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ORIGINAL PAPER

Changes in Lipid Class and Fatty Acid Composition of Cultures of *Pavlova lutheri*, in Response to Light Intensity

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Abstract Aquaculture is a growing commercial activity worldwide, which resorts more and more often to microalgae as feed; the lipid composition of such microalgae is a critical factor with regard to the fish growth rate upon ingestion. The aim of this work was thus to study the influence of light intensity on the lipid profile of a known microalga, *Pavlova lutheri*. Several semi-continuous cultures were carried out, and biochemical parameters such as lipid, protein, carbohydrate, and chlorophyll contents were quantified. Lipids were specifically fractionated into classes by TLC, and those in each class were subjected to GC afterwards in an attempt to ascertain their fatty acid profile. Evidence was consequently provided which showed that cultures grown under low light intensity (9 W m^{-2}) possess a higher fraction of eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids esterified in polar classes—which are those with a more favorable role in aquaculture. It was also demonstrated that intermediate levels of light intensity (19 W m^{-2}) may be misleading in terms of favorable effects upon EPA and DHA contents—because there is an increase in their total yields and productivities, but they appear mostly esterified into triacylglycerols; this may be a favorable deed for production and purification, but is metabolically not so effective in aquaculture. The highest EPA and DHA productivities attained were 1.29 and 0.69 mg L^{-1} day^{-1}, respectively, at intermediate levels of light intensity (19 W m^{-2}).

Keywords EPA · DHA · Chromatographic analysis · Microalgae · Processing factors

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Introduction

Microalgae are widely employed in the aquaculture industry to directly feed crustacean, fish and bivalves—or as feed for zooplankton, which are in turn used as part of the diet of fish and crustaceans [1, 2]. Therefore, studies pertaining to the biochemical composition of microalgae which will be eventually employed as feed are desirable, as their nutritional quality plays a crucial role in the growth rate of animals reared thereon.

Several studies encompassing polyunsaturated fatty acids (PUFA)—especially eicosapentaenoic acid [EPA—20:5(n-3)] and docosahexaenoic acid [DHA—22:6(n-3)], have established their relevance to human health [3–5]; they have also supported their practical importance for commercial microalga production, should they be used as primary source of nutrients in aquaculture [2]. It should be remembered that fish are still at present the single most important source of PUFA in the human diet; however, fish can hardly synthesize EPA and DHA themselves [6, 7], instead they accumulate those compounds from their diet—mainly PUFA-rich microalgae. Therefore, it is more logical to act at the basis of the food chain, in attempts to optimize performance thereafter, eventually at the human nutrition level. On the other hand, it is known that PUFA esterified in polar lipid classes are more effective for nutrition of

cultured fish than those esterified in neutral lipids [8]. Hence, studies pertaining to microalgal fatty acid composition—bearing in mind the actual lipid classes where they appear, will be an important tool for the optimization of their nutritional value as feed for fish, and directly or indirectly as vectors of PUFA for the human being.

In fact, most studies pertaining to microalgal lipids have so far focussed only on total lipids or on total individual fatty acids. However, a better understanding of their metabolic performance demands extra information on the distribution of such important PUFA as EPA and DHA within the lipid pool. On the other hand, despite realization that microalgae can be used as an industrial source of PUFA, this is not yet economically feasible—owing to productivity constraints, coupled with downstream concentration and purification requirements; the distribution of EPA and DHA within the various types of microalgal lipids, and their companion fatty acids in the lipid molecules are thus critical issues for the market applicability of microalgae [8]. In particular, preferential distribution of given fatty acid residues among the various existing lipid classes may contribute to designing more effective separation strategies, especially aimed at eventual scale-up.

Pavlova lutheri—a small flagellate; is one good example of a microalga able to produce PUFA to a considerable extent; hence, it is often used in aquaculture [9–11]. It can be cultivated in bioreactors under controlled conditions—which permit consistent rates of biomass production and metabolism, irrespective of the outer environmental parameters and the geographical location. Manipulation of such processing conditions as light intensity, nutrient status or temperature then allows modulation of the lipid composition of the final microalgae [2, 12–14], and consequent optimization of their overall yield and productivity. This optimization can follow the objective function of improving the nutritional value of microalgal food (for aquaculture) and/or boosting the PUFA overall production (for the pharmaceutical industry).

As mentioned above, several environmental factors (viz. temperature, salinity, light and nutrients); as well as culture time affect the growth and biochemical composition of microalgae [15, 16]. Significant variations in lipid contents and fatty acid profiles have indeed been observed in response to distinct growth conditions [17, 18]. However, most data available in the literature pertain to the influence of only the temperature on the fatty acid composition of microalgae [19, 20]. It is generally recognized that an increase in PUFAs is one of the ways used by marine microalgae to acclimatize to low-temperature conditions, and still maintain membrane fluidity [21, 22]. Furthermore, the fatty acid composition is also known to be affected by the level of irradiance [23, 24] to extents that are species-specific [25]. High PUFA levels were observed under low light, thus suggesting that the biochemical composition of chloroplasts adapts to low irradiance intensities via increasing PUFA synthesis [26]. However, the highest proportions of EPA were obtained at low levels of irradiance, whereas DHA levels were highest under high light intensities in most species [25, 27].

The major goal of this research effort was therefore to assess the influence of incident light intensity on growth and biochemical composition of *P. lutheri,* especially in terms of lipids and fatty acid residues—as well as in terms of proteins, chlorophylls and carbohydrates, in controlled, bench-scale photobioreactors operated semi-continuously. The distribution of fatty acids (with a special emphasis on EPA and DHA) was ascertained by thin layer and gas chromatographies. The production rates of both EPA and DHA by this microalga were also assayed, under various levels of light and at several physiological states, using a light control device described in detail elsewhere [28].

