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Culture of the marine diatom *Phaeodactylum tricornutum* with different nitrogen sources: Growth, nutrient conversion and biochemical composition

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Abstract: Cultures of the marine diatom *Phaeodactylum tricornutum* were grown in nitrate, nitrite, ammonia or urea media. Nitrogen transformation efficiencies were close to 100% in cultures with nitrate, nitrite or urea, with productivities in dry biomass between 2.2 $g.l^{-1}$ (urea) and 2.8 $g.l^{-1}$ (nitrate). However growth in ammonia cultures ceased due to a drop in pH of the medium. The biochemical profile was more affected by the growth phase than by the N source. Cellular contents of chlorophylls *a* and *c* decreased and the chlorophyll *a/c* ratio increased when cultures entered the stationary phase. Protein and RNA were higher during exponential growth (about 30% and 8% ash free dry weight (AFDW), respectively), but carbohydrates increased up to 220% in the stationary phase. Although significant differences were observed in cellular contents, the biochemical composition (as % AFDW) of the nitrate, nitrite and urea-grown cells was very similar. The highest total fatty acids and polyunsaturated fatty acids (PUFAs) contents in the stationary phase were obtained in the urea-grown cells, with an eicosapentaenoic acid (EPA) content of 26.8 $mg.g^{-1}$ of dry biomass.

Résumé : Des cultures de la diatomée marine *Phaeodactylum tricornutum* ont été réalisées dans des milieux contenant du nitrate, du nitrite, de l'ammonium ou de l'urée. Dans les milieux contenant nitrate, nitrite ou urée, l'efficacité de transformation de l'azote a été proche de 100 %, avec des rendements en biomasse sèche allant de 2,2 $g.l^{-1}$ (urée) à 2,8 $g.l^{-1}$ (nitrate), alors que la croissance en présence d'ammonium a été inhibée à la suite d'une chute de pH du milieu. La phase de croissance a eu une influence plus grande sur le profil biochimique que la source d'azote. Les contenus cellulaires en chlorophylle *a* et *c* ont diminué et le rapport chlorophylle *a /c* a augmenté pendant la phase stationnaire. La quantité de protéines et d'ARN a été plus élevée pendant la croissance exponentielle (environ 30 % et 8 % du poids sec sans cendres (AFDW) respectivement), tandis que les hydrates de carbone ont augmenté de plus de 220 % durant la phase stationnaire. Malgré les différences significatives observées pour les contenus cellulaires, la composition biochimique des cellules (exprimée en % de AFDW) était comparable dans les milieux contenant nitrate, nitrite ou urée. Le maximum en acides gras totaux et en acides gras polyinsaturés (PUFAs) pendant la phase stationnaire a été observé en présence d'urée, avec un contenu en acide eicosapentaénoïque (EPA) de 26.78 $mg.g^{-1}$ de biomasse sèche.

Keywords: *Phaeodactylum*, nitrogen sources, growth phase, biochemical composition, EPA.

Introduction

The marine diatom *Phaeodactylum tricornutum* Bohlin (Bacillariophyceae) is widely used in crustacean and mollusc aquaculture (De Pauw & Persoone, 1988); its importance is enhanced by other potential applications of microalgae as feed supplements or as a source of chemicals (Richmond, 1986). *P. tricornutum* has been reported to have high eicosapentaenoic acid (EPA) content (Veloso *et al.*, 1991; Yongmanitchai & Ward, 1991; Chrismada & Borowitzka, 1994).

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Growth and biochemical content of microalgae can vary with changes in the environmental conditions. After carbon, nitrogen is quantitatively the most important element contributing to the dry matter of algal cells. Among the different components of the culture medium, the source and concentration of nitrogen can provoke important changes in the growth and biochemical composition of microalgal species (Kaplan *et al.*, 1986; Tadros & Johansen, 1988; Fabregas *et al.*, 1989; Levasseur *et al.*, 1993). A variety of nitrogen compounds, both inorganic and organic, can serve as sole nitrogen sources for the growth of various microalgae. The ability to use nitrate (NO_3^-), nitrite (NO_2^-) or ammonium (NH_4^+) appears to be general among microalgae (Kaplan *et al.*, 1986), although ammonium is usually the nitrogen source preferred. The most generally used nitrogen source in microalgal culture is nitrate, but the algae encounter ammonia, released as an excretory product by the animals (bivalves, rotifers and fish) and utilized preferentially. Nitrite can serve as a N source for many species of marine microalgae (Kaplan *et al.*, 1986) and *Dunaliella tertiolecta* Butcher (Chlorophyceae) showed similar growth in nitrite and nitrate media (Fabregas *et al.*, 1989). Different organic substrates can also be used as nitrogen source. Urea appears as a good potential nitrogen source (Herrero *et al.*, 1994), especially for immobilized microalgae (Mak & Trevan, 1989). The ability of *D. tertiolecta* for growing in urea with a better growth than in nitrate and nitrite has been reported (Fabregas *et al.*, 1989).

