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Long chain polyunsaturated fatty acid production and partitioning to triacylglycerols in four microalgae

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Abstract

Gas chromatographic profiling of fatty acids was performed during the growth cycle of four marine microalgae in order to establish which, if any, of these could act as a reliable source of genes for the metabolic engineering of long chain polyunsaturated fatty acid (LC-PUFA) synthesis in alternative production systems. A high-throughput column based method for extraction of triacylglycerols (TAGs) was used to establish how much and at what stage in the growth phase LC-PUFAs partition to storage lipid in the different species. Differences in the time course of production and incorporation of docosahexaenoic acid (22:6n-3, DHA) and eicosapentaenoic acid (20:5n-3, EPA) into TAGs were found in the marine microalgae *Nannochloropsis oculata* (Eustigmatophyceae), *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bacillariophyceae), and the Haptophyte *Pavlova lutheri*. Differences were not only observed between species but also during the different phases of growth within a species. A much higher percentage of the total cellular EPA was partitioned to TAGs in stationary phase cells of *N. oculata* compared to *P. tricornutum*. Although *P. tricornutum* produces DHA it does not partition it to TAGs. Both *T. pseudonana* and *P. lutheri* produce EPA and DHA and partition these to TAGs during the stationary phase of growth. These two species are therefore good candidates for further biochemical and molecular analysis, in order to understand and manipulate the processes that are responsible for the incorporation of LC-PUFAs into storage oils.

Keywords: Marine microalgae; Acyl lipid composition; LC-PUFAs; EPA; DHA; Triacylglycerols; Batch culture

1. Introduction

There is currently a resurgence of interest in the fatty acid (FA) composition and associated metabolism of marine microalgae that produce the long chain polyunsaturated fatty acids (LC-PUFAs) docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3) since these compounds are now recognised as having a number of important neutraceutical and pharmaceutical applications (Shahidi and Wanasundara, 1998; Horrocks and Yeo, 1999). Investigations into the roles of LC-PUFAs and associated eicosanoid metabolites have highlighted their involvement in homeostatic function in animals and consequently several national agencies have approved the use of LC-PUFAs as food additives (Gill and Valivety, 1997). For example, the requirement for DHA in the diet of infants in order to achieve full developmental potential has been claimed on the basis of reports that infants fed breast milk, that naturally contains DHA, perform better on tests that assess neurodevelopmental outcomes than do infants fed on formula milk that lack this fatty acid. Although the data from such tests do not conclusively prove that these LC-PUFAs are essential, consistent findings such as the lower levels of cerebral DHA in the brains of formula-fed infants relative to those fed human breast milk has provided a compelling case for LC-PUFA supplementation of formula milk (Gibson and Makrides, 2001). Other claimed health benefits for DHA include the prevention and treatment of chronic diseases such as coronary heart disease, hypertension, type II diabetes, ocular diseases, arthritis and cystic fibrosis (Simopoulos, 1999).

The principal dietary source of DHA and EPA is fish. However, fish accumulate pollutants, the extracted oil has unpleasant odour and the proportion of specific

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fatty acids in the lipids from this source are difficult to control. Fish are also a declining resource and there are serious environmental consequences related to the continued exploitation of fish-stocks in order to meet the demands of an expanding market. Because of these drawbacks, new sources of LC-PUFAs are needed. Attempts to enhance the LC-PUFA content of meat products by manipulating animal feed have met with limited success. Commercial cultivation of marine microorganisms which biosynthesise DHA has also been evaluated. Although there have been some successes using this approach, cultivation of algae is technically demanding and costly (Molina Grima et al., 1995).

The development of new, low-cost, environmentally friendly production systems for LC-PUFAs such as DHA and EPA is therefore desirable. In recent years advances in biotechnology have resulted in plants that have been genetically altered to produce new compounds. Particular attention has focused on the genetic modification of fatty acid metabolism in order to produce economically valuable oils for food and non-food industrial uses in oilseed crops (Ohlrogge, 1994; Thelen and Ohlrogge, 2002). Plants therefore represent an attractive alternative production system to algae for the production of LC-PUFAs such as EPA and DHA. In order to realise this target it is essential that genes responsible for the various steps involved in LC-PUFA synthesis, including substrate specific desaturases and elongases, are isolated and shown to be functional in plant species (Napier et al., 1999; López Alonso and García Maroto, 2000). Microalgae that produce EPA and DHA represent a useful source of such genes. Furthermore, it is desirable that LC-PUFAs such as DHA and EPA are produced in the seed oils of plants, which aids harvesting and provides a concentrated source of FAs for extraction. Acyltransferases are responsible for the transfer of fatty acids into triacylglycerols (TAGs), which are stored in oil bodies in both algae and the seeds of oilseed crops. Therefore, to enable the commercially successful transfer of genes that produce DHA and EPA from algae into crops, acyltransferases also need to be identified that are responsible for the incorporation of these FAs into TAGs.

The LC-PUFA content of microalgae and most importantly, the partitioning of LC-PUFAs into TAGs depend not only on the species, but also on factors related to culture condition including composition of the medium, aeration, light intensity, temperature and age of culture (Dunstan et al., 1993). The aim of the current work was to perform a comprehensive study of the production of LC-PUFAs and their partitioning into TAGs during the growth cycle of four species of microalgae from three different classes, so that we could identify the best strain and growth stage for the isolation of LC-PUFA related genes. Significant differences in the time course of production and incorporation of DHA and EPA into TAGs were found in *Nannochloropsis oculata* (Eustigmatophyceae), *Phaeodactylum tricornutum* and *Thalassiosira pseudonana* (Bacillariophyceae), and the Haptophyte *Pavlova lutheri,* thus highlighting the importance of comprehensive metabolite analysis prior to embarking on gene discovery programmes.