Materials and Methods

Culture Conditions

Pavlova lutheri SMBA 60 was kindly supplied by IPIMAR (Instituto Português de Investigação MARítima, Portugal). The culture medium was artificial sea water ASW [12]; a working volume of 1.5 L was utilized in a 2-L Biostat B fermentor (Braun, Germany), operated semi-continuously. The temperature was kept at 20 ± 0.5 °C via a refrigeration jacket, stirring was at 50 rpm, and pure air enriched with 0.3% (v/v) CO_2 was bubbled in at the bottom at a volumetric flow rate of 0.8 L min^{-1}; as soon as admission of CO_2 started, the pH was maintained at 8.00 via automatic addition of 1 M NaOH.

Quasi-steady-state (QSS) cultures were achieved with batch-fed daily dilutions, and adjusted every day so as to keep essentially the same initial biomass concentration at each new stage. For each average light intensity level tested (viz. 9, 19 and 30 W m^{-2}), several stages were conducted until three consecutive equal dilutions resulted; the culture was then considered to have attained QSS. For each light intensity under a given QSS, three samples were taken sequentially for biochemical assaying; these samples were freeze-dried and stored under nitrogen, until analysis was carried out.

Analytical Assays

The biomass was monitored on-line by a flow injection analysis (FIA) device containing two sample loops, each providing its own dilution rate of the culture medium. To

calculate the biomass concentration, the detector response was calibrated previously with data pertaining to both viable cell numbers and ash-free-dry-weight (AFDW). The type of loop and the tube length used for dilution, as well as the flow rate of the fluid carrier had been optimized so as to provide the best combination of two dilutions that would cover the whole growth range within the linear correlation zone—as described previously to some length [29]. Off-line measurements of AFDW were done by filtering 5 mL of culture through preconditioned GF/C glass fiber filters (Whatman, UK), drying at 100 °C to constant weight and finally heating to 550 °C for 1 h.

The total lipid extracts were obtained from duplicate, spray-dried samples (200 mg), after five to seven sequential extractions, each with a 2-mL mixture of *n*-hexane/2-propanol (3:2, v/v), as described elsewhere [30].

Samples were then dried in a rotavapor, weighed, resuspended in a mixture of chloroform/methanol (2:1, v/v), and finally stored at −30 °C under nitrogen (to prevent lipid oxidation).

The aforementioned lipid extracts were separated into polar and nonpolar fractions, using solid-phase extraction: 500-mg LC-Diol cartridges (Sigma, USA) were equilibrated with 2 mL of methanol and 2 mL of chloroform; ca. 50 mg of the oil sample dissolved in chloroform was placed in the cartridges using a micropipette; and the nonpolar fraction was then eluted with 2.5 mL of chloroform, whereas the polar fraction was eluted with 7 mL of a 100:0.5 (v/v) mixture of methanol/25% (v/v) aqueous ammonia.

Both polar and nonpolar fractions were fractionated afterwards, for lipid class separation and identification, by one-dimensional thin-layer chromatography using TLC plates (20 × 20 cm) coated with silica gel 60 (Merck). Polar lipids were eluted with a 5-component mixture of chloroform/acetone/methanol/acetic acid/water (50:20:10:10:5, v/v), and nonpolar lipids with a complex mixture of petroleum ether (boiling point range 60–80 °C)/diethyl ether/acetic acid (80:20:1, v/v). Each individual class was then subjected to gas chromatography for quantification of its fatty acid profile, after direct transmethylation. The resulting esters were analyzed in a gas chromatograph (Perkin-Elmer), using detection by flame ionization. Pure standards of fatty acids (Sigma) were injected for tentative identification based on retention time—following the protocol reported in full by Meireles et al. [31].

Proteins were determined by the Lowry method [32], after hydrolysis with 2 M NaOH for 15 min in an ultrasonicator, followed by heating to 100 °C for 5 min. Carbohydrates were determined using the phenol/sulfuric acid method [33], after calibration with glucose. Chlorophyll *a* was extracted with 90% (v/v) acetone, and quantified by spectrophotometry at 664 and 630 nm [34].

Statistical Analyses

Analysis of variance (ANOVA) and Tukey's tests (when ANOVA indicated at least one significantly different result) were applied to the experimental data set of total lipids, proteins and carbohydrates. Each datum point was replicated three times—so as to obtain a representative measure of variability, thus allowing ANOVA to pinpoint differences between the various parameters that characterize the biochemical profile. These true replicates used in turn two analytical replicates, for each chemical assay.

Results and Discussion

Biomass Growth

The typical biomass figures generated in the various semi-continuous stages are depicted in Fig. 1. After a starting batch period, the culture was maintained in semi continuous mode for several days, and the dilution rate was adjusted daily so as to periodically recover the initial biomass concentration. The biomass interval was kept within the range 1.3–2.0 × 10^7 cell mL^{-1}, for all conditions tested, so that it would be possible to assess quantitative differences in the biochemical profiles that were directly influenced by the prevailing light intensity.

Biochemical Profile

The biochemical profile—in terms of total lipids, proteins and carbohydrates, is depicted in Fig. 2. No significant ($P < 0.05$) differences could be found in the total amount of proteins and carbohydrates versus light intensities—

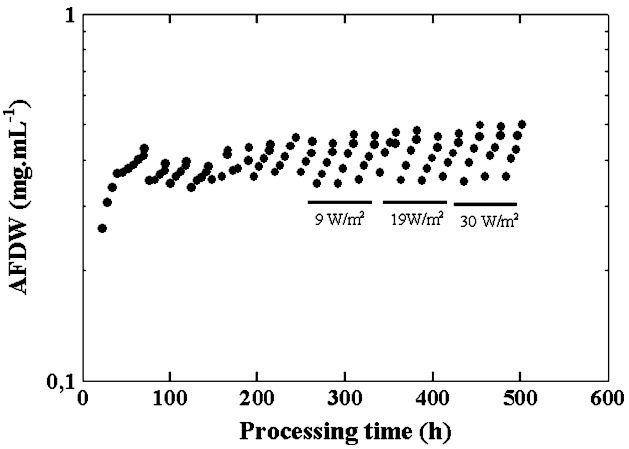

Fig. 1 Variation of overall biomass, as ash-free-dry-weight (AFDW), of *P. lutheri* with the processing time in cultures grown under sequential QSS semi-continuous stages at 20 °C, pH 8, 50 rpm and bubbling of air enriched with 0.3 % (v/v) CO_2