We report here the growth and nutrient conversion ability of *P. tricornutum* cultured with different nitrogen sources (nitrate, nitrite, ammonia and urea). Biochemical composition was determined for each nitrogen source at different growth phases, with special emphasis on fatty acids.

Materials and methods

Phaeodactylum tricornutum was isolated from Ria de Arousa waters (NW Spain) by Dr. Fábregas (University of Santiago, Spain). Cultures were grown in 0.45 µm-filtered seawater (salinity 36 $g.l^{-1}$), autoclaved at 120 °C for 20 min, and enriched with $NaH_2PO_4.2H_2O$ 0.2 mM, ferric citrate 40.0 µM, $ZnCl_2$ 2.0 µM, $MnCl_2.4H_2O$ 2.0 µM, $Na_2MoO_4.5H_2O$ 2.0 µM, $CoCl_3$ 0.2 µM, $CuSO_4.5H_2O$ 0.2 µM, thiamine 70.0 $\mu g.l^{-1}$, biotine 10.0 $\mu g.l^{-1}$, vitamin B_{12} 6.0 $\mu g.l^{-1}$, EDTA 92.8 µM, TRIS-HCl (pH 7.4) 1.0 mM. Nitrogen sources used were: nitrate, as $NaNO_3$, nitrite, as $NaNO_2$, ammonia, as $(NH_4)_2SO_4$, and urea ($(NH_2)_2CO$), all of them at a concentration of 4 mg.atom $N.l^{-1}$.

Cultures were grown in a controlled environmental incubator at 18±1 °C and illuminated with Mazda Fluor C7 TF40 fluorescent lamps, at an irradiance of 115 $\mu mol.m^{-2}.s^{-1}$. A 12:12 light-dark cycle was maintained in order to obtain synchronous cultures. Cultures were continuously bubbled with air at a rate of 10 $l.min^{-1}$. Axenic stock cultures were grown in nitrate media. Inocula were taken from the same stock culture at the late exponential phase of growth, and initial densities were adjusted to $1.5x10^6$ $cells.ml^{-1}$. Algal cells were not preadapted to the new N source. Experiments were carried out in triplicate.

Cell numbers were measured daily using an electronic particle counter (Coulter Electronics Ltd.), pH was recorded, and instantaneous growth rates (μ) were calculated.

Samples for biochemical analysis were taken at the 4th day of exponential growth (log phase samples), and when µ was lower than 0.1 day^{-1} (stationary phase samples). Because biochemical composition varies along the daily cycle (Ganf *et al.*, 1986), samples were always collected just after four hours from the beginning of the ligth period. The dry weight was determined according to Vonshak (1986). Ash content was determined by ashing at 540 °C in a muffle oven, and the ash free dry weight (AFDW) was calculated by subtraction. Samples for protein and carbohydrates were treated according to Fábregas *et al.* (1989), and analyzed using the dye-binding method (Bradford, 1976) for protein and the phenol-sulphuric acid method (Kochert, 1978a) for carbohydrates. After extraction with methanol:chloroform:water, lipids were measured by a quantitative carbonization assay (Marsh & Weistein, 1966). RNA was extracted with perchloric acid, and determined by the method of Kochert (1978b). Pigments were extracted in 90% acetone at 4 °C for 24h. The extracts were filtered through a Fluoropore Millipore filter for clarification, and chlorophyll concentrations were determined according to the equations of Jeffrey & Humphrey (1975).

In the stationary phase, biomass was collected by centrifugation and freeze-dryed after washing with 0.9% (P:V) ammonia formiate. N was determined in freeze-dryed biomass by the micro-Kjedhal method in a TECATOR 1030 autoanalyzer. Fatty acid methyl esters were analyzed from freeze-dryed biomass following the procedure described by Miller (1984) using a Perkin-Elmer 8310 gas-liquid chromatograph with FID detector equipped with a 30 m fused silica capillary column SP-2330 (Supelco). Identification of fatty acid methyl esters was accomplished by comparing the retention times of experimental samples of those of known standards. Identification was verified by GC-MS using a Hewlett-Packard gas-liquid chromatograph connected to an HP5972 mass selective detector using both SP-2330 and Omegawax 250 (Supelco) analytical columns.

Data were analyzed using ANOVA and the Duncan multiple range test ($\alpha = 0.05$).

Results

Growth of *Phaeodactylum tricornutum* with the four nitrogen sources is shown in Figure 1. Populations entered

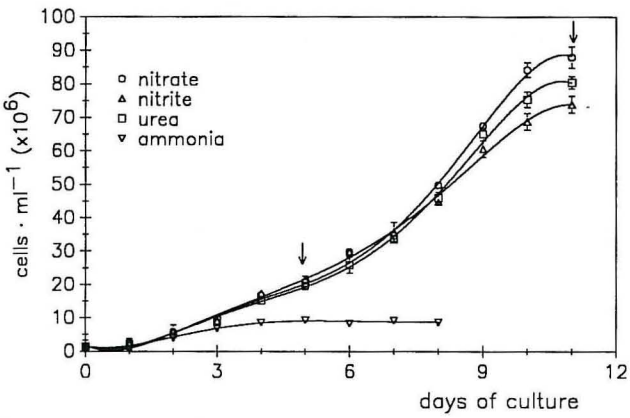

Figure 1. Growth curves of *Phaeodactylum tricornutum* cultured with different nitrogen sources. The values presented are means of replicates and standard errors. Arrows indicate the sampling for biochemical analysis.