2. Results and discussion

2.1. TAG extraction method

In order to establish what proportion of the total fatty acids composition per algal cell is present in TAGs, a quantitative TAG based extraction method was developed. Bond Elut 1 ml solid phase extraction (SPE) columns with 100 mg Si packing were used to partition TAGs from other lipids in algal extracts. To test the stringency of partitioning, a mixture of 50 μg tripentadecanoin (15:0 TAG) and 50 μg of a representative next least polar lipid, the diacylglycerol, dipalmitin (16:0 DAG), were combined in 100 μl hexane. This was loaded onto the column and progressively eluted with 1 ml aliquots of increasing concentrations of chloroform in hexane, and the collected fractions derived to FAMEs for GC-FID analysis (Fig. 1A). The composition of the fractions determined that TAGs could be exclusively eluted, without contamination from DAGs or more polar lipids, using 30–40% chloroform in hexane (v/v). This was also found to be true for *Arabidopsis thaliana* leaf lipid extracts spiked with 15:0 TAG (Larson and Graham, unpublished). A similar experiment was done to determine the optimal solvent volume and composition for complete TAG elution (Fig. 1B). This was determined to be 40% (v/v) chloroform in hexane (2:3 v/v by ratio) using an elution volume of 1.5 ml for 97% TAG recovery. Using this solvent, DAG contamination was 0.6% for equal concentrations of TAG and DAG applied to the column. These optimised extraction conditions were used for the algal samples.

2.2. Fatty acid composition

Table 1 shows the FA composition during the exponential and stationary phases of growth of each of the four microalgae used in this study. In all cases except the exponential phase of growth of *P. lutheri*, the FAs shown in the table represent at least 80% of the total FA extracted. In *P. lutheri*, a high percentage of short and medium chain FAs (6:0, 8:0, 10:0 and 12:0) amounting to 25% in the total extract and 60% in the TAG extract were detected in the exponential growth sample (data not shown). Changes in overall FA chain

length and degree of unsaturation were revealed by a comparison of the ratio of unsaturated FA/saturated FA and the ratio of $(C_{20:5}+C_{22:6})/(C_{16:0}+C_{16:1})$ in the exponential and stationary phase cultures. In *P. tricornutum* and *T. pseudonana* there was an overall increase in the amount of saturation and a decrease in chain length as cells shifted from the exponential to stationary phase of growth and this is reflected in the TAG extracts (Table 1). However, in *P. lutheri* there was an overall increase in the amount of unsaturation and chain length in the total FA content as the cells shifted from exponential to stationary phase. However, ratio analyses such as these can be dominated by changes in the most abundant FAs. As a result, changes in lower abundance fatty acids can be masked. For example, 20:5 EPA increased rather than decreased in abundance in *P. lutheri* stationary phase samples compared to exponential phase samples, whereas in the other species EPA decreased upon transition to stationary phase.

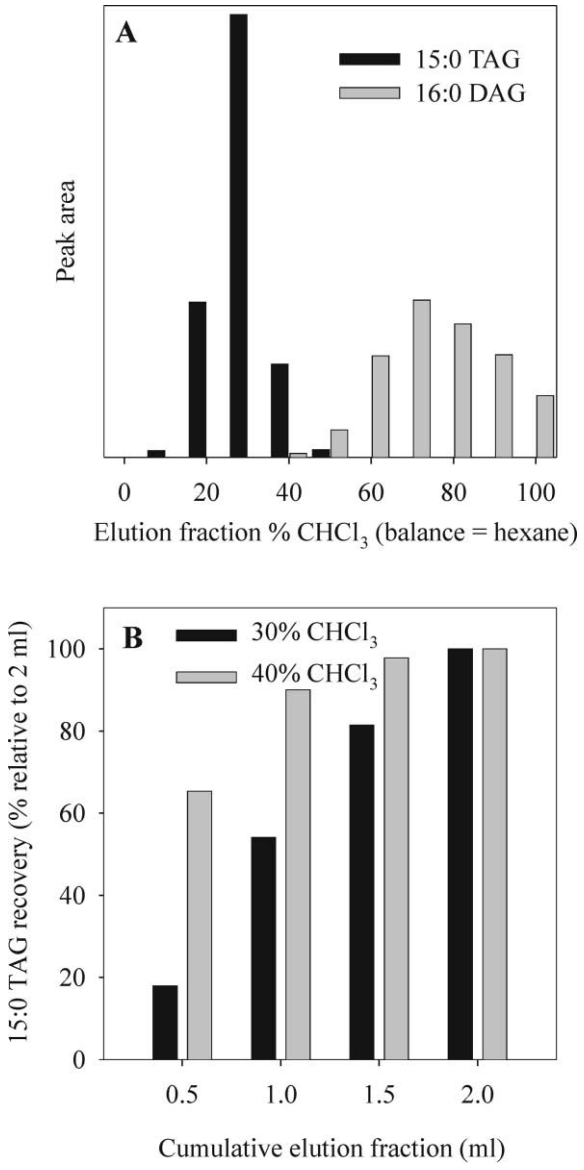

Fig. 1. Separation of triacylglycerols from other lipids using solid phase extraction. **(A)** Stringency test for separation of 15:0 TAG and 16:0 DAG. **(B)** Determination of optimal solvent volume and composition for complete TAG extraction.

The predominant FAs in all four species were 16:0 and 16:1. In *N. oculata*, the amount of 16:1 decreased concomitantly with an increase of 16:0 and 18:1 upon the transition from exponential to stationary phase and this pattern was seen in both the total and TAG extract. This change is possibly due to the elongation of 16:1 to 18:1 as the cells enter stationary phase. As previously observed (Volkman et al., 1989; Zhukova and Aizdaicher, 1995) the amount of 16:1 was significantly greater than 16:0 in *P. tricornutum* extracts. TAG composition in this species changes significantly upon entry to stationary phase with 18:1 increasing and EPA showing a dramatic decrease in concentration. The FA profile of *T. pseudonana* showed less variation between the two phases of growth than that observed for the other diatom, *P. tricornutum*.

In addition to detection of high levels of EPA and DHA, with EPA always more abundant than DHA, our analysis also showed the presence of other PUFAs, including 18:2, 18:3, and 20:3 but at levels less than 1%. EPA and DHA were the main PUFAs detected in both the total and TAG extracts in all species except *N. oculata*. This failure to detect DHA in *N. oculata* is in agreement with other published works (Hodgson et al., 1991; Schneider et al., 1995) but disagrees with previous work that reported production of DHA by *N. oculata*, albeit under different growth conditions (Vazhapilly and Chen, 1998). We found that *P. tricornutum* grown in batch culture produced DHA but did not incorporate this LC-PUFA into TAGs, which is in contrast to the report of López Alonso et al. (2000) who found a low quantity of DHA in the storage lipids under continuous culture conditions. It therefore appears that culture growth conditions have significant effects on FA content and partitioning. Interestingly, DHA produced in *T. pseudonana* and *P. lutheri* was only partitioned to TAGs during the stationary phase of growth.

P. lutheri was the only species used in the current study where EPA and DHA levels increased in both the total and TAG FA extract upon the transition to the stationary phase. A recent study of the FA composition of *P. lutheri* (Carvalho and Malcata, 2000) grown in various media and sampled at the transition point between exponential and stationary phase found lower percentages of 16:0 and 16:1 and higher levels of PUFAs (mainly EPA) than we report here. Culture growth conditions are again the most likely factor responsible for the different results. In the present study we found that EPA and DHA represented close to 20% of total FA content in *P. lutheri*, which is characteristic of the Haptophyceae (Okuyama et al., 1992). The

Table 1
Fatty acid composition (molar %) of four microalgae at two stages of their growth cycle (each value represents the mean ± SD of two replicates)

Fatty acids		*Nannochloropsis oculata*		*Phaeodactylum tricornutum*		*Thaslassiosira pseudonana*		*Pavlova lutheri*	
		E[a]	S[b]	E	S	E	S	E	S
Total extract	14:0	4.88±0.05	6.11±0.03	11.12±0.34	8.70±0.17	7.28±0.01	8.53±0.03	8.04±0.99	11.26±0.62
	16:0	26.65±0.13	35.88±0.01	13.41±0.05	25.34±0.33	22.64±0.09	27.19±0.36	14.14±1.36	22.48±0.04
	16:1	38.12±0.31	32.91±0.05	29.30±0.09	42.92±1.03	26.15±0.03	25.46±0.36	11.09±1.09	26.97±0.69
	18:0	2.42±0.10	0.92±0.01	0.69±0.04	0.68±0.07	1.94±0.11	1.31±0.10	10.39±0.90	1.89±0.25
	18:1	9.14±0.61	11.69±0.01	5.31±0.04	4.34±0.12	5.30±0.26	2.31±0.05	6.59±1.40	11.78±2.03
	20:5	12.13±0.24	8.01±0.02	29.98±0.10	11.57±0.58	16.66±0.20	12.29±0.17	5.19±0.70	11.60±0.03
	22:6	ND[c]	ND	3.10±0.02	1.00±0.04	4.54±0.08	3.40±0.03	4.23±0.75	9.16±0.71
	[d]unsat. FA/sat. FA	1.75	1.23	2.68	1.72	1.65	1.17	0.83	1.67
	[e]$(C_{20:5}+C_{22:6})/(C_{16:0}+C_{16:1})$	0.19	0.12	0.77	0.18	0.43	0.30	0.37	0.42
TAG extract	14:0	5.78±0.16	6.30±0.08	4.07±5.75	6.70±0.75	6.45±0.06	5.98±0.01	2.31±0.29	10.64±0.03
	16:0	30.71±0.32	37.98±0.03	28.15±1.31	30.59±0.12	35.83±033	29.43±0.09	6.27±0.41	25.63±0.28
	16:1	48.44±1.31	34.96±0.02	48.06±3.91	51.91±0.55	39.82±1.59	28.77±0.09	11.92±1.67	39.95±0.38
	18:0	0.57±0.15	0.83±002	ND	ND	0.46±0.65	0.36±0.05	1.64±1.02	0.69±0.06
	18:1	5.50±0.33	11.49±0.07	ND	3.61±0.51	2.03±1.14	2.00±0.10	2.88±1.63	3.85±0.07
	20:5	4.08±0.24	5.25±0.02	16.08±5.67	5.83±0.37	7.74±0.13	7.51±0.02	0.58±0.10	8.67±0.10
	22:6	ND	ND	ND	ND	ND	0.69±0.04	ND	2.61±0.09
	unsat. FA/sat. FA	1.57	1.15	1.99	1.65	1.16	1.09	1.5	1.49
	$(C_{20:5}+C_{22:6})/(C_{16:0}+C_{16:1})$	0.05	0.07	0.21	0.07	0.10	0.14	0.03	0.17

[a] E, sample withdrawn during the exponential growth phase.
[b] S, sample withdrawn during the stationary phase.
[c] ND, not detected.
[d] unsat. FA/sat. FA, ratio between the unsaturated FA and the saturated FA.
[e] $(C_{20:5}+C_{22:6})/(C_{16:0}+C_{16:1})$, ratio between the sum of LC-PUFAs (20:5 EPA, 22:6 DHA) and C_{16} fatty acids (16:0, 16:1).

apparent difference of incorporation of LC-PUFAs into TAGs by the different species suggests that the mechanism responsible for this process is not universal or is perhaps regulated under different growth conditions or stages of growth. Such a regulation could operate for example at the level of expression of genes encoding acyltransferases or other proteins involved in the process.

2.3. Temporal production of EPA and DHA and partitioning into TAGs

Since the single time point analysis of exponential and stationary phase cultures revealed significant differences in the production and partitioning of EPA and DHA into TAGs we decided to perform time course studies throughout the exponential and stationary phases of growth. Nitrate is the major limiting nutrient for growth of the microalgae under the conditions employed in the current study. Nitrate concentration was monitored to provide an indication of nutrient status of the media and to establish if there is a correlation between nitrate depletion and the transition into the stationary phase of growth.

In *N. oculata*, after an initial lag phase, nitrate levels fell to a minimum after 350 h and this coincided with the cell population reaching a maximum density of 1×10^8 cell ml^{-1} (Fig. 2A). After a slight decrease during the exponential phase, the total FA content increased rapidly to a maximum of 2000 fg cell^{-1} at the end of the stationary phase (Fig. 2A). Of the total FA 90% was found to be incorporated in TAGs (data not shown). FA accumulation in TAGs in stationary phase batch culture of *N. oculata* was previously reported by Hodgson et al. (1991), but in that study only 25% of the total FA was partitioned into TAGs. The difference may at least partly be due to the point in the stationary phase when the cells were sampled. As noted above, *N. occulata* produced EPA but not DHA under the conditions employed in the present study. Levels of EPA remained fairly constant during the exponential phase but showed a significant increase when the cells entered stationary phase. At the end of stationary phase 68% of the EPA was present in TAGs compared with much lower levels (the lowest being 8%) during the exponential phase of growth (Fig. 2B). *N. occulata* therefore shows an induction of EPA production upon the transition to stationary phase and also an increase in the partitioning

of EPA into TAGs. This suggests that the pathways responsible for the synthesis of EPA and partitioning into TAGs are induced upon the transition from exponential to stationary phase of growth.

The growth curve obtained for *P. tricornutum* (Fig. 3A) shows that, unlike *N. occulata*, cell division continued for approximately 100 h after nitrate reaches a minimum in the growth medium. The continued growth in nitrate depleted medium is possibly due to the cells utilising endogenous stores of nitrogen. The amount of total FAs did not change significantly during the growth phase but increased strongly at the beginning of the stationary phase where 75% of the total FA was incorporated in TAGs. This value was significantly lower than that described above for *N. occulata*. *P. tricornutum* produces both EPA and DHA, but from our initial studies only EPA was partitioned into TAGs (Table 1). The amount of EPA per cell did not show any significant increase upon the transition to stationary phase (Fig. 3B) and DHA levels actually showed a slight decrease (Fig. 3C). However, there was a significant induction in the incorporation of EPA into TAGs, and this increased from a minimum of 3% in exponential phase cells to 40% of the total EPA in the stationary phase cells. The absolute levels of DHA per cell were 5–10 fold lower than those of EPA. None of the DHA appeared to be partitioned into TAGs in either exponential or stationary phase cells which could be due to the EPA out-competing the DHA in the acyltransferase reaction involved in incorporation of these LC-PUFAs into TAGs.

T. pseudonana is a diatom as is *P. tricornutum* but with *T. pseudonana* growth ceased as soon as nitrate became depleted (Fig. 4A). Total FA content showed a significant increase after the cells were in stationary phase for 150 h. At this stage, 74% of the total FAs were incorporated in TAGs. This increase was mirrored by the levels of EPA (Fig. 4B). The amount of EPA partitioning into TAGs also showed a significant increase, from 16 to 67% after 150 h in stationary phase. Overall DHA levels were 2–4 fold less than EPA levels but a significant amount of DHA did partition into TAGs (Fig. 4C). Levels of DHA in TAGs increased from zero at the beginning of the exponential phase to

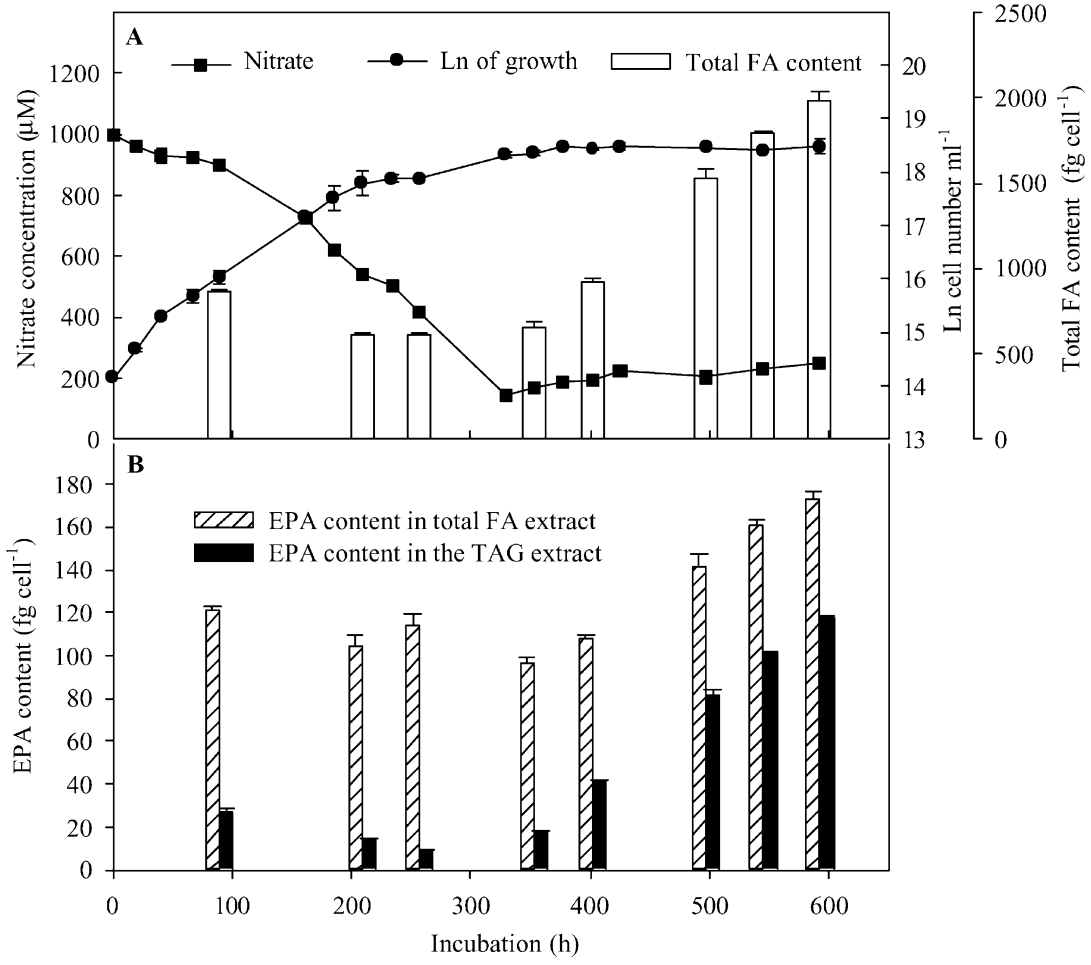

Fig. 2. Growth, nitrate consumption and changes in the fatty acid content of *Nannochloropsis oculata* CCAP 849/1. Each value represents the mean ± SD of two replicates. Where error bars are not visible, they fit in the symbols. **(A)** Cell number, nitrate consumption and total FA content per cell. **(B)** EPA content per cell in the total FA extract and in the TAG extract.

30% of total DHA at the last point sampled in stationary phase. The ability of *T. pseudonana* to partition both DHA and EPA to TAGs compared to *P. tricornutum* which only partitions EPA raises the question of what is regulating this process. It is possible that acyltransferases with different substrate specificities operate in these two species.

Of the four species analysed in the current study *P. lutheri* was the slowest growing with nitrate depletion of the media and cells entering stationary phase occurring after 330 h (Fig. 5A). Total FA content per cell showed an overall decrease upon entry to stationary phase, which is the opposite to that observed for the other three species. At the end of the stationary phase, 52% of the total FAs were incorporated in TAGs. Similar levels of total FAs in stationary phase *P. lutheri* cells have previously been reported by Volkman et al. (1991). In contrast to the overall decrease in FA levels both EPA