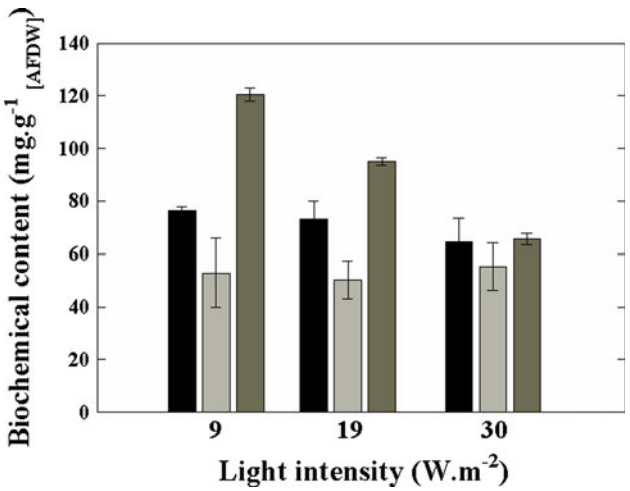

Fig. 2 Variation of biochemical composition of *P. lutheri* with different light intensities: *black-shaded bar* protein content, *light gray-shaded bar* carbohydrate content and *gray-shaded bar* total lipid content (means ± standard deviation of 3 replicates)

except for carbohydrates at 30 W m^{-2}, for which there was a slight increase ($P < 0.05$). This overall trend is probably due to the similarity in the amounts of nutrients provided to all cultures; it is known that such parameters as nitrogen and phosphorus levels can influence protein and carbohydrate syntheses [35], and there was no carbon- or nitrogen-limitation in our experiments. In terms of lipids, there was a significant decrease ($P < 0.05$) in their total amount as light intensity increased. This type of observation—which is usually species-specific, has been recorded previously for other microalgal species [36]; its rationale lies on the fact that lipids are major constituents of chloroplasts, so an increase in light intensity overcomes the need for a high chloroplastidial activity—as further confirmed by the cellular chlorophyll *a* content (see Fig. 3).

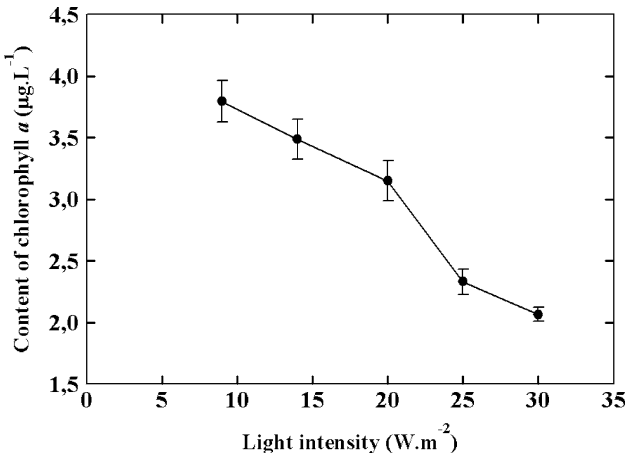

Fig. 3 Variation of chlorophyll *a* content of *P. lutheri* with light intensity at 20 °C, pH 8, 50 rpm and bubbling of air enriched with 0.3% (v/v) CO_2 (means ± standard deviation of 3 replicates)

Lipid Class Composition

The lipid class composition, under various light intensities, is presented in Table 1—whereas the fatty acid profile of each class is presented in Tables 2, 3 and 4, for light intensities of 9, 19 and 30 W m^{-2}, respectively. The major constituents of *P. lutheri* were nonpolar lipids—composed of mono-, di- and triacylglycerols (MAG, DAG and TAG, respectively), and glycolipids (GL)—composed of mono- and digalactosylacylglycerols, and acylated steryl glycosides (MGDG, DGDG and SG, respectively). Phospholipids (PL)—composed of phosphatidylglycerol (PG) and diphosphatidylglycerol (DPG), as well as sulfolipids (mainly sulfoquinovosylacylglycerol, SQDG) were also present but at smaller levels, as described by other authors [21, 37, 38]. Surprisingly, only trace amounts of betaine lipids (BL) were found, and only in cultures subjected to the lowest light level. However, owing to its lipid profile, the unknown class (UN) may eventually encompass BL [31]; note that UN was present at all light intensities.

From 9 to 19 W m^2, there was a dramatic change in the lipid composition of *P. lutheri*: the amount of TAG increased from 23.3 to 78.3% of the total fatty acids (TFA). Despite the decrease in MAG and DAG contents, the amount of nonpolar lipids increased from 62.5 to 90.0%, owing to the aforementioned increase in TAG. Similar amounts of TAG had already been described in cultures of *Phaedactylum tricornutum* grown under nitrogen deprivation [39]. The amount of DAG found in cultures exposed to 9 and 19 W m^2 was unusually high, yet the amount of TAG at 9 W m^{-2} was rather low; keeping the culture at a very low light intensity may have interfered with conversion of DAG to TAG in the lipid biosynthesis pathway. Our experimental data provide evidence for the actual presence of DAG in microalgal lipids—a matter that has often been controversial, because the small amounts (when found) have classically been attributed to analytical artifacts [8, 31]. A further increase in light intensity to 30 W m^{-2} produced a decrease in the total amount of TAG, which is probably explained by the lower amounts of total lipids; it is important to note that the fraction of TAG in the total lipids increased always with increasing light intensities.