Figure 1. Courbe de croissance de *Phaeodactylum tricornutum* cultivé en présence de différentes sources d'azote. Les valeurs présentées correspondent à la moyenne des mesures et à leur écart-type. Les flèches indiquent le moment du prélèvement pour l'analyse biochimique.

exponential growth after a one-day lag phase. In ammonia cultures growth stopped rapidly (day 4) and the culture pH fell to below 5, whereas the remaining cultures reached the stationary phase on day 10. Maxima instantaneous growth rates (μ) were 1.01 day^{-1} for nitrate and nitrite, 0.84 for urea, and 0.68 for ammonia media (Table 1). Higher cellular densities and dry weight (DW) yields in the stationary phase were reached in nitrate cultures: 88 x 10^6 cells.ml^{-1} and 2.84 g.l^{-1} DW. Lower yields were obtained in ammonia cultures.

Table 1. Maximal instantaneous growth rate (μ_{max}), cell densities and dry biomass yielded in the stationary phase for *P. tricornutum* cultured with different N sources. The values presented are means of two replicates and standard deviations.

Tableau 1. Taux de croissance instantanés maximaux (μ_{max}), densités cellulaires et biomasses sèches obtenus pendant la phase stationnaire pour *P. tricornutum* cultivé en présence de différentes sources d'azote. Les valeurs présentées correspondent à la moyenne et à l'écart type de deux mesures.

	Nitrate	Nitrite	Urea	Ammonia
μ_{max} (d^{-1})	1.01	1.01	0.84	0.68
cell.ml^{-1} (x10^6)	87.98 ± 4.40	74.01 ± 2.85	80.88 ± 2.50	8.72 ± 0.30
dry matter (g.l^{-1})	2.84 ± 0.01	2.56 ± 0.03	2.18 ± 0.02	0.58 ± 0.02

According to the cell quota concept of Droop (1975), in batch cultures growth rate decreases and growth ceases when the nutrient in shortest supply, relative to the metabolic needs of the algal population, is exhausted. Nitrogen transformation efficiencies (NTE) (percentages of N in the media converted into cellular-N) may be used to characterize N depletion. A mathematic relation can be established between nitrogen transformation efficiencies (NTE) and the logistic formulation. The logistic formulation, limitation = (C - N) / C = 1 - (N/C), where N is the population and C the carrying capacity of the medium, may be transformed to the expression:

Lim (NUTE) = 1 - [(PHYTO x NUTIR) / NUTT] (Morrison *et al.*, 1987)

where Lim (NUTE) is the limitation on growth due to the nutrient NUT; PHYTO, biomass concentration (mg.l^{-1}); NUTIR, the internal nutrient concentration per unit biomass concentration (mg nutrient.mg $biomass^{-1}$); and NUTT, the total usable concentration of nutrient. NUTI, the internal nutrient concentration into biomasas (mg.l^{-1}), is equal to PHYTO x NUTIR, and NUTT = NUTE + NUTI, NUTE being the external usable concentration of nutrient (mg.l^{-1}).

From the NTE definition, this can be formulated as follow:

Efficiency = ($NUTI_f$ - $NUTI_i$) / $NUTE_i$

where $NUTI_f$ and $NUTI_i$ are the organic N concentrations (mg.l^{-1}) at the stationary phase and in the inocula respectively, and $NUTE_i$ is the N concentration supplied in the medium. Because $NUTI_i$ <<< $NUTI_f$, $NUTI_i$ may be equaled to 0; thus, $NUTI_f$ - $NUTI_i$ = $NUTI_f$= NUTI, and $NUTE_i$= NUTT.

Efficiency = NUTI/NUTT = (PHYTO x NUTIR) / NUTT

Lim (NUTE) = 1 - [(PHYTO x NUTIR) / NUTT] = 1 - Efficiency

Lim (NUTE) takes values between 0 and 1, being 0 when biomass equals carrying capacity, indicating limitation due to nutrient NUT (Morrison *et al.*, 1987). This means that efficiencies close to 1 (i.e., 100%) indicate limitation due to nitrogen. Internal concentration of nitrogen was estimated in the stationary phase. In the cultures with nitrate, nitrite and urea, NTE were 102.1, 101.3 and 87.5%, respectively. Therefore, these cultures entered the stationary phase by N depletion (Lim (NUTE) about 0). However, in ammonia cultures NTE was 22.8%, and growth ceased due to a drop in pH. Ammonia cultures were not considered for biochemical analysis because another parameter (pH) interacted with the nitrogen source.

In each growth phase, significant differences occurred in dry weights, ash percentages and organic matter (AFDW) of cells grown with different N-sources (Table 2). Cells grown in urea had lower dry weights. Ash content was higher in cells cultured with oxidized nitrogen in both growth phases: about 40% of dry weight in the logarithmic phase and 36% in the stationary phase, against 29.8% of dry weight in both

Table 2. Dry weight (DW), ash content and ash-free dry weight (AFDW) of *P. tricornutum* grown in different N sources. The values presented are means of two replicates and standard deviations.