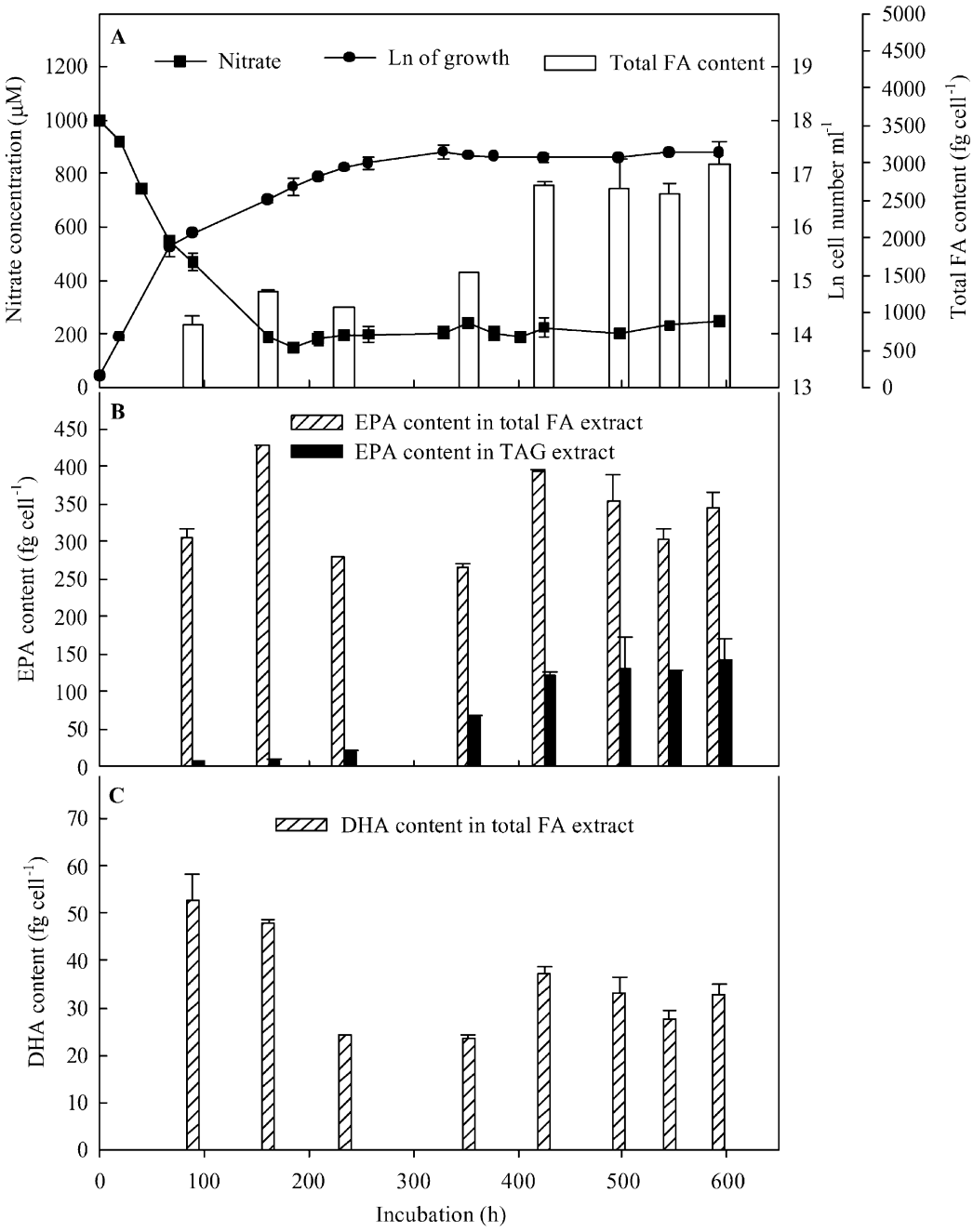

Fig. 3. Growth, nitrate consumption and changes in the fatty acid content of *Phaeodactylum tricornutum* CCAP 1052/1A. Each value represents the mean±SD of two replicates . Where error bars are not visible, they fit in the symbols. **(A)** Cell number, nitrate consumption and total FA content per cell. **(B)** EPA content per cell in the total FA extract and in the TAG extract. **(C)** DHA content per cell in the total FA extract and in the TAG extract.

and DHA content increased during the incubation period (Fig. 5B, C). In fact DHA increases in stationary phase *P. lutheri* cells to levels that are greater than in any of the other species analysed in the current study. Interestingly, EPA incorporation in TAGs begins at the mid-exponential phase of growth whereas DHA incorporation begins at the end of the exponential phase. Total incorporation of EPA into TAGs is also significantly greater than DHA (40% of EPA and 17% DHA in TAGs) despite the fact that in this species the total content of EPA and DHA are similar. In *T. pseudonana* (Fig. 4C) EPA incorporation into TAGs was also significantly greater than that of DHA but in that case the overall levels of EPA in total FAs was also significantly greater than those of DHA.

In conclusion, this study clearly demonstrates the importance of performing detailed time course analysis of FA production and partitioning in selected algal species under controlled growth conditions. Significant differences in PUFA production and partitioning into TAGs occur not only between species but also during the different phases of growth within a species. This is