Concerning GL, it is interesting to observe that MGDG was a major constituent of the microalgae subject to 9 W m^{-2}, second only to TAG—but it decreased to a very low level when light intensity was increased to 19 W m^{-2}; a further increase in light had no effect. On the other hand, the amounts of DGDG remained essentially unchanged under all light intensities. It was suggested [40, 41] that MGDG (which encompasses a considerable amount of EPA) is one of the major components of chloroplast membranes; as discussed previously, an increase in light intensity precludes the need for highly efficient

Table 1 Variation of total mass of lipid classes of *P. lutheri* with different light intensities (means ± standard deviation of 3 replicates)

Total concentration (mg g_{AFDW}^{-1})											
Light intensity (W m^{-2})	SQDG	DGDG	DPG	MGDG	PG	SG	UN	MAG	DAG	TAG	FFA
9	0.247 ± 0.069	0.114 ± 0.008	0.109 ± 0.016	1.232 ± 0.325	0.166 ± 0.061	0.122 ± 0.001	0.151 ± 0.024	0.294 ± 0.112	2.068 ± 0.155	1.422 ± 0.222	0.174 ± 0.081
19	0.161 ± 0.015	0.119 ± 0.049	0.085 ± 0.029	0.055 ± 0.000	0.102 ± 0.028	0.238 ± 0.003	0.053 ± 0.012	0.214 ± 0.115	0.965 ± 0.026	7.607 ± 0.554	0.116 ± 0.030
30	0.145 ± 0.000	0.151 ± 0.000	0.069 ± 0.011	0.066 ± 0.000	0.055 ± 0.010	0.268 ± 0.055	0.066 ± 0.001	0.251 ± 0.004	0.446 ± 0.000	4.446 ± 1.906	0.302 ± 0.250

DAG diacylglycerols, *DGDG* digalactosylacylglycerols, *DPG* diphosphatidylacylglycerols, *FFA* free fatty acids, *MAG* monoacylglycerols, *MGDG* monogalactosylacylglycerols, *PG* phosphatidylglycerols, *TAG* triacylglycerols, *SG* acylated sterol glycosides, *SQDG* sulfoquinovosyldiacylglycerols, *UN* unidentified phospholipid class

chloroplasts, so a reduction in PUFA content is expected [40]—indeed, the dramatic decrease in total MGDG is mostly accounted for by the decrease of EPA. Unlike MGDG, generation of SG increased consistently with increasing light intensity; this increase was, however, chiefly due to increases in saturated fatty acid residues. The contents of GL were normally much higher under higher light intensity; this might be because MGDG, DGDG and SQDG are strongly associated with photosynthetic membranes in microalgae [42].

Phospholipids—an essential component of the cell membranes, represented ca. 5% of the TFA in cultures subject to 9 W m^{-2}, but decreased in concentration with increasing light intensity; again, this decrease could be rationalized by a decrease in PUFA, mainly DHA—remember that EPA is apparently more concentrated in GL, and DHA more concentrated in PL [31].

The SQDG class presented a typical behavior that was similar to that of PL. It was reported above that an increase in TAG produces a decrease in the former, yet this decrease was more intense from 9 to 19 W m^{-2}; although the total amount decreased, the fraction of SQDG in TFA barely changed from 19 to 30 W m^{-2}, when it accounted for 1.3 and 1.1% of TFA, respectively.

Finally, assuming that UN is essentially constituted by a Betaine lipid, a decrease in BL with increasing light intensity was eventually recorded.

It should be further emphasized that the decrease in GL, PL and SQDG, observed when the culture was changed from 19 to 30 W m^{-2}, was simply a result of the reduction of TFA—since the fraction of TFA in this class remained essentially unchanged. In fact, a strong variation in said class distribution only occurred when the culture was changed from 9 to 19 W m^{-2}; this observation may imply that the culture was near light saturation at 19 W m^{-2}, and was accordingly characterized by a particularly low biomass concentration—otherwise, a similar substantial decrease in the polar classes should have resulted, when going from 19 to 30 W m^{-2}.

Fatty Acid Composition

Changes in the fatty acid composition in microalgae are often related to the relative amounts of the various lipid classes present—which exhibit distinctive fatty acid profiles [14]. In general, cells grown under high light intensity are characterized by extensive triacylglycerol synthesis, which leads to a low PUFA content and high levels of 16:0 and 16:1n-7 fatty acids [14].

It has been found that *P. lutheri* contained higher levels of the dominant fatty acids 16:0 and DHA, and lower levels of 18:4n-3 and EPA when light intensity was higher, thus suggesting that the increase in TFA was correlated

Table 2 Concentration by lipid class, of each type of fatty acid, in *P. lutheri* (means ± standard deviation of 3 replicates), and corresponding mass percent (means, in italic) in cultures grown at a light intensity of 9 W m^{-2}

