THE EFFECTS OF ENVIRONMENTAL VARIABLES UPON THE LIPID CLASS AND FATTY ACYL COMPOSITION OF A MARINE MICROALGA: *NANNOCHLOROPSIS OCULATA* (Droop) Eustigmatophyceae. Hibberd.

Thesis presented for the degree of
Doctor of Philosophy
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by

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To my Parents.

DECLARATION

I hereby declare that this thesis has been composed entirely by myself and has not been submitted in any previous application for a degree.

The work of which it is a record has been carried out by myself. The nature and extent of any work carried out by, or in conjunction with, others has been specifically acknowledged by reference.

ACKNOWLEDGEMENTS

First and foremost I wish to express my gratitude to my supervisors Dr. R. J. Henderson, Dr. J. W. Leftley and Professor J. R. Sargent of the University of Stirling and Dunstaffnage Marine Laboratories for their advice and encouragement throughout the duration of this project, and for their constructive criticism of draft versions of this thesis.

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I also wish to thank the Natural Environment Research Council for the provision of a research studentship allowing this work to be undertaken.

Cheers to the members of AC/DC and Iron Maiden; their music boosted me through many a late night session.

ABSTRACT

Detailed analyses of the lipid class and fatty acid composition were carried out for the marine microalgal species *Nannochloropsis oculata* (Droop) (CCAP strain no. 849/1) of the division Eustigmatophyceae (Hibberd). The alga was grown in batch and continuous culture using a novel culturing apparatus, the cage culture turbidostat, the construction of which is detailed in full.

The total lipid extract yielded by the alga varied in a growth-phase dependent manner within the range 25 % to 80 % of the lyophilised cell mass. Of this between 40 % and 70 % was recovered as fatty acid methyl esters (FAME) upon transesterification. The total fatty acid composition of *N. oculata* consisted mainly of 16:0, 16:1 and 20:5(*n*-3), these three fatty acids often accounting for greater than 80 % of the total fatty acid mass. Between 9 % and 50 % of the mass of total FAME was accounted for by 20:5(*n*-3), the balance being accounted for by variations in the relative proportions of 16:0, 16:1, 18:1, 18:2 and 20:4.

During periods of low cellular division rate, such as the lag- and stationary-phases, the proportion of polyunsaturated fatty acids (PUFA) (mainly 20:5(*n*-3)) decreased. The total fatty acids became increasingly saturated as higher proportions of shorter chain length fatty acids accumulated, mainly in triacylglycerols (TAG). Increased cellular proportions of total lipid resulted from TAG accumulation which occurred on account of preferential partitioning of carbon into TAG biosynthesis whilst cellular division was suspended. The fatty acid composition of the TAG was more saturated at high synthesis rate and *vice-versa* at lower rates. The galactolipids, monogalactosyldiacylglycerol (MGDG) and digalactosyldiacyl-glycerol (DGDG) were rich in 20:5(*n*-3) during exponential cell division containing up to 77 % and 53 % 20:5(*n*-3) respectively. Phosphatidylcholine (PC) was the only class to contain significant proportions of C_{18} fatty acids during exponential growth, thus implicating its involvement in the acyl chain elongation reactions between the C_{16} and C_{20} fatty acids.

Culture incubation temperature in the range 5 °C to 25 °C did not influence the fatty acid composition of *N. oculata*. The effect of temperature upon culture dynamics at the lower culture incubation temperatures gave an apparent decrease in the PUFA content of the total fatty acid at a given point on the cultures growth curves. By expressing the data in terms of culture doubling periods during the exponential-phases of growth it was found that temperature had no real effect upon fatty acid unsaturation or chain length, at either the total or the individual lipid class FAME level after the cells had passed through five doubling periods.

Increasing the culture medium salinity from one quarter to one and a half times that of normal seawater decreased the unsaturation and chain length of the fatty acids at both total and individual lipid class levels. The change resulted from the progressive accumulation of 18:1 and 18:2 at the expense of 20:5. Variation of salinity did not affect the dynamics of the cultures in the same respect as temperature in that a lag-phase was not observed on the cultures growth curves. However, such a phase was evident in the fatty acid profile of the cells in the period following inoculation.

The effects of culture illumination intensity in the range 45 $\mu E\ m^{-2}\ sec^{-1}$ to 170 $\mu E\ m^{-2}\ sec^{-1}$ were examined under continuous culture conditions using the cage culture turbidostat. Accumulation of saturated TAG by the cells at the higher illumination intensities gave an apparent decrease in the rate of PUFA biosynthesis. The polar lipid classes were found to be more highly unsaturated at higher illumination intensities. At lower illumination intensity TAG accumulation was reduced and the total fatty acid composition was accordingly more unsaturated. The fatty acid composition of the TAG component was more unsaturated but those of the polar lipid classes were less unsaturated than at higher illumination intensity. Increased illumination increased the degree of unsaturation of the polar lipid classes. Excess fixed carbon was partitioned into TAG biosynthesis, primarily as 16:0 and 16:1. The net accumulation of this lipid class even at high cell division rates resulted in a low overall unsaturation level.

The effects of decreasing nitrate concentration in the range 1.0 mM NO_3^- to 0.001 mM NO_3^- had a similar basis to those of illumination in that the changes in the total fatty acid composition were largely governed by the rate of TAG accumulation. At high nitrate concentrations the cellular division rate was relatively high and the proportion of TAG in the total lipid extract was low. Consequently, both total and individual lipid classes contained high proportions of unsaturates, particularly 20:5(*n*-3). However, when the nitrate concentration was decreased, such that it began to limit the rate of cellular division, TAG accumulated.

Cursory analyses of the molecular species of the galactolipid classes, MGDG and DGDG, and phospholipid class PC are presented. The effects of environmental variables are discussed in terms of the changes which may occur in the growth phase distribution of the cells in asynchronous culture, along with the concommitant changes in the lipid composition of the cells. The potential linkage of the elongation and desaturation reactions with both MGDG and PC is also discussed briefly with reference to future research.

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LIST OF ABBREVIATIONS & SYMBOLS USED.

ACP - Acyl carrier protein.
$AgNO_3$ - Silver nitrate.
BHT - Butylated hydroxy-toluene.
Car. - Carotenoids.
CCT - Cage culture turbidostat.
$CHCl_3$ - Chloroform.
Chl. a. - Chlorophyll a.
Chol. - Cholesterol.
Co-A - Coenzyme A
DAG - Diacylglycerol.
DGDG - Digalactosyldiacylglycerol.
EtOH - Ethanol.
ER - Endoplasmic reticulum.
FAME - Fatty acid methyl ester.
FFA - Free fatty acid.
FID - Flame ionisation detection.
G-3-P - *sn*-Glycerol-3-phosphate.
GC-MS - Gas chromatography-mass spectrometry.
GLC - Gas liquid chromatography.
HAc - Glacial acetic acid.
HPLC - High performance liquid chromatography.
HPTLC - High performance thin layer chromatography.
HUFA - Highly unsaturated fatty acids.
iso-PrOH - Propan-2-ol.
LPC - *Lyso*-phosphatidylcholine.
MCM - Mean cellular mass.
MeCN - Acetonitrile.
MeOH - Methanol.
MAG - Monoacylglycerol.
MGDG - Monogalactosyldiacylglycerol.
Mol.spp. - Molecular species.
OFN - Oxygen free nitrogen
PC - Phosphatidylcholine.
PE - Phosphatidylethanolamine.
PG - Phosphatidylglycerol.
PI - Phosphatidylinositol.
PUFA - Polyunsaturated fatty acids.
PS - Phosphatidylserine.
RFE - Rotary film evaporator.
RI - Refractive index.
SE - Sterol ester.
SQDG - Sulphoquinovosyldiacylglycerol.
ST - Sterol.
TAG - Triacylglycerol.
TLE - Total lipid extract.
TLC - Thin layer chromatography.
WE - Wax ester.
§ - prepared using Chemdraw™.
¶ - prepared using CricketGraph™.
† - prepared by hand.

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SECTION 1: INTRODUCTION.

1.1 LIPIDS IN GENERAL.

The term 'lipid', applied very broadly, describes biomolecules distinguishable from proteins, carbohydrates and nucleic acids by their solubility in organic solvents such as hexane, diethyl ether, chloroform and methanol and insolubility in water. The solvent mixture used in their extraction from tissues determines to a certain extent the composition of the lipid extract yielded (*cf.* Chuecas & Riley, 1969; Guckert *et al.* 1988). However, as no strict definition has been internationally agreed upon, the interpretation used here restricts 'lipids' to a narrowed field of compounds. Specifically these are long chain ($\geq C_{14}$) fatty acids and their glycerol ester derivatives, or glycerolipids. These are often also referred to as the 'acyl' or 'saponifiable' lipids, because fatty acid moieties are released as free fatty acids upon acid hydrolysis or as soaps upon alkaline hydrolysis.

Examples of the non-acyl, or non-saponifiable, groups which were beyond the scope of the present study are steroids, terpenes, carotenoids and photosynthetic pigments. These were not dealt with in any detail, but they were co-extracted with the acyl lipids by general extraction procedures employed here (*e.g.* Bligh & Dyer, 1959; section 3.2). Their presence, often making a significant contribution to 'total lipid' mass, consequentially had to be considered. The term 'total lipid extract' (TLE) was applied to the fraction yielded by the initial organic extraction of cellular material. The most significant non-acyl contributors to total lipid mass in the case of *N. oculata* were the sterols and photosynthetic pigments.

1.2 FATTY ACID STRUCTURE & NOMENCLATURE.

There are several systems of nomenclature regarding fatty acids and because of the variety of usage throughout the literature, the reader should be aware of these. In a thesis such as this, full systematic names are cumbersome and the exactitude they imply is not justified by the GLC-FID analysis techniques employed. Trivial names are often confusing

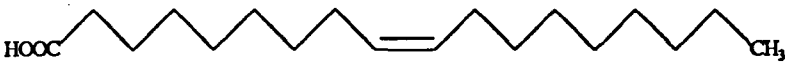
as they bear no systematic relationship to fatty acyl structure, although they are still in common use amongst the 'old hands' and in the older literature.

A mixture of systematic and shorthand notation has become commonplace and it is that which has been adopted here. Unless stated otherwise, it is generally accepted that the double bonds are of the methylene ($-CH_2-$) interrupted *cis* conformation (Gurr & James, 1980; Christie, 1982; Gunstone, 1986). This assumption holds true for marine lipids containing PUFA (see Ackman *et al*, 1964; Pohl & Zurheide, 1979) ultimately derived from marine microalgae. By stating the carbon chain length, number of double bonds and the position of the first double bond, from either terminus, the structure of the common fatty acids may be adequately described. Branched chains are denoted by the *iso-* or *anteiso-* prefix.

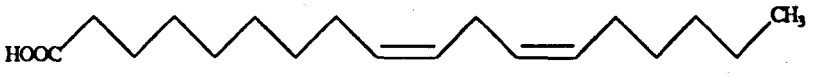
There are alternative notations for the position of the first double bond. The Δ notation designates the terminal carboxyl group carbon atom as C_1 whereas the ω and (*n*-*) notations designate the terminal methyl carbon atom as C_1. The (*n*-*) nomenclature advocated by the IUPAC-IUB Commission on Biochemical Nomenclature (1967) was used throughout this study due to the ease by which the fatty acids of similar biosynthetically derived families may be distinguished (see 1.4 & Gunstone, 1986). The (*n*-3) series of unsaturated fatty acids predominate in the marine environment in contrast with the (*n*-6) series which dominate in terrestrial and freshwater environments (Sargent & Henderson, 1986; Wood, 1988).

i) 16:1(*n*-13)*trans*; *trans*-3-hexadecenoic acid; 16:1ω13; 16:1Δ^{3}

ii) 18:1(*n*-9); oleic acid; *cis*-9-octadecenoic acid; 18:1ω9; 18:1Δ^{9}



iii) 18:2(*n*-6); linoleic acid; *cis,cis*-9,12-octadecadienoic acid; 18:2ω6; 18:2$\Delta^{9,12}$



iv) 18:3(*n*-3); linolenic acid; all-*cis*-9,12,15-octadecatrienoic acid; 18:3ω3; 18:3$\Delta^{9,12,15}$

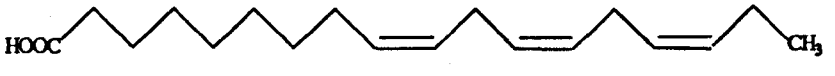


Figure 1.2.1: The relationships between different nomenclature systems for unsaturated fatty acids: (shorthand (*n*-*); trivial; full systematic; shorthand ω; and Δ notations)[1].

1.3 LIPID CLASSES.

The acyl lipids are routinely divided at several structural levels. A common division is into the 'simple' and 'complex' lipids, also termed 'neutral' and 'polar' lipids. The basis of this seems to correlate with their behaviour on silica gel TLC plates. The neutral classes are moved from the origin using a low polarity solvent mixture, whereas the polar classes remain at the origin. However, the terms simple and complex have a more direct chemical basis. Simple lipid classes yield either one or two products upon complete hydrolysis; *i.e.* fatty acids and the moieties to which they were previously esterified, a sterol, methanol, glycerol or fatty alcohol group. Complex lipid classes yield three or more hydrolysis products; fatty acids, three carbon alcohol backbone and head group (see Gurr & James, 1980; Christie, 1982).

Each of the simple lipid classes may be subdivided into individual classes. Examples of those of major quantitative importance are shown in Figure 1.3.1. The simple class of importance in the present study is triacylglycerol (TAG). It is the main simple acyl lipid class and represents a potential sink for photosynthetically derived carbon under certain environmental conditions (see below).

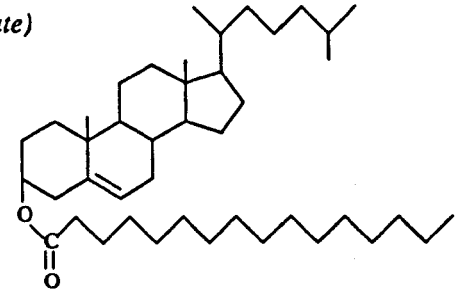
Of the complex lipids, two groups are of importance in the present study: the glycolipids and phospholipids. Both of these groups, derived *via* initially similar biosynthetic pathways, are structural components of cellular membranes and potential substrates in polydesaturation pathways (see Section 1.4). The glycolipids of microalgae are, for the most part, associated with the plastid membranes. They consist of a glycerol backbone to which a mono or dimeric non-phosphorylated sugar moiety is glycosidically linked at the *sn*-3 position (small amounts of tri, tetra and pentameric sugar chains are often also detectable) (Joyard & Douce, 1987). Acyl moieties are esterified at the *sn*-1 and *sn*-2 positions. There are three main glycolipids present in significant proportions in the majority of microalgal species. These are monogalactosyldiacylglycerol, digalactosyldiacylglycerol

and sulphoquinovosyldiacylglycerol (MGDG, DGDG and SQDG respectively). Structural examples are shown in Figure 1.3.2. Other additional sulphur containing lipid classes are present in minor proportions in a wide variety of microalgal species (Kates & Volcani, 1966; Opute, 1974; Mercer & Davis 1975; 1979).

The phospholipid classes consist of a glycerol backbone phosphorylated at the *sn*-3 position and the phosphate may be linked to a range of small molecules including organic bases, amino acids and alcohols. As with the galactolipids, fatty acids may be esterified at both the *sn*-1 and *sn*-2 positions. General structural examples of each of the quantitatively significant phospholipid classes found in the present study are given in Figures 1.3.3 and 1.3.4.

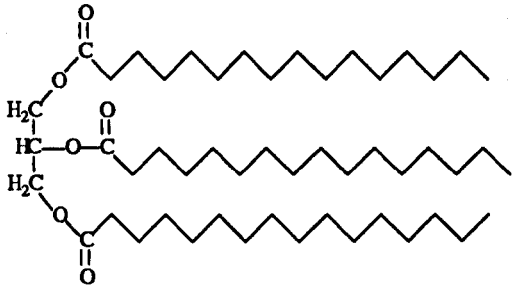
Kates & Volcani (1966) characterised the lipid classes of several diatom species using radiolabelling techniques. These authors showed that the algae have very similar qualitative compositions to those of the leaves of higher plants. The same major structural lipid classes MGDG, DGDG, SQDG, PG, PI & PC being present.

i) **Steryl esters.**
(Cholesteryl palmitate)

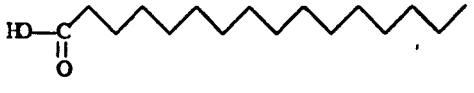


ii) **Methyl esters.**
(Methyl palmitate)

iii) **Triacylglycerols.**
(Tripalmitin)



iv) **Free fatty acids.**
(Palmitic acid)



v) **Sterols.**
(24-methylcholesta-5,22E-dien-3β-ol)
"Crinosterol"

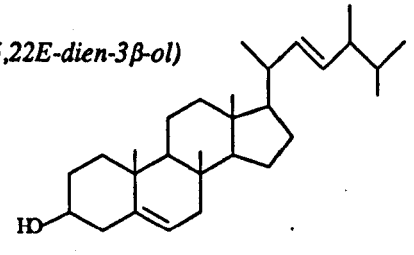
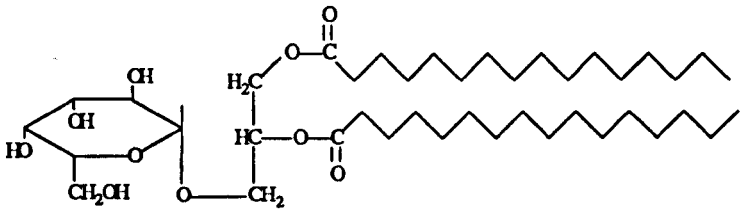


Figure 1.3.1: Structural representations of examples of simple lipid classes commonly found in lipid extracted from microalgal cells[4].

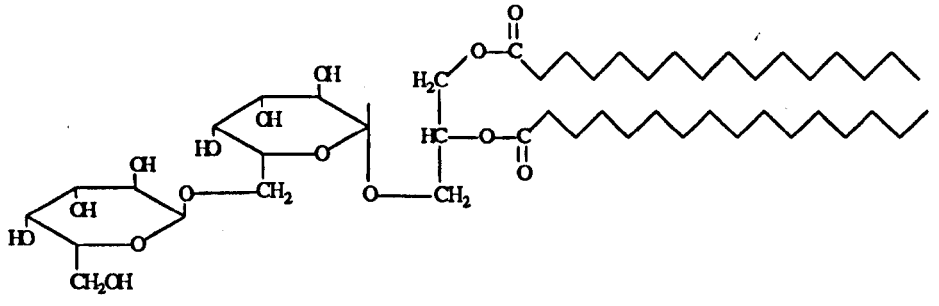
i) Monogalactosyldiacylglycerols.

(1,2-dihexadecanoyl[α-D-galactopyranosyl(1'-3')]-sn-glycerol)



ii) Digalactosyldiacylglycerols.

(1,2-hexadecanoyl[α-D-galactopyranosyl-(1'-6')-β-D-galactopyranosyl(1'-3')]-sn-glycerol)



iii) Sulphoquinovosyldiacylglycerols.

(1,2-dihexadecanoyl[6-sulpho-α-D-quinovosyl-(1'-3')]-sn-glycerol)

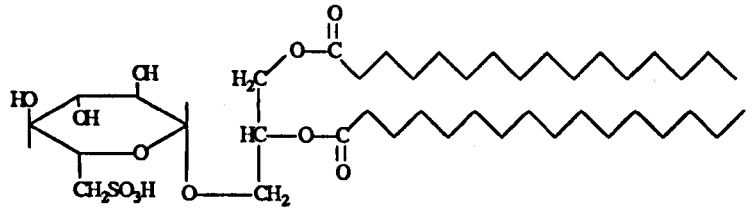
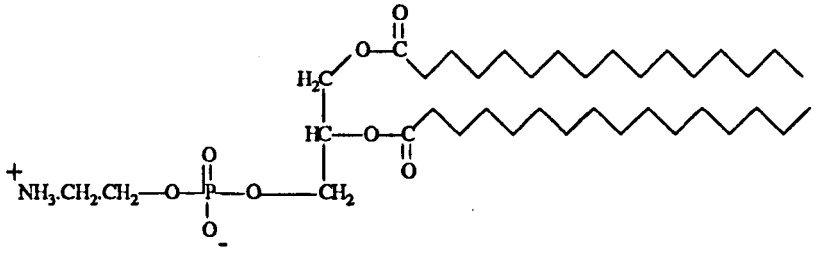
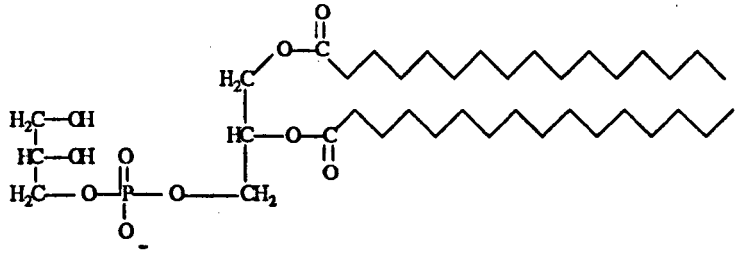


Figure 1.3.2: Structural representations of examples of glycolipid classes commonly found in lipid extracted from microalgal cells[8].

i) **Phosphatidylethanolamines.**



ii) **Phosphatidylglycerols.**



iii) **Phosphatidylinositols.**

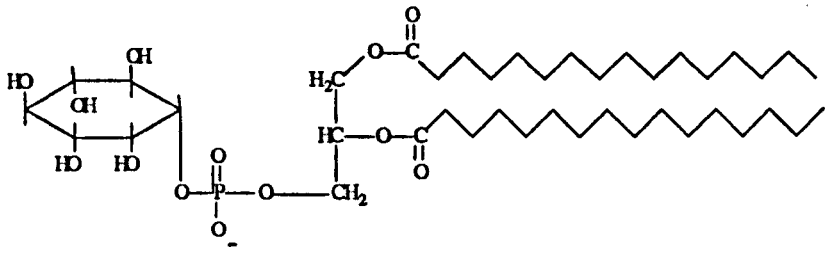
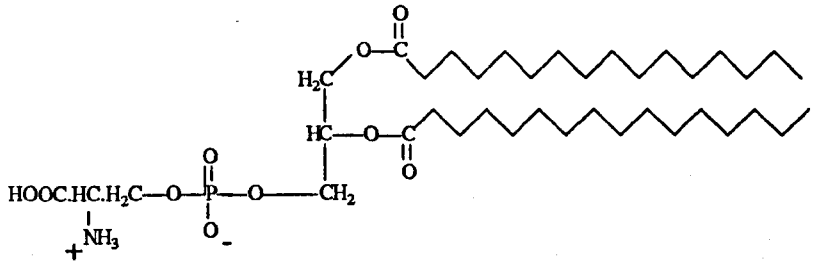
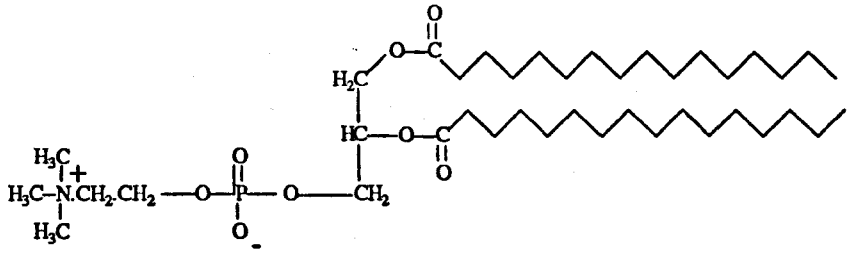


Figure 1.3.3: Structural representations of examples of phospholipid classes commonly found in lipid extracted from microalgal cells[5].

i) **Phosphatidylserines.**



ii) **Phosphatidylcholines.**



iii) ***Lyso*-phosphatidylcholines.**

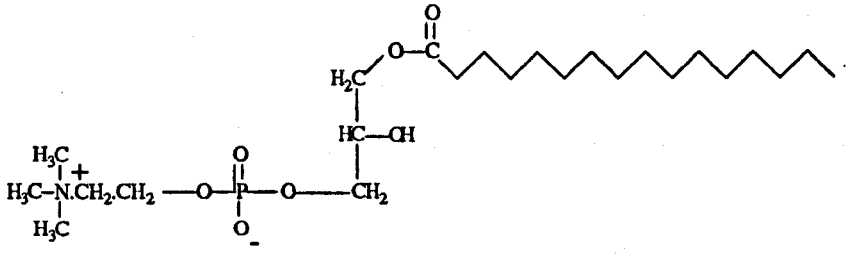


Figure 1.3.4: Structural representations of examples of phospholipid classes commonly found in lipid extracted from microalgal cells[§].

1.4 BIOSYNTHETIC ORIGINS.

1.4.1 *De novo* fatty acid synthesis in eukaryotic plants and algae.

In higher plants the proplastids and plastids, or chloroplasts, are considered to be the sole sites of *de novo* fatty acid synthesis. The basis of this is the localisation of acyl carrier protein (ACP) in these organelles (Ohlrogge *et al.*, 1979; Stumpf, 1980). The detail of the general sequence of reactions (Figure 1.4.1) involved in the *de novo* synthesis of 16:0 may be found in standard biochemistry texts (*e.g.* Lehninger, 1975; Metzler, 1977; Stryer, 1981; Zubay, 1988) as well as the more specific lipid biochemistry texts (*e.g.* Gurr & James, 1980; Harwood & Russell, 1984; Vance & Vance, 1985). Pyruvate, derived from glucose *via* glycolysis (Miernyk & Dennis, 1982), is converted to acetyl-CoA by the pyruvate dehydrogenase complex (PDC) (Miernyk & Dennis, 1982). Evidence indicates that other TCA cycle intermediates (*e.g.* propionate) are not major sources of C_2 or C_3 units for incorporation into fatty acids (Yamada & Nakamura, 1975).

The subcellular source of the acetyl-CoA units is still unclear. They may originate directly from proplastid / chloroplast glycolysis as reported for spinach chloroplasts, castor oil seed proplastids and pea chloroplasts (Yamada & Nakamura, 1975; Miernyk & Dennis, 1982; Camp & Randall, 1985). Miernyk & Dennis (1982) showed the enzymes of the proplastid pathway to be isozymes of the corresponding cytosolic enzymes. Yamada & Nakamura (1975) working upon spinach chloroplasts found a complete complement of glycolytic enzymes enabling the following pathway to operate in the chloroplasts:

PGA ⟶ PEP ⟶ pyruvate ⟶ acetyl-CoA ⟶ fatty acids

In contrast, Liedvogel & Stumpf (1982), unable to demonstrate spinach chloroplast PDC activity, proposed an alternative pathway for acetyl-CoA derivation. Pyruvate derived *via* the cytosolic glycolytic pathway enters the mitochondria where the sequential action of mitochondrial PDC and acetyl-CoA hydrolase yields inert free acetate. This diffuses from the

mitochondria, *via* the cytosol, into the chloroplasts where it is re-esterified by acetyl-CoA synthetase localised in the stroma phase to form reactive acetyl-CoA. However, this pathway may be specific to spinach chloroplasts. The presence of mitochondrial acetyl-CoA synthetase in other plant species has been questioned and evidence for the presence of plastid PDC is reportedly becoming more widespread (Camp & Randall, 1985).

The acetyl-CoA moieties are carboxylated by acetyl-CoA carboxylase to yield malonyl-CoA (Yamada & Nakamura, 1975). These two esters are substrates for the fatty acid synthetase (FAS) complex localised in the stroma of the chloroplast (see Stumpf, 1984; 1987). The final product of the FAS elongation cycle (Figure 1.4.1) is 16:0-ACP. This is readily elongated to 18:0-ACP by 16:0-ACP elongase which is often a major contaminant of FAS preparations (Harwood, 1975). This accounts for the incorporation of ^{14}C-acetate into the C_{18} fractions of FAS incubations. The sites of FAS and ACP localisations vary between plants (in the chloroplasts) and animals and prokaryotes (in the cytoplasm), indicating different sites of *de novo* fatty acid synthesis. The biosynthetic pathways leading to saturated fatty acids remain essentially similar in prokaryotes and eukaryotes. However, divergence occurs at the desaturation stages.

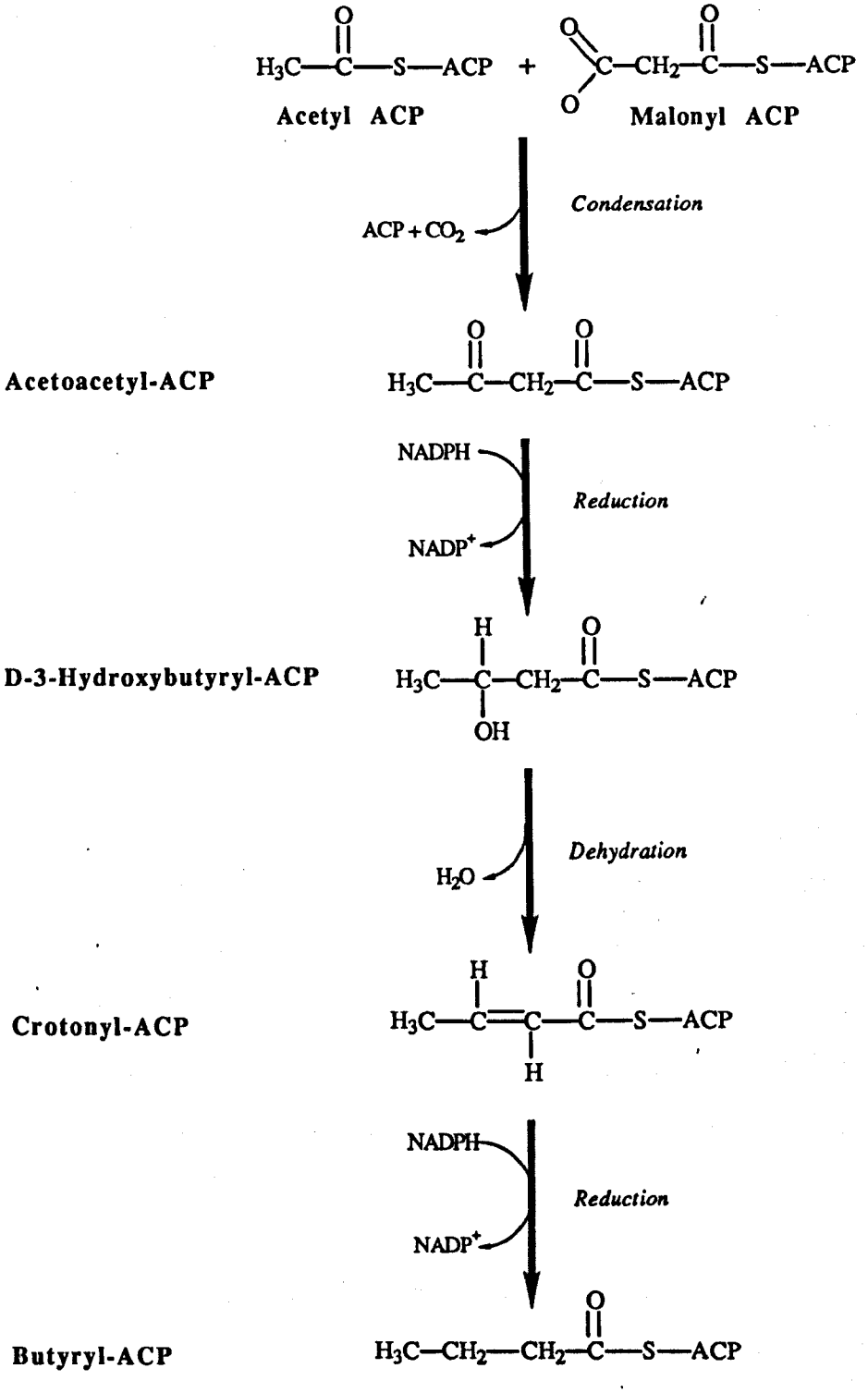


Figure 1.4.1: Reaction sequence for the *de novo* synthesis of fatty acids: condensation, reduction, dehydration, and reduction. Intermediates for first round of synthesis (After Stryer, 1981)[8].

1.4.2 Monounsaturated fatty acid biosynthesis.

Two types of desaturation, the introduction of a *cis* double bond into a hydrocarbon chain, are known; these are the oxygen independent and dependent (the so called anaerobic and aerobic) mechanisms. The anaerobic mechanism, although it will proceed in the presence of molecular oxygen, does not require it, the double bond being introduced by a β-hydroxydecanoyl-ACP dehydrase in prokaryotes. The major products of this enzyme are 16:1(*n*-7) and 18:1(*n*-7). The aerobic mechanism requires a reductant (NADPH or NADH: Photosystems I & II may also donate electrons), an electron carrier (ferredoxin), a desaturase enzyme and molecular oxygen. All evidence indicates the aerobic mechanism as the principal desaturation mechanism for the synthesis of unsaturated fatty acids. None of the existing evidences supports the anaerobic mechanism in plants (Stumpf, 1980).

The *in vitro* aerobic desaturation of 18:0 to 18:1(*n*-9) in eukaryotes has been studied in detail (see Jaworski, 1987). The enzyme involved, 18:0-ACP desaturase, is localised in solution in the stroma of the chloroplasts. Its solubility was a key factor in its isolation and purification using DEAE and affinity column (Sepharose 4B to which ACP was bound) chromatography (McKeon & Stumpf, 1982). Further desaturation, or polydesaturation, is less well understood. The difficulties encountered in purification of further active desaturases for *in vitro* study probably originate in part from their being membrane associated. The substrates for these enzymes are not well defined and may well take the form of complex glycerolipid classes (see Section 1.5).

Evidence from the prokaryotic cyanobacteria *Anabaena variabilis* shows that newly synthesised 18:0 is esterified not to ACP but to monoglucosyldiacylglycerol (MGlcDG) prior to desaturation to 18:1(*n*-9). The MGlcDG is subsequently epimerised to MGDG as a final step (Lem & Stumpf, 1984; Sato *et al.*, 1986). Similar reactions may be observed in another prokaryotic alga: *Prochloron* sp. (Murata & Sato, 1983). *A. variabilis* shows no detectable 18:0-ACP desaturase, 18:0-CoA desaturase or acyl-CoA transferase activities (Lem & Stumpf, 1984; Murata & Nishida, 1987). In the light of the current status of the

cyanobacteria as putative progenitors of eukaryotic cell chloroplasts, the absence of these activities, when comparing prokaryotic systems with eukaryotic systems, links them with the later evolution of flux control mechanisms integrating fatty acid metabolism between organelles (Lem & Stumpf, 1984).

1.4.3 Desaturation capabilities of animals, higher plants & algae.

Higher plants and animals exhibit distinctly different polydesaturation pathways, whereas exceptions such as the phytoflagellate *Euglena gracilis* and many marine algal species exhibiting a combination of both types (see Figure 1.4.2). Higher plants and chlorophytic algae normally introduce double bonds between the terminal methyl group and an existing double bond. Animals introduce double bonds between the terminal carboxyl group and an existing double bond. The major fatty acyl substrates for polydesaturation pathways are the products of the Δ^9 desaturases, 18:1(*n*-9) and to a lesser extent 16:1(*n*-7).

Animals possess the Δ^4, Δ^5, Δ^6 and Δ^9 desaturases (Figure 1.4.2). Because they are unable to desaturate fatty acids beyond the Δ^{9-10} position, and retroconversion and elongation occur at the carboxyl terminal, the (*n*-*) notation is not subject to change by elongation or animal desaturation pathways. A characteristic plant fatty acid, 18:2(*n*-6), is essential to the healthy development and growth of mammals and other vertebrates as a precursor of eicosanoids and other biologically active biomolecules, (*n*-3) series fatty acids are essential in neural and male reproductive tissue. Thus the (*n*-6) and (*n*-3) series PUFA are ultimately, if not always directly, derived *via* the food chain from plant primary production. The dietary origins of certain non-interchangeable fatty acid families, notably the (*n*-6) and (*n*-3) families, become more obvious through the use of the (*n*-*) notation.

When compared with the characteristic higher plant and animal pathways described above, the marine microalgae exhibit a broader range of desaturase activities (see Figure 1.4.2). This is manifest in the presence of 18:4(*n*-3), 20:5(*n*-3) and 22:6(*n*-3). These acids cannot be synthesised from 18:1(*n*-9) using the Δ^{12} and Δ^{15} desaturases alone. The range of

desaturases was illustrated for a characteristic diatom species *Phaeodactylum tricornutum* (Moreno *et al.*, 1978). The labelling patterns produced after incubation of algal cultures in the presence of various ^{14}C labelled fatty acid substrates enabled these authors to conclude that *P. tricornutum* must possess Δ^6, Δ^5 and Δ^4 desaturase activities. The relevance of this is that many marine microalgae are able to desaturate to either side of the initial double bond at the Δ^9 position of the 18:1(*n*-9) substrate (also 16:1(*n*-7) but to a lesser extent). They therefore possess desaturase activities usually considered as characteristic of both higher plants *and* animals. The *de novo* synthesis and accumulation of (*n*-3) highly unsaturated fatty acids (HUFA) (*i.e.* acids with more than 4 double bonds per carbon chain) is a general, but not universal, characteristic of marine as opposed to freshwater phytoplankton (see reviews by Pohl & Zurheide, 1979; Wood, 1988).

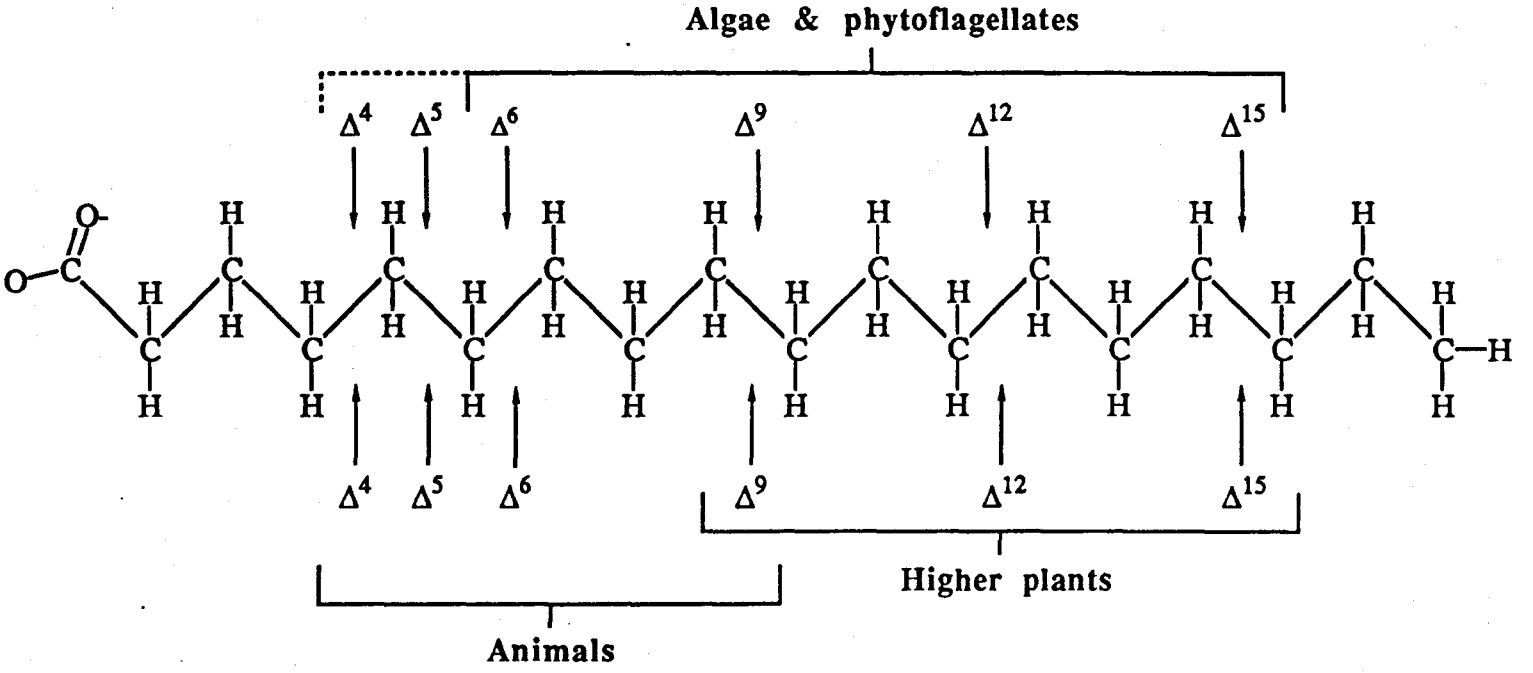


Figure 1.4.2: Positions of fatty acyl chain desaturase enzyme action in animals, higher plants, algae and phytoflagellates. (After Cook, 1985)[8].

1.5 INTEGRATION OF POLYDESATURATION PATHWAYS WITH COMPLEX GLYCEROLIPID BIOSYNTHETIC PATHWAYS.

1.5.1 Is the microalgal cell analogous to the higher plant leaf cell ?

In a section such as this it is not possible to deal in depth with a subject to which volumes have already been dedicated (see Stumpf, 1980; 1987; Harwood, 1979; Mazliak, 1980). Instead, a brief overview of potential pathways is presented to summarise lipid class - lipid class acyl transferase catalysed exchanges, diacylglycerol exchanges and to give perspective to existing algal lipid biosynthesis studies as well as data presented in later sections of this thesis.

To avoid problems regarding the lack of cellular homogeneity and physiological problems associated with wound damage *etc.* in leaf slices from higher plant tissues (Roughan & Slack, 1982) early radiolabelling experiments were conducted using *Chlorella* spp. (Nichols, 1966; Nichols *et al.*, 1967). Records of such strategies go back much further in the field of photosynthetic carbon fixation. The work of Warburg (1919) paved the way for that of Calvin & Benson (1948).

The culture itself could be regarded as a homogeneous organism which could be subdivided and sampled without damage. Precursors and cofactors could be conveniently supplied in the culture medium therefore avoiding excision of leaves and other wounding procedures to provide experimental material. The results obtained from such experiments were extrapolated to higher plants and as a result the question above was asked. The consensus, apparently, was that the metabolism of leaf and seed tissues were sufficiently different from microalgae to warrant surmounting, rather than skirting, the problems originating from the use higher plant tissues. With the subsequent advancement of subcellular fractionation techniques, better isolated chloroplast preparations were obtained. Much work was concentrated here due to the greater commercial importance of higher plants in terms of vegetable oil and crop production. Unfortunately this has led to little

advancement in the knowledge of algal lipid biosynthesis over the last decade or so. Therefore, there is a comparative paucity of direct information. However, the interest generated by the recent revelation of the essential fatty acid requirements of commercially farmed marine fin and shellfish species has resulted in an increased publication of algal lipid compositional data in aquaculturally biased journals.

A plethora of theoretical pathways exists for the further desaturation of 18:1(*n*-9) through 18:2(*n*-6) to 18:3(*n*-3) and beyond, following elongation. These are further compounded by differential derivation sites for various molecular species of diacylglycerol (DAG) moieties within the galactolipid and phospholipid classes amongst the genera of higher plants and model algae (*e.g. Chlorella* & *Chlamydomonas* spp.). Most of the literature is concerned with higher plants and chlorophytic algae, both having maximum chain lengths up to C_{18} *and* similar PUFA profiles. These studies in effect deal with desaturation alone as the potential for further elongation to C_{20} fatty acids does not exist. Information regarding biosynthetic pathways in the non-chlorophytic freshwater or marine algae with $C_{20/22}$ fatty acids is scarce and usually limited to compositional data at single time points. The potential for integration of the elongation and desaturation pathways may be more fully studied through the use of algal systems. Work with isolated algal chloroplasts is complicated by difficulties in obtaining clean subcellular fractions from species with inorganic cell walls or frustules (*e.g.* diatoms) since procedures used to rupture these structures normally disrupt any membranes within the cell (Nichols & Appleby (1969). As a result, most of the algal lipid biosynthetic studies use *in vivo* radiolabelling techniques and these are inherently limited in the type of information they yield.

Despite this, early work upon *Chlorella vulgaris* (Nichols, 1966; Nichols *et al.*, 1967; Gurr *et al.*, 1969; Safford & Nichols, 1970) had provided the origins of concepts widely adopted in higher plant polydesaturation pathways. These include the involvement of complex glycerolipids as substrates for desaturases, acyl turnover and diacylglycerol exchange interactions, and the chain length positional specificity of glycerolipid classes (see

Section 1.5.2). The assumption of microalgal / higher plant analogy may not be entirely accurate. However, there is sufficient overlap to provide a framework for further speculation and extrapolation from the *Chlorella* spp., which closely resembles higher plants with respect to qualitative fatty acyl composition, to the marine species of other genera.

1.5.2 Derivation & subcellular origins of *sn*-1,2-diacylglycerol moieties.

The higher plant chloroplasts are well established as the sole sites of *de novo* fatty acid synthesis (Roughan & Slack, 1982; 1984). Fatty acyl-ACP / CoA esters were thought to be translocated out of the chloroplast prior to esterification to *sn*-glycerol-3-phosphate (G-3-P) by acyltransferase enzymes associated with microsomal endoplasmic reticulum (ER). These enzymes form the basis of the Kennedy (Harwood & Russell, 1984) or Kornberg-Pricer (Joyard & Douce, 1987) pathway in which G-3-P is sequentially acylated to form lyso-phosphatidate (LPA) and phosphatidate (PA), and then dephosphorylated to yield the *sn*-1,2-diacylglycerol (DAG) moieties used in complex glycerolipid biosynthesis and possibly TAG biosynthesis (Kennedy, 1961).

Ferrari & Benson (1961) studied the incorporation of $^{14}CO_2$ into the lipids of *C.pyrenoidosa* and concluded that phospholipid and TAG were not derived from the same DAG pool due to differences in the turnover rate of the radio-label incorporated into the fatty acyl moieties of phospholipid and TAG. The findings presented in this thesis suggest for *Nannochloropsis oculata* that both TAG and the phospholipid classes share a common diglyceride pool. Some of the effects of environmental variables are due to modifications of the fluxes from this intermediate DAG pool into the committed pathways of either complex or TAG biosynthesis. The evidence supports the idea that the fatty acyl elongation and desaturation reactions leading to C_{20} PUFA are the result of acyl transfer reactions between the chloroplast lipid class monogalactosyldiacylglycerol and phosphatidylcholine as has been suggested by several authors.

By virtue of their highly characteristic lipid class and fatty acid compositions it seems

logical that the chloroplasts be actively involved in the biogenesis of their own membrane lipids. Glycolipids constitute 80% of the polar thylakoid lipid classes (Joyard & Douce, 1977), 90% of these being MGDG and DGDG (Wintermans, 1960; Benson, 1971). Since the early elucidation of the Kornberg-Pricer / Kennedy pathway (Kornberg & Pricer, 1953; Kennedy , 1961) in guinea pig liver extracts, similar enzyme activities were demonstrated for avocado mesocarp (Barron & Stumpf, 1962) and spinach microsomal ER (Chenia, 1965; Sastry & Kates, 1966). Later such activities were also shown in association with plant cell organelles including the Golgi apparatus, mitochondria and chloroplasts (Boehler & Ernst-Fonberg, 1976; Jelsema *et al.*, 1982). Studies using higher plant (*i.e.* spinach) chloroplasts showed the envelope membrane, of which MGDG is a major component, as the sole site of PA synthesis (Joyard & Douce, 1977; 1979). PA may be sequentially dephosphorylated and galactosylated in the presence of UDP-galactose at the envelope membrane to form MGDG. However, Jelsema *et al.* (1982) working with both mutant and wild type strains of *Chlamydomonas reinhardtii* provide evidence that both envelope and thylakoid (where DGDG is localised) membranes are capable of incorporating *sn*-glycerol-3-phosphate into their lipids. Whether this contrasting evidence is an artifact of subcellular fractionation or a true phylogenic difference is still debatable.

The hydrolysis of phosphatidate is a significant branching point in the biosynthesis of galactolipids, sulpholipids and phospholipids. Although the biosynthetic pathways of diacylglycerol moieties of all complex glycerolipids are apparently similar the characteristic fatty acid profiles of individual lipid classes must reflect some mechanism of control. There are several potential levels of control, a well documented example of this originating in the acylation of G-3-P in different cellular compartments of higher plants and chlorophytic microalgae. Two discrete pools of DAG have been recognised. These may be differentiated by the distribution of C_{16} and C_{18} fatty acids at the *sn*-2 position of DAG.

The chloroplasts synthesise DAG with C_{16} fatty acids esterified at the *sn*-2 position and either C_{16} or C_{18} fatty acids at the *sn*-1 position. An analogous pattern is observed in the

cyanobacteria, the putative progenitors of higher plant chloroplasts in the endosymbiotic theory of organelle evolution. Hence, this pattern is known as the 'prokaryotic' pattern. The resultant 18:1/16:0 MGDG molecular species (mol.sp.) is subsequently sequentially desaturated at both positions yielding the characteristic 18:3/16:3 MGDG mol.sp.. The alternative pool of DAG is characterised by C_{18} fatty acids at the *sn*-2 position and C_{16} or C_{18} fatty acids at the *sn*-1 position. The 18:1/18:1 or 16:0/18:1 DAG mol.sp. have the 18:1 moiety desaturated yielding the characteristic eukaryotic DAG pattern with C_{18} PUFA at the *sn*-2 position.

These pathways operate simultaneously but in different cellular compartments. In some cases it is possible to distinguish groups of plants by the proportions of the specific DAG types present in their MGDG. These have become known as the '16:3' plants (*e.g.* spinach, *Chlorella* spp. & *Ulva* spp.) and '18:3' plants (*e.g.* maize and legumes generally) (Roughan & Slack, 1982; 1984). The '16:3' plants have proportionately more prokaryotic type DAG whereas the '18:3' plants have proportionately more eukaryotic type DAG in their MGDG. The reasons behind this and the mechanisms by which the proportions are controlled are still unknown. There is, however, an apparent taxonomic linkage, down to the division level at least.

The marine algae add a certain amount of depth to the patterns above due to their abundance of the characteristic marine fatty acid 20:5(*n*-3) in their galactolipids. Analyses of the molecular species compositions and fatty acyl positional distributions for non-chlorophytic algae are rare. However, existing data hint that 20:5(*n*-3) may be regarded as analogous to the 18:3(*n*-3) moieties of higher plants and Chlorophyta. Arao *et al.* (1987) working with the marine diatom *P. tricornutum* showed 20:5(*n*-3) to be localised at the *sn*-1 positions in all complex glycerolipid classes with the exception of PC wherein it was distributed between both *sn*-1 and *sn*-2 positions. C_{16} PUFA were concentrated at the *sn*-2 position of MGDG indicating *P. tricornutum* to be a '16:3' or prokaryotic type species. In a following publication (Arao & Yamada, 1989) several algal species from the Rhodophyta,

Phaeophyta and Chlorophyta were shown to have differing chain length positional specificities at the *sn*-1 and *sn*-2 positions of their galactolipids.

The findings of the current thesis suggested that *N. oculata* was an eukaryotic type species. Due to the abundance of 20:5(*n*-3) in its MGDG (up to 75 %) it was inevitable that it should have a substantial proportion of the di-20:5 molecular species. This was supported by a cursory analysis of the molecular species composition of the galactolipid classes undertaken as part of the current study. This information, linked with further data gathered during the course of this study and that of Nichols *et al.* (1967), Safford & Nichols, (1970), Arao *et al.* (1987) and Arao & Yamada (1989), lent itself to the formation of conclusions regarding the potential pathways of elongation and desaturation and their association with lipid class and *sn*-positionally specific reactions to account for the fatty acyl distributions encountered both experimentally and in the literature. These pathways are tentatively proposed in later sections of this thesis.

SECTION 2: MICROALGAL FATTY ACIDS & THE EFFECTS OF ENVIRONMENTAL VARIABLES: A LITERATURE REVIEW.

2.1 Introduction: A Brief History of Algal Lipidology.

The aim of the following section was to provide a brief overview of the chain of events which led to the initial research upon algal biochemistry. The progression from that point to the present day where the importance of algal lipids and fatty acids covers such diverse fields of interest as pure biochemistry, marine ecology, aquaculture, mariculture, biotechnology, alternative energy sources, petrochemicals and even space research. Not all these fields are *directly* concerned with the subject matter of the current study but they serve to illustrate the wide ranging implications of algal lipid composition. It is mainly the data and / or the analytical techniques they contain that are relevant here, rather than the research topics with which they are involved. However, such studies are, in part, responsible for the data contributing to the reasons behind the initiation of studies such as the one presented in this thesis.

Korn (1964) observed that biomolecules such as the purine and pyrimidine bases, amino acids and sugars, with rare exceptions, are universally abundant in most living organisms (*cf.* Chuecas & Riley, 1969). In comparison, fatty acids have a great capacity for structural diversity in terms of combinations of chain length, number and position of double bonds. Consequentially, specific and characteristic distribution patterns are often displayed upon examination. This factor has become the cornerstone of many studies in the past forty to fifty years. A wide variety of data has become available as a result of the studies concerned with the fatty acid composition of marine and freshwater microalgae, or phytoplankton. As the techniques of both lipid class and fatty acid analyses have developed and improved, a concurrent increase in the detail and accuracy of such analyses has also occurred. To a large extent, such changes have resulted in a retrospective pattern of analysis, followed by later reanalyses of the same few species (*e.g.* Schlenk *et al.*, 1960; also *cf.* Pohl

& Zurheide, 1979). It is probable that fewer than forty species of marine microalgae have been subjected to any more than a superficial analysis in as many years (*cf.* Borowitzka, 1988). Considering the range of species and strains available this is barely a representative cross-section (*cf.* Pohl & Zurheide, 1979; Thompson *et al.*, 1988).

Early work showed great insight into the partitioning of metabolic intermediaries during divisional and non-divisional growth phases of both *Chlorella* and diatom species (Beijerinck, 1890; 1904; *cf.* Soeder, 1986; Fogg, 1988). However, the very limited analytical technology and algal species in pure culture available did not allow the provision of the hard data necessary for firm conclusions to be formed. At this time the techniques applied to bacteria by microbiologists were being applied to the microalgae with some success. As a result, significant developments were made in the artificial culture of microalgae (*e.g.* Beijerinck, 1890; Pringsheim, 1947). Interest in the algae as biochemical tools was initiated by research into photosynthesis using the single celled alga *Chlorella vulgaris* (Warburg, 1919). After thirty to forty years of development this culminated in the Nobel prize-winning work of Calvin & Benson (1948), elucidating the pathway of photosynthetic carbon fixation. Thus, *Chlorella* was established as a valuable experimental organism for laboratory studies of plant biochemistry in such fields as respiration, nitrogen assimilation and growth kinetics. A more detailed summary may be found in the work of Soeder (1986) and Fogg (1988).

In the years following the 1939 - 45 war, feared shortages of both protein and fat prompted many countries, including the U.K., Germany and Japan, to develop single cell protein (SCP) and oil products (Harder & von Witsch, 1942). Although sound in theory, these projects never came to fruition due to economic factors (Soeder, 1986); *Chlorella* protein of the quality for human consumption reportedly costing more than beefsteak (Fogg, 1988). The only economic market for such expensive products being that of healthfood suppliers (Seto et al., 1984; Cojocaru *et al.*, 1988; Henderson & Sargent, 1989; Yongmanitchai & Ward, 1989)). Out of such work came other ideas for the uses of

microalgal products. In particular, the finding that *Chlorella* could accumulate up to 85 % of its biomass as lipid even led to the issue of a patent on the discovery, although it was put to no use.

To some extent the modern algal lipidology is still confirming theories tentatively proposed many years earlier, and adding the 'icing on the cake'. One often finds that, following the introduction of a new technique, a plethora of publications emerge applying the technique to various kinds of samples. Occasionally the technique proves to be particularly suitable to a specific type of analysis. A classic recent example of such a situation was the introduction of the Iatroscan TLC-FID which resulted in a mass of literature originating from several sources (see Section 3). Several other recent additions to the battery of analytical technology are discussed more fully in Section 3. Examples of other recent advances in the field of lipidology may be found by consulting several of the following references (Christie, 1985; 1986; Kesselmeier & Heinz, 1985; Baty *et al.*, 1986; Gustavsson, 1986; Kovacs *et al.*, 1986; Conte, & Bishop, 1988; Cojocaru *et al.*, 1988; Nagata *et al.*, 1988; Stefanov *et al.*, 1988; Olsen & Henderson, 1989; Henderson & Tocher, 1990.)

The present situation is such that, a great deal of data is available for the same few species. The majority of this data concerns the fatty acid compositions of microalgae, little information is available regarding the quantities of the lipid classes of which the fatty acids are components. This was because no satisfactory method of quantification was available, and is a problem discussed in more detail in Section 3.

A major reason for the continuing studies of microalgal lipids in the past may be attributed to their possession of two superficially contradictory properties. 1) The specific qualitative and quantitative distribution profiles of the fatty acids within microalgal taxonomic groupings led to an interest in their potential use as indices around which taxonomic classification could be constructed. 2) The quantitative intra-species variation in

fatty acid profiles of different cultures found during these studies led to many attempts to examine the effects of the culture regime and how they were able to modify the microalgal acyl profiles.

The former property also had useful implications in the fields of biogeochemistry and food chain / food web interaction and energetics studies. The specificity of the distribution of the lipid classes and acyl moieties had the potential to be used as biomarkers. Using these molecules it was thought possible that the origins and fluxes of lipid contributions of the planktonic communities to marine sediments and food chains could be identified and quantified (Mayzaud *et al.*, 1976; Volkman *et al.*, 1981; Morris *et al.*, 1985; Venkatesan & Kaplan, 1987; Conte & Bishop, 1988). These areas were essentially the keys to the work conducted in the 1960's and 1970's, with more diversification of interest occurring in the later 1970's & 1980's.

The latter property frustrated many of the attempts to form more than the most generalised chemotaxonomic index, such as the differentiation between the Bacillariophyceae and the Chlorophyceae. Because such emphasis was placed upon the use of identical culture conditions, many workers failed to recognise an important fact. Although many species of algae will grow in a single generalised culture medium, they must be adapting physiologically and are therefore not dividing at their potential peak rate (*i.e.* where exogenous factors are not affecting growth rate (Raven, 1988)) (Shaw *et al.*, 1989). The challenge is to find the specific conditions under which such growth will occur, unfortunately, in practice this would in all probability lead to a vast array of different culture conditions (see references to the work of Fogg & Collyer below.). This would, of course, be equally unsuitable for mass chemotaxonomic purposes. Lechevalier & Lechevalier (1988), in an overview of the chemotaxonomic use of microbial lipids, concluded that only very rarely could convincing taxonomic conclusions be based upon lipid composition. Even prokaryotes and eukaryotes are not universally differentiated by such a system. However, in combination with the present classifications based upon pigment and morphological

characteristics it may be possible to differentiate subclassifications based upon lipid / fatty acyl compositions. There are two levels at which such a system is useful. For example, at the general level it is possible to distinguish the algae from the fungi (*cf.* Harwood & Russell, 1984). And at the genus level it is possible to distinguish apparent anomalies in the accepted classifications (*e.g.* Menzel & Wild, 1989; see Section 5.2).

Several review authors (Cowey & Sargent, 1977; Watanabe, 1982; Bell *et al.*, 1985 a / b; Bell *et al.*, 1986; Teshima *et al.*, 1987; Sargent *et al.*, 1989) observed the dietary requirement by certain commercially important species of both fin and shellfish for the (*n*-3) series of fatty acids, particularly 20:5 and 22:6. The fatty acid composition of the food of these organisms therefore assumed a high level of significance. Several species of microalgae fed to the commercially farmed consumer organisms were shown to increase growth and survival in larval and juvenile stages. To a large extent these effects were correlated with those species of algae having high proportions of $C_{20/22}$ (*n*-3) series PUFA (Davis & Guillard, 1958; Scott & Middleton, 1979; Ewart & Epifanio, 1981; Pillsbury, 1985). This of course led to screening of the commonly grown species to determine their fatty acid compositions (see De Pauw & Persoone, 1988 and Regan, 1988 for species summaries). Many of these species were essentially from marine or euryhaline habitats (*e.g. Brachiomonas submarina*, *Isochrysis galbana* and *Nannochloropsis oculata*).

Although some of the original research upon *Chlorella* species had touched upon the uses for several of the products from microalgae (see above). This was a sphere of interest that gained more widespread support in the 1980's with the advent of commercial scale 'Biotechnology'. The definitive work '*Algae Biomass: Production & Use*', edited by Shelef & Soeder (1980), was followed by several others (*e.g.* Richmond, 1986; Borowitzka & Borowitzka, 1988) which gathered together much of the literature regarding the current state algal biotechnology. The algae, along with many other microorganisms, were exploited for the commercially valuable products they were able to produce (*cf.* Borowitzka, 1988a/b). Amongst these products were the lipids and fatty acids. In this context the emphasis is upon

the product *i.e.* the fatty acids themselves, rather than the algae. The commercial aspects of this work occasionally place restrictions upon the free flow of information concerning such research. Many of the species screened in this respect were inhabitants of extreme environments such as hypersaline pools. The potential for the use of these algae at hot arid sites to make use of the high sunlight availability would be important to achieve maximal production rates in otherwise poor, agricultural locations (Ben Amotz *et al.*, 1985; Tadros & Johansen, 1988, Sriharan *et al.*, 1989).

2.2 The Development of Algal Lipid / Environment Interaction Research.

Since the pioneering research of Spoehr & Milner (1949) it has become widely accepted that environmental factors modify the chemical composition of both marine and freshwater microalgae, to a greater or lesser degree. This includes the proportions of the various lipid classes and their constituent fatty acids. Spoehr & Milner (1949) working with the freshwater species *Chlorella pyrenoidosa* (Emerson) (*C. emersonii* var. *globosa*, Shihira & Krauss, 1965) determined the degree of reduction of the cellular material from its elementary chemical composition and attributed it an R-value based upon their analyses. From contemporary literature they calculated R-values for carbohydrate, protein and total lipid (these being 28, 42 & 67.5 respectively). Using percentage protein (calculated from cellular nitrogen) in combination with individual and total R-values, they were able to estimate percentages of carbohydrate and lipid using simultaneous equations. A near linear relationship between increasing R-value, increased percentage lipid and decreased percentages of carbohydrate and protein was demonstrated.

The effects of various factors (*e.g.* atmospheric composition, concentrations of nutrients and cations, illumination and temperature) were tested and expressed in terms of the R-values of cultures. Values between 38, (equivalent to 58 % protein, 37.5 % carbohydrate, 4.5 % lipid) and 63, (equivalent to 8.7 % protein, 5.7 % carbohydrate 86.5 % lipid) were obtained. No details of changes in lipid classes or fatty acids were given. In general, sparging with 5 % CO_2 in air improved the mass yield of cells unit culture^{-1} unit

time^{-1}. Medium depleted of fixed nitrogen resulted in cells of higher R-value (*i.e.* a higher percentage of total lipid). Temperature and illumination had little effect on R-values. Changes in pigment composition accompanied the onset of increasing R-value implying that the cells had entered a non-division phase of growth. Cells of high R-value were found to possess 1/500th to 1/2000th of the pigment of low R-value cells. In this early work the emphasis was upon mass yield efficiency with illumination rather than cellular division rates. Cells of high R-value by definition, represent a high proportion of stored energy. With hindsight this is probably in the form of the lipid TAG. The data of Milner (1948) corroborates this point. This author found that cells with a high lipid content also had a higher proportion of saponifiable material per unit mass of total lipid. This may have been possible through an increased proportion of TAG having a mole ratio of fatty acids : glycerol of 3:1, compared with structural phospholipids and glycolipids having fatty acid : glycerol mole ratios of 2:1. A decrease in a non-acyl component of the total lipid, such as photosynthetic pigments, would have a similar effect. Increased levels of unesterified fatty acids would also produce such an effect although this is one of the least likely alternatives, with unesterified fatty acids usually being minor components of total lipid.

In the post 1939-45 war years increasing attention was turned towards the unicellular algae as commercial sources of fats and oils. Paschke & Wheeler (1955) in a discussion of the fatty acid content of *C. pyrenoidosa* noted nitrogen replete cells as having a relatively low lipid content of high unsaturation and *vice versa* in depleted cells. This abundance of unsaturated fatty acids was considered to be potentially problematical if the algal material was to be used as livestock foodstuff. Only ruminants, such as cattle, capable of modifying dietary fats, *via* biohydrogenation by the rumen flora (see Harwood & Russell, 1984), could be fed this way. Otherwise, the products from these animals would have been unusually susceptible to auto-oxidation upon storage. Another use, particularly for the unsaturated fatty acids, was in air drying coatings such as paints.

Studies to compare the lipid production of several species were begun with the

prospect of large scale commercial culturing of microalgae for biomass. It was realised that for a true comparison the effects of different culturing conditions would have to be minimised. Fogg & Collyer (1953) pointed out that material used for analysis should ideally represent equivalent stages of growth. The different requirements of many algae meant that culturing them using identical conditions was impossible. The approach these authors adopted was to grow each algae in the most suitable medium (*i.e.* chemical environment) whilst other factors were maintained as similar as possible. The few axenic cultures available showed an increase in fatty acid content with culture age. It was concluded that a major factor influencing the accumulation of lipid was the cellular quota of combined nitrogen. The amount of nitrogen available to the algal cells was thought to be determining the lipid accumulation in two ways. Firstly, by limiting growth photosynthate may have been redirected into the formation of storage products. Secondly, by changes in the enzymatic proteins of the biosynthetic pathways of lipid and carbohydrate biosynthesis. The overall finding was that lipid accumulation conditions varied widely with species, *C. pyrenoidosa* having the greatest ability for accumulation within the range of species tested.

Iwamoto *et al.* (1955) followed a similar course of investigation to Spoehr & Milner (1949) using *Chlorella ellipsoidea* as the single species for investigation. Cells were grown in normal medium and then diluted with nitrogen free medium in which it was claimed they were still able to grow and multiply. Again, nitrogen deficient medium supported the greatest lipid accumulation. The intensity of illumination apparently plays an important role here. Strong illumination retarded the accumulation of lipid in later stages of culture, possibly due to the effect of changes in pigmentation upon C-fixation. A following publication (Iwamoto & Nagahashi, 1955) attempted to determine a rudimentary pathway for the partitioning of photosynthetically fixed carbon between protein, carbohydrate and lipid. The changes in protein and lipid proportions were almost complementary in the early stages of deficiency - the proportion of carbohydrate changing little. In later stages the proportion of carbohydrate fell with its conversion into lipid. Zhukova *et al.* (1967) described two different response patterns from nitrogen starved *Chlorella* spp.. Strains of *C. pyrenoidosa* and *C. ellipsoidea*

synthesised predominantly fatty acids. These strains showed increased cellular division at the onset of N-starvation, similar to that reported by Iwamoto *et al.* (1955), resulting in the formation of smaller lipid rich cells. Other strains of the same species were characterised by carbohydrate accumulation, these showed lesser cellular division and resulted in much larger cells. Klyachko-Gurvich *et al.* (1969) noted that the pattern of fatty acid distribution in the total lipids of N-starved *Chlorella* spp. closely resembled that of its TAG. This tends to indicate that a major portion of cellular lipid was in the form of TAG. This storage material would presumably be consumed upon renewed cell division.

An apparently parallel cycling of the proportions of lipid classes may be seen at a different level of growth. Otsuka & Morimura (1966) observed the accumulation of neutral lipid classes during the cell cycle of synchronous cultures of *C. ellipsoidea*. These classes, mainly TAG of high 16:0 and 18:1 content, accumulated during the illuminated periods and were consumed during cellular division regardless of illumination. Thus, accumulation of TAG may be viewed as a preparation for cell division, either as an energy store for catabolism or as a fatty acid sink for their later mobilisation and incorporation into biomembranes.

O'Brien & Benson (1964) purified three characteristic chloroplast lipids, mono and digalactosyldiacylglycerol and sulphoquinovosyldiacylglycerol from *C. pyrenoidosa*. They reported the fatty acid compositions as consisting mainly of 18:1 and 18:3 in the galactolipid, the sulpholipid was more saturated with a higher proportion of 16:0. A study of greater depth (Nichols, 1966) fractionated the total lipids of *C. vulgaris* grown under photo-autotrophic, photoheterotrophic and heterotrophic conditions into individual classes for fatty acid analysis. A comparison was drawn between the leaf lipids of higher plants and those of *C. vulgaris*. When grown photoautotrophically the lipid classes of *C. vulgaris* were similar to those found in leaves. Under different culture regimes very different total fatty acid profiles were obtained. A considerable increase in the fatty acid 18:3(*n*-3) - characteristic of the chloroplast lipids of higher plants (Joyard & Douce, 1987; Mudd & Kleppinger-Sparace,

1987) was recorded upon photoautotrophic growth. This was apparently at the expense of 18:1(*n*-9) when compared with photoheterotrophic and heterotrophic cultures. Similar phenomena were described for *C. fusca* and *C. emersonii* at the apparent expense of both C_{16} and C_{18} unsaturated fatty acids (Dickson *et al.*, 1969; Wright *et al.*, 1979). When grown in organic medium *C. vulgaris* still synthesised galactolipids but their proportion of 18:3(*n*-3) was greatly reduced. The fatty acid 16:1(*n*-13) *trans* was also absent from another chloroplast associated lipid class, phosphatidylglycerol. This indicated that it was not light that was the sole determining factor in the synthesis of the fatty acids associated with photosynthetic fixation of carbon but a combination of light with the presence *or absence* of some chemical factor.

This work was followed up by Nichols *et al.* (1967) with a series of radiolabelling experiments. *Chlorella vulgaris* cells previously grown in the dark were incubated for periods of up to 48 hrs in the presence of ^{14}C-acetate. When incubated in the dark maximal labelling occurred in DAG and TAG over the short term (1 - 4 hrs). The membrane lipids were labelled much more slowly. 18:0, present only in low quantity, had the highest specific activity whereas the largest proportion of the label was incorporated into 18:1(*n*-9). Very little activity progressed into 18:2(*n*-6) and 18:3(*n*-3). On illumination an overall higher rate of incorporation into all classes occurred. Initially neutral glycerides, PG, PC and MGDG showed the highest proportion of labelling. Maximal turnover was found in the neutral glyceride fraction. In the MGDG fraction the desaturation of 18:1(*n*-9) and 18:2(*n*-6) to 18:3(*n*-3) was indicated by the complementary decrease and increase in these acids. By following the trends of labelling and changes in the specific activity of lipid classes and fatty acids and using data from the literature Nichols *et al.* (1967) proposed several schemes of de- and reacylation of specific lipid classes to account for the patterns they found. These involved specific molecular species of the lipid classes with the higher turnover rates being significantly metabolically active (see Section 1.4) as well as structurally important components of photosynthetic membranes.

The elucidation of the Calvin-Benson Cycle (Calvin & Benson, 1948) using *Chlorella* sp. made it clear that the lipids were not direct products of inorganic carbon fixation and consequently interest in algal lipid biochemistry waned in some quarters. These studies did much to increase the acceptance of algal cell material as experimental systems. However the fatty acids of marine phytoplankters were the subject of studies of the role of lipids as a medium of energy transfer through the trophic levels of marine food webs. The early 1960's saw the emergence of a series of studies to characterise the lipids of marine origin at various trophic levels to elucidate the fluxes and modifications which may have occurred. To some extent this was a post-war resurgence of work conducted in the mid to late 1930's, although these earlier works predominantly emphasised the higher trophic levels (*i.e.* fishes.) (Lovern, 1936; 1942) (see the review of Henderson & Tocher, 1987 for details of earlier references). Improvement upon analytical techniques extended the scope of the latter studies particularly the introduction of GLC for the separation of FAME.

Until this point in time almost all the available data was for the various strains of *Chlorella*, relatively little data being available for the major marine phytoplanktonic species. An early proximate analysis of several marine microalgal species by Parsons *et al.*, (1961) indicated the potential variety of quantitative composition of biochemical constituents amongst different species during exponential growth. Even at this early stage the nutritional significance of the microalga *Monochrysis lutheri* in the diet of shrimp and bivalve species had been recognised by Davis & Guillard (1958) and Shiraishi & Provasoli (1959) (Parsons *et al.*, 1961). Although the reasons for this were unknown at the time, this species was later found to be rich in the (*n*-3) PUFA 20:5(*n*-3). Several more studies focussed upon the fatty acid composition of phytoplankters and have become the backbone of the literature pertaining to the lipids and fatty acids of marine microalgae. Ackman *et al.* (1964) analysed the fatty acids of the diatom *Skeletonema costatum* cultured in artificial media for various time periods. Significantly, these authors also noted the decrease in unsaturation and acyl chain shortening with increased culture age, 20:5(*n*-3) being replaced by 14:0. They suggested several reasons for this, depletion of nitrate, changes in pH or

pO_2 or accumulation of excreted metabolites in batch cultures. A further suggestion based upon the data of Brockerhoff *et al.* (1963) was that fatty acids synthesised in excess of the structural lipid class requirements were used for depot fat biosynthesis, *i.e.* the potentially saturated TAG. The drawbacks of extrapolating the data obtained from cultures to the natural environment were pointed out with respect to the variations and shifts in biosynthetic pathways. Brockerhoff *et al.* (1964) determined the distribution of fatty acids within lipid classes. The PUFA, consisting mainly of 22:6(*n*-3) and 20:5(*n*-3) in the depot TAGs of marine fishes, were found to be located at the β or *sn*-2 position of the glycerol backbone (see Section 1). This was observed to be a continuation of a pattern originating in both the zooplankton and some phytoplankton species such as *S. costatum*. The *sn*-2 distribution of PUFA was relatively, but not completely, conserved through the processes of digestion and absorption in the guts of marine fishes (Brockerhoff & Hoyle, 1963; Brockerhoff *et al.*, 1964). This finding had the obvious implication of a potential for the use of such conserved fatty acids to determine the source and flux of lipid through experimental and natural food chains. The ultimate source of the PUFA may be determined from their (*n*-*) number. Due to further elongation and desaturation by the consumers metabolism clues to the origin of unradiolabelled fatty acids do not point to the chain lengths consumed unless the composition of the food source is also known. Fatty acids specific to each group of organisms in the food chain *e.g.* the differences between the phytoplankton and zooplankton with respect to chain length and unsaturation distribution.

Williams (1965) carried out a study to characterise the fatty acids derived from various sources to ascertain the origins of fatty acids found in seawater and marine sediments as part of a biogeochemical project. This author analysed the total FAME composition of six representative marine microalgal species. In retrospect these analyses are somewhat suspect in that they show species such as *Phaeodactylum tricornutum* and *M. lutheri* to have no C_{20} or C_{22} PUFA. This finding was later contradicted by many analyses. Kates & Volcani (1966) found *P. tricornutum* to have high proportions of both 20:5(*n*-3) and 22:6(*n*-3) as did Ackman *et al.* (1968) for both *P. tricornutum* and *M. lutheri*. Kates &

Volcani (1966) conducted the first detailed study of marine diatom species, characterising their fatty acid and individual lipid class composition *via* radiolabelling studies. Using ^{32}P, ^{35}S and ^{14}C in combination with TLC autoradiography these authors identified the major lipid classes of five selected species of marine diatom species and a single freshwater species. All six species had qualitatively similar fatty acyl and lipid class profiles. Their lipid class compositions compared closely with those of the green algae (*e.g. Chlorella* spp.) and the leaves of higher plants. However, in addition to the major sulphur-containing lipid class SQDG, the diatoms also contained minor sulpholipid classes which were not identified. Mercer & Davis (1975) reported the occurrence of chlorosulpholipids in several species of microalgae but later studies failed to locate them in any of the marine species studied (Mercer & Davis 1979). Although there have been many reports of minor sulpholipids (Kates & Volcani, 1966; Opute, 1974) it is rare that any chemical characterisation or identification is offered. All the species had relatively high proportions of 20:5(*n*-3) and C_{16} saturates and monounsaturates. The freshwater species *Navicula pelliculosa* possessed the highest mole percentage of 16:3 in contrast with the marine species which generally possessed a greater proportion of 16:0. Unlike *Chlorella* spp. C_{18} PUFA were present in low proportion only. Kates & Volcani (1966) suggested that the involvement of unsaturated lipids in the Hill Reaction, the photolysis of water evolving free oxygen, necessitated a certain *degree* of unsaturation per mole of fatty acid rather than any specific PUFA. This they suggested was achieved with 20:5(*n*-3) rather than the combination of 16:3 and 18:3 as is the norm in higher plants and green algae (*cf.* Stumpf & Conn, 1984; 1987). Ackman *et al.* (1968) cultured twelve marine microalgal species from several taxa under similar conditions. The study examined several aspects of the fatty acyl composition of these species and related it to taxonomy, food web interrelationships and the effects of incubation temperature and culture age. Similar observations to previous studies were made. The Chlorophyceae, or green algae, had no C_{20} PUFA and a similar qualitative profile to higher plants with C_{16} and C_{18} PUFA predominant. The Bacillariophyceae, or diatoms, had relatively high proportions of C_{14} and C_{16} saturates and monounsaturates and high proportions of C_{20} PUFA. Other taxa

had a general range of fatty acids. Parsons (1963) concluded that the phytoplanktonic fatty acids made a significant contribution to the saturates and monounsaturates of the C_{14}, C_{16} and C_{18} chain lengths 'dissolved' in sea water with the other longer chain, more unsaturated acids being the subject of auto-oxidation. Even so, local conditions may have a significant effect upon the acyl composition of both the dissolved and particulate fractions of seawater through both changes in the species composition and succession as well as the effects of changing environment, particularly during a bloom (Parsons, 1963; Parrish, 1987, Claustre *et al.*, 1988). The potential contributions therefore are subject to both long and short term variation with the oceanic seasons (*cf.* Tait, 1981; Barnes & Hughes, 1982; Morris *et al.*, 1985; Dubinsky, 1986; Fogg & Collyer, 1987; Mayzaud *et al.*, 1990).

Ackman *et al.* (1968) examined the effects of culture age and incubation temperature upon three marine microalgal species, *Dunaliella tertiolecta, Porphyridium* sp. and *M. lutheri*. Culture age appeared to have a significant but inconsistent effect when inter-specific comparisons were made. In both *D. tertiolecta* and *Porphyridium* sp. chain lengthening from C_{16} to C_{18} was observed whilst in *M. lutheri* the opposite was observed with C_{16} and C_{22} PUFA increasing at the expense of the C_{20} chain lengths. Because some of this data is incomplete it is difficult to correlate changes in the fatty acid profiles with lipid yield or growth phase. These authors did however recognise the limitations of chemotaxonomy, using fatty acyl profiles as an additional aid to classification only.

Nichols & Appleby (1969) analysed *Porphyridium cruentum* and reportedly obtained results similar to those found for other species of red algae analysed by Klenk *et al.* (1963). Their results also stand up to comparison with those of Ackman *et al.* (1968). Although, as one might expect there are quantitative differences in the fatty acid profiles. However, Nichols & Appleby (1969) fractionated the total lipid of *P. cruentum* into its component lipid classes and then analysed the fatty acid profile of each class. On the basis of the two data sets (Ackman *et al.*, 1968; Nichols & Appleby, 1969) it is straightforward to attribute the changes observed with culture age by Ackman and co-workers to increasing proportions of

TAG, the fatty acyl profile of which was provided by Nichols & Appleby, particularly with respect to the quantitative changes in the proportions of 18:2, 20:4 and 20:5 in the total lipid. Examination of the two lipid extracts using TLC densitometry (see Section 3.2.2) would have confirmed or dismissed this conclusion. Similar studies identified changes in both the quantitative total lipid yield and qualitative fatty acyl profile of microalgal cells in artificial culture and from the natural environment to be attributable to TAG accumulation (Klyachko-Gurvich *et al.*, 1967; Dickson *et al.*, 1969; Harrington *et al.*, 1970; Shifrin & Chisholm, 1981; Hitchcock, 1983; Piorreck & Pohl, 1984; Piorreck *et al.*, 1984; Robinson *et al.*, 1987). Thus it seems that under conditions of nutrient stress such as would exist in an old or nutrient limited culture TAG is an important determinant of the relative unsaturation of the total FAME of the algal cells.

Actively dividing cells were observed to have a much lower proportion of TAG by comparison to their polar, structural lipid classes. This implies that whilst the environmental conditions favour cell division polar lipid classes are synthesised in preference to the neutral lipid classes. This reflects a differential partitioning of the products of fatty acyl biosynthesis under certain culture conditions. How this is achieved by the cells is poorly understood and remains a topic for speculation; as do the mechanisms of the other effects of culture conditions. The fact that fatty acyl elongation and desaturation seem to be linked to the biosynthesis of polar lipid classes lends credence to the theories that these reactions are in some way complex lipid-linked (see Section 1).

Chuecas & Riley (1969) in a classic study covering the relationships of fatty acid profiles of twenty-seven marine microalgal species with their taxonomy do not commit themselves to a chemotaxonomic index with such a basis. As with the previous studies they point out the variation in culture conditions as being contributory to the differences observed when inter-study comparisons were attempted. De Mort *et al.* (1972) in a smaller scale study of ten species of estuarine phytoplankters used a diversity index described by M^{c}Intosh (1967) (see below) to compare the similarity of quantitative and qualitative fatty acid

composition by pairwise comparison of the species involved.

$$D_{jh} = \left[\sum_{i-1}^{n} (\rho_{ij} - \rho_{ih})^2\right]^{\frac{1}{2}}$$

Where D_{jh} = the degree of difference between the j^{th} and h^{th} species

ρ_{ij} = the percentage of the total fatty acid represented by the i^{th} fatty acid in the j^{th} species.

They do however outline the importance of another factor. These authors tested lipid extraction procedures used by several other authors and found that these determined to a significant extent the distribution of the proportions of fatty acid chain lengths present in the final extract. They showed that this was particularly the case with the data of Williams (1965) who found low proportions of C_{20} fatty acids to be present in species later found to be abundant in them (see above).

Two freshwater species and one marine diatom species were examined by Opute (1974) in a similar vein to that of Kates & Volcani (1966). Opute, however, took the analyses one stage further and fractionated the total lipid and analysed the fatty acid profiles of the major individual lipid classes. All three species of the genus *Navicula* were found to have similar qualitative compositions of both lipid classes and fatty acids to those of other species examined by Kates & Volcani (1966). Whilst the lipid classes themselves resembled those of the higher plants and green algae (Chlorophyceae) the fatty acids showed distinct distribution patterns within the individual lipid classes. The galactolipids MGDG and DGDG showed characteristically high unsaturation (see Stumpf & Conn, 1984; 1987). However, the diatom galactolipids possessed high proportions of C_{16} and $C_{20/22}$ PUFA (mainly 16:3 and 20:5). Similarly, the data of Nichols & Appleby (1969) for the unrelated species *Porphyridium cruentum* showed the galactolipids to possess high proportions of C_{20} PUFA. This is common feature amongst many groups of the marine microalgae and macroalgae (Jamieson & Reid, 1971; Johns *et al.*, 1979; Pohl & Zurheide, 1979), but in model systems

described for higher plants C_{20} PUFA are never found in significant quantities (see Section 1).

Shaw *et al.* (1989) in a study of four clones of the characteristic diatom species *Skeletonema costatum* examined the diversity of their fatty acid profiles in a chemotaxonomic context. The clones, isolated from different environments (*i.e.* coastal, estuarine, and oceanic) showed significant (although not major) differences in growth kinetics and quantitative acyl composition when cultured under identical conditions. These authors used the data of Ackman *et al.* (1964) and Chuecas & Riley (1969) as a base for comparison and found adequate but not identical agreement, the differences being attributable to the analytical techniques employed. Shaw *et al.* (1989) suggested that fatty acid composition was a potentially important criterion in natural selection processes for a specific environment. Although the limited scope of their study did not allow the confirmation of this. They found that the total saturated fatty acids were the best distinguishing feature between the clones from the different environments. Claustre *et al.* (in press) reported the presence of two sub-populations in a mono-specific bloom of the marine diatom *Nitzschia seriata*. The smaller sized cells showed compositional characteristics similar to those of aged cultures whilst the larger cells resembled actively dividing cells. Fisher & Schwarzenbach (1978) also working with a diatom species, *Thalassiosira pseudonana*, reported *quantitatively* different acyl compositions for each of two clones under identical culture conditions. This illustrates the problems of extrapolating laboratory studies into the natural environment. In addition to the effects of changes in environmental conditions different species and even clones of the same species may exhibit different responses and compositions to the same environmental conditions.

Apart from studies upon the chemotaxonomic aspects of the acyl compositions of the different microalgal species a large proportion of the existing literature is concentrated on relating the effects due to changes in culture conditions of well characterised 'model' microalgal species. As was discussed earlier in this section, much of this data is concentrated

upon the various species of *Chlorella*. With the realisation in the mid-1970's that detailed characterisation of the lipid and fatty acyl compositions of major microalgal species present in oceanic blooms was of little chemotaxonomic value, a change of emphasis occurred. Early work in the marine science field laid important foundations for following studies into the nutritional significance of the (*n*-3) series of fatty acids in the food chains of marine organisms. It was not until the advent of commercial scale aquaculture that the manipulation of fatty acyl composition of microalgae by modifying the environmental conditions used in its culture became a feasible option. It has been to this end and that of understanding both the elongation and desaturation mechanisms *and* the benefits to the cells of such changes that further studies have been conducted. Many authors have varied one or two environmental factors and then analysed the acyl compositions of the cells cultured under the applied conditions. By far the most popular factors for investigation have been nitrogen supply, illumination intensity and temperature. These were recognised as easily modifiable factors as well as having significant effects upon the growth of the cells. Several key publications have determined the direction of the research.

The homeoviscous adaptation of membranes to temperature fluctuations by modification of their fatty acid composition as proposed for *E. coli* by Marr & Ingerham (1964) has been generally accepted for many organisms including marine microalgae and fishes after further investigation by many other authors. Patterson (1970) conducted one of the earliest *detailed* studies of the effects of microalgal culture incubation temperature on fatty acyl composition using the freshwater species *Chlorella sorokiniana*. This author noted that increasing the incubation temperature from 14 ^{o}C to 22 ^{o}C *increased* the unsaturation of the fatty acids, further increases in the incubation temperature up to 38 ^{o}C resulted in decreasing unsaturation. Notably, increased temperature always resulted in a shorter mean acyl chain length. Both decreasing chain length and increasing unsaturation result in a lower membrane viscosity. The peak unsaturation of the lipids of *C. sorokiniana* occurred at 22 ^{o}C, this also coincided with the minimum lipid yield from the cells although this was not correlated with the peak growth rate which was observed at 38 ^{o}C. Similar observations

were made by Teshima *et al.* (1983) using a marine species *Chlorella saccarophila.* Maximum total lipid unsaturation for this species was recorded between 21 °C and 24.7 °C. Seto *et al.* (1985) reported that another marine *Chlorella* species, *Chlorella minutissima,* showed a marked response to changes in its incubation temperature. Again the trend was one of increased unsaturation with a temperature decrease from 25 °C to 20 °C. Seto *et al.* (1985) also suggest a mechanism to account for the changes in unsaturation based upon the work of Brown & Rose (1969) involving temperature dependence of oxygen solubility.

Both of the marine *Chlorella* species discussed above have acyl compositions radically different from that of the freshwater species in that they contain very low proportions of C_{18} fatty acids and high proportions of C_{20} PUFA. These species have acyl compositions very similar to those of the diatoms and the subject of the present study, *Nannochloropsis oculata*. The occurrence of C_{20} and C_{22} PUFA is more common amongst marine species than those from freshwater.

The red algae are represented most frequently by the macrophyte *Porphyra* (Kayama *et al.*, 1985) and the microalga *Porphyridium cruentum* (Cohen *et al.*, 1987; Rezanka *et al.*, 1987). The effect of different incubation temperatures upon the fatty acyl composition of this group of algae has been investigated by these authors and those working on other macrophytic species (Pettitt *et al.*, 1989). Kayama *et al.* (1985) and Rezanka *et al.* (1987) used artificially cultured material in their studies and concluded that the proportion of PUFA increased with decreased culture temperature. Cohen *et al.* (1987) using outdoor cultures concluded that at supra-optimal temperatures during the summer the proportion of 20:4 was increased at the expense of 20:5. During the winter the proportion of 20:5 was greater than that of 20:4. A more subtle balance was also achieved by the manipulation of cell density to modify the relative proportions of these two fatty acids. Pettitt et al. (1989) also monitored the changing acyl compositions of *Chondrus crispus* and *Polysiphonia lanosa.*from the shore of the South Wales Coast during the months of January and August. Their studies showed that the degree of unsaturation of the algae's fatty acids increased during the winter.

In laboratory studies of the same species these authors noted that increasing incubation temperature increased the proportion of [^{14}C] acetate incorporated into 14:0 and 16:0 whilst decreasing the proportion found in 18:1. The findings were again discussed in terms of both homeoviscous adaptation and decreased solubility of oxygen at elevated temperatures (Rebeille *et al.*, 1980). Henderson & Mackinlay (1989) studied the effects of incubation temperature upon the fatty acyl composition of the cryptomonad *Chroomonas salina.* They concluded that, whilst the PUFA content remained similar, although somewhat increased at decreased temperatures, the chain length distribution was modified resulting in increased proportions of C_{20} PUFA amongst the phospholipids and to a lesser extent in the galactolipid classes. The neutral lipid class TAG became more monounsaturated (increased 18:1(*n*-9)) at lower temperature although at the expense of saturated fatty acids (16:0), rather than PUFA. Therefore unsaturation was also increased.

A strain of *Nannochloropsis* was examined by James *et al.* (1989) to ascertain the effects of growth in the temperature range 15 °C to 35 °C. These authors found that the maximum proportion of (*n*-3) PUFA occurred at 25 °C and decreased at temperatures above or below this in a similar manner to that described by Patterson (1970) for *C. sorokiniana.*. The same authors also examined a species of *Chlorella* which they found to contain increasing proportions of unsaturated fatty acids with decreasing temperature.

Nutrient limitation is an important factor influencing primary production in the marine environment. It drives the seasonal cycling of the phytoplankton population in the temperate oceans. The term 'nutrient' may encompass such compounds as nitrate, phosphate, silicate, vitamin B12 and iron, amongst many others, which are required by many algal species for cell division (Davies, 1988). Three macro-nutrients have been widely examined with respect to their effect upon the lipid and fatty acyl composition of microalgae. Two of these are nitrogen (nitrate and the related nitrogenous compounds ammonium, nitrite and urea) and phosphorus (phosphate). In addition, the diatoms have a requirement for silicate from which they form their siliceous frustules. The specific growth rate of algal cells

is a hyperbolic function of the cell quota of the rate limiting nutrient (Davies, 1988). Importantly, Liebig's law of the minimum states that only one nutrient is ever limiting for a monospecific algal population. Although the cellular quota of nitrogen has been shown to affect the rate and efficiency of algal photosynthesis (Rijstenbil, 1989) a deficiency of this essential nutrient results in a greater decrease in the cellular division rate. Thus, neutral lipid has been found by many workers to act as a sink for the excess carbon fixed during photosynthesis under conditions of nitrogen deficiency. The depletion of an essential nutrient is an important factor in determining the onset of the stationary phase in batch cultures. The depletion of essential nutrients most probably contribute to the changes in algal lipid class and acyl composition associated with the 'aging' or senescence of cultures.

Fogg & Collyer (1953), Iwamoto & Nagahashi (1955), Klyachko-Gurvich *et al.* (1967) and Zhukova *et al.* (1969) working with various strains of freshwater *Chlorella* noted that the total fatty acyl composition of these strains under conditions of nitrogen deficiency was similar to that of their TAG. That is to say they became increasingly saturated. These workers concluded that nitrogen deficiency increased the rate of 18:0 and 18:1 biosynthesis and the rate of TAG biosynthesis resulting in its accumulation to become the major influence upon the fatty acyl composition of the algal cells. Conover (1975) examined the partitioning of carbon and nitrogen in cultures of the diatom *Thalassiosira fluviatilis* using various sources of nitrogen (nitrate, ammonium and urea). This author concluded that changes in the C:N and lipid:protein ratios of the cells reflected the onset of nitrogen deficiency irrespective of the nitrogen source used. Both of these ratios increased as the cultures aged indicating the accumulation of intracellular carbon as lipid relative to nitrogen represented by the proportion of protein. Conover (1975) did not fractionate the total lipid into its component classes or determine the fatty acyl composition of the cells.

A later study of another marine alga from the same genus, *T. pseudonana*, (Fisher & Schwarzenbach, 1978) associated culture aging with nutrient depletion and established that changes in fatty acyl unsaturation and chain length occurred as a result of nutrient depletion.

Several other workers have also observed this association (Pugh 1971; Opute, 1974; Pugh 1975; Volkman *et al.*, 1980). They found that the percentage of the dry cell mass accounted for by fatty acids increased with culture age and the fatty acyl chain length and unsaturation decreased. These changes were accounted for by increased proportions of the fatty acids 16:0 and 16:1 and negatively correlated changes in the proportions of 16:3 and 20:5.

Piorreck *et al.* (1984) and Piorreck & Pohl (1984) examined the effects of nitrogen regime on several species of freshwater green and blue-green algae (cyanobacteria). As found in previous studies the proportion of cellular total lipid in the green algae increased with decreasing medium nitrogen levels. This was accounted for by increased proportions of the neutral lipid class TAG which contained high proportions of the fatty acids 16:0 and 18:1. At higher nitrogen concentrations the proportion of total lipid was decreased, accounted for by predominantly unsaturated polar lipid classes. The cyanobacteria, having the capacity to fix nitrogen were unaffected by variations in the medium nitrogen concentration. Similar findings were recorded for outdoor cultures of *Chlorella* and *Scenedesmus* species by El-Fouly *et al.* (1985), for declining blooms of sea ice diatom communities (Nichols *et al.*, 1988)and for enclosed planktonic assemblages by Mayzaud *et al.* (in press).

Suen *et al.* (1987) showed that the lipid accumulation in a *Nannochloropsis* species under conditions of nitrogen deficiency was primarily due to *de novo* CO_2 fixation rather than the conversion of other cellular components such as carbohydrate. This showed that the accumulation of the neutral lipid class TAG was a result of the partitioning of excess carbon into this class. In this alga which is a member of the same genus as the subject of the present thesis nitrogen deficiency resulted in higher proportions of the fatty acids 14:0, 16:0, 16:1 and 18:1 than under nitrogen replete conditions. Henderson & Sargent (1989) noted that the amount of ^{14}C incorporated into TAG increased with culture age, whether supplied as CO_2, acetate or 18:1(*n*-9). A possible mechanism relating the partitioning of fixed carbon between phospholipid and TAG biosynthesis *via* a common diacylglycerol precursor was suggested by Parrish (1987). A similar line of reasoning was followed by Henderson & Sargent

(1989) who concluded that as phospholipid biosynthesis decreased the products of the FAS complex were incorporated into neutral lipid without elongation and desaturation. Thus if acyl biosynthesis actually remained the same then the sum of phospholipid fatty acids and TAG fatty acids would always be constant. However this is not the case, the accumulation of the mass of TAG is usually greater than the decrease in phospholipid mass due to increased cell mass. Although the accumulation of lipid is a general feature of cells grown under conditions of nitrogen deficiency it is not a universal one (Shifrin & Chisholm, 1981). Some algal species such as *Dunaliella* spp. reportedly accumulate carbohydrate instead (Ben Amotz *et al.*, 1985).

Although the effects of nitrogen supply upon lipid and fatty acyl composition of microalgae have been widely investigated the effects of other nutrient factors have not been so well documented. The effects of changes in another important macro-nutrient, phosphorus, have been examined by Siron *et al.* (1989). These authors observed that the effects of phosphorus deficiency on two marine algal species, *Dunaliella tertiolecta* and *Phaeodactylum tricornutum* were similar to those observed during nitrogen deficiency. The total fatty acid content of the cells was increased as was the proportion of 18:1 whilst 20:5 the predominant PUFA in *P. tricornutum* was decreased in proportion. This led these authors to speculate as to its significance as an intermediate in the pathways leading to C_{20} PUFA.

The effects of silicate deficiency upon diatoms have been investigated by Shifrin & Chisholm (1981), Taguchi *et al.* (1987), Mortensen *et al.* (1988) and Roessler (1988). Again it was found that the effect of the limiting nutrient was to increase the cellular proportion of lipid but in a more rapid manner than was associated with nitrate deficiency (Mortensen *et al.*, 1988). Roessler (1988) showed that $^{14}CO_2$ was rapidly incorporated into TAG. This tends to indicate that excess photosynthetically fixed carbon is incorporated more rapidly due to the fact that silicate deficiency does not affect the rate of photosynthesis in the same way as nitrogen deficiency.

A topic which has recently come to light with the current interest in marine CO_2 fixation in the context of the bioremoval of greenhouse gases by microalgal blooms in polar waters, is that of iron deficiency. Apparently, iron is a potentially limiting nutrient in such environments due to the absence of terrestrial run-off which would otherwise act to replenish it (Davies, 1990; Christopher, 1990). Such phenomena do not have any supporting data with respect to the lipid and fatty acyl compositions of microalgae at present.

The effects of illumination regime upon the lipid and fatty acyl compositions of microalgal cells in the natural environment are coupled to the changes in the seasons and the availability of limiting nutrients in temperate and polar waters. In temperate waters the breakdown of the thermocline over the winter period supplies nutrients to the algal cells and in combination with the increasing duration and intensity of illumination results in the spring blooms (Cohen *et al.*, 1987; Mayzaud *et al.*, 1989; Mayzaud *et al.*, in press). In polar waters the change from continual darkness in winter to continual illumination in summer also results in growth surges in the phytoplankton population (Nichols *et al.*, 1988; Palmisano *et al.*, 1988). Because the lipids are mainly composed of carbon, photosynthesis rate plays an important role in their formation. The chloroplasts, the organelles responsible for the photosynthetic fixation of carbon, are highly membranous. Their membranes are composed of the galactolipids, these lipid classes being characterised by their high content of (*n*-3) PUFA. Therefore the interest in the effect of illumination intensity is in effect two-fold and in one respect closely linked to the effects of nutrient limitation. In aging cultures the coincidence of declining nutrient concentrations and decreasing illumination cellular irradiance due to increasing culture turbidity is common. During this period the fatty acyl composition of the cells becomes less unsaturated.

The rate of carbon incorporation into microalgal cells is determined by their rate of photosynthesis. At high rates of photosynthesis the cells usually grow and divide more rapidly. As has been described above and in the previous section during periods of high cell division the acyl composition of the cells is generally more unsaturated. Because the rate of

photosynthesis increases with illumination intensity at sub-saturating illumination intensities many authors have attempted to link the effect with increasing proportions of the highly unsaturated galactolipids.

Nichols (1965) showed that dark-grown *Chlorella vulgaris* possessed much lower proportions of the PUFA associated with the chloroplast lipids. In the absence of illumination the fatty acids synthesised from organic carbon sources were more saturated and monounsaturated. Similarly Dickson *et al.* (1969) observed reduced proportions of 18:3 and total absence of 16:4 when *Chlorella fusca* was grown in the dark and compared with illuminated cultures. Other authors (Opute, 1974; Wright *et al.*, 1980) have found similar results for other species of microalgae. Orcutt & Patterson (1974) working with the diatom species *Nitzschia closteridium* grew their cultures under regimes of high and low illumination intensity rather than in either light or dark. They found that at low illumination intensity the polar lipid classes, diacylglycerols and unesterified fatty acids contained higher proportions of PUFA whereas the triglycerides were more highly unsaturated and *vice versa* at high illumination intensity. The overall fatty acyl composition was more saturated at high illumination intensity due to the accumulation of saturates in the TAG fraction. They concluded that the differences in the TAG compositions of these cultures were a response due to the cells encountering 'more optimal environmental conditions'. Similar findings have been recorded by Seto & Hesseltine (1984) for *Chlorella minutissima* although the findings were not discussed in this context. Partially contradictory findings have been presented for the Cryptomonads (Beach *et al.*, 1970), diatom species *Chaetoceros gracilis* (Mortensen *et al.*, 1988) and the red alga *P. cruentum* by both Rezanka *et al.* (1987) and Cohen *et al.* (1988). Higher illumination intensities produced higher proportions of 20:5 and lower proportions of 16:0. At the lower illumination intensities, increased proportions of 18:2 and 20:4 maintained the proportions of PUFA in the total lipid. No detail of the lipid class compositions were given. In the most recent publication to date (Thompson *et al.*, 1990) eight species commonly used in aquaculture were examined with respect to their response to illumination intensity. These authors found that most species had their greatest proportion of

20:5 at low illumination intensities. They suggested that the fatty acid composition of algal cells responded significantly to the illumination regime employed and supported the idea that the accumulation of 16:0 and 16:1 at higher illumination intensities was due to its accumulation in TAG.

Data documenting the effects of salinity upon the lipid class and fatty acyl composition of microalgae is rare by comparison to that available for other factors. This is possibly due to the inconclusive data presented in early publications (Pugh, 1971, 1975) which did not find a readily observable trend of change in fatty acyl unsaturation or chain length distribution. Lee *et al.* (1989), in keeping with the findings of Pugh (1971, 1975) reported that *Porphyridium cruentum* showed a peak of polyunsaturation in its fatty acids the proportions of which decreased at salinities above and below this.

As a general summary of the state of knowledge of the effects of culture conditions or environmental variables on the lipid class and fatty acyl composition the following consensus is presented. It is important to remember that this may vary due to natural variation and sub-populations.

1) *Temperature*: Apart from decreasing the growth rate of the algal cells decreasing culture incubation temperature increases the proportion of PUFA in the membrane lipid classes.

2) *Nutrients*: If cells are grown under conditions of nitrogen, phosphorus or silicate stress both chain length and fatty acyl unsaturation decrease whilst the proportion of cellular lipid and the C/N ratio of the cells increase. The increased proportion of lipid is characterised by the accumulation of neutral lipid classes such as TAG, wax ester and in some extreme cases hydrocarbons. Similar effects have been noted for 'aging' cultures. This is probably due to the depletion of one nutrient such that the growth of the culture becomes limited.

3) *Illumination intensity*: In the case of this variable the consensus is that increasing illumination intensity increases the PUFA content of the polar lipid classes most notably the

galactolipids. When cells are grown in the dark using organic carbon sources they generally have decreased proportions of (*n*-3) PUFA. However, the total fatty acid composition may show decreasing proportions of PUFA due to the accumulation of TAG or other neutral lipid classes which apparently act as a sink for fixed carbon in excess of the requirements of cell division.

4) *Salinity*: Increasing culture medium salinity generally results in an increase in the unsaturation of the fatty acids of microalgal cells. However, at high and low salinities the effect is to decrease the proportion of PUFA.

SECTION 3: ANALYTICAL METHODOLOGY & RESULTS.

3.1 QUALITATIVE LIPID CLASS ANALYSIS

3.1.1 Introduction

A combination of well established and more recently developed methodology was employed. TLC and GLC analysis are so universally familiar as to require little explanation. During the course of this study time was devoted to the assessment of the mass detector. High performance liquid chromatography (HPLC) was applied to the separation of algal lipid classes along with quantification by the mass detector. The quantitative aspects of the techniques of HPLC and HPTLC (high performance thin layer chromatography) separation are the subject of Section 3.2.

3.1.2 Lipid extraction

A known mass of lyophilised microalgal cells was resuspended in 10 ml distilled water. 10 ml *iso*-propanol (*iso*-PrOH) were added and the suspension homogenised for 1 min in a teflon-glass homogeniser. Following this 26.6 ml chloroform ($CHCl_3$) and 3.3 ml *iso*-propanol (*iso*-PrOH) were added. The suspension was rehomogenised for 1 min. Phase separation was aided by centrifugation at 1000 rpm for 1 min. The aqueous upper phase was removed and retained. The remaining organic phase was filtered into a round bottomed flask through a prewashed (50 ml $CHCl_3$:*iso*-PrOH 2:1 v/v) Whatman No.1 filter paper. The residue from the filter paper was returned to the aqueous layer and re-extracted with the mixture of $CHCl_3$ and *iso*-PrOH as above. The two filtrates were combined and evaporated to dryness under reduced pressure in a rotary film evaporator (RFE). The extract was redissolved in 5 ml $CHCl_3$ / *iso*-PrOH (1:1 v/v) and passed through a 0.22 μm pore size filter (Acrodisc-CR™, Gelman Sciences Inc.) into a tared glass vial. Solvents were evaporated under oxygen-free nitrogen (OFN). The extract was then dried to constant mass under vacuum desiccation. The mass of the extract was determined gravimetrically and redissolved in 0.22 μm filtered $CHCl_3$ / *iso*-PrOH (2:1 v/v) at a concentration of 10 mg ml^{-1}.

3.1.2.1 Lipid extraction efficiency

Several extraction procedures were examined before accepting any particular one. Fraser (1986) tested a $CHCl_3$ / MeOH procedure against a $CHCl_3$ / hot *iso*-PrOH procedure as a check for possible hydrolysis of lipid classes by solvent activated plant lipases (Harwood, 1980). This may result in the liberation of unesterified fatty acid moieties. Slight differences occurred in the fatty acid composition of the extract but no apparent difference in unesterified fatty acid composition was noted. However, no consideration was given to other hydrolysis products such as phosphatidic acid or diacylglycerols. The effectiveness of hot *vs.* cold *iso*-PrOH was tested here with a view towards both the efficiency of total lipid extraction and quantitative extraction of lipid classes.

The effects of using lyophilised algal cells as opposed to 'wet' cells, in terms of lipid yield and quantitative class extraction were also examined.

3.1.2.1.1 Hot *vs.* cold *iso*-PrOH extraction

The cold method used was as detailed above. The hot procedure was as follows.

10 ml *iso*-PrOH at 70 °C were added to *ca.* 40 mg lyophilised stationary phase batch cultured *Nannochloropsis oculata.* The suspension was homogenised for 20 sec in a teflon glass tissue grinder. The homogenate was transferred to a stoppered tube and maintained at 70 °C for 30 min after which it was transferred back to the tissue grinder. 5 ml $CHCl_3$ were added followed by rehomogenisation. 5 ml $CHCl_3$, 5 ml 0.88 % KCl (w/v) and 2 drops 1 N HCl (to aid recovery of acidic polar lipids) were added. The mixture was shaken and centrifuged to separate the organic and aqueous phases. The organic phase was filtered through a prewashed Whatman No.1 paper and solvents removed by evaporation under OFN. The extract was dried to constant mass under vacuum desiccation in a tared glass vial. The mass of extract was determined gravimetrically.

3.1.2.1.2 Lyophilised *v s.* 'wet' algal cells

A batch culture of *Nannochloris atomus* (CCAP Strain No. 251/4B) grown at S.M.B.A. Oban was divided into two equal volumes. Each was harvested by centrifugation. One pellet was extracted according to the procedure of Bligh & Dyer (1967) substituting *iso*-PrOH for MeOH. The remaining pellet was lyophilised and extracted as above. Quantitative efficiency was determined gravimetrically.

Each of the lipid extracts was analysed by HPLC with mass detection to determine the relative qualitative and quantitative compositions of the lipid extracts from each procedure. The relative efficiencies of each extraction and the proportions of the lipid classes obtained are presented in Figure 3.1.2. The lyophilised tissue yielded *ca.* 5 - 6 times more lipid and a negligible amount of free fatty acids by comparison to the *iso*-PrOH fixed cells (0 % *vs.* 16 %). A portion of each total lipid extract was transesterified and analysed by GLC in order to determine their total fatty acid compositions. These compositions are shown in Table 3.1.1. Major differences occurred in the proportions of 18:1(*n*-9) and total PUFA, the *iso*-PrOH fixed cells yielding a higher proportion of PUFA. However, in view of the vast differences in quantitative yield between the two cell treatments, lyophilisation was regarded as the more effective of the two and was therefore used throughout the remainder of the study.

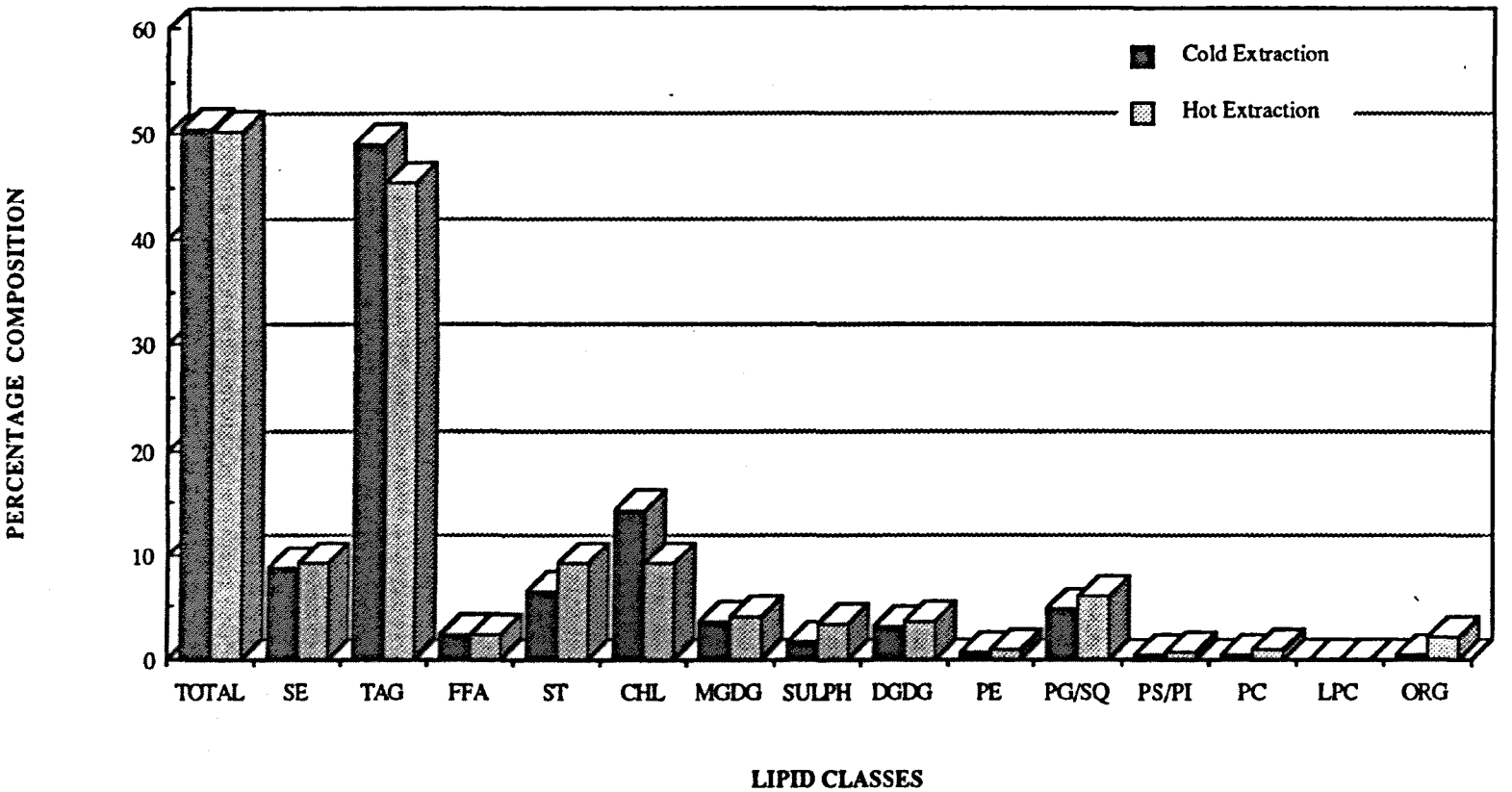


Figure 3.1.1 : The effect of hot and cold *iso*-propanol extraction procedures upon total lipid extract from lyophilised *N. oculata* cells expressed as a percentage of dry cell mass (TOTAL) and qualitative yield of the various lipid classes contained therein expressed as percentages of the total lipid mass extracted (see text for details)[1].

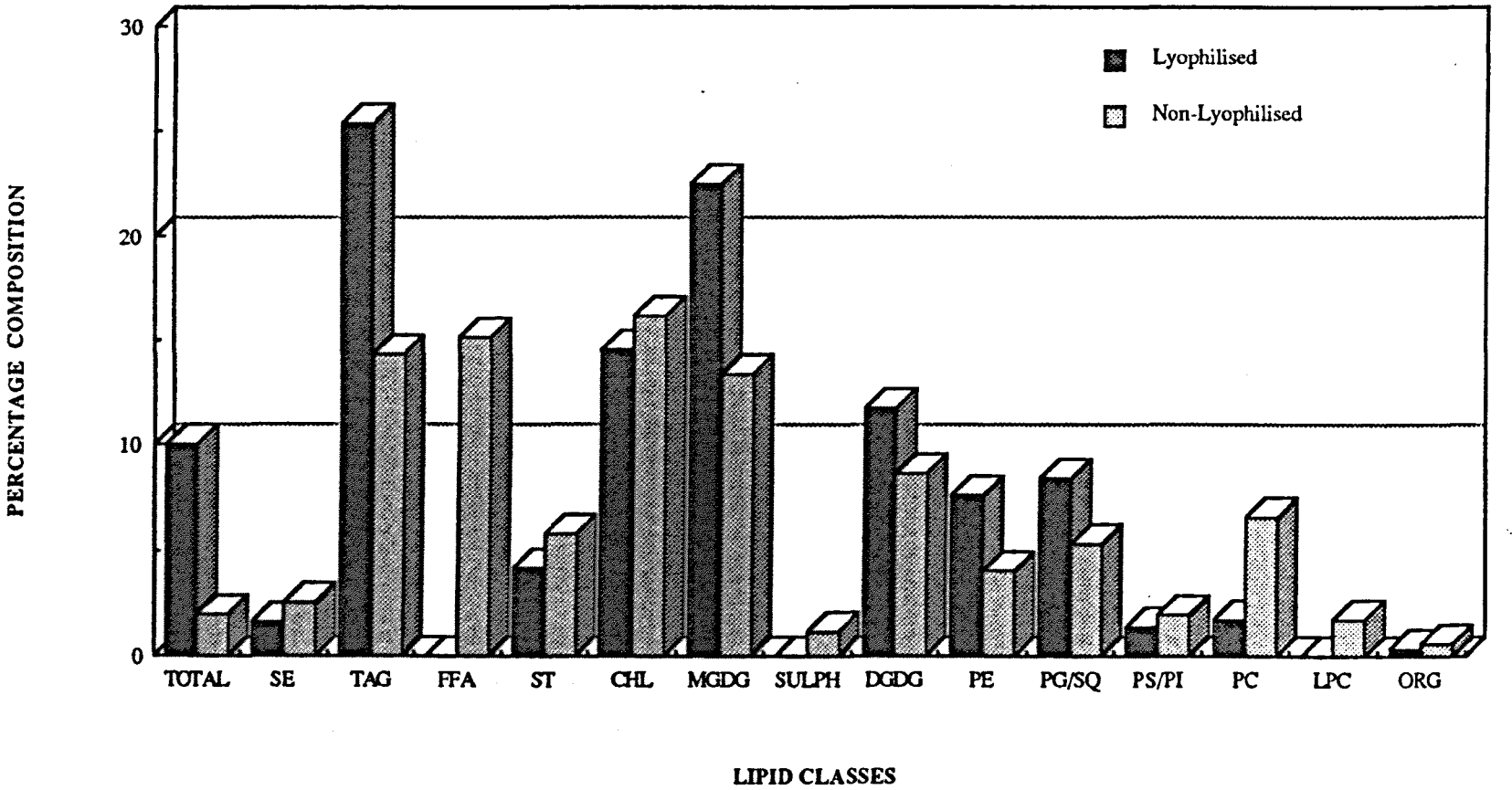


Figure 3.1.2 : The effect of sample pre-extraction treatment of *N. atomus* cells upon total lipid yield expressed in mg (TOTAL) and the qualitative yield of the various lipid classes contained therein expressed as percentages of the total lipid mass when extracted using a cold chloroform / *iso*-propanol mixture (see text for details)[1].

TABLE 3.1.1: Total FAME composition of *N. atomus* extracted prior to, and after lyophilisation

FATTY ACID	Unlyophilised	Lyophilised
14:0	1.3	0.9
15:0	.	.
16:0	22.7	19.7
16:1(*n*-7)	4.4	3.3
16:1(*n*-13)*t*.	.	.
16:2	4.7	4.8
17:0	.	.
16:3	9.3	7.2
16:4	.	.
18:0	1.2	1.2
18:1(*n*-9)	20.2	32.6
18:1(*n*-7)	.	.
18:2(*n*-6)	11.4	13.7
18:3(*n*-6)	.	.
18:3(*n*-3)	10.5	9.1
18:4(*n*-3)	1.8	0.8
20:0	.	.
20:2	.	.
20:3(*n*-6)	.	.
20:4(*n*-6)	0.3	0.7
20:3(*n*-3)	.	.
20:4(*n*-3)	.	.
20:5(*n*-3)	4.9	2.0
Unidentified:	7.4	3.9
Total sats :	25.2	21.8
Total monos :	24.5	35.9
Total PUFA :	42.9	38.4
Total (*n*-9) :	20.2	32.6
Total (*n*-6) :	11.7	14.4
Total (*n*-3) :	17.2	12.0
(*n*-3)/(*n*-6) :	1.5	0.8
16:1/16:0 :	0.2	0.2
20:5/16:0 :	0.2	0.1
ΣC_{16}:	41.1	35.0
ΣC_{18}:	45.1	57.4
ΣC_{20}:	5.2	2.7

Abbreviations & Symbols.
Data expressed in terms of mass percentage of total FAME.
tr.,<0.1%
„ not detected

3.1.3 Thin layer chromatographic (TLC) separations

3.1.3.1 Neutral lipid classes

Neutral lipid classes were separated on glass plates (20 x 20 cm, 0.25 mm layer thickness and 10 x 10 cm, 0.1 mm layer thickness) coated with silica gel 60 (E. Merck, Darmstadt, West Germany.). Plates were developed using the solvent system: hexane / diethyl ether / glacial acetic acid (40:10:1 v/v/v). (see Plate 3.1.1)

3.1.3.2 Galactolipids

Galactolipids were separated on silica gel 60 plates using the solvent system: acetone / benzene / water (90:30:8 v/v/v) (Pohl *et al.*, 1970). The galactolipids were also separated using the solvent system described below.

3.1.3.3 Polar lipid classes

Phospho- and galactolipids were separated on silica gel 60 plates activated at 160 °C for 1 hr. Various solvent systems were used in single dimension (1-D) and two dimensional (2-D) separations in filter paper lined chromatography tanks.

Methyl acetate / propan-1-ol / chloroform / methanol / 0.25 % (w/v) KCl (25:25:25:10:9 by vol.) Plate 3.1.2 (Vitiello & Zanetta, 1978).

Chloroform / acetone / methanol / glacial acetic acid /water (40:15:13:12:8 by vol.). (Heim *et al.*, 1987).

Chloroform / methanol / 7 M ammonium hydroxide (65:30:4 v/v/v) in 1st dim. Chloroform / methanol / glacial acetic acid / water (170:25:25:4 by vol.). in 2nd dim. (Christie, 1982)

3.1.3.4 FAME separation by argentation ($AgNO_3$) TLC

FAME were separated according to the number of double bonds in the carbon chain by TLC on silver nitrate impregnated TLC ($AgNO_3$-TLC) plates. Silica gel 60 plates were predeveloped in $CHCl_3$ / MeOH (2:1 v/v). The plates were then sprayed with 0.4 g $AgNO_3$ in 15 ml MeCN (for 10 x 20 cm HPTLC plates) or 2 g $AgNO_3$ in 25 ml MeCN (for 20 x 20 cm TLC plates). The plates were dried at 110 °C for 5 min after which they were wrapped in aluminium foil and stored in the dark prior to use. The plates were developed in benzene /

ethyl acetate (90:10 v/v) in darkness. FAME bands were visualised as per Sections 3.1.4.1 2, 3 or 4. This system may also separate FAME with respect to chain length in some instances *e.g.* 16:1 & 18:1.

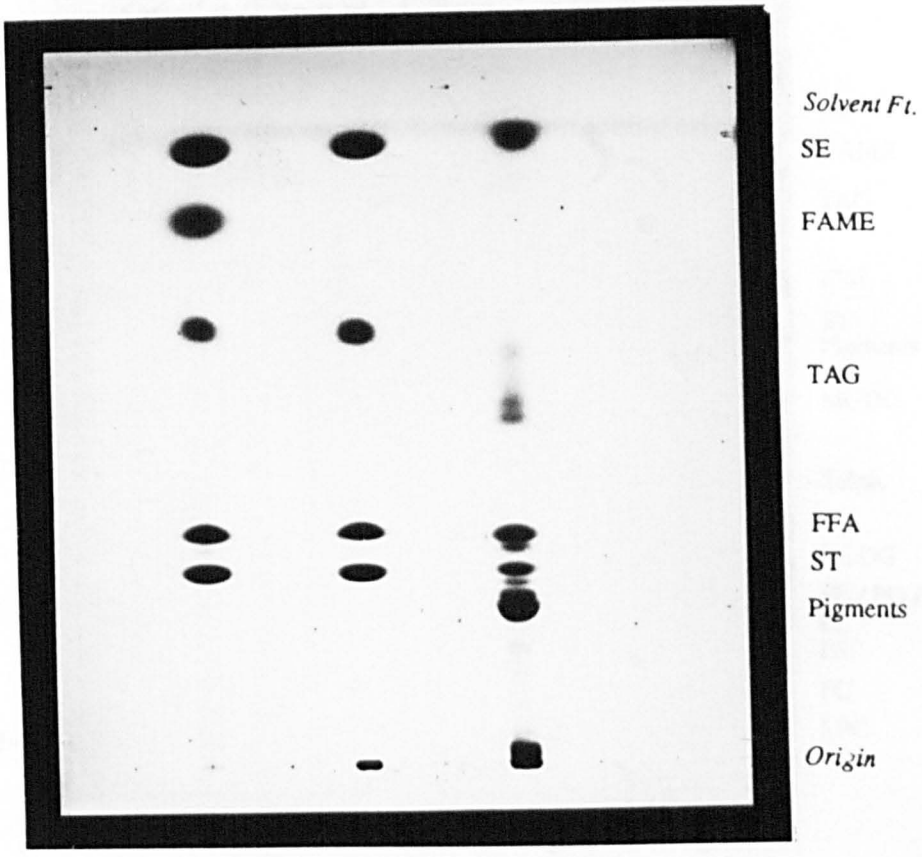


Plate 3.1.1 : HPTLC (10 x 10 cm) separation of (left to right) a neutral lipid class standard, a mixed neutral and phospholipid class standard and total lipid extract from *Nannochloropsis oculata*. Solvent system: hexane / diethyl ether / glacial acetic acid (40:10:1.5 v/v/v). Lipid classes visualised using cupric acetate reagent as per the TLC-densitometry protocol (see Section 3.2.2.1). Note the effect of acyl unsaturation upon the spot shape of the classes from the natural sample particularly that of TAG.

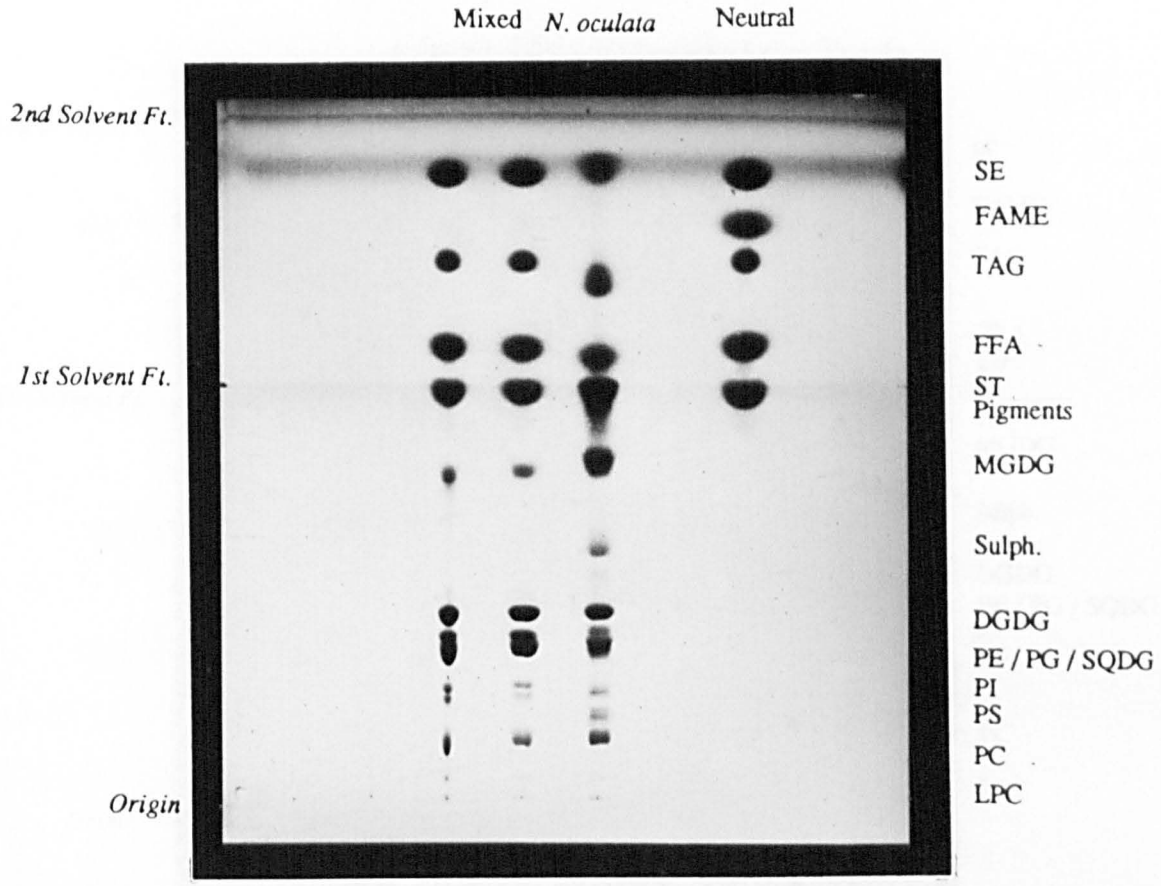


Plate 3.1.2 : Single dimensional double development HPTLC (10 x 20 cm) separation of (left to right) mixed neutral lipid, galactolipid & phospholipid standards loaded as a spot and a streak, a total lipid extract from *Nannochloropsis oculata* and a mixed neutral lipid class standard. Lipid classes separated & visualised using cupric acetate reagent as per the TLC-densitometry protocol (see Section 3.2.2.1). Note the unidentified band between the two galactolipid classes.

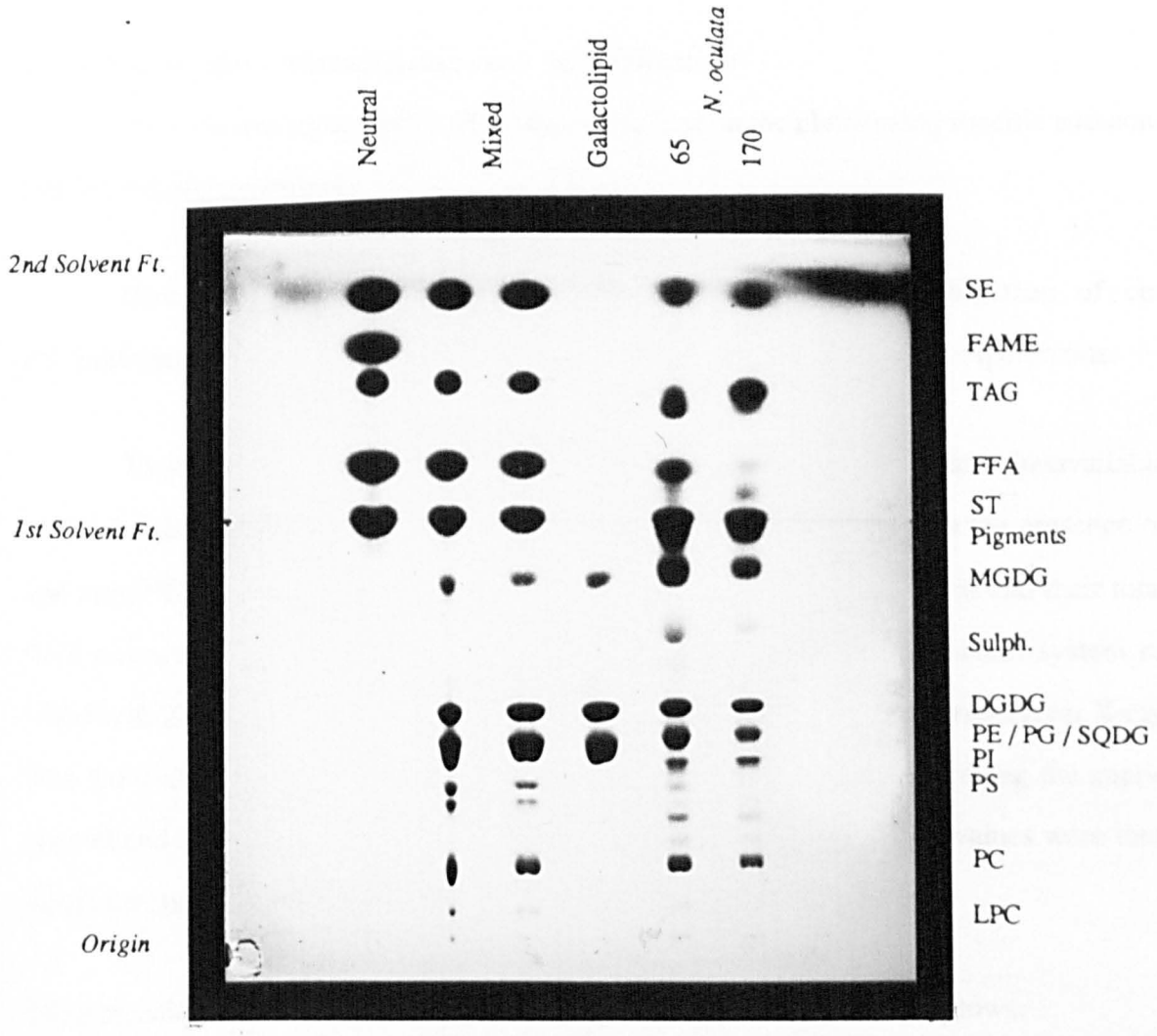


Plate 3.1.3 : Single dimensional double development HPTLC (10 x 10 cm) separation of (left to right) a neutral lipid class standard, a mixed neutral, galactolipid and phospholipid standard loaded as a spot and a streak, a mixed galactolipid and phosphatidylglycerol standard and total lipid extracted from *Nannochloropsis oculata* cultured at illumination intensities of 65 & 170 $\mu E\ m^{-2}\ sec^{-1}$. Lipid classes separated & visualised using cupric acetate reagent as per the TLC-densitometry protocol (see Section 3.2.2.1). Note the difference in staining for the two galactolipids loaded at the same mass (*cf.* section 3.2.2)

3.1.4 Lipid class visualisation and identification

Lipid classes separated by TLC were visualised on the plates using specific and non-specific staining techniques.

Identification of lipid classes was achieved using a combination of co-chromatography with known standards, specific staining and radiolabelling experiments.

To achieve a positive identification of sulphur-containing lipid classes (unavailable commercially), particularly SQDG, a culture of *N. oculata* was grown in the presence of sodium (^{35}S) sulphate (Amersham International plc.). Cells were harvested and their total lipid extracted. The extract was chromatographed using TLC by the solvent system of Vitiello & Zanetta (1978). The developed chromatogram was autoradiographed on X-ray film for 7 days at -80 °C. (Plate 3.1.4). The plates were then visualised using the cupric acetate and 1-Napthol reagents detailed in the following section. The R_f values were then noted for comparison.

Most techniques of visualisation involved spray reagents. These were as follows:

3.1.4.1 Non-specific techniques

1. Copper acetate: Developed chromatograms were sprayed with a solution of 3 % (w/v) Cu acetate in 8 % H_3PO_4 (v/v) (Fewster *et al.*, 1969) until saturated. They were then dried in a stream of warm air followed by charring at 160 °C for 20 min. The lipids show as blue-black spots on a white background. Sterol containing classes showed earliest as intense blue spots. This technique may be used to differentiate between steryl and wax esters.

2. Iodine solution: Developed chromatograms were sprayed lightly with 1 % (w/v) iodine in $CHCl_3$. The iodine vaporised from the silica gel in several minutes at room temperature but remained bound to the double bonds in unsaturated fatty acid moieties. Lipids showed as orange brown spots. This technique did not visualise saturated lipids well.

3. Iodine vapour: Developed chromatograms were exposed to iodine vapour in a TLC tank. Lipids showed as described in Section 3.1.4.1.2 in *ca.* 10 - 15 min.

4. 2,7-Dichlorofluorescein (2',7'-DCF): Developed chromatograms were sprayed with 0.1 % (w/v) 2',7'-DCF in 95 % MeOH containing 0.05 % (w/v) BHT and viewed under U.V. light. Lipids showed as fluorescent yellow-green spots. This is a non-destructive technique. Lipids stained in this way were used in further analysis.

3.1.4.2 Specific staining techniques

1. Unesterified fatty acids: Developed chromatograms were sprayed sequentially with 2,7-DCF, 1 % (w/v) aluminium chloride in 90 % ethanol (v/v) and 1 % (w/v) ferric chloride $_{(aq)}$. The unesterified fatty acids showed as pink spots on a yellow-orange background.

2. Glycolipids: Developed chromatograms were sprayed lightly with 0.5 % (w/v) 1-napthol in 50 % MeOH (v/v) and allowed to air dry. The plates were then sprayed with 95 % H_2SO_4 and heated in an oven at 120 °C. The glycolipids showed as blue-grey spots and other classes showed as faint yellow spots (Christie, 1982). Sulphoquinovosyl-diacylglycerol showed as a pink-red spot distinctly different in colour from monogalactosyl-diacylglycerol and digalactosyldiacylglycerol.

3. Phospholipid: Reagent was prepared as follows: Solution A - 4 g ammonium molybdate was dissolved in 30 ml distilled water. 2.5 ml mercury, followed by 10 ml conc. HCl were added to 20 ml of solution A. The mixture was shaken vigorously for 30 min in an arm shaker then filtered through Whatman No. 54 paper. The filtrate was added to the remaining 10 ml of solution A and 50 ml conc. H_2SO_4 were added. The resulting mixture was diluted to 250 ml with distilled water. Developed chromatograms were sprayed lightly with the reagent. Phospholipids showed as blue spots after *ca.* 10 min at room temperature (Christie, 1982).

4. Phosphatidylcholine: (and other classes containing a quaternary ammonium group - Dragendorff stain). Reagents were prepared as follows; Solution A, 1.7 g bismuth nitrate

were dissolved in 100 ml 20 % aqueous acetic acid (v/v). Solution B, 10 g potassium iodide were dissolved in 25 ml distilled water. Prior to use 20 ml A and 5 ml B were mixed and diluted to 70 ml with distilled water. Developed chromatograms were sprayed lightly with the reagent. Quaternary ammonium groups showed as orange spots.

5. Phosphatidylethanolamine and phosphatidylserine: (classes containing an amino group). Developed chromatograms were sprayed with a solution of 0.2 % (w/v) ninhydrin in water saturated butanol. Amino groups showed as purple spots after *ca.* 10 min at 120 °C.

6. Phosphatidylinositol, phosphatidylglycerol and glycolipids: Developed chromatograms were sprayed with 0.2 % (w/v) sodium periodate and left at room temperature for 15 min. Excess reactant was destroyed by exposure of the plates to SO_2 in a sealed TLC tank. The plates were then sprayed with commercially available (Sigma Chemical Co.) Schiff's reagent. Vicinal diol groups showed as blue-purple spots after *ca.* 10 min at room temperature

3.1.4.3 Autoradiographic techniques

Radiolabelled lipids and fatty acids upon TLC plates were detected using X-ray film following separation. For the separation of total lipid extracts, aliquots of *ca.* 150 x 10^3 cpm were loaded per lane on 10 x 10 cm HPTLC plates, or 500 x 10^3 cpm for 20 x 20 cm TLC plates. The plates were stamped with ^{14}C-labelled ink on a convenient unused edge as an aid to later comparisons between the plates and their autoradiograms. The chromatograms were developed in the appropriate solvent mixture(s) and either air dried or dried *in vacuo* prior to analysis of the acyl composition of the fractions by radio-GLC. In a photographic darkroom using 'Safelite' illumination X-ray film (Konica) was placed in contact with the silica surface of the TLC plate. This was held in place using a specialised light-proof cassette. The closed cassette was transferred to a -80 °C freezer and the X-ray film exposed to the radiolabelled chromatogram for 24 hr (or longer if radioactivity was less than the limits above).

Following exposure the film, or autoradiogram, was developed (under 'Safelite' illumination) using Kodak LX 24 developer (393 ml in 878 ml distilled water) for 5 min. The film was then transferred into Kodak FX 40 fixer (400 ml in 1600 ml distilled water) for 5 min. After being well rinsed under running water the autoradiogram was hung to air dry and harden. The locations of radiolabelled bands were visible as fogged areas on the film. The areas stamped with radiolabelled ink were also visualised on the film by this procedure. The locations of bands of radiolabelled lipid classes or FAME were matched with the corresponding areas of the chromatograms by aligning the ink stamped areas with their outline on the autoradiograms. By using specific radioisotopes it was possible to identify the lipid classes with which they are associated. SQDG was identified by the detection of ^{35}S-labelled lipid classes (see Plate 3.1.4).

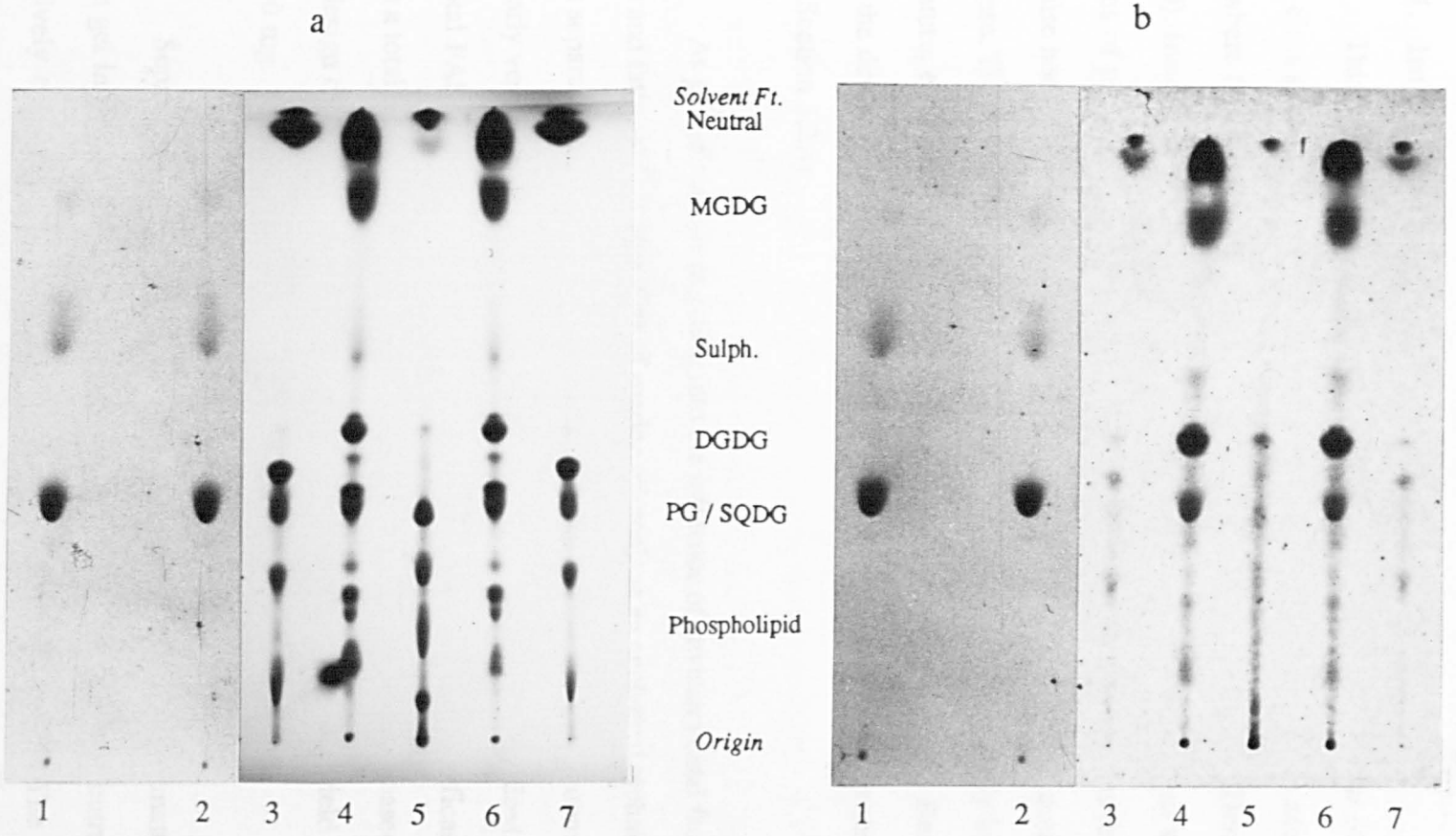


Plate 3.1.4 : Autoradiographic development of ^{35}S labelled total lipid extract of *N. oculata* separated using HPTLC. HPTLC plates then visualised using a) Cu Acetate and, b) 1-napthol. Lanes 1 & 2 are ^{35}S labelled total lipid from *N. oculata* visualised using autoradiography. Lanes 3, 5 & 7 are mixed neutral and phospholipid standards, and lanes 4 & 6 are total lipid from *N. oculata* visualised using the staining technique a) or b) as above.

3.2 QUANTITATIVE LIPID CLASS ANALYSIS.

3.2.1. Introduction.

This section was not intended to be a comprehensive review of chromatographic lipid class separation and quantification techniques. These have been adequately described elsewhere (Christie, 1982; Gunstone *et al.*, 1986; Hamilton, 1990; Henderson & Tocher, 1990). Instead, a brief overview of the techniques used in the present study is given as a means of pointing out their advantages and disadvantages. This was felt to be necessary because none was ideal, each had its particular limitations, and future users should be aware of these. The mass detector was a recent development and consequently had little supporting literature, especially in the fields of marine and algal lipid research. Because this was the case the device was the subject of a more detailed treatment in a later section of this thesis (see Section 3.2.4).

As part of the investigation into the influence of environmental factors upon the lipid class and fatty acyl composition of marine microalgae an analytical technique enabling lipid class separation and their subsequent quantification was required. A constraint imposed by the early version of the cage culture turbidostat (see Appendix B) required that a full analysis of total FAME, individual lipid class FAME, and lipid class quantification be carried out upon a total lipid extract sample of *ca.* < 5 mg. This restriction was eased after a change in the design of the culture vessel (see Section 4.3) enabled the lipid yield to be increased to *ca.* 10 mg.

Separation of the lipid classes is a straightforward enough matter, possible using silica gel in the form of a column or coated onto glass plates. The neutral lipid classes are relatively easily separated using TLC in a single development. The galactolipids and phospholipids, or polar classes, are more problematical. Using various combinations of organic solvents, the sequential elution of individual lipid classes is possible. Column chromatography does not have as high a degree of resolution as thin layer chromatography

and so the latter technique is generally the more common of the two. The introduction of small prepackaged columns has found favour with several authors (Ben-Amotz, 1985; Norman & St. John, 1986). These authors tended to designate fractions by reference to their specific solvent / eluant (*e.g.* acetone, chloroform or methanol) rather than the lipid classes themselves. The separations of neutral, galactolipid and phospholipid classes attempted during the early stages of the present study using the method of Norman & St. John (1986) showed considerable cross contamination upon analysis of the fractions using TLC. This technique was therefore abandoned in favour of preparative TLC in two dimensions (Christie, 1982). Whilst good separations were achieved, identification and quantification still presented substantial problems. The quantities of lipid separable on a single plate developed in two dimensions do not always allow fatty acid analyses, and there is little scope for scaling up the procedure. Inconsistencies in the R_f values of the lipid classes occurred from plate to plate, even when batches of plates were developed concurrently in the same solvent tank. This meant that reference standards, run on a separate plate, were unreliable as a means of identification for sample classes. Several two-stage, single dimension development techniques allow the separation of lipid classes varying in polarity from steryl esters to lyso-phosphatidylcholine on a single high-performance TLC (HPTLC) plate (Yao & Rastetter, 1985; Kovacs *et al.* 1986; Olsen & Henderson, 1989). This type of development has the advantage that, up to eighteen samples / replicates may be applied per plate. Identification of lipid classes can be achieved by simultaneous chromatography of standards on the same plate (*cf.* Yao & Rastetter, 1985). The use of the whole of the lower edge of the plate for sample loading also allows a much increased sample mass to be applied when compared with two dimensional separations (Leray *et al.*, 1987)

Once separated, quantification may take several forms. Firstly, the lipid classes can be charred using oxidising agents either on, or after elution from, the plate. The amount of carbon is then determined by photodensitometry. The previously mentioned single dimension separation techniques lend themselves very well to this type of analysis. Because

the samples have a well defined 'lane' on the plate, an automated scanner is easily able to follow them (Conte & Bishop, 1987; Olsen & Henderson, 1989). Secondly, the lipids can be sprayed using a dye and determined using fluorimetry, after scraping the adsorbent from the glass plate. Theoretically, this technique may be adapted to use the same scanner as for photodensitometry, after fitting it with a more specialised excitation lamp. Thirdly, the lipid classes may be eluted from the silica gel, using organic solvents such as chloroform or chloroform / methanol mixtures, and determined using techniques specific to the chemistry of the classes (*e.g.* Raheja *et al.*, 1973; Christie, 1982; Henderson & Tocher, 1990; see Section 3.1.4.2). This technique is capable of producing accurate results but is time consuming. Some of these techniques have also been modified for TLC-densitometry (Gustavsson, 1986). Quantification by gravimetry was eliminated due to the projected low sample masses, the presence of silica in the sample following elution from the adsorbent and potentially variable recovery efficiencies. The integration of neutral lipid class data derived from a separate TLC separation was surmountable using an adaptation of the calculation technique used by Fraser (1987) for Chromarod separations. Christie *et al.* (1970) described a method based upon calculation from quantification of the derivatised acyl moieties of the separated lipid classes following 'spiking' with an internal acyl standard and separation by gas-liquid chromatography. This method was again heavily dependent upon the reliability of the recovery efficiencies for the separated classes.

A logical progression of TLC was TLC - flame ionisation detection (TLC-FID) marketed commercially as the Iatroscan TH-10 by Iatron laboratories Inc. of Japan. The lipid classes were separated by TLC on thin quartz rods externally coated with silica gel (Chromarods) and subsequently quantified by passing the individual rods through the FID, thus eliminating the effects of variable recovery efficiencies. The attraction of such a system to many workers was the TLC separation system. However, conventional solvent systems for use on silica coated glass plates did not lend themselves directly to Chromarod separations. Fortunately, the solvent systems required for the separation of a wide range of lipid classes have been well documented (Christie & Hunter, 1979; Hirayama & Morita,

1980; Kramer *et al.*, 1980; Innis & Clandinin, 1981; Banerjee *et al.*, 1985; Kramer *et al.*, 1986). The popularity of TLC-FID amongst lipid researchers in the marine field has also been well documented (Parrish & Ackman, 1983; Fraser *et al.*, 1985; Morris *et al.*, 1985; Parrish, 1986; Fraser, 1987; Parrish *et al.*, 1988a; 1988b; Nichols *et al.*, in Press). Therefore, Iatroscan TLC-FID seemed to be the most favourable technique to use.

To some extent the Iatroscan TLC-FID filled a void within the range of techniques for the routine quantification of lipid classes. However, in the author's experience, the reproducibility and resolution of Chromarod TLC separation of the polar lipid classes was sadly lacking, particularly when compared to HPTLC. This also seems to be the case with other studies. Although it is never pointed out, the Iatroscan TLC-FID is rarely used to separate and quantify polar lipids, a scan of the origin usually suffices as 'polar lipids' (*cf.* Parrish & Ackman, 1983; Morris *et al.*, 1985; Parrish, 1986; Parrish *et al.*, 1988a/b; Nichols *et al.*, in Press). In addition, solvent focussing, complex multiple developments and partial scanning techniques described by Parrish (1986) ultimately result in lengthy analysis times and detract from the simplicity of the TLC-FID procedure; a major attraction in routine analysis. Even so, a considerable time saving may be made by the processing of ten Chromarods simultaneously - potentially replicate or individual samples.

The HPLC - mass detector separation and quantification system described in Section 3.2.3 presented problems which are discussed therein. At this point it is sufficient to say that mass detector quantification did not prove to be reliable enough for its long term application. Ultimately it was decided to adopt HPTLC - densitometry as the technique to monitor the changes occurring in the lipid classes of the algal cells during the culture periods. This technique is discussed in the section below.

3.2.2. TLC - photodensitometry.

The technique of thin layer chromatography - photodensitometry proved to be the most amenable method of lipid class separation and quantification. The separation system

was based upon the methods of Vitiello & Zanetta (1978), Yao & Rastetter (1985), Kovacs *et al.* (1986) and Olsen & Henderson (1989). Previous techniques were reliant upon two dimensional development for the separation of the 'polar' phospholipids and galactolipids. Because the lipid classes are thereby distributed over a wide portion of the chromatogram, quantification by densitometry is problematical and alternative methods must be applied (Tocher & Sargent, 1984). The solvent mixtures used by Vitiello & Zanetta (1978) enabled a satisfactory resolution of the phospholipids in a single dimensional development to be achieved. Yao & Rastetter (1985) modified this technique using a double development to separate firstly the polar classes, then the neutral classes, on a single plate. The separated classes, after visualisation, could be scanned densitometrically and quantification of the total lipid components achieved.

The advantages of single dimension double development TLC - photodensitometry are severalfold. The most attractive of these, for analysis of large numbers of samples, are the speed and simplicity of the separation procedure. During the development of the TLC procedure 10 x 10 cm HPTLC plates were used. However, during the later experimental analyses the use of 10 x 20 cm HPTLC plates was adopted to handle the large numbers of time course samples . In the 'short & wide', or landscape, orientation this allowed 17 to 18 samples to be separated on a single plate, thereby keeping time course point samples together. Such a strategy was important for comparative examination of the data obtained from densitometric analyses (see below). Using the same plate type in its 'tall & narrow', or portrait, orientation did not improve resolution. The increased development time and lateral diffusion of the sample during development resulted in a less satisfactory variation in the technique.

The separation of the phospholipids, particularly in the region of PE and PG (see Plates 3.1.2 & 3.1.3) was apparently loading dependent. Increased sample mass resulted in excessive tailing of the separated lipid classes causing them to merge into a poorly resolved multiple spot. The phenomenon was more noticeable when the sample was loaded as a small

spot (*cf.* Olsen & Henderson, 1989). By reducing the loaded sample mass increased resolution was achieved at the expense of faint spots for minor components such as PI, PS and LPC. However, if the sample was loaded as a short streak *ca.* 3 mm in length instead of a concentrated spot the same mass of sample could be loaded without loss of resolution or sample numbers fitted onto the plate. Whilst this did not improve the visibility of the faint spots to the naked eye, the densitometer operating in the zig-zag mode (see Appendix C) yielded larger peak areas and thus increased signal to background noise ratios. The effect of the streaking technique was similar to that described by Conte & Bishop (1988) for plates with a preadsorbent zone.

An additional refinement of the quantification procedure was attempted by constructing calibration curves using lipid class mixtures made up from commercially available standards. Standard mixtures were separated on HPTLC plates using the appropriate solvent mixtures and visualised by the method of Fewster *et al.* (1969). Variation in peak area for a given mass of lipid was *ca.* 1 % on an intra-plate basis but was higher on an inter-plate basis at *ca.* 15 %. To eliminate this effect, calibration was constructed from single points on one HPTLC plate. The response in the range 0.5 μg to 5 μg per lipid class was linear (see Figures 3.2.1 & 3.2.2). Similar results may also be found in the work of Conte & Bishop (1988) and Olsen & Henderson (1989). Although the work of Olsen & Henderson (1989) was conducted in the same laboratory as the present study these authors used an earlier model of densitometer. During the recalibration of the newer model the effects of fatty acid unsaturation upon the density of staining by the cupric acetate reagent were noted to be significant. Because the lipids of *N. oculata* were known to have high proportions of PUFA, the standards purchased were purified from natural sources, preferably of plant origin, wherever possible. Even so, significant differences were found in the staining density per unit mass when MGDG and DGDG standards were compared (see Plate 3.1.3). After transesterification and GLC analyses the two standards were determined to have different fatty acyl compositions. The MGDG standard was found to contain mostly C_{16} and C_{18} saturates whereas the DGDG standard was found to possess higher proportions

of C_{16} and C_{18} PUFA. Both standards were purified from wheat. Given that the 'authentic' standards were of poor quality or unavailable as in the case of SQDG, this method was abandoned as impracticable during the remainder of the project. As with previous studies, and at the suggestion of Olsen & Henderson (1989), the percentage compositions obtained from the scanning densitometer were used directly. This of course placed limitations upon interpretation of the values presented due to the known, yet unavoidable, differential staining densities of the individual lipid classes.

Quantification of lipid classes using segments of calibration curves constructed on every plate was evaluated during the technique's assessment. This could have effectively circumvented interplate variation in staining density per unit mass. Calibration curves were linear over the concentration ranges used (see Figures 3.2.1 & 3.2.2). Although they remained linear from chromatogram to chromatogram, the gradients, and the regression equations, of the curves varied from both lipid class to lipid class and from chromatogram to chromatogram.

Because the calibration curves may be shifted vertically upon their axes, the use of standard conversion factors based upon a 'one-off' calibration performed upon separate plates was precluded. The proposed calibration technique accounted for this. Using three calibration lanes to cover the expected ranges of lipid mass each plate had in effect an individual calibration for each major lipid class loaded alongside the experimental samples. Because every single HPTLC plate required its own set of calibration curves, interpretation of the data, particularly using manual calculation was bound to cause problems. Fortunately, an adaptation of a similar computer programme previously written for the Iatroscan TLC-FID could be made to handle the data. The programme was designed to perform the necessary calibration and interpolation after the raw data had been keyed into the microcomputer (see Appendix A).

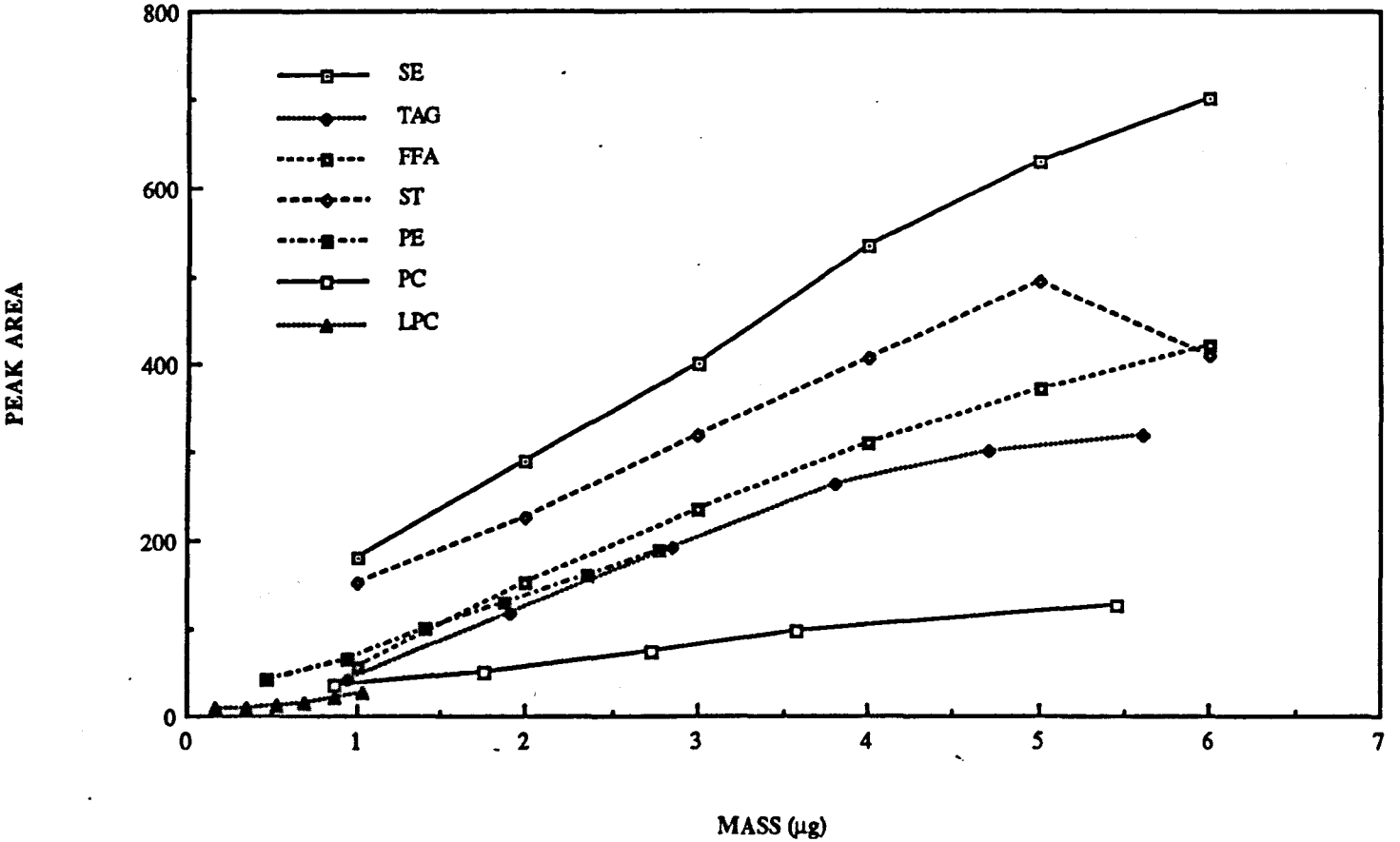


Figure 3.2.1 : An example standard calibration curve array for the single dimension double development HPTLC separation technique[1].

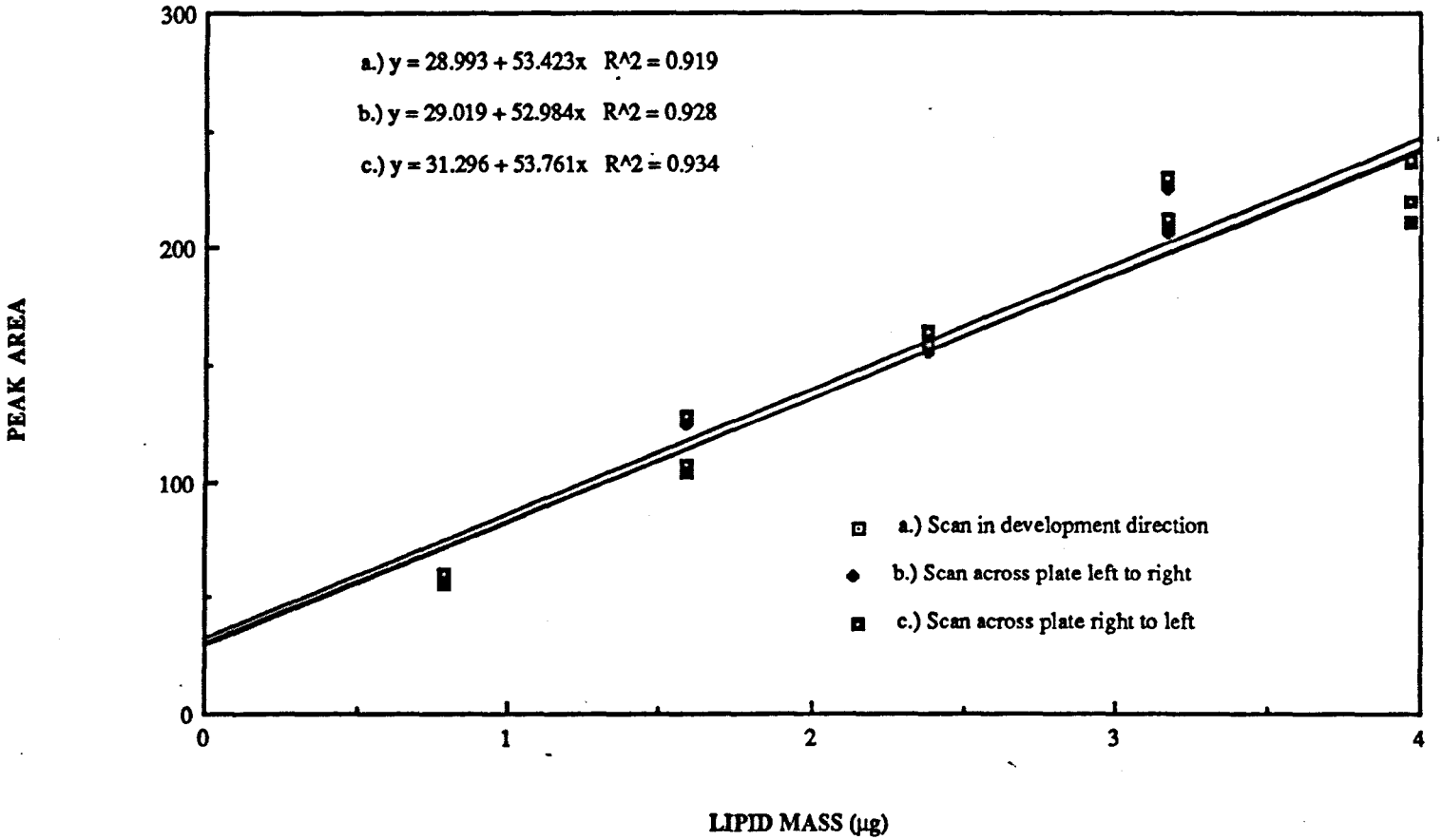


Figure 3.2.2 : The effect of the direction of scanning on the peak area of triolein separated from a standard lipid mixture using single dimensional double development HPTLC. Equations are the linear regression equations for each line[1].

Further problems were encountered in addition to those of the chemical properties of the lipid class standards themselves (*i.e.* fatty acyl unsaturation and standard unavailability). The calibration standard mixtures were accurately made up, by necessity. The continual use of the standard mixtures increased the rate of evaporation of the volatile solvents which resulted in them increasing in concentration and therefore inaccuracy. The problems caused by evaporation of the standard mixture during long periods of storage, even at -20 °C, meant that this technique, although theoretically applicable, was impractical on a routine basis. During the course of the calibration it was found that there were two *major* sources of inaccuracy. These were, firstly, the differences in unsaturation of the acyl groups of the standards and the experimental samples and secondly, the application of the standards and samples to the plate. If these two factors could be overcome, in combination with the calibration technique above, a workable and accurate method of lipid class quantification by HPTLC densitometry would probably be produced.

3.2.2.1 TLC - photodensitometry protocol

The HPTLC plates to be used were predeveloped in $CHCl_3$ / MeOH / H_2O (5:5:1 v/v/v) after which the solvents on the plates were allowed to evaporate at room temperature for 1 - 2 min. Following this treatment the plates were stored over NaOH pellets *in vacuo* for *ca.* 24 hr prior to being used. Immediately before use the contaminated, pre-development stage solvent front (*i.e.* the top 5 - 6 mm of the silica on the plate) was removed. The plate was then turned though 90 ° so its full 10 cm length of the was again available in the direction of development. In the case of 10 x 20 cm HPTLC plates pre-development was in the 'tall and narrow' orientation the plates then being turned to the 'short and wide' orientation following contaminant removal.

Samples of 10 - 20 μg total lipid extract (TLE) were applied to the HPTLC plates as 3 - 4 mm streaks 6 mm above the lower edge of the glass plate. The plates were redesiccated *in vacuo* for 30 min and then developed to 60 mm above the lower edge of the plate in the first solvent (methyl acetate / *iso*-propanol / chloroform / methanol / 0.25 % (w/v aq.) KCl as described above. The semi-developed chromatogram was dried using a stream of heated air directed onto the glass side of the plate and desiccated for 45 min *in vacuo*. The chromatograms were developed to *ca.* 98 mm above the lower edge of the plate in the second, and final solvent system. This required a modification of the neutral lipid solvent mixture described in Section 3.1.3.1. When the unmodified solvent system was used, the TAG and steryl ester / hydrocarbon were poorly resolved as were sterols and unesterified fatty acids. Addition of extra acetic acid increased the R_f value of the unesterified fatty acids and a decreased proportion of diethyl ether decreased the R_f value of TAG. The modified mixture was hexane / diethyl ether / glacial acetic acid 40:6:2 v/v/v). Traces of solvents were removed from the developed chromatogram using heated air, as detailed previously. The separated lipid classes were visualised on the plate by spraying it to saturation with the cupric acetate reagent followed by charring in an oven at a temperature of 160 °C for 10 min. After it had cooled sufficiently to handle the chromatogram was scanned immediately using the densitometer.

Scanning was by a Shimadzu dual wavelength flying spot scanner Model CS-9000 (Shimadzu Corp., Kyoto, Japan) operating in zig-zag mode. Quantification was by a Shimadzu DR-13 recorder and GDU-10C display linked to the scanner (see Appendix C for operating parameters). The chromatograms were always scanned in the same direction, from below the origin to above the solvent front.

3.2.2.2 Results.

The Figures 3.2.3 & 3.2.4 illustrate the type of trace produced by the scanning densitometer for lipid classes separated by single dimensional double development HPTLC. Figure 3.2.3 is the trace obtained from a standard mixture. This was prepared from purchased standards, a similar chromatogram is illustrated by Plate 3.1.2. The second figure, Figure 3.2.4 is a trace of the separation obtained from the lipid extract of the experimental microalga *N. oculata.* Note how the pigments co-chromatograph with the sterols in the latter trace, also note the unidentified sulphur-containing lipid components between the MGDG and DGDG peaks.

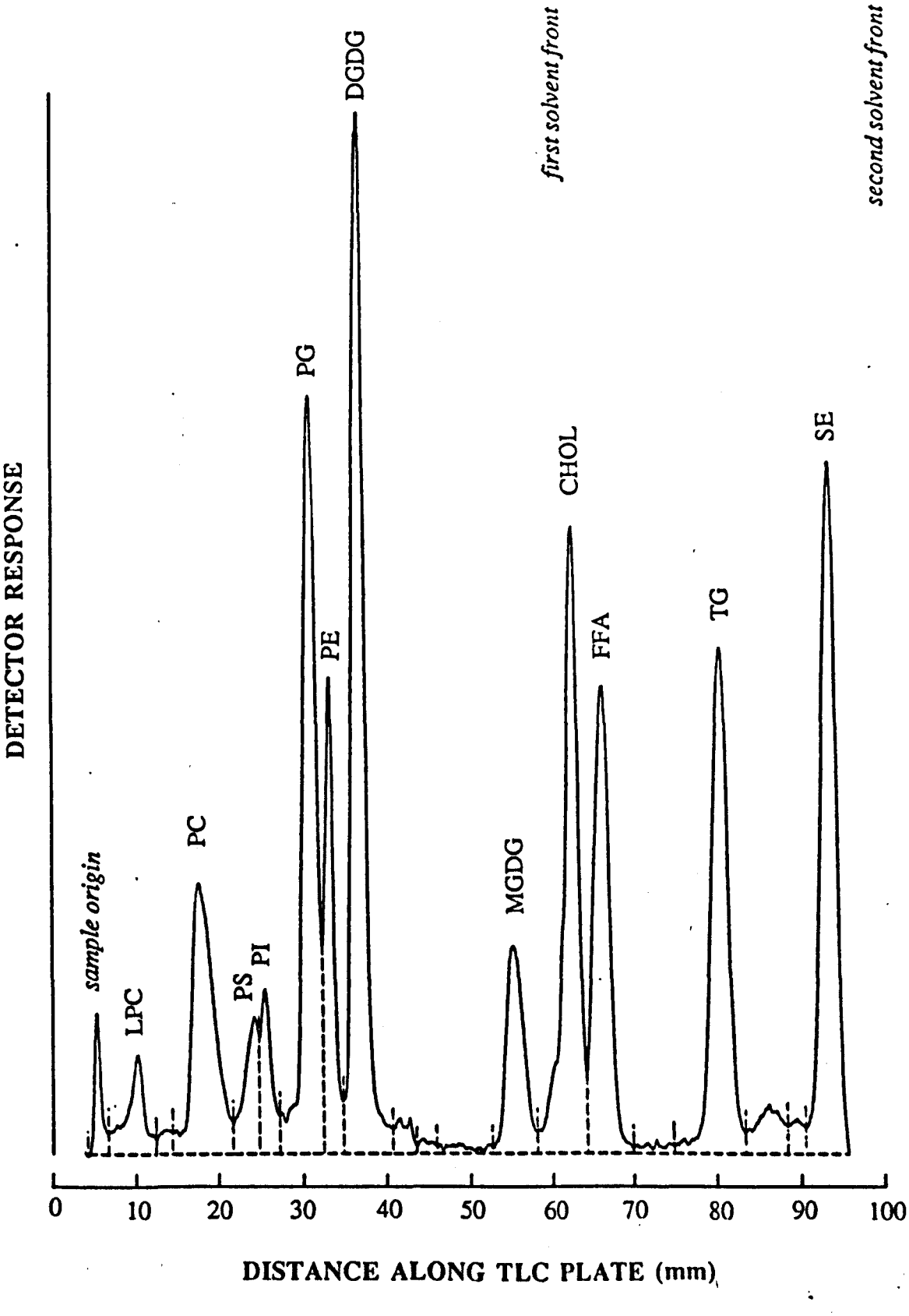


Figure 3.2.3 : TLC-densitometer trace showing the separation of a lipid class standard mixture using a double development technique (see text) on a 10 x 10 cm HPTLC plate followed by staining with cupric acetate (Fewster *et al.*, 1969)†.

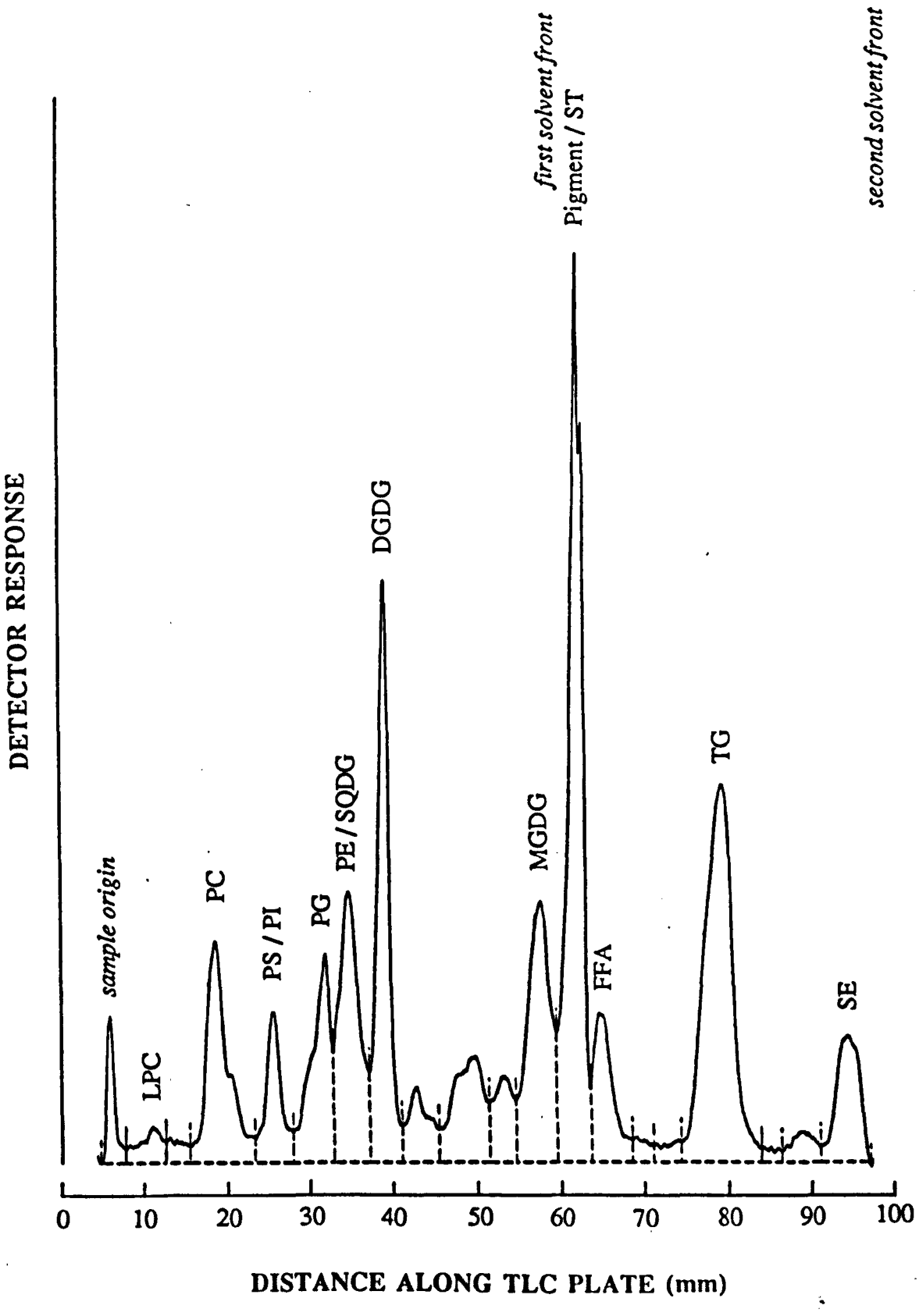


Figure 3.2.4 : TLC-densitometer trace showing the separation of the total lipid extracted from *N. oculata*, using a double development technique (see text) on a 10 x 10 cm HPTLC plate followed by staining with cupric acetate (Fewster *et al.*, 1969)†.

3.2.3 HPLC - Mass detection

3.2.3.1 Introduction

Conventional column chromatography has been limited in its use for lipid class separation by its lack of resolution compared to TLC. Small cartridge type columns, such as the Sep-Pak™ (Waters Associates Inc.) and Bond-Elut™ (Analytichem International), are finding increasing use for subfractionation of crude lipid extracts into neutral, glyco and phospholipid (Ben-Amotz, 1985; Norman & St. John, 1986). Larger columns are used in preparative scale procedures where gravimetric quantification is practical. On the whole, column chromatography is rarely used in lipid class quantification. HPLC columns enable increased resolution - for a smaller sample mass. Gravimetric determination of mass therefore becomes problematical.

The major reason HPLC has not been more readily adopted for separation of lipid classes is the lack of a universal detection system. The variable wavelength ultra-violet (U.V.) absorption detector is by far the most common means of detection; others include the differential refractometer (Macrae *et al.*, 1982) and moving wire / belt FIDs' (Aitzetmuller, 1977; Dixon, 1983). The U.V. detector and refractometer both limit usable solvent mixtures to those with low U.V. absorbances between 200 - 210 nm, or refractive indices (R.I.s') even in isocratic elution schemes. The changing physical properties (U.V. abs. / R.I.) of the solvents in a gradient system preclude their use with such detectors. Derivatisation procedures altering the absorption properties of the lipid classes prior to separation cannot be used (compared with molecular species analyses) because they require the substitution of the head group, upon which chromatographic separation characteristics depend, by a universal chromaphore. Thus a new problem of detection is created. FID devices eliminate many solvent restrictions. The only requirement is that the solvents be sufficiently volatile to evaporate completely before reaching the FID (Dixon, 1983). Custom built devices (Stolyhwo & Privett, 1973; Stolyhwo *et al.*, 1973; Privett & Erdahl, 1978; Phillips & Privett, 1981; and Phillips *et al.*, 1982) have failed to find a commercial market and are reputedly complex in operation (Aitzetmuller, 1977).

The evaporative analyser (Charlesworth, 1978), marketed as the mass detector (Applied Chromatography Systems, Macclesfield, Cheshire, U.K.) has been found by the author to be convenient to use if not quite as quantitatively reliable as alternative techniques on a long term basis. Briefly the principle of operation is as follows; the column effluent solvent stream is 'nebulised' at the top of the device in a stream of heated air. The volatile solvents evaporate leaving the less volatile solutes as minute droplets. The droplets scatter light supplied from a projector bulb via a collimating lens. The scattered light falls onto a photomultiplier producing a signal of magnitude related to the amount of solute present. The solvent limitations are similar to those of the FID, *i.e.* they must be more volatile than the solutes.

Solvent systems for the elution of lipid classes of widely varying polarity (*i.e.* as found in crude total lipid extracts) from silica gel columns have been described for use in conjunction with the mass detector (Christie, 1985; and 1986). The advantages of this system over TLC and TLC-FID are:

i) separation, quantification and fraction collection can be achieved simultaneously by installing a stream-splitting T-piece post-column and linking a recording integrator to the mass detector. This represents a substantial time saving over TLC, plate scraping and elution. The separate fractions collected can be transesterified directly for GLC analysis which is not possible with TLC-FID or TLC - photodensitometry.

ii) HPLC is a versatile technique requiring only a change in column packing and solvents to convert to analysis of chlorophyll / carotenoids (Mantoura & Llewelyn, 1983), sugars, proteins, amino / nucleic / fatty acids etc.

If fractions are not required for further analysis routine separation of large numbers of samples by HPLC becomes time consuming due to its ability to handle one sample at a time compared to TLC or the Iatroscan's ten. This can be overcome using an autoinjector

(and programmable fraction collector) to run the equipment 24 hours per day - although the hardware is somewhat expensive !

3.2.3.2 HPLC mass detector system

Solvent delivery was by a system comprising one Waters 501 and two Waters M-45 HPLC pumps, all controlled via a Waters 680 Automated Gradient Controller (Waters Chromatography Division, Millipore (U.K.) Ltd., Harrow, Middlesex, U.K.). Samples were introduced via a Rheodyne Model 7125 syringe loading sample injector equipped with a 20 µl loop (Anachem Ltd., Luton, Bedfordshire, U.K.). The column; 100 mm x 4.6 mm i.d. was packed with 3 µm Spherisorb™ silica gel (HPLC Technology, Macclesfield, Cheshire, U.K.). This was protected on the upstream end by an Upchurch™ precolumn filter equipped with a 2 µm pore replaceable stainless steel frit (Anachem Ltd.). Detection was by an ACS Model 750/14 Mass Detector connected to a Gast™ Model DAA-P125-700B-EG-1 twin cylinder air compressor equipped with a TUMD 106/1 reservoir (Applied Chromatography Systems). Instrument settings were as follows: Internal air pressure = 26 psi, Compressor supply pressure = 42 psi, Evaporator set = 50, Attenuation range = 8, Photomultiplier sensitivity = 3, Time constant = 5.

Quantification was by a Shimadzu C-6RA recording integrator (Shimadzu Corp.) using the following settings: Width = 5, Slope = 1500, Drift = 0, Min.Area = 10,000, T.dbl = 1000, Stop Tm. = 25, Atten. = 7, Speed = 5, Method$ = 41, Format = 0, Spl.Wt. = 100, Is.Wt. = 0.

3.2.3.3 Solvents and Gradient Elution Programme

Each of the three HPLC pumps was responsible for delivering one solvent mixture from:-

A - hexane / diethyl ether / tetrahydrofuran / 2 % acetic acid in diethyl ether (95:5:1:0.2 v/v/v/v)

B - *iso*-propanol / chloroform (4:1 v/v)

C - *iso*-propanol / 0.5 mM D-serine $_{(aq)}$ (adjusted to pH 7.5 with ethylamine (Sigma Chemical Co.)) (1:1 v/v)

to form a ternary gradient system. The proportions of solvents were controlled by the gradient controller according to the programme in Table 3.2.1 and illustrated in Figure 3.2.5. All solvents were of the HPLC grade (Rathburn Chemicals Ltd., Walkerburn, Scotland, U.K.) and were filtered through an 0.45 μm membrane filter (Millipore HVHP 0.45, Millipore (U.K.) Ltd.) prior to use. The water used in solvent C contained 0.5 mM D-serine and was adjusted to pH 7.5 with ethylamine (Sigma Chemical Co. Ltd., Poole, Dorset, U.K.) (Christie, 1986).

The system did not elute the neutral lipid-classes from the column in the manner described by Christie (1986). This was assumed to be due to inter-column packing differences. The retention times of unesterified fatty acids (FFA) and cholesterol were shorter than reported by Christie (1986) resulting in their simultaneous elution from the column. It was found necessary to modify solvent A, adding 5 % diethyl ether and 0.2 % of 2 % glacial acetic acid in diethyl ether in order to achieve a satisfactory separation of FFA, DAG, cholesterol and MAG (see Figure 3.2.6).

Table 3.2.1 : Programme used to control solvent proportions in the ternary gradient elution system.

Time (min)	Flow (ml min^{-1})	%A	%B	%C	Curve
Initial	0.00	0	0	0	*
1.00	2.00	100	0	0	6
5.00	2.00	80	20	0	6
5.10	2.00	42	52	6	6
20.00	2.00	32	52	16	6
20.10	2.00	30	70	0	6
25.00	2.00	100	0	0	6
31.00	2.00	100	0	0	6
31.02	0.00	0	0	0	6

N.B. Curve 6 denotes a linear gradient from the previous set conditions.

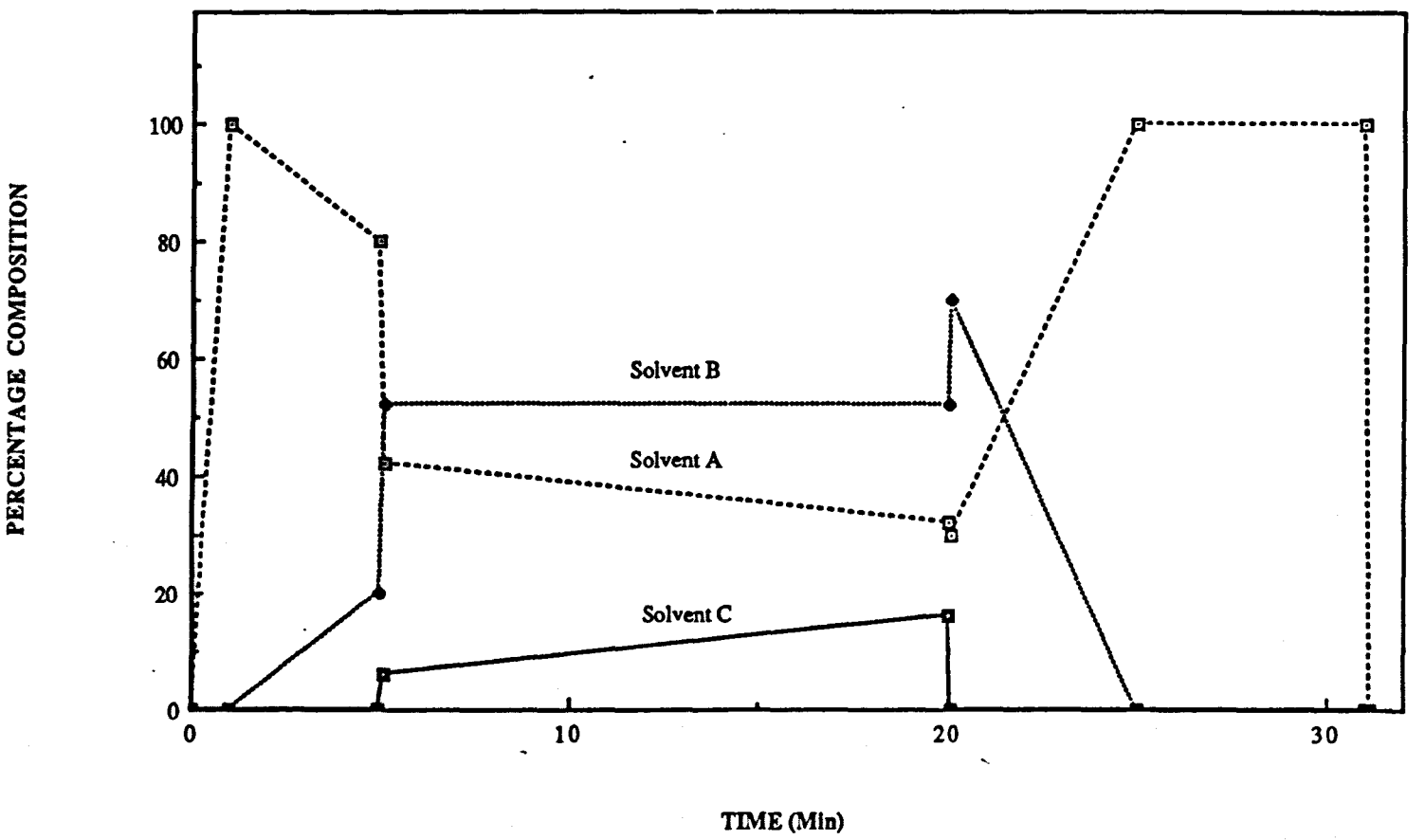


Figure 3.2.5 : A graphical representation of the ternary gradient elution profile used in the HPLC separation of total lipid for quantification using the mass detector[1].

3.2.3.4 Calibration & results

The type of traces generated by the mass detector are illustrated in Figures 3.2.6 and 3.2.7. The former figure illustrates the range of lipid classes separable using the HPLC technique. The sample used was a lipid class standard mixture containing a wide range of both polar and neutral lipid classes. The resolution achieved for the neutral lipid classes was relatively poor when compared with both the published data and alternative techniques involving the various types of TLC. However, the resolution of the polar lipid classes was at least on par with that expected from HPTLC and a distinct improvement over that obtained with Chromarod-TLC. It was found that improvements in the resolution of the sterols and the mono and diglycerides could be achieved with minor adjustments in the proportions of the solvents used during the early stages of the elution gradient.

Figure 3.2.7 illustrates the application of the HPLC mass detector separation and quantification technique to an experimental sample extracted from *N. oculata*. The fatty acyl profile of the natural lipid extract was thought to detract from the quality of the separation resulting in broader, less resolved peaks. A further problem which was encountered was that of the accumulation of the neutral lipid class TAG during some growth phases of the algal cells. Although specific examples have not been included, the mass of total lipid required to be loaded onto the HPLC column to obtain detectable peaks for the polar lipid classes in such cases was so great that resolution amongst the neutral lipid classes decreased to unacceptable levels. Unfortunately the technique was found to be unsuitable for the particular requirements of the present thesis.

The following two figures (Figures 3.2.8 & 3.2.9) illustrate tentative attempts at the construction of calibration curve arrays for the mass detector which were made during the course of the present thesis. The curves show another major factor which made the technique unsatisfactory for routine lipid class quantification. Although calibration produced a complex array of curves the standard deviations associated with some the calibration were as great as ± 50 %.

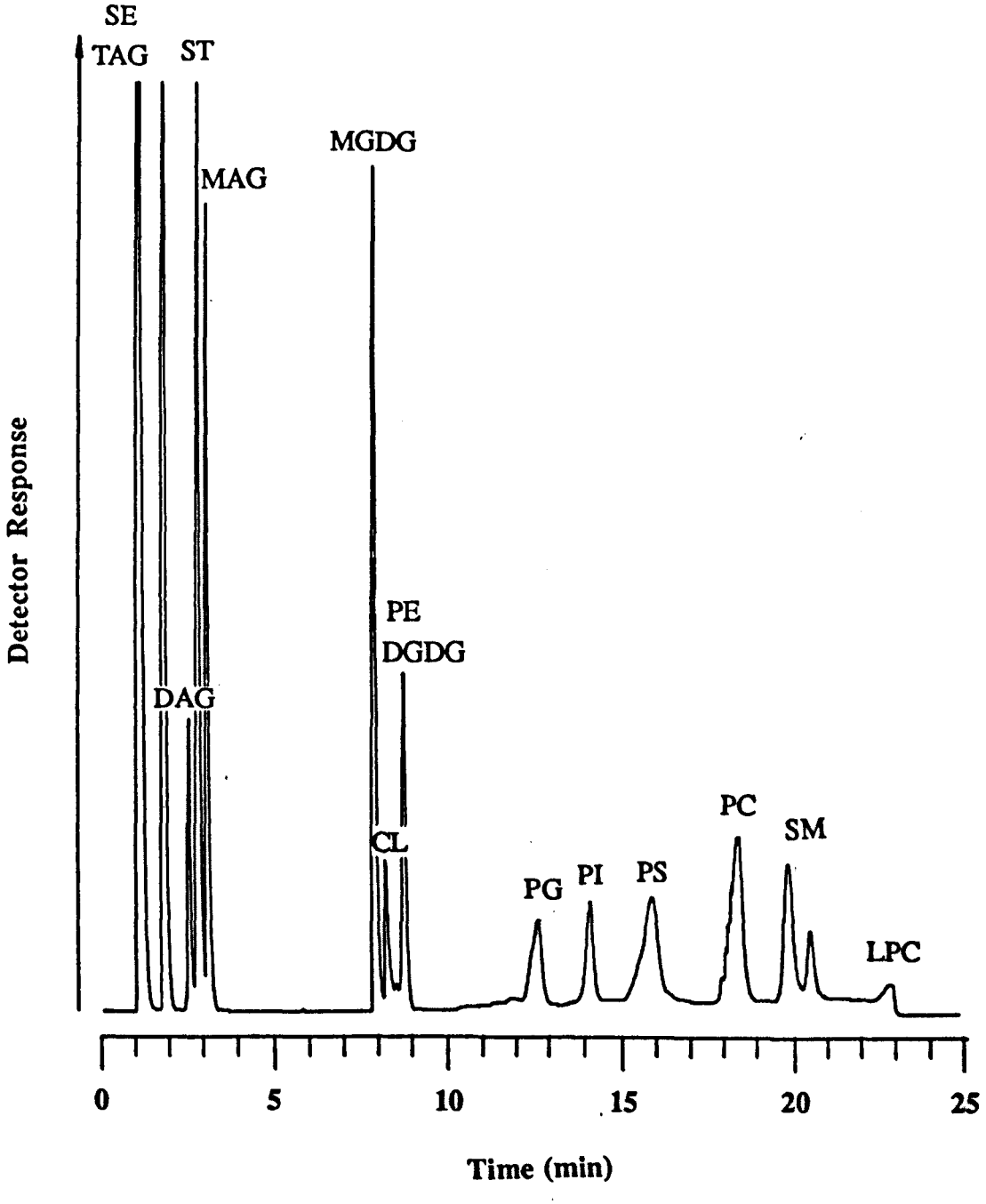


Figure 3.2.6 : HPLC - Mass detector trace showing the separation of a lipid class standard mixture using ternary gradient elution†.

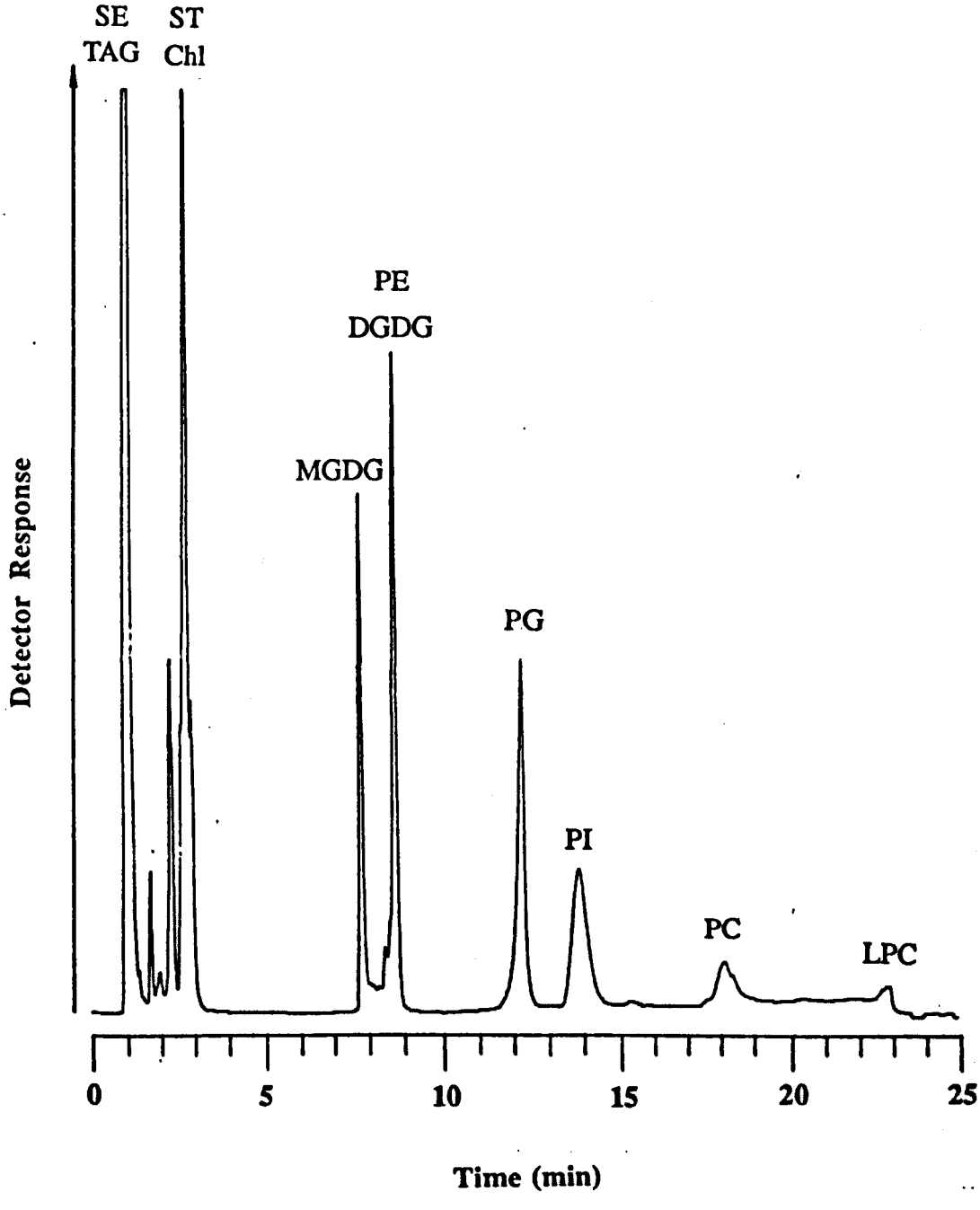


Figure 3.2.7 : HPLC - Mass detector trace showing the separation of total lipid extracted from *N. oculata* using ternary gradient elution†.

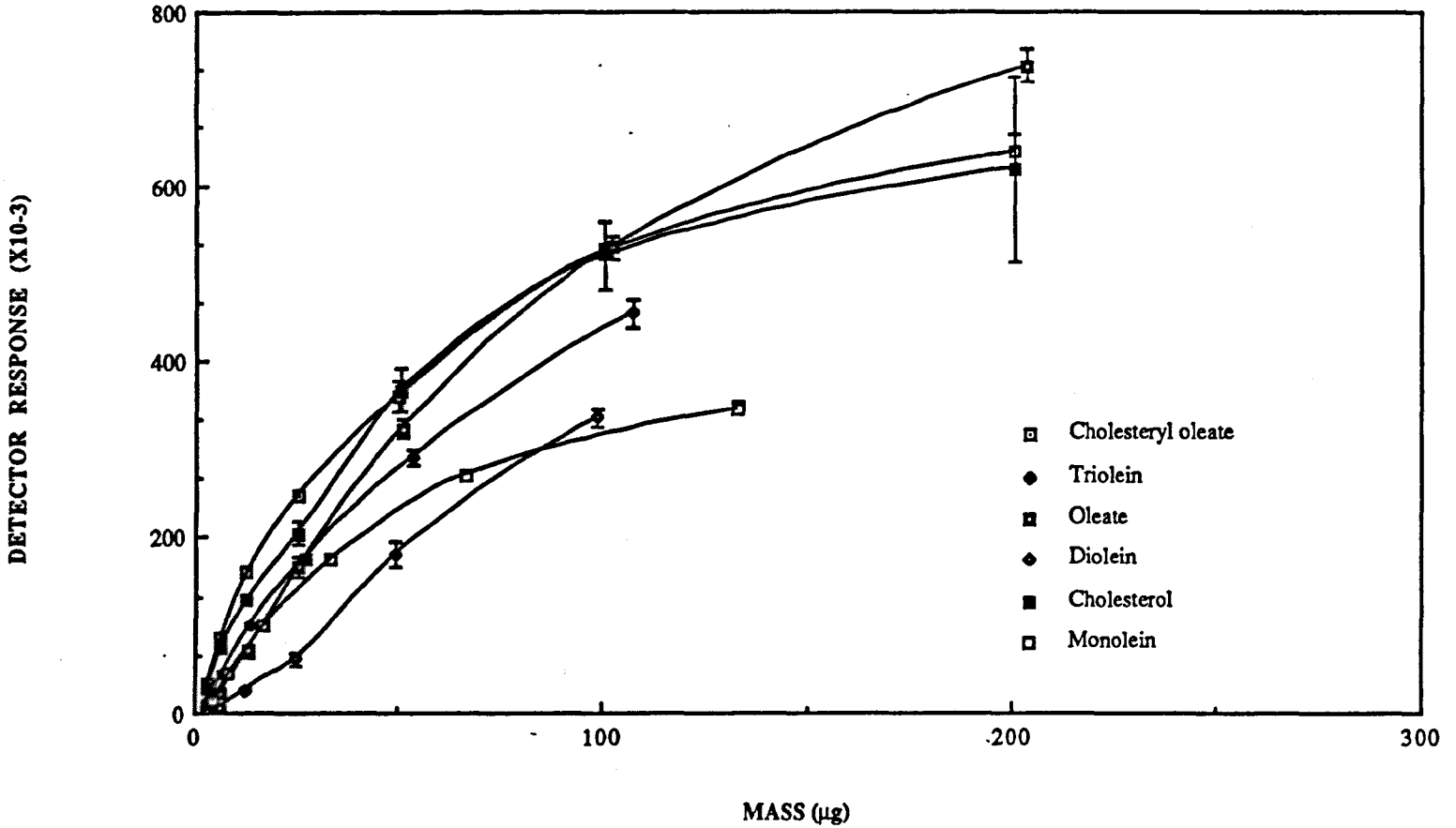


Figure 3.2.8 : Neutral lipid class calibration curves for the 'mass detector'. Points are the mean of 5 determinations ± s.d.1.

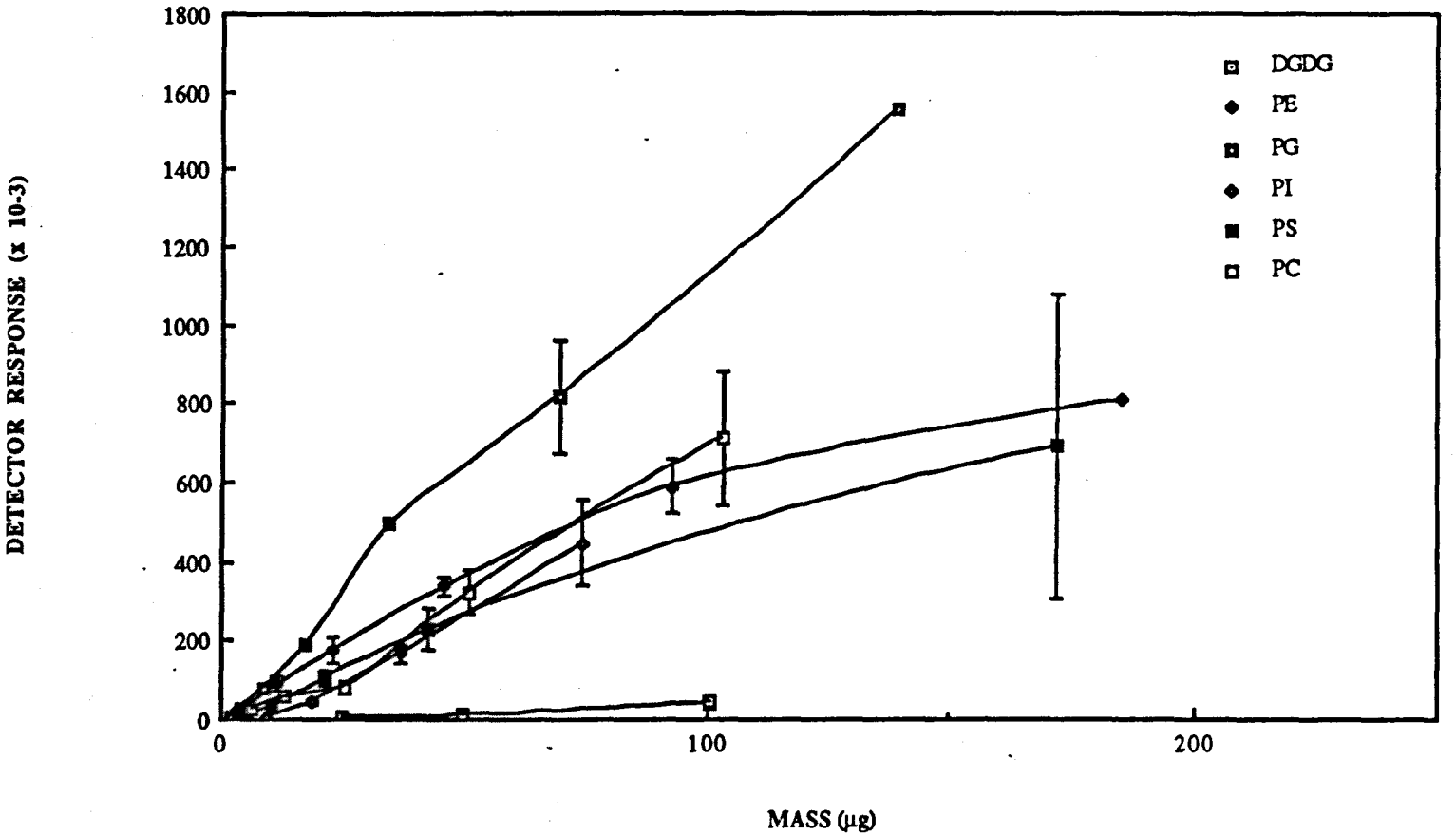


Figure 3.2.9 : Polar lipid class calibration curves for the 'mass detector'. Points are means of 5 determinations ± s.d.¶.

3.3 FATTY ACID ANALYSIS

3.3.1 Transesterification and FAME purification

Lipid classes separated by HPLC were transesterified directly after removal of solvent under OFN. Lipid classes separated by TLC were transesterified on silica gel after scraping it from the plate. Sterol esters were however eluted from the adsorbent prior to transesterification (Christie, 1982).

2 ml 1 % H_2SO_4 in MeOH (v/v) (plus 1 ml toluene to solubilise TAG) were added to the lipid and the mixture incubated at 50 °C overnight (*ca.* 15 hours) in stoppered tubes flushed with OFN. FAME were extracted with 2 x 5 ml volumes of hexane containing 0.01 % BHT (w/v). The extract was washed and neutralised with 3.5 ml 2 % $KHCO_3$ (w/v) and the upper organic layer removed. Hexane was removed by evaporation under OFN.

FAME were purified using Bond-Elut NH_2 aminopropyl mini-columns. The columns were preconditioned with 8 ml hexane. Samples of up to 25 mg dissolved in hexane were applied to the top of the column and forced through with gentle air pressure. 4 ml hexane were passed through the column to elute adsorbed FAME. The effluent was collected and hexane removed by evaporation under OFN. The FAME was then redissolved in hexane at the rate of 1 mg ml^{-1} and stored at -20 °C in screw topped vials prior to GLC analysis. The columns were regenerated, removing adsorbed unesterified fatty acids, by passing 8 ml 2 % glacial acetic acid in diethyl ether (v/v) followed by 8 ml hexane through them.

3.3.2 GLC analysis of FAME

Analyses of FAME were carried out using a Packard 436 gas chromatograph (Packard Instruments Ltd., Caversham, U.K.). This was equipped with an open fused silica capillary column 50 m in length, 0.32 mm i.d. and coated with CP Wax 51 liquid phase (Chrompack, Middelburg, The Netherlands). The FID was operated at 250 °C. Sample

injection was via an on column injector. The oven was programmed to hold oven temperature at 50 °C for 0.1 min post injection increasing to 190 °C at rate 39 °C min^{-1}, then increasing at rate 1.5 °C min^{-1} to a final temperature of 225 °C which was held for 18 min.

Component peaks were identified by comparison of relative retention times with those of a GC-MS characterised fish oil (Marinol) standard (see Figure 3.3.1). Quantification was by a Shimadzu C-R3A recording integrator connected to the GC.

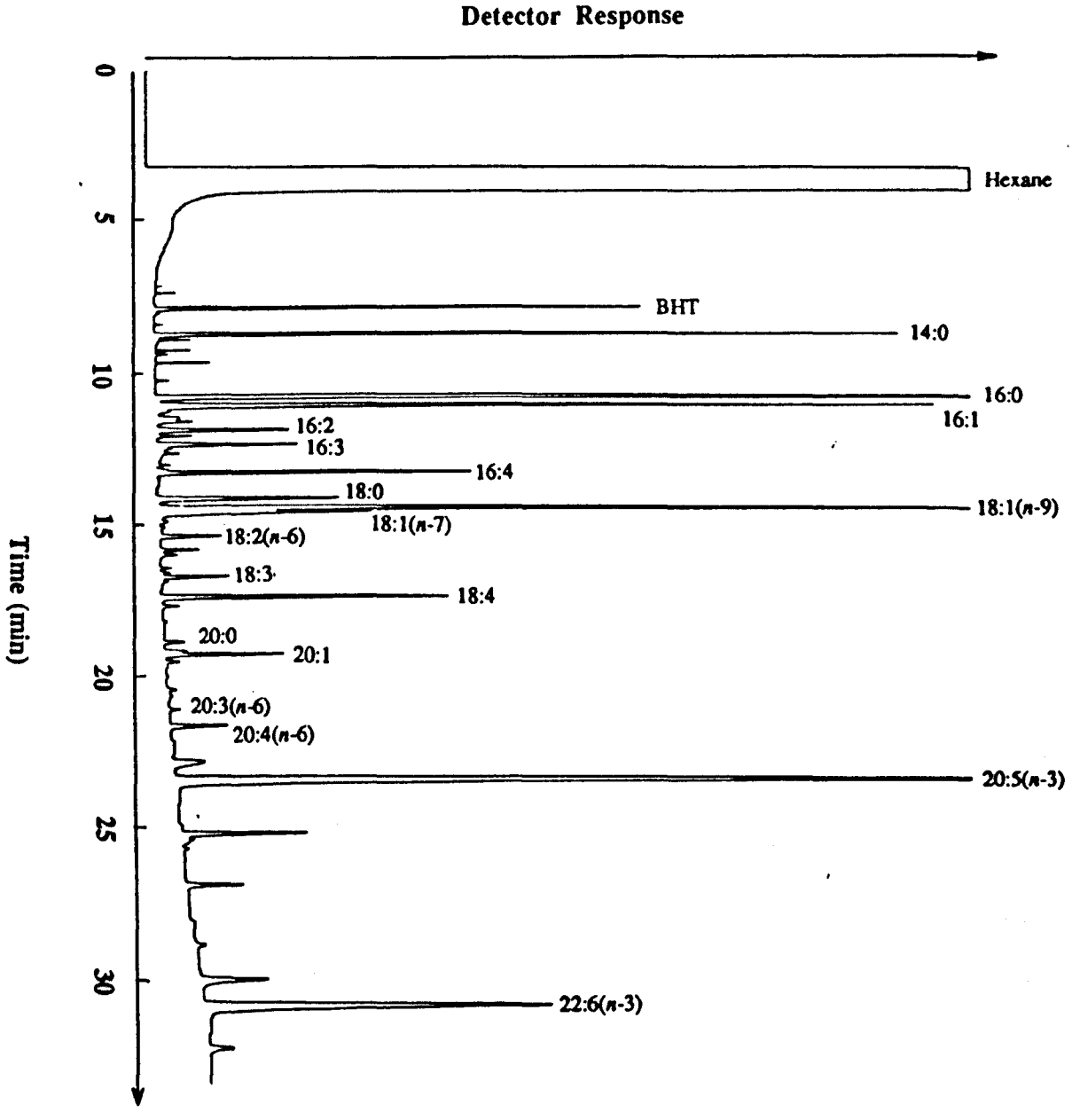


Figure 3.2.6 : GLC trace showing the separation of 'Marinol' FAME (as described in the text above)†.

3.4 COMPLEX LIPID CLASS MOLECULAR SPECIES & FATTY ACID POSITIONAL DISTRIBUTION ANALYSIS.

3.4.1 Bulk separation of lipid classes.

A combination of column and thin layer chromatography was used for the bulk separation of lipid classes to be used in detailed molecular species and positional distribution analyses. Removal of the neutral lipids and interfering pigments using a diethyl ether elution simplified later preparative TLC purification of MGDG, DGDG and PC. Total lipid (extracted as per Section 3.1.2) was redissolved in 2 - 3 ml diethyl ether. A glass column 1.5 cm x 10 cm was packed to a depth of 5 cm with diethyl ether washed 70 - 230 mesh silica gel 60 (Merck). The packed column was then washed with 200 ml diethyl ether. The sample total lipid extract was loaded onto the top of the column and allowed to wash down onto the adsorbent under gravity. The column was sequentially eluted with 50 ml diethyl ether and 50 ml $CHCl_3$ / MeOH / H_2O (5:5:1 v/v/v), the respective eluates being collected in round bottomed flasks. Solvents from both fractions were removed by RFE under reduced pressure. The remaining lipid was transferred to tared vials and desiccated *in vacuo* overnight prior to reweighing. The two lipid fractions were redissolved in $CHCl_3$ / *iso*-PrOH at the rate of 25 mg ml^{-1}.

Predeveloped ($CHCl_3$ / MeOH 2:1 (v/v)) 20 x 20 cm glass TLC plates were loaded with 6 mg of the $CHCl_3$ / MeOH / H_2O (5:5:1 v/v/v) extract as a 15 cm streak. The chromatograms were developed using the solvent mixture of Vitiello & Zanetta (1979). Separated lipid classes were visualised under U.V. light after spraying the developed chromatograms with 2,7-DCF. Silica gel corresponding to the lipid class bands was scraped from the plate and eluted with 3 x 25 ml $CHCl_3$ / MeOH / H_2O (5:5:1 v/v/v). The combined eluates were filtered through prewashed (50 ml $CHCl_3$ / MeOH 2:1 (v/v)) Whatman No.1 filter papers and solvents were removed by RFE under reduced pressure. The purified lipid classes were transferred to tared vials and desiccated *in vacuo* overnight prior to reweighing. An aliquot (5 μg) of each fraction was rechromatographed alongside authentic lipid class

standards using 10 x 10 cm HPLTC plates. The galactolipids gave a single spots whereas PC gave its characteristic pair of spots. A further aliquot (500 µg) of each class was used for FAME preparation to ascertain the general fatty acyl composition of each fraction.

3.4.2 *Sn*-1,2-diacylglycerol liberation.

3.4.2.1 From galactolipid classes.

The procedure used for liberation of DAG from MGDG and DGDG was essentially that of Heinze *et al.* (1984). Periodate oxidation of the glycosyl moieties was followed by reaction with dimethylhydrazine. Aliquots of 1 - 2 mg of individual lipid classes prepared as above were evaporated to dryness under OFN and taken up in 1 ml MeOH. 1 ml freshly prepared 0.2 M periodic acid in MeOH was added and the mixture incubated in stoppered OFN flushed tubes in darkness for 90 min. Following incubation 4 ml $CHCl_3$ and 1.5 ml 0.45 % (w/v) NaCl were added and mixed. The aqueous upper phase was removed to waste. 1.5 ml 5 % (w/v) ethyleneglycol (Sigma Chemical Co.) were added, the aqueous phase was again removed to waste. A further 1.5 ml 0.45 % (w/v) NaCl were added and mixed. The aqueous phase was discarded. Five drops of *iso*-PrOH were added to the remaining organic phase after which the solvents were removed under OFN. The products were dissolved in 0.5 ml freshly prepared 1 % (w/v) 1,1-dimethylhydrazine (BDH) in *iso*-PrOH / $CHCl_3$ / glacial acetic acid / H_2O (3.5:3:1.5:1 v/v/v/v). The mixture was incubated in stoppered OFN flushed tubes in darkness for 20 hr in cases of both MGDG and DