italics

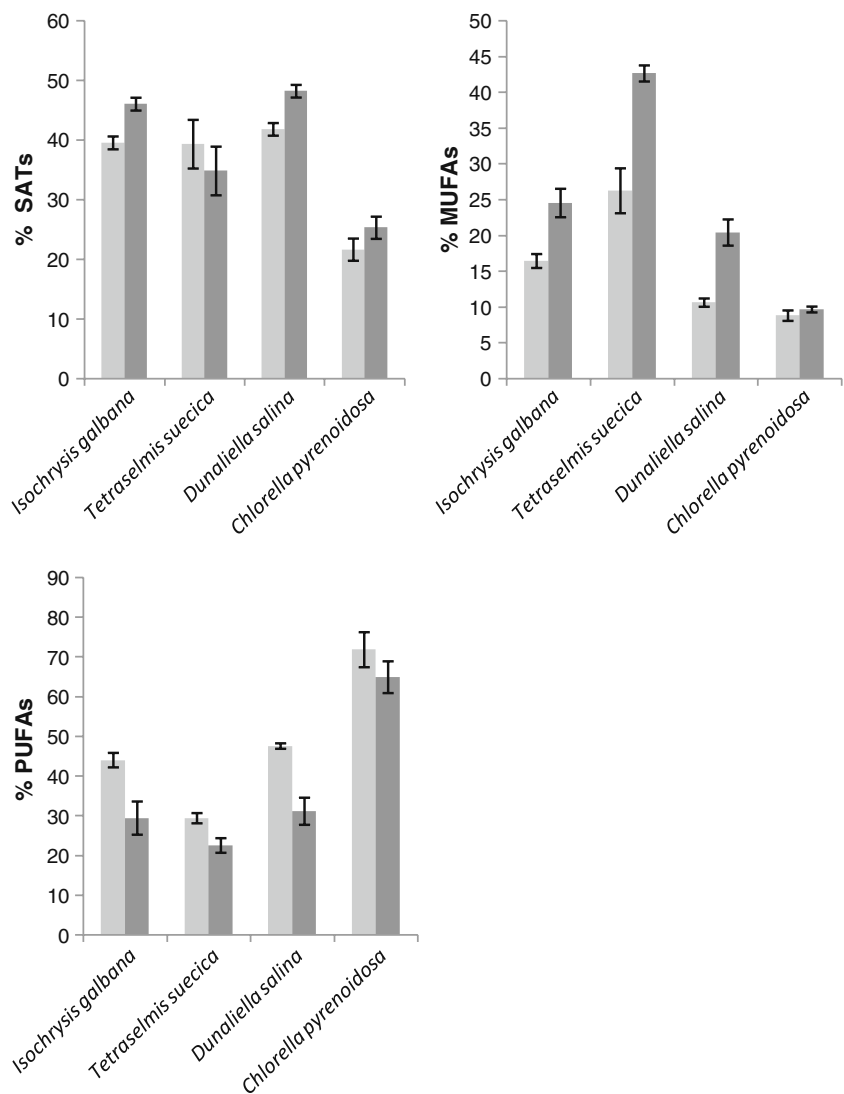
polar/neutral ratio is an indirect indicator of relative fatty acid composition in microalgae, the ability to quickly estimate this ratio by cytometry on a large number of samples in a short space of time (>3,000 events min⁻¹) and associate analyses with automatic cell sorting makes cytometry and the polar/neutral ratio an optimum method for conducting mass microalgae screening programmes for PUFA production or for other applications, e.g. biodiesel production where parameters such as the unsaturation index are extremely important (Ferrari and Sousa 2009).

In previous works, the inability to use the Nile Red method to quantify cellular polar lipids in microalgae has been indicated because overlap of fluorescent signal of chlorophyll and Nile Red in this lipids exists (Chen et al. 2009). This is true in the case of spectrofluorometry analysis, but in the flow cytometry the overlap of fluorescent is less decisive; since it is possible that an active compensation of the different signals, besides the usual problems of residual fluorescent in the spectrofluorometry analysis, is less important in flow cytometry, the data generated correspond

to an aggregate of individual fluorescents signals measured by cells. Another problem associated with the use of Nile Red in quantifying lipid in microalgae is related with the interspecific differences observed in the degree of cell permeation of Nile Red (Chen et al. 2009), but the present results suggest that this does not affect the relative values of fluorescence; the ratio of FL3/FL2 is significantly correlated with the ratio of polar/neutral lipids. However, despite these results, it is necessary to note that the proposed technique is mainly qualitative and that its application is mainly orientated to the work of mass screening of microalgae.