Fatty acid	Lipid class (mg g_{AFDW}^{-1})/(%, *w/w*)										
	SQDG	DGDG	DPG	MGDG	PG	SG	UN	MAG	DAG	TAG	FFA
C14:0	70.58 ± 28.42	17.81 ± 1.63	4.35 ± 0.15	45.65 ± 13.62	15.70 ± 8.06	24.27 ± 10.73	11.10 ± 2.88	tr	56.37 ± 1.20	97.46 ± 33.03	tr
	30.80	*43.86*	*5.72*	*4.08*	*12.79*	*24.84*	*10.35*	–	*9.68*	*7.24*	–
C16:0	77.41 ± 35.22	21.44 ± 2.05	16.94 ± 0.76	45.57 ± 22.94	22.28 ± 14.82	25.23 ± 5.51	37.60 ± 1.66	33.61 ± 31.52	42.13 ± 11.29	252.33 ± 152.43	10.53 ± 6.16
	33.78	*0.47*	*22.28*	*4.07*	*18.16*	*25.82*	*35.04*	*33.34*	*7.23*	*18.74*	*44.38*
C16:1(n-7)	19.84 ± 10.62	16.08 ± 5.98	2.53 ± 0.93	21.30 ± 4.55	24.01 ± 21.18	7.09 ± 6.68	6.78 ± 3.02	19.64 ± 11.62	101.66 ± 3.56	469.41 ± 72.54	16.37 ± 17.18
	8.66	*0.42*	*3.32*	*1.90*	*19.56*	*7.26*	*6.32*	*19.48*	*17.45*	*34.87*	*15.77*
C18:0	5.94 ± 0.27	4.33 ± 0.78	5.20 ± 0.51	14.44 ± 6.65	4.13 ± 1.10	6.14 ± 1.56	4.88 ± 1.15	8.09 ± 1.36	14.39 ± 0.60	21.63 ± 5.59	8.01 ± 1.30
	2.59	*0.28*	*6.84*	*1.29*	*3.36*	*6.28*	*4.55*	*8.02*	*2.47*	*1.61*	*7.72*
C18:1(n-9)	5.67 ± 1.53	4.84 ± 1.03	4.44 ± 1.52	6.01 ± 1.44	5.97 ± 0.81	7.74 ± 2.49	5.59 ± 0.20	6.76 ± 2.39	258.04 ± 32.24	26.65 ± 8.60	3.56 ± 1.15
	2.47	*0.30*	*5.83*	*0.54*	*4.86*	*7.92*	*5.21*	*6.71*	*44.31*	*1.98*	*3.43*
C18:1(n-7)	3.54 ± 0.29	1.68 ± 0.77	1.12 ± 0.37	9.56 ± 5.53	3.90 ± 1.48	2.19 ± 1.04	5.12 ± 1.41	tr	18.30 ± 0.10	12.51 ± 5.37	tr
	1.55	*0.18*	*1.47*	*0.85*	*3.18*	*2.24*	*4.78*	–	*3.14*	*0.93*	–
C18:2(n-6)	4.32 ± 4.42	8.26 ± 3.09	5.24 ± 5.80	11.77 ± 4.13	17.61 ± 3.40	11.47 ± 5.44	12.92 ± 2.00	4.77 ± 2.24	29.58 ± 7.34	49.03 ± 7.60	1.97 ± 0.14
	1.89	*0.38*	*6.89*	*1.05*	*14.35*	*11.74*	*12.04*	*4.73*	*5.08*	*3.64*	*1.90*
C18:3(n-6)	3.26 ± 0.54	2.91 ± 0.23	2.79 ± 0.33	43.44 ± 23.21	3.06 ± 1.14	3.05 ± 0.38	3.15 ± 0.45	7.18 ± 1.34	20.99 ± 20.29	31.81 ± 6.95	6.36 ± 0.03
	1.42	*0.23*	*3.67*	*30.88*	*2.49*	*3.12*	*2.93*	*7.12*	*3.60*	*2.36*	*6.13*
C18:3(n-3)	0.10 ± 0.17	tr	tr	138.92 ± 86.81	2.59	0.69 ± 0.31	0.54 ± 0.20	6.95 ± 5.12	19.02 ± 8.00	17.87 ± 6.43	tr
	0.04	–	–	*12.40*	*2.11*	*0.70*	*0.51*	*6.89*	*3.27*	*1.33*	–
C18:4(n-3)	0.82 ± 0.29	2.18 ± 1.42	tr	335.71 ± 85.20	0.94 ± 1.33	1.61 ± 1.11	0.75 ± 0.12	4.82 ± 4.05	6.26 ± 1.65	76.23 ± 13.84	tr
	0.36	*0.17*	–	*29.97*	*0.77*	*1.65*	*0.70*	*4.78*	*1.07*	*5.66*	–
C20:5(n-3)	31.69 ± 3.84	30.80 ± 8.01	2.22 ± 1.14	441.09 ± 66.23	16.31 ± 3.17	2.12 ± 1.62	4.83 ± 2.49	4.73 ± 1.99	7.37 ± 0.16	151.46 ± 69.68	2.09 ± 0.66
	13.83	*0.54*	*2.92*	*39.39*	*13.29*	*2.17*	*4.50*	*4.69*	*1.27*	*11.25*	*2.01*
C22:6(n-3)	5.95 ± 3.00	5.84 ± 4.62	31.20 ± 5.87	6.52 ± 1.47	6.22 ± 5.09	6.10 ± 5.58	14.04 ± 0.14	4.27 ± 1.83	8.29 ± 6.31	139.96 ± 2.45	19.37 ± 12.58
	2.60	*0.27*	*41.04*	*0.58*	*5.07*	*6.25*	*13.08*	*4.23*	*1.42*	*10.40*	*18.66*
TFA	229.12 ± 46.98	116.17 ± 11.92	76.03 ± 8.58	1119.98 ± 143.35	122.72 ± 28.07	97.70 ± 16.27	107.30 ± 5.83	100.82 ± 34.54	582.40 ± 41.87	1346.35 ± 186.87	68.26 ± 22.24
	100.00	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*

tr traces, *TFA* total fatty acids, *DAG* diacylglycerols, *DGDG* digalactosylacylglycerols, *DPG* diphosphatidylacylglycerols, *FFA* free fatty acids, MAG monoacylglycerols, *MGDG* monogalactosylacylglycerols, *PG* phosphatidylglycerols, *TAG* triacylglycerols, *SG* acylated sterol glycosides, *SQDG* sulfoquinovosyldiacylglycerols, *UN* unidentified phospholipid class

Table 3 Concentration by lipid class, of each type of fatty acid, in *P. lutheri* (means ± standard deviation of 3 replicates), and corresponding mass percent (means, in italic) in cultures grown at a light intensity of 19 W m^{-2}