Tableau 2. Poids sec (DW), contenu en cendres (ash) et poids sec sans cendres (AFDW) de *P. tricornutum* cultivé en présence de différentes sources d'azote. Les valeurs présentées correspondent à la moyenne et à l'écart type de deux mesures.

	Nitrate	Nitrite	Urea
Logarithmic phase			
DW (pg.cell^{-1})	48.75 ± 0.40	41.13 ± 0.39	39.12 ± 0.55
ash (% DW)	40.7 ± 0.5	39.0 ± 0.5	29.8 ± 0.2
AFDW (pg.cell^{-1})	28.91 ± 0.24	25.10 ± 0.24	27.48 ± 0.39
Stationary phase			
DW (pg.cell^{-1})	32.24 ± 0.04	34.55 ± 0.45	26.93 ± 0.27
ash (%)	35.9 ± 0.1	35.9 ± 0.2	29.8 ± 0.3
AFDW (pg.cell^{-1})	20.68 ± 0.02	22.13 ± 0.29	18.90 ± 0.19

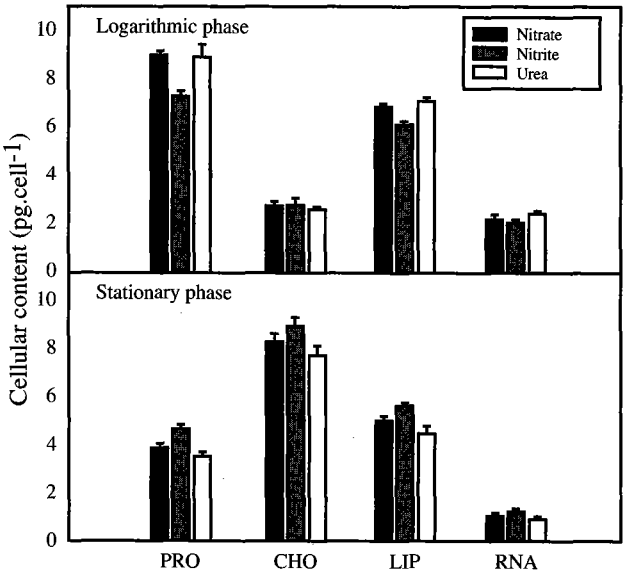

Figure 2. Cellular contents (pg cell^{-1}) in protein (PRO), carbohydrates (CHO), lipids (LIP), and RNA, of *P. tricornutum* cells grown with different N sources, during the logarithmic and stationary phases of growth. The values presented are means of two replicates and standard deviations.

Figure 2. Contenus cellulaires (pg cell^{-1}) en protéines (PRO), hydrates de carbone (CHO), lipides (LIP) et ARN, de cellules de *P. tricornutum* cultivées avec différentes sources d'azote, durant les phases logarithmique et stationnaire de croissance. Les valeurs présentées correspondent à la moyenne et à l'écart type de deux mesures.

phases in cells cultured with urea. Organic cell weights (AFDW) were very similar for cells grown in nitrate, nitrite and urea in each growth phase. Considering the effect of the growth phase, dry weights and organic weights were significantly higher in the logarithmic than in the stationary phase for all cultures. Ash content was higher in the logarithmic phase in nitrate and nitrite cultures, but there were no differences between both growth phases in urea cultures (Table 2).

Cellular contents of protein, carbohydrates, lipids and RNA, of *P. tricornutum* grown with different nitrogen sources in the logarithmic and in the stationary phases are represented in Fig. 2. Cellular contents of chlorophylls are shown in Table 3, and biochemical composition as percentage of AFDW in Table 4.

Table 3. Cellular contents of chlorophylls *a* and *c*, and ratio between them in the logarithmic and stationary phases of *P. tricornutum* cultured with different N sources. The values presented are means of three replicates and standard deviations.

Tableau 3. Contenus cellulaires en chlorophylles *a* et *c*, et leur rapport durant les phases logarithmique et stationnaire de *P. tricornutum* cultivé en présence de différentes sources d'azote. Les valeurs présentées correspondent à la moyenne et à l'écart-type de trois mesures.

	Nitrate	Nitrite	Urea
Logarithmic phase			
Chlorophyll *a*	0.61 ± 0.01	0.52 ± 0.02	0.68 ± 0.01
(pg.cell^{-1})	a-b	b	a
Chlorophyll *c*	122.4 ± 5.9	94.9 ± 1.9	149.7 ± 3.0
(pg.cell^{-1}x10^{-3})	x-y	x	y
Chl *a/c*	5.00	5.47	4.52
Stationary phase			
Chlorophyll *a*	0.24 ± 0.01	0.21 ± 0.02	0.22 ± 0.01
(pg.cell^{-1})	c	c	c
Chlorophyll *c*	44.9 ± 2.2	37.3 ± 2.4	38.9 ± 1.2
(pg.cell^{-1}x10^{-3})	z	z	z
Chl *a/c*	5.33	5.51	5.64

Means were compared using the multiple range test of Duncan (α = 0.05). Differences were not significant for groups with the same letter.

Les moyennes sont comparées en utilisant le test de Duncan (α = 0.05). Les différences ne sont pas significatives pour les groupes désignés par la même lettre.

In the logarithmic phase, cells cultured in urea showed higher values of both chlorophylls, *a* and *c*, than those cultured in nitrate and nitrite, whereas ratios between them were higher in cultures with nitrate and nitrite (Table 3). In

Table 4. Biochemical composition, as %AFDW, of *P. tricornutum* cells cultured with different N sources and in different phases of the growth cycle. The values presented are means of two replicates and standard deviations.

Tableau 4. Composition biochimique (% du poids sec sans cendres) de cellules de *P. tricornutum* cultivées en présence de différentes sources d'azote et durant différentes phases de la courbe de croissance. Les valeurs présentées correspondent à la moyenne et à l'écart-type de deux mesures.

	Nitrate	Nitrite	Urea
Logarithmic phase			
chlorophyll *a*	2.11 ± 0.04	2.08 ± 0.10	2.46 ± 0.01
chlorophyll *c*	0.42 ± 0.03	0.38 ± 0.01	0.54 ± 0.02
protein	30.84 ± 0.39	28.54 ± 0.25	31.68 ± 0.67
carbohydrate	9.57 ± 0.54	11.13 ± 1.14	9.17 ± 0.14
lipid	23.32 ± 0.09	24.00 ± 0.11	25.79 ± 0.54
RNA	7.73 ± 0.25	7.92 ± 0.01	8.49 ± 1.34
Stationary phase			
chlorophyll *a*	1.17 ± 0.05	0.93 ± 0.05	1.15 ± 0.02
chlorophyll *c*	0.22 ± 0.02	0.18 ± 0.02	0.21 ± 0.01
protein	18.88 ± 0.54	20.80 ± 0.67	18.69 ± 1.06
carbohydrate	39.48 ± 1.00	39.93 ± 1.12	40.32 ± 0.21
lipid	24.19 ± 0.52	25.03 ± 1.09	23.63 ± 0.10
RNA	5.11 ± 0.17	5.34 ± 0.27	5.00 ± 0.17

the stationary phase there were no significant differences in chlorophyll contents among nitrate, nitrite and urea cultures. Cellular contents of chlorophylls *a* and *c* decreased and chlorophylls *a/c* ratio increased when populations entered the stationary phase. Chl *a* as proportion of ash free dry weight (AFDW) declined from values about 2% in the logarithmic phase to values about 1% when growth ceased (Table 4). In the logarithmic phase, there were no significant differences in the protein, carbohydrates, lipids and RNA concentrations between cells grown in nitrate and urea (Fig. 2A), but cells grown in nitrite had significantly lower protein, lipid and RNA contents. Protein was the main constituent in this phase in all cultures and protein concentration varied between 7.16±0.31 pg.cell^{-1} (nitrite) and 8.71(±0.58)-8.92(±0.25) pg.cell^{-1} (urea and nitrate). Lipid content ranged between 6.02 pg.cell^{-1} (nitrite) and 6.75-7.09 pg.cell^{-1} (nitrate and urea). RNA was highest in urea cultures (2.33±0.07 pg.cell^{-1}), significantly different to the nitrite cultures (1.99±0.05 pg.cell^{-1}). Carbohydrates showed less variation during the exponential growth, without significant differences among the nitrate, nitrite and urea-grown cells (2.52 - 2.79 pg.cell^{-1}, $P > 0.05$).

Despite variations in cellular contents, in the logarithmic phase little differences occurred in the biochemical composition expressed as %AFDW (Table 4). In this phase, differences did not occur between nitrate and nitrite cultures, which show lower % of chlorophylls, protein, lipids and RNA than urea cultures (Table 4).

In the stationary phase (Fig. 2B) there were no significant differences ($P > 0.05$) between protein contents in urea (3.53 ± 0.23 pg.cell^{-1}) and nitrate (3.90 ± 0.17 pg.cell^{-1}), and between nitrate and nitrite (4.59 ± 0.31 pg.cell^{-1}), but differences were significant between urea and nitrite. As occurred in the logarithmic phase, in the stationary phase there were no significant differences in carbohydrates concentration among these three sources of N, with values in the range 7.62 - 8.64 pg.cell^{-1} Lipid content ranged between 4.46 - 5.54 pg.cell^{-1}. RNA content ranged between 0.95 ± 0.05 pg.cell^{-1} (urea) and 1.18 ± 0.06 pg.cell^{-1} (nitrite). In the stationary phase, there were no differences among nitrate, nitrite and urea cultures in the biochemical profile as % AFDW (Table 4). In this phase the main biochemical constituents were carbohydrates (40% AFDW) (Table 4).

The biochemical cell profile was more affected by the growth phase than by the nitrogen source. Cellular contents in protein (Fig. 2) and protein percentage of ash free dry weight (Table 4) decreased in the stationary phase. Protein accounted for 28.5 - 31.7% AFDW during exponential growth, decreasing to 18.7 - 20.8% AFDW in the stationary phase. Cellular contents of carbohydrates increased as a result of nutrient exhaustion (Fig. 2) and differences up to 220% between both growth phases were observed. Carbohydrates, as percentage of AFDW, also increased from 9 - 11% in the logarithmic phase to about 40% in the stationary phase (Table 4), in which carbohydrates were the main chemical fraction in these cultures.

Cellular contents in lipids (Fig. 2) decreased in the stationary phase. However, in both growth phases lipids represented about 23 - 26% AFDW (Table 4), being the second cellular fraction after proteins in the logarithmic phase and after carbohydrates in the stationary phase. RNA concentrations decreased in the stationary phase (Fig. 2); they represented 7.7 - 8.5% AFDW during the exponential growth, and about 5% when growth ceased (Table 4).

Ratios between different cellular constituents have been proposed as indicators of physiological state of microalgal populations. Different ratios were calculated and are presented in Table 5. Protein/carbohydrate (PRO/CHO) ratios decreased in the stationary phase. PRO/CHO ratios were higher than 2 in the logarithmic phase, for all the nitrogen sources used, decreasing to 0.5 in the stationary phase. Protein/lipid (PRO/LIP) ratios were also higher than 1 during the exponential growth, with the highest value for nitrate (1.32), and decreased below 1 in the stationary phase. The ratio of proteins to total storages (PRO/CHO+LIP) was between 0.82 - 0.94 in the logarithmic phase, declining to 0.29 - 0.32 at the stationary phase, without differences among N sources. Lipid/carbohydrate (LIP/CHO) ratio fell

from values > 2 in the logarithmic phase, to values about 0.6 in the stationary phase for nitrate, nitrite and urea cultures. Protein/RNA ratio was very similar among the different nitrogen sources in both growth phases.

The fatty acid composition of *P. tricornutum* at the beginning of the stationary phase is given in Table 6. The major fatty acids were 14:0, 16:0, 16:1ω7, 16:2ω4, 16:3ω4, 18:2ω6 and 20:5ω3. Higher total fatty acid content (120 mg.g^{-1} DW) and higher contents of polyunsaturated fatty acids (PUFAs) (50.4%) occurred in urea cultures. Therefore the ratio saturated fatty acids + monounsaturated fatty acids (SFA+MUFAs) to PUFAs was lower in urea cultures (0.98) than in nitrate and nitrite cultures (2.77 - 1.27). Ratio between ω6- and ω3-PUFAs was higher in nitrate and nitrite cultures (0.64-0.31) than in urea cultures (0.22). These ratios are considered of nutritional value in bivalve nutrition. Higher contents of eicosapentaenoic acid (EPA, 20:5ω3), were obtained in cultures with urea: 22.4% of total fatty acids, and 26.8 mg.g^{-1} DW. The highest concentration of docosahexaenoic acid (DHA, 22:6ω3) was also obtained in cultures with urea: 1.29 mg.g^{-1} DW (Table 5).

Table 5. Ratios between cellular constituents of *P. tricornutum* cultured with different N sources (PRO: protein, CHO: carbohydrate, LIP: lipid).

Tableau 5. Rapports des constituants cellulaires de *P. tricornutum* cultivé en présence de différentes sources d'azote (PRO: protéines; CHO: hydrates de carbone; LIP: lipides).

	Nitrate	**Nitrite**	**Urea**
Logarithmic phase			
PRO/CHO	3.23	2.60	3.49
PRO/LIP	1.32	1.19	1.24
PRO/CHO+LIP	0.94	0.82	0.92
LIP/CHO	2.45	2.19	2.81
PRO/RNA	3.99	3.60	3.77
Stationary phase			
PRO/CHO	0.48	0.52	0.47
PRO/LIP	0.78	0.83	0.79
PRO/CHO+LIP	0.30	0.32	0.29
LIP/CHO	0.61	0.63	0.59
PRO/RNA	3.69	3.90	3.74

Table 6. Fatty acid composition of *P. tricornutum* grown with different N sources at the beginning of the stationary phase. The values presented are means of two replicates. SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

Tableau 6. Composition en acides gras de *P. tricornutum* cultivé avec différentes sources d'azote au début de la phase stationnaire. Les valeurs présentées correspondent à la moyenne de deux mesures. SFA : acides gras saturés ; MUFA : acides gras monoinsaturés ; PUFA : acides gras polyinsaturés.

	nitrate		**nitrite**		**urea**	
FA	% total	mg.g^{-1} DW	% total	mg.g^{-1} DW	% total	mg.g^{-1} DW
14:0	11.40	7.28	8.30	7.40	6.20	7.43
15:0	2.54	1.62	0.45	0.40	0.27	0.32
16:0	17.35	11.07	11.68	10.41	10.74	12.86
16:1ω7	34.65	22.11	30.14	26.88	28.88	34.60
16:2ω7	0.85	0.55	0.70	0.63	1.00	1.20
16:2ω4	4.55	2.90	3.69	3.29	4.32	5.17
16:3ω4	6.26	3.99	9.29	8.28	11.92	14.28
16:4ω1	0.82	0.53	1.11	0.99	1.18	1.41
18:0	0.87	0.56	0.19	0.17	0.27	0.32
18:1ω9	1.53	0.98	1.17	1.04	0.80	0.96
18:1ω7	1.08	0.69	0.61	0.54	0.55	0.65
18:2ω6	2.57	1.64	3.96	3.53	3.23	3.87
18:3ω6	0.54	0.34	0.59	0.52	0.35	0.42
18:3ω3	0.68	0.44	1.71	1.53	1.47	1.76
18:4ω3	0.23	0.14	1.02	0.91	0.33	0.39
18:4ω1	0.58	0.37	1.46	1.30	0.53	0.63
20:0	0.60	0.38	-	-	0.26	0.32
20:1ω9	0.18	0.12	0.45	0.40	0.29	0.35
20:2ω6	0.26	0.17	0.66	0.59	0.25	0.30
unknown 1	1.16	0.74	0.91	0.81	0.41	0.49
unknown 2	0.45	0.29	tr	tr	0.34	0.41
20:4ω6	0.36	0.23	0.54	0.49	0.40	0.48
unknown 3	0.23	0.14	0.34	0.30	0.96	1.15
20:4ω3	0.30	0.19	0.74	0.66	0.27	0.32
20:5ω3	6.18	3.94	16.01	14.28	22.36	26.78
22:0	0.41	0.26	1.01	0.90	tr	tr
22:6ω3	0.50	0.32	1.26	1.12	1.08	1.29
24:0	2.87	1.83	2.02	1.80	1.34	1.60
total (mg.g^{-1} DW):		63.82		89.19		119.78
%SFA:	36.04		22.65		19.08	
%MUFA:	37.43		32.36		30.53	
%PUFA:	26.53		43.98		50.39	
SFA+MUFA/PUFA:	2.77		1.27		0.98	
ω6/ω3:	0.64		0.31		0.22	

Discussion

Nitrate, nitrite and urea were suitable nitrogen sources for *Phaeodactylum tricornutum*, whereas poor growths were obtained using ammonia as N source. These results are in accordance with other published data; microalgae belonging to different taxonomic groups can take and grow with different nitrogen compounds (Kaplan *et al.*, 1986), although differences in their ability for growing under the different sources using them have been reported (Kaplan *et al.*, 1986; Tadros & Johansen, 1988; Fábregas *et al.*, 1989; Levasseur *et al.*, 1993). Although algal cells were not pre-adapted to the new nitrogen source, duration of the lag phase was the same in the four media (24 hours), and probably related to the physiological state of inocula (early stationary phase cells).

Ammonia is a common nitrogen source for microalgal cultivation, but ammonia uptake by microalgal cells tends to result in a drop in pH of the medium (Kaplan *et al.*, 1986), which inhibited algal growth. 1 mM TRIS included in the culture media was sufficient to stabilize the pH in nitrate, nitrite and urea cultures, in which pH was maintained at the optimum values; but this TRIS concentration was insufficient to maintain the pH in ammonia cultures, in which pH tends to decline. Therefore, higher concentrations of TRIS would be needed in ammonia cultures. In cultures of the marine microalga *Dunaliella tertiolecta* with ammonia, pH was maintained within the optimum range using TRIS at a concentration as high as 15 mM (Abalde *et al.*, 1991).

Nitrogen transformation efficiency (NTE) is an important parameter in mass culture optimization, because biomass production must not be limited by nutrient supply but, on the other hand, residual unmetabolized nutrients make the product more expensive. High NTE does not necessarily denote metabolic need. Luxury nitrogen consumption and storage in protein have been observed in several microalgae under conditions of N abundance (Wikfors, 1986), when no other factors are limiting. Such consumption probably accounts for the high NTE obtained in nitrate, nitrite and urea cultures of *P. tricornutum*. NTE values in nitrate, nitrite and urea cultures proved that these cultures entered into stationary phase by nitrogen depletion, as was confirmed by biochemical data.

The nitrogen source used affected the biochemical composition of *P. tricornutum* cells. Changes in cellular composition have also been reported for *D. tertiolecta* (Fábregas *et al.*, 1989; Abalde *et al.*, 1991) cultured with different N sources. However, biochemical composition was more affected by the growth phase. Biochemical composition of microalgae is very important in mollusc larvae and spat feeding (Enright et *al.*, 1986).

Chlorophyll decreased and chlorophyll *a/c* ratio increased in the stationary phase for nitrate, nitrite and urea cultures (Table 3). Similar decline in the chlorophyll concentration has been observed in several microalgae, both under N limitation and in aged cultures (Roy, 1988; Falkowski *et al.*, 1989). It has been attributed to mobilization of N of pigment-protein complexes when cellular reserves of N diminish. Protein pools of ligth-harvesting complexes (Roy, 1988) and RuBisCO (Falkowski *et al.*, 1989) have been proposed as a transient N reservoir as cells became N limited. A relationship between nitrogen availability and chlorophyll levels was also reported either in continuous culture (Roy, 1988) and during the exponential growth in mass cultures (Fábregas *et al.*, 1985).

The increase in the chl *a/c* ratio associated to slower growth observed in our cultures was also reported by Roy (1988) and attributed to changes in the turnover rates of both pigments.

Chl *a* as proportion of ash free dry weight (AFDW) has been found to be extremely sensitive to physiological state in *P. tricornutum*, decreasing below 1% when nutrients become exhausted (Veloso *et al.*, 1991). In our experiments, this percentage declined from values about 2% in the logarithmic phase to values about 1% when growth ceased (Table 4).

Unicellular algae grown under nutrient sufficient conditions commonly assimilate the photosynthetic fixed carbon into protein to support growth and division. However, when an algal culture is deprived of an essential nutrient, cell division promptly ceases and the fraction of carbon allocated into storage macromolecules can be greatly increased at the expenses of protein synthesis that decreases due to the low availability of N (Myklestad, 1988). Microalgal cells with low protein and high carbohydrate contents have been often associated with nitrogen deficience, both in culture and *in situ* under blooms conditions (Wikfors, 1986; Myklestad, 1988). Low protein and high carbohydrate contents were found in nitrate, nitrite and urea cultures at the stationary phase (Table 4, Fig. 2), confirming the nutrient depletion deduced by the NTE values. Carbohydrates are the main carbon reserve in many diatoms (Myklestad, 1988). However carbohydrates have been found to act as an intermediate storage in several microalgae (Utting, 1985), because a time-lag is required after nitrogen exhaustion for the production of the enzymes essential for lipid synthesis (Richmond, 1986).

The PRO/CHO ratio has been suggested as a practical indicator of physiological state in marine diatom populations (Myklestad, 1988) and a number of other microalgal species (Ganf *et al.*, 1986; Wikfors, 1986). PRO/CHO ratio serves to nutritive status characterization of the cells for the more commonly limiting nutrients, nitrogen and phosphorus (Ganf *et al.*, 1986), and this ratio decreases under 2 when these nutrients are exhausted. For marine diatoms, ratios <1 are considered to indicate nutrient deficiency (Myklestad,

1988). In our experiments, PRO/CHO ratio took values less than 1 in the stationary phase in nitrate, nitrite and urea cultures, in relation with nutrient exhaustion (Table 5).

The fatty acid composition of *P. tricornutum* at the beginning of the stationary phase (Table 6) is in general agreement with the results of Thompson *et al.* (1992) for this diatom, although variations due to temperature were reported. Highest fatty acid content (120 $mg.g^{-1}$ DW) were obtained in urea cultures, followed by nitrite cultures (89 $mg.g^{-1}$ DW) and nitrate cultures (64 $mg.g^{-1}$ DW). These results are in general concordance with those found by Yongmanitchai & Ward (1991) for *P. tricornutum* according to the nitrogen source; they found higher total fatty acid contents in urea than in nitrate media. Cultures with urea presented higher contents of PUFAs (Table 6). Among these, eicosapentaenoic acid (EPA) (20:5ω3), and docosahexaenoic acid (DHA) (22:6ω3), have potential pharmaceutical uses. The relative amount of EPA of the urea-grown cells was between 1.4 and 3.6 times higher than EPA amounts of the oxidized N-grown cells. Considering EPA concentrations per dry weight of algae, differences were higher between urea (26.78 $mg.g^{-1}$ DW) and nitrate (3.94 $mg.g^{-1}$ DW) cultures. Higher EPA concentrations were also obtained in urea media than in nitrate media by Yongmanitchai & Ward (1991). Values of DHA comprised from 0.50 to 1.26% of total fatty acids, and greatest concentrations per dry weight of algae were obtained in urea media (about four times the DHA content of the nitrate-grown cells).

Lower ratios SFA+MUFAs to PUFAs, and ω6- to ω3-PUFAs, were also obtained in urea cultures, followed by nitrite cultures. These ratios are considered of nutritional value in bivalve nutrition. Diets with lower values of SFA+MUFAs to PUFAs and ratios $\omega 6/\omega 3 < 0.5$ were optimal for juvenile oysters (Enright *et al.*, 1986). Therefore, *P. tricornutum* cells grown in urea appear as the most suitable for aquaculture, related to their fatty acid composition.

The use of nitrate, nitrite or urea generated a slight increase in the pH, but productivities were not reduced whereas growth in ammonia cultures was depressed by acidity of medium. The use of this N-source for mass culture requires higher buffer concentrations, increasing strongly the production costs.

Urea growth was very similar to that obtained in nitrate, with very similar biochemical composition, but contents of total fatty acids, PUFAs and EPA were higher in urea than in nitrate cultures. Therefore, urea could be a good nitrogen source to culture *P. tricornutum* for lipid obtention. Urea is one of the lowest cost nitrogen source for microalgal culture, making urea-based medium an ideal growing medium for microalgal mass cultivation and could be the best medium to immobilized microalgae (Mak & Trevan, 1989); therefore it could be possible the use of immobilized *P. tricornutum* cells grown on urea to obtain EPA or other products of interest.

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