Despite the differences observed in the species studied, most of them follow a similar pattern in the variation of fatty acid content, which agrees with earlier findings for other species. Therefore, in restricted growth conditions such as nitrogen starvation, most species show a decrease in the unsaturation index (Roessler 1990). *C. pyrenoidosa* was the exception; no significant variation was observed in the content of fatty acids in both experimental conditions; probably, the culture of this species required a more prolonged exposure to the condition

Fig. 5 Variation in fatty acid content between control cultures (dark grey bars) and $-N$ cultures (light grey bars). Data expressed as percent of total fatty acids



of nitrogen starvation to be able to observe significant variations in the fatty acid composition.

Variations in the unsaturation index between the marine and hypersaline species studied occurred primarily in relation to variation in PUFA and MUFA content, whereas SATs showed high stability. This may suggest that PUFA and MUFA mobility in response to variations in culture conditions is more simple or more active, although it is also possible that mobility could be associated with greater impact of nitrogen starvation in the cultures on these fatty acids, which could be linked to the strong effect of nitrogen starvation on the photosynthesis system (Rao and Terry 2000). In this sense, PUFAs are the predominant fatty acids in the composition of structures with chloroplast membrane, and their role in membrane behaviour is decisive (Yokthongwattana et al. 2005; Wu et al. 2009). Earlier studies detected variation patterns in PUFA and MUFA content, particularly in the case of 18:1, in response to nitrogen starvation and activation of photoadaptation strategies (Mendoza et al. 1999). 18:1 is the initial fatty acid

in $-\omega 3$ PUFA synthesis and predominates in the composition of the species studied, in whose synthesis thylakoid membranes play a major role (Mustardy et al. 1996; Andreu et al. 2007). The metabolic relation between MUFAs (primarily oleic acid) and $-\omega 3$ PUFAs would explain why the greatest variations in response to nitrogen starvation are detected in the latter. The increase in MUFAs (primarily 18:1) in cultures with reduced nitrogen may correspond to a reduction in the synthesis activity of the PUFAs.

In general, as the results of this study show, fatty acid composition in microalgae has high plasticity. Production can be efficiently altered by modifying culture conditions, to the point that this method can be a decisive factor in producing fatty acids and biodiesel from microalgae (Gouveia et al. 2009). A readily available technique to quickly estimate the lipid composition of microalgae would be a key element in optimising cultures. In this sense, using Nile Red with spectrofluorimetric analysis techniques and relatively simple and inexpensive equipment could consti-

tute an effective alternative to traditional analysis techniques. Moreover, in just a few seconds, cytometry could provide a wide range of information on other essential aspects of culture viability, such as cell integrity, chlorophyll content and the presence of pollutants.

Acknowledgments This study was conducted with the support of the Spanish Ministry of Science and Innovation, through the PSE microalgae project (PSE-440000-2008-1-FEDER).