Fatty acid	Lipid class (mg g_{AFDW}^{-1})/(%, w/w)										
	SQDG	DGDG	DPG	MGDG	PG	SG	UN	MAG	DAG	TAG	FFA
C14:0	38.03 ± 4.48	36.89 ± 2.77	22.79 ± 9.11	7.80 ± 1.46	7.55 ± 1.94	42.91 ± 3.57	9.49 ± 0.46	17.49 ± 7.97	40.79 ± 8.48	185.37 ± 56.64	tr
	27.15	*19.21*	*21.70*	*13.61*	*8.12*	*17.71*	*18.46*	*13.61*	*7.90*	*5.54*	–
C16:0	7.11 ± 0.40	27.00 ± 1.23	35.32 ± 19.58	14.99 ± 4.73	35.68 ± 9.80	38.29 ± 0.93	15.48 ± 1.04	22.40 ± 6.98	39.84 ± 0.19	635.08 ± 110.12	14.14 ± 2.52
	5.08	*14.06*	*33.63*	*26.13*	*38.37*	*15.80*	*30.10*	*17.43*	*7.72*	*19.00*	*26.60*
C16:1(n-7)	5.27 ± 0.51	12.42 ± 7.27	8.60 ± 2.19	19.18 ± 0.86	12.53 ± 6.64	132.91 ± 20.23	3.40 ± 0.43	33.87 ± 24.43	55.64 ± 21.08	1.051.60 ± 251.49	6.02 ± 1.42
	3.76	*6.47*	*8.19*	*33.45*	*13.48*	*54.85*	*6.62*	*26.36*	*10.78*	*31.46*	*11.32*
C18:0	4.60 ± 0.84	4.09 ± 1.12	6.25 ± 4.40	4.27 ± 1.22	5.56 ± 1.56	4.63 ± 1.70	4.95 ± 0.41	12.15 ± 2.25	13.08 ± 1.72	37.06 ± 11.32	5.92 ± 0.26
	3.29	*2.13*	*5.95*	*7.44*	*5.98*	*1.91*	*9.63*	*9.45*	*2.53*	*1.11*	*11.14*
C18:1(n-9)	3.96 ± 1.68	4.18 ± 1.22	4.04 ± 0.63	4.97 ± 0.95	6.65 ± 0.71	7.37 ± 1.40	3.89 ± 0.31	6.10 ± 0.13	243.53 ± 9.83	55.99 ± 7.59	2.98 ± 0.18
	2.83	*2.18*	*3.84*	*8.66*	*7.16*	*3.04*	*7.57*	*4.75*	*47.16*	*1.67*	*5.61*
C18:1(n-7)	1.43 ± 0.33	2.79 ± 0.49	2.55 ± 1.09	1.64 ± 0.53	12.88 ± 4.08	3.16 ± 0.74	1.25 ± 0.31	0.99 ± 0.79	20.54 ± 2.00	29.57 ± 4.82	4.43 ± 1.79
	1.02	*1.45*	*2.43*	*2.86*	*13.85*	*1.30*	*2.43*	*0.77*	*3.98*	*0.88*	*8.34*
C18:2(n-6)	3.24 ± 1.58	2.77 ± 0.13	5.26 ± 3.04	tr	tr	tr	3.02 ± 0.19	5.37 ± 0.78	27.03 ± 1.91	149.02 ± 36.49	2.56 ± 0.86
	2.31	*1.44*	*5.01*	–	–	–	*5.87*	*4.18*	*5.24*	*4.46*	*4.81*
C18:3(n-6)	tr	2.08 ± 0.73	3.72 ± 1.57	3.11 ± 0.62	2.67 ± 0.04	2.82 ± 0.22	2.71 ± 0.31	6.52 ± 0.20	6.18 ± 0.46	10.84 ± 6.33	6.44 ± 0.48
	–	*1.08*	*3.54*	*5.42*	*2.87*	*1.16*	*5.26*	*5.08*	*1.20*	*0.32*	*12.12*
C18:3(n-3)	tr	0.53 ± 0.18	tr	tr	0.68 ± 0.11	1.29 ± 0.55	tr	2.15 ± 0.99	4.73 ± 0.28	168.88 ± 86.11	tr
	–	*0.28*	–	–	*0.73*	*0.53*	–	*1.67*	*0.92*	*5.05*	–
C18:4(n-3)	6.28 ± 1.54	4.97 ± 2.06	tr	tr	tr	3.19 ± 2.09	tr	5.66 ± 4.93	14.13 ± 1.30	221.73 ± 93.33	2.37 ± 0.22
	4.49	*2.59*	–	–	–	*1.32*	–	*4.40*	*2.74*	*6.63*	*4.46*
C20:5(n-3)	66.27 ± 5.07	91.11 ± 23.39	3.00 ± 1.37	0.17 ± 0.24	4.63 ± 1.67	4.32 ± 2.07	1.27 ± 0.50	13.14 ± 11.79	42.07 ± 17.29	537.09 ± 175.74	3.07 ± 1.26
	47.31	*47.46*	*2.86*	*0.30*	*4.98*	*1.78*	*2.47*	*10.22*	*8.15*	*16.07*	*5.78*
C22:6(n-3)	3.88 ± 0.91	3.16 ± 2.22	13.49 ± 2.85	1.23 ± 0.39	4.15 ± 0.07	1.42 ± 0.12	5.97 ± 2.43	2.66 ± 1.09	8.80 ± 4.02	260.93 ± 75.53	5.22 ± 0.96
	2.77	*1.64*	*12.85*	*2.14*	*4.46*	*0.58*	*11.60*	*2.07*	*1.70*	*7.80*	*9.82*
TFA	140.07 ± 7.45	191.99 ± 24.94	105.02 ± 22.67	57.36 ± 5.34	92.98 ± 12.90	242.31 ± 20.91	51.43 ± 2.85	128.5 ± 29.68	516.36 ± 30.67	2291.56 ± 364.52	53.15 ± 22.24
	100.00	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*

tr traces, *TFA* total fatty acids, *DAG* diacylglycerols, *DGDG* digalactosylacylglycerols, *DPG* diphosphatidylacylglycerols, *FFA* free fatty acids, *MAG* monoacylglycerols, *MGDG* monogalactosylacylglycerols, *PG* phosphatidylglycerols, *TAG* triacylglycerols, *SG* acylated sterol glycosides, *SQDG* sulfoquinovosyldiacylglycerols, *UN* unidentified phospholipid class

Table 4 Concentration by lipid class, of each type of fatty acid, in *P. lutheri* (means ± standard deviation of 3 replicates), and corresponding mass percent (means, in italic) in cultures grown at a light intensity of 30 W m^{-2}