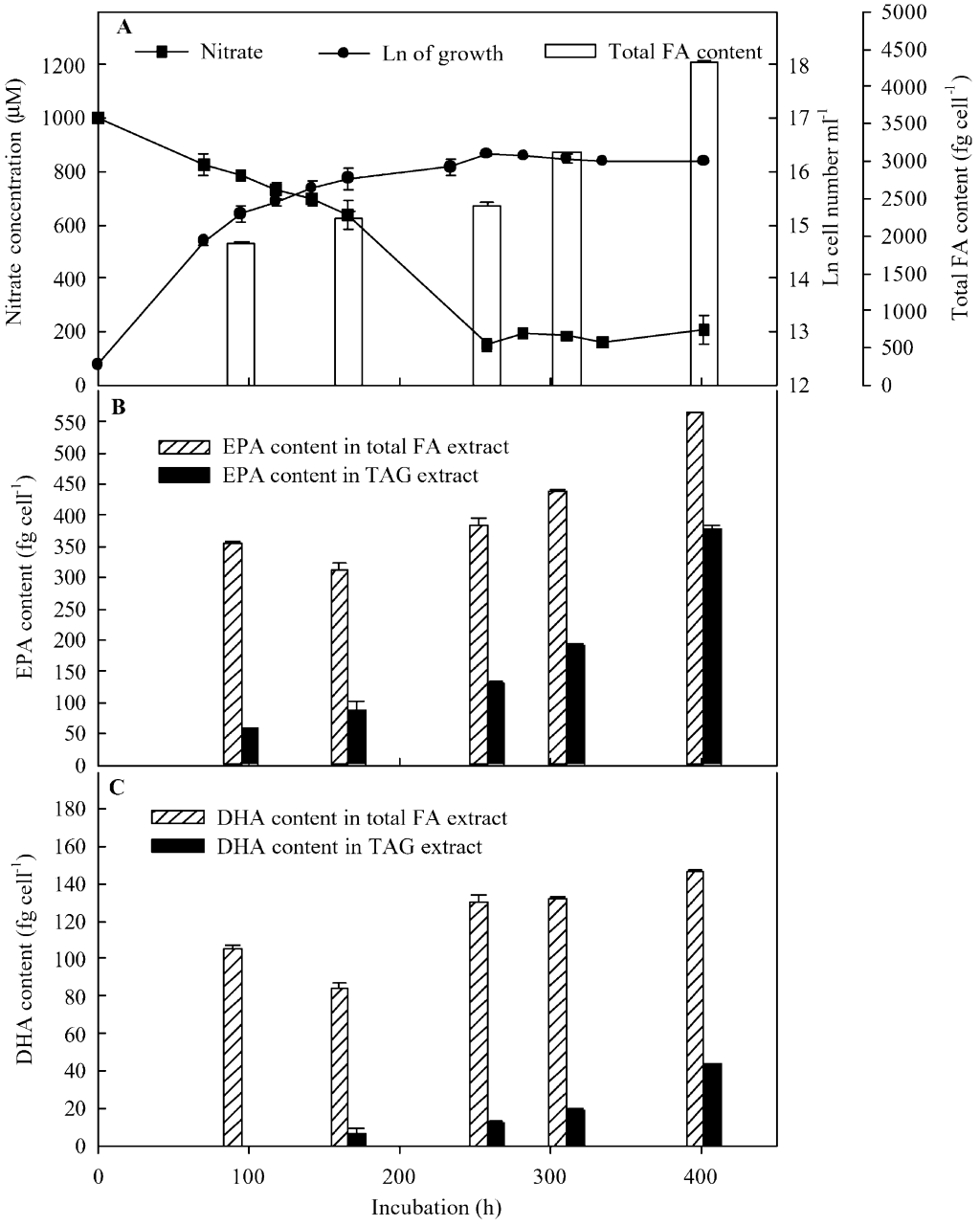

Fig. 4. Growth, nitrate consumption and changes in the fatty acid content of *Thalassiosira pseudonana* CCAP 1058/12. Each value represents the mean±SD of two replicates. Where error bars are not visible, they fit in the symbols. **(A)** Cell number, nitrate consumption and total FA cell content per cell. **(B)** EPA content per cell in the total FA extract and in the TAG extract. **(C)** DHA content per cell in the total FA extract and in the TAG extract.

particularly relevant in work that is aimed at understanding the biochemical processes relating to LC-PUFA production and partitioning and the discovery of related genes. For example, this study reveals that stationary phase cells of *N. oculata* may be preferable to *P. tricornutum* for the identification of genes involved in EPA partitioning into TAGs since a much higher percentage of the total EPA is present in TAGs of *N. oculata*. The study of *P. tricornutum* further revealed that although this species produces DHA it does not partition it to TAGs therefore it is probably not a useful source of material for genes involved in DHA partitioning into TAGs. Both *T. pseudonana* and *P. lutheri* produce EPA and DHA and partition these LC-PUFAs into TAGs in the stationary phase of growth, with a higher proportion in *T. pseudonana* than in *P. lutheri*. These two species are therefore good candidates for further biochemical and molecular analysis, in order to

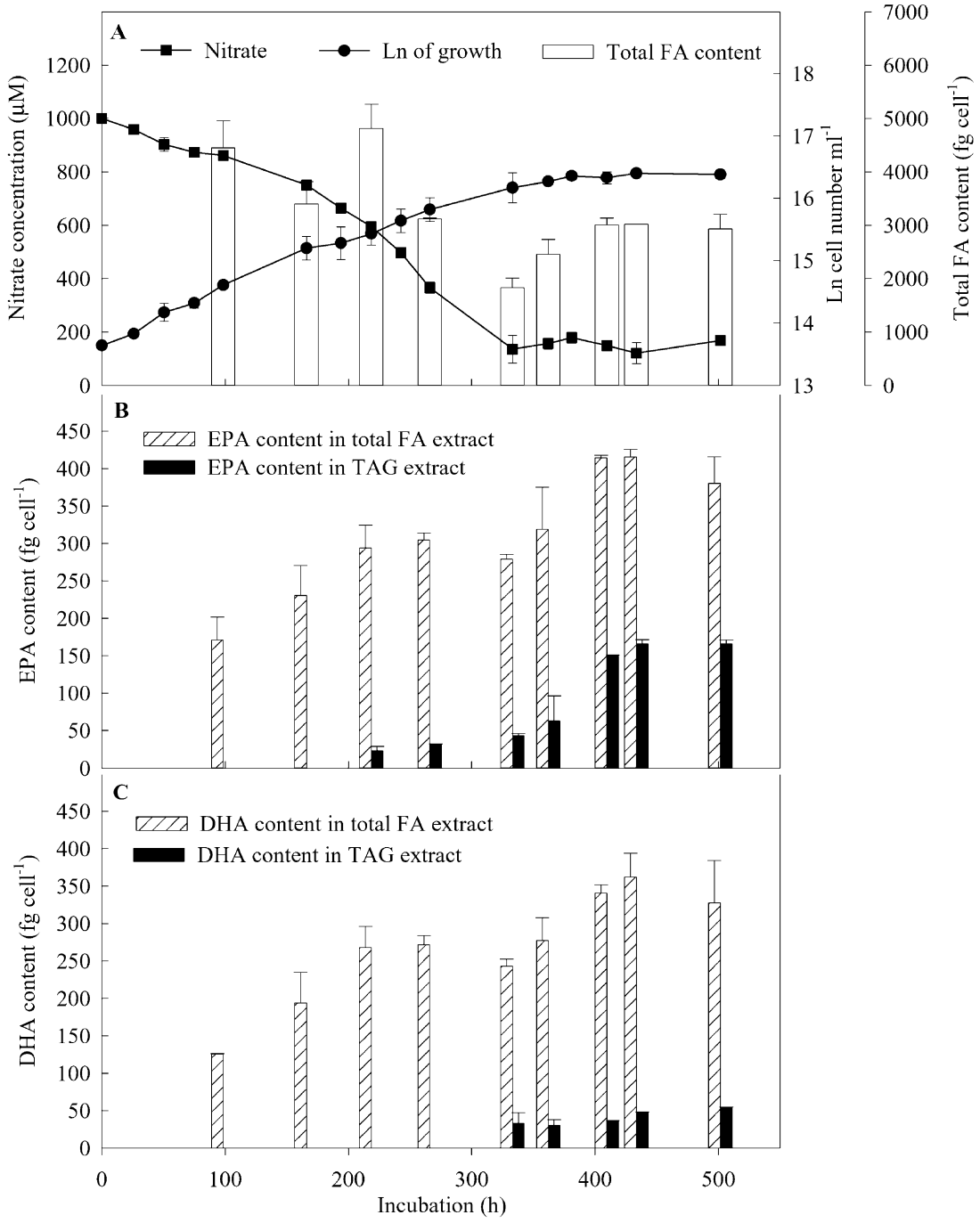

Fig. 5. Growth, nitrate consumption and changes in the fatty acid content of *Pavlova lutheri* CCAP 931/1. Each value represents the mean SD of two replicates. Where error bars are not visible, they fit in the symbols. **(A)** Cell number, nitrate consumption and total FA content per cell. **(B)** EPA content per cell in the total FA extract and in the TAG extract. **(C)** DHA content per cell in the total FA extract and in the TAG extract.

understand and manipulate the processes that are responsible for the incorporation of LC-PUFAs into storage oils.

3. Experimental

3.1. Strains and culture conditions

Cultures of *Nannochloropsis oculata* (CCAP 849/1), *Phaeodactylum tricornutum* (CCAP 1052/1A), *Thalassiosira pseudonana* (CCAP 1085/12, non-axenic culture), and *Pavlova lutheri* (CCAP 931/1) were obtained from the Culture Collection of Algae and Protozoa (Dunstaffnage Marine Lab., Oban, PA34 4AD, Scotland, UK).

The growth medium used in all experiments was enriched artificial seawater medium (EASW), made up in 20-l batches as described by Harrison et al. (1980), and modified by Thompson et al. (1991). The medium was further modified by increasing the macronutrient concentrations of $NaNO_3$ and $Na_2SiO_3{\cdot}9H_2O$ to 1 mM, and NaH_2PO_4 to 200 μM. The silicate was dissolved separately in deionized ultrafiltered water and the pH adjusted to approximately 8.0 with 50% HCl before it was added to the medium. This medium was buffered to pH 8.0 by adding 20 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES) and 20 mM NaOH. The freshly prepared medium was filtered through a 0.22 μM Millipore™ GS membrane filter into a 20 l sterile polypropylene reservoir. It was then dispatched as 0.5 l aliquots in 1-l conical glass flasks and sterilised by autoclaving (30 min, 120 °C). Batch cultures were grown at 18 °C with 240 μE m^{-2} s^{-1} constant illumination, and aeration provided by shaking the flasks at 150 rpm. At least two flasks for each strain were incubated in the different conditions described above.

Cell density was monitored by counting cells with a haemocytometer. Since the *Pavlova lutheri* cells are motile, they were first incubated in 20 mM sodium azide to immobilise the cells before counting. Although the stock of *T. pseudonana* was described as non-axenic by the supplier (Dunstaffnage Marine Lab., Oban, PA34 4AD, Scotland, UK), no growth of any other microorganism was detected in this culture.

The nitrate concentration was determined periodically during the culture period by measuring the change in the medium absorbance at 220 nm, according to the method described by Collos et al. (1999).

3.2. TAG extraction and fatty acids analysis

Algal cells (2 ml of culture medium) were harvested during the experimental period by centrifugation at 13,000 rpm for 15 min. Tripentadecanoin (50 μg; 15:0-TAG) were added to the pellet as an internal standard. The pellet was then resuspended in 1 ml of 2:1 chloroform:methanol (v/v) and frozen in liquid nitrogen. After 1 h at 4 °C, the cell debris was discarded by centrifugation and 0.3 ml of 0.9% KCl added to the supernatant. After centrifugation, the bottom phase was transferred into a 2 ml microfuge tube and the KCl rinsed with 0.5 ml of chloroform. The chloroform phases were pooled and dried under vacuum. The lipid extract was suspended in 0.2 ml of hexane, and this volume was divided into 2 samples of 0.1 ml. The first sample was dried, and the lipid extract suspended in 0.2 ml of hexane. This represented the total lipid extract. The second sample was used to isolate the TAGs by hydrophobic column chromatography. Bond Elut (Varian, Surrey, UK) 1 ml solid phase extraction (SPE) columns with 100 mg Si packing were used to partition TAGs from other lipids in algal extracts. This was adapted from a method described by Yongmanitchai and Ward (1992). The eluate was dried and the TAG extract suspended in 0.2 ml of hexane. From the two samples, lipids were transmethylated to fatty acid methyl esters, and analysed by GC as described previously by Larson and Graham (2001).

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