References

- Alonzo F, Mayzaud P (1999) Spectrofluorometric quantification of neutral and polar lipids in zooplankton using Nile Red. *Mar Chem* 67:289–301
- Andreu V, Collados R, Testillano PS, Risueño MC, Picorel R, Alfonso M (2007) In situ molecular identification of the pastid ω 3 fatty acid desaturase FAD7 from soybean: evidence of thylakoid membrane localization. *Plant Physiol* 145:1336–1344
- Bai J, Pegano RE (1997) Measurement of spontaneous transfer and transbilayer movement of BODIPY-labeled lipids in lipid vesicles. *Biochemistry* 36(29):8840–8848
- Chen W, Chengwu Z, Song L, Sommerfeld M, Hu Q (2009) A high throughput Nile red method for quantitative measurement of neutral lipids in microalgae. *J Microbiol Meth* 77:41–47
- Chisti Y (2007) Biodiesel from microalgae. *Biotechnol Adv* 25:294–306
- Cooksey KE, Guckert JB, Williams GA, Callis PR (1987) Fluorometric determination of the neutral lipid content of microalgal cells using Nile Red. *J Microbiol Methods* 6:333–346
- Da Silva TL, Santos CA, Reis A (2009) Multi-parameter flow cytometry as a tool to monitor heterotrophic microalgal batch fermentations for oil production towards biodiesel. *Biotechnol Bioprocess Eng* 14(3):330–337
- Davey HM, Kell DB (1996) Flow cytometry and cell sorting of heterogeneous microbial populations—the importance of single-cell analyses. *Microbiol Rev* 60:641–696
- de la Jara A, Mendoza H, Martel A, Molina C, Nordström C, de la Rosa V, Díaz R (2003) Flow cytometric determination of lipid content in marine dinoflagellate *Cryptocodinium cohnii*. *J Appl Phycol* 15:433–438
- Ferrari RA, de Souza WL (2009) Evaluation of oxidation stability of sunflower oil biodiesel with antioxidants. *Quim Nova* 32(1):106–111
- Gouveia L, Marques AE, da Silva TL, Reis A (2009) *Neochloris oleoabundans* UTX#1185: a suitable renewable lipid source for biodiesel production. *J Ind Microbiol Biotechnol* 36:821–826
- Griffith MJ, Harrison TL (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *J Appl Phycol* 21:493–507
- Guillard RRL (1975) Culture of phytoplankton for feeding marine invertebrates. In: Smith WL, Chanle MH (eds) *Culture invertebrate animals*. Plenum, New York, pp 26–60
- Guimarães ARP, Costa Rosa LFBP, Sitnik RH, Curi R (1991) Effect of polyunsaturates (PUFA n-6) and saturated fatty acid-rich diets on macrophage metabolism and function. *Biochem Int* 23:1739–1751
- Hu Q, Sommerfeld M, Jarvis E, Ghirardi E, Posewitz M, Seibert M, Darzins A (2008) Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. *Plant J* 54:621–639
- Juaneda P, Rocquelin G (1985) Rapid and convenient separation of phospholipids in rat heart using silica cartridges. *Lipids* 20:40–41
- Li Q, Du W, Liu D (2008) Perspective of microbial oils for biodiesel production. *Appl Microbiol Biotechnol* 80:749–756
- Mendoza H, Martel A, Jiménez del Río M, García-Reina G (1999) Oleic acid is the main fatty acid related with carotenogenesis in *Dunaliella salina*. *J Appl Phycol* 11:15–19
- Mendoza H, de la Jara A, Carmona Duarte L, Freijanes Presmanes K (2009) Estimate by means of flow cytometry of variation in composition of fatty acids from *Tetraselmis suecica* in response to culture conditions. *Aquac Int*. doi:10.1007/s10499-008-9235-1
- Muller-Feuga A (2000) The role of microalgae in aquaculture: situation and trends. *J Appl Phycol* 12:527–534
- Mustardy L, Los DA, Gombos Z, Murata N (1996) Immunocytochemical localization of acyl-lipid desaturases in cyanobacterial cells: evidence that both thylakoid membranes and cytoplasmic membranes are sites of lipid desaturation. *Proc Natl Acad Sci USA* 93:10524–10527
- Patil V, Källqvist T, Olsen E, Vogt G, Gislerød HR (2007) Fatty acid composition of 12 microalgae for possible use in aquaculture feed. *Aquac Int* 15:1–9
- Pulz O, Quac W (2004) Valuable products from biotechnology of microalgae. *J Appl Microbiol Biotechnol* 65:635–648
- Rao IM, Terry N (2000) Photosynthetic adaptation to nutrient stress. In: Yunus M, Pathre U, Mohanty P (eds) *Probing photosynthesis mechanisms, regulation and adaptation*. New York, Taylor & Francis, pp 380–397
- Roessler PG (1990) Environmental control of glycerolipids metabolism in microalgae: commercial implications and future research directions. *J Phycol* 26:393–399
- Semenenko VE, Abdullaev AA (1980) Parametric control of β -carotene biosynthesis in *Dunaliella salina* cells under conditions of intensive cultivation. *Sov Plant Physiol* 27:22–30
- Thi P, Borrel-Flood C, Vieira da Silva J, Justin AM, Mazliak P (1987) Effects on lipid metabolism in cotton leaves. *Phytochemistry* 24:723–727
- Ward OP, Singh A (2005) Omega-3/6 fatty acids: alternative sources of productions. *Proc Biochem* 40:3267–3652
- Wikfors GH, Ohno M (2001) Impact of algal research in aquaculture. *J Phycol* 37:968–974
- Wu Q, Liu T, Liu H, Zheng GC (2009) Unsaturated fatty acid: metabolism, synthesis and gene regulation. *Afr J Biotechnol* 8:1782–1785
- Yokthongwattana K, Savchenko T, Polle JEW, Melis A (2005) Isolation and characterization of a xanthophylls-rich fraction from the thylakoid membrane of *Dunaliella salina* (green algae). *Photochem Photobiol Sci* 4:1028–1034
- Yongmanitchai W, Ward OP (1992) Separation of lipid classes from *Phaeodactylum tricornutum* using silica cartridges. *Phytochemistry* 31:3405–3408