Fatty acid	Lipid class (mg g^{-1}_{AFDW})/*(%, w/w)*										
	SQDG	DGDG	DPG	MGDG	PG	SG	UN	MAG	DAG	TAG	FFA
C14:0	43.56 ± 4.58	9.47 ± 3.16	22.12 ± 4.27	1.33	7.41 ± 2.21	66.87 ± 6.19	17.44 ± 5.11	4.07	28.78 ± 21.15	156.47 ± 53.08	tr
	32.63	*7.17*	*32.24*	*9.95*	*13.55*	*25.22*	*29.11*	*2.83*	*8.38*	*6.78*	–
C16:0	62.87 ± 7.30	71.52	24.45 ± 4.95	3.35	22.52 ± 5.86	57.88 ± 7.84	23.71 ± 3.21	18.75 ± 0.05	52.04 ± 26.30	466.21 ± 159.76	16.18 ± 4.47
	47.10	*54.17*	*35.64*	*25.11*	*41.17*	*21.83*	*39.59*	*13.04*	*15.15*	*20.21*	*7.45*
C16:1(n-7)	7.80 ± 2.76	19.44 ± 12.39	5.39 ± 0.56	1.55	5.01 ± 0.59	118.19 ± 37.24	3.77 ± 2.10	7.05 ± 1.89	51.63 ± 30.87	774.36 ± 285.03	6.49 ± 1.78
	5.84	*14.72*	*7.85*	*11.62*	*9.15*	*44.58*	*6.29*	*4.90*	*15.03*	*33.56*	*2.99*
C18:0	6.43 ± 0.16	7.69 ± 1.55	4.60 ± 1.03	1.12	5.23 ± 1.58	6.41 ± 0.58	5.75 ± 0.13	12.54 ± 3.24	14.18 ± 4.97	20.64 ± 8.99	9.01 ± 0.09
	4.81	*5.82*	*6.70*	*8.40*	*9.56*	*2.42*	*9.59*	*8.72*	*4.13*	*0.89*	*4.15*
C18:1(n-9)	4.28 ± 0.16	6.47 ± 1.12	3.62 ± 0.09	0.92	3.67 ± 0.40	6.96 ± 0.83	4.88 ± 0.64	40.76 ± 21.84	80.72	38.84 ± 17.15	8.09 ± 5.60
	3.20	*4.90*	*5.27*	*6.87*	*6.70*	*2.62*	*8.14*	*28.34*	*23.49*	*1.68*	*3.73*
C18:1(n-7)	3.62 ± 0.26	14.16 ± 3.75	1.69 ± 0.45	0.00	3.81 ± 1.02	3.29 ± 0.87	tr	4.67 ± 2.34	21.16 ± 17.34	18.12 ± 6.80	10.40 ± 3.46
	2.71	*10.72*	*2.46*	*0.00*	*6.97*	*1.24*	–	*3.25*	*6.16*	*0.79*	*4.79*
C18:2(n-6)	tr	tr	tr	3.42	1.94 ± 0.15	tr	tr	7.62 ± 0.15	34.65 ± 31.71	115.96 ± 65.53	4.89 ± 0.34
	–	–	–	*25.62*	*3.55*	–	–	*5.29*	*10.09*	*5.03*	*2.25*
C18:3(n-6)	2.07 ± 0.40	2.40 ± 0.39	2.66 ± 0.27	0.64	2.45 ± 0.21	2.66 ± 0.25	2.48 ± 0.04	8.66 ± 4.23	8.20 ± 3.75	173.22	10.54 ± 6.21
	1.55	*1.82*	*3.87*	*4.77*	*4.47*	*1.00*	*4.14*	*6.02*	*2.39*	*7.51*	*4.85*
C18:3(n-3)	tr	0.89 ± 0.07	tr	0.00	tr	1.11 ± 0.70	tr	1.21	3.21 ± 2.05	38.90 ± 13.87	5.07
	–	*0.68*	–	*0.00*	–	*0.42*	–	*0.84*	*0.93*	*1.69*	*2.34*
C18:4(n-3)	tr	tr	tr	0.37	tr	tr	tr	2.54 ± 1.90	13.47 ± 9.88	93.29 ± 41.58	75.5
	–	–	–	*2.78*	–	–	–	*1.77*	*3.92*	*4.04*	*34.77*
C20:5(n-3)	2.86	tr	1.46 ± 0.18	0.53	1.61 ± 0.64	1.73 ± 0.93	0.86 ± 0.19	1.31 ± 0.11	16.52 ± 3.34	243.43 ± 117.77	55.06
	2.14	–	*2.12*	*4.00*	*2.94*	*0.65*	*1.43*	*0.91*	*4.81*	*10.55*	*25.36*
C22:6(n-3)	tr	tr	2.64 ± 0.50	0.12	1.06 ± 0.33	tr	1.03	34.65	19.00 ± 18.92	167.63 ± 92.32	15.91 ± 10.34
	–	–	*3.85*	*0.88*	*1.94*	–	*1.71*	*24.09*	*5.53*	*7.27*	*7.33*
TFA	133.49 ± 9.06	132.04 ± 13.47	68.63 ± 6.68	13.33	54.71 ± 6.62	265.10 ± 38.60	59.92 ± 6.43	143.83 ± 22.76	343.56 ± 62.51	2307.07 ± 372.31	217.14 ± 14.56
	100.00	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*	*100.00*

tr traces, *TFA* total fatty acids, DAG, diacylglycerols, *DGDG* digalactosylacylglycerols, *DPG*, diphosphatidylacylglycerols, *FFA* free fatty acids, *MAG* monoacylglycerols, *MGDG* monogalactosylacylglycerols, *PG* phosphatidylglycerols, *TAG* triacylglycerols, SG acylated sterol glycosides, *SQDG* sulfoquinovosyldiacylglycerols, *UN* unidentified phospholipid class

with the decrease in total PUFA under high irradiance levels [43]. With regard to cellular Chl *a*, the data reported in Fig. 3 show a higher content at a lower irradiance. This phenomenon has also been observed in other microalgae [42]; under low light intensity, the cells do increase their photosynthetic pigments (as is the case of Chl *a*), in order to maximize their ability to harvest light [14]. An association between fatty acids and pigments has been also suggested an underlying positive correlation between cellular Chl *a* content and PUFA levels [14, 25].

The fatty acid composition of nonpolar lipids, as obtained in our research, was in agreement with various other authors [8, 21, 31]; it was explained by large amounts of saturated residues, mainly C14:0 and C16:0, as well as of monounsaturated ones, viz. C16:1(n-7). The fatty acid composition of GL at 9 W m^{-2} was also as expected [21, 40]—with MGDG and DGDG encompassing large amounts of unsaturated fatty acid residues (and particularly rich in EPA and DHA). However, when the light intensity was increased from 9 to 19 W m^{-2}, there was a dramatic shift in the degree of EPA and DHA esterification—which was more notable in TAG. At the highest light intensity, the relative distribution of unsaturated residues was maintained relative to that prevailing at lower light intensities—but there was a substantial decrease in the total amount (note that the highest fractions of EPA and DHA were recorded in TAG).

At higher light intensities (i.e., 19 and 30 W m^{-2}), the lipid profiles of MGDG and DGDG exhibited high fractions of saturated fatty acids—which is not at all uncommon in those classes. It should be remembered that PUFA esterified in non-polar lipid classes (viz. TAG) are not particularly suitable for aquaculture; the quality of the microalgae as feed would be consequently poorer, even though the total EPA yield at 19 W m^{-2} was slightly higher than that at 9 W m^{-2}.

Concerning the other GL, the lipid profile of SG was similar to that previously described [31]—with large amounts of medium-chain saturated fatty acids; it is interesting to point out the huge increase in C16:1(n-7), from ca. 8 to 55%, which was obviously associated with the increase in light intensity.

The variation of the fatty acid profile of PL was similar to that of GL, in that unsaturated fatty acids were reduced when light intensity was increased. In this case, there was a major reduction in the total amount of DHA present in DPG—which accounted for 41% of the total PL class at 9 W m^{-2}, but feel down to a mere 13 or 4%, at 19 and 30 W m^{-2}, respectively.

At lower light intensities, the SQDG class behaved untypically; this class does in fact usually exhibit a negligible amount of unsaturated fatty acids. In our case, EPA accounted for 13% of SQDG at 9 W m^{-2}, and 42% at 19 W m^{-2}; when light was increased to 30 W m^{-2}, there was a substantial shift in the opposite direction, so SQDG showed a typical profile characterized by very small amounts of unsaturated fatty acid residues. The presence of high levels of EPA in SQDG had already been reported [8, 21], but not at such high levels as found in the present work.

EPA and DHA Productivity

The total EPA and DHA productivities in cultures at QSS—grown under 9, 19 and 30 W m^{-2}, are tabulated in Table 5. It is apparent that the maximum productivities were attained at 19 W m^{-2}, with a significant increase ($P < 0.01$) from 9 to 19 W m^{-2}; there was also a significant difference ($P < 0.05$) between cultures grown at 9 and 30 W m^{-2}, but only in what concerns EPA. Furthermore, under both conditions, the major fraction of either EPA or DHA was esterified in TAG—which may be a favorable process, should one seek production (and purification) of PUFA from microalgae. If, on the other hand, *P. lutheri* is seen primarily as a feed source, the increase in production may not be desirable at all, because (as stated before) the fatty acids esterified in non-polar classes are poorly effective in aquaculture—owing to metabolic constraints in fish. This realization backs up the importance of detailed studies on lipid composition, because a simplistic determination of the overall fatty acid profile of the biomass may lead to erroneous decisions in terms of processing afterwards.

PUFAs may also help to protect microalgae against photooxidation reactions associated with photosynthetic activity. According to previous studies [25], the highest proportions of DHA are indeed obtained under saturating irradiance in most species. This could be explained by the fraction of DHA found in each lipid class (ca. 12% in non-polar lipids, ca. 4% in GL and ca. 20% in PL—Table 3), which was at a maximum under 19 W m^{-2} light intensity. Therefore, high DHA levels in PL, and reduction thereof during light limitation, could play a central role in the physiology of the plasma membrane and in the mechanism of energy storage.

Table 5 Variation of productivity of EPA and DHA in *P. lutheri* with different light intensities (means ± standard deviation of 3 replicates)

Light intensity (W m^{-2})	Productivity (mg L^{-1} day^{-1})	
	EPA	DHA
9	0.687 ± 0.032	0.197 ± 0.039
19	1.289 ± 0.041	0.693 ± 0.006
30	0.377 ± 0.071	0.275 ± 0.057

In our study, when the cells were cultured under a low light intensity, the highest levels of such PUFA as EPA were predominantly found in GL—which constitutes a major component of the lipid membranes of chloroplasts [14, 25, 43]. The results reported here indicate that the galactolipids in *P. lutheri* have been enriched with EPA to adapt the cells to light intensities as low as 9 W m^{-2}. An accumulation of n-3 fatty acids has accordingly been observed in thylakoid membranes, which is a clue to thylakoid expansion as an adaptation of the cells to specific environmental conditions of light intensity [10, 14]. Hence, EPA may preserve the fluidity of the aforementioned thylakoid membranes, and thus the rate of the electron flow involved in photosynthesis [10].

Note that our experimental results are based on average light intensity rather than total incident light—because the light actually available to microalgae depends on the biomass concentration in the reactor: Thomson et al. [10] tested the influence of light intensity on *P. lutheri* used to feed Pacific oysters (*Crassostrea gigas*), in an 8-L turbidostat operated at high cell densities (1.2–2.6 × 10^9 cell mL^{-1})—and found that the best performance, in terms of PUFA, was at 30 W m^{-2}; however, at the biomass levels thus attained, the incident light of 30 W m^{-2} yields much lower values in terms of average intensity of light received—in fact, much closer to the 9 W m^{-2} intensity experimented with in our study, and leading to a similar fatty acid distribution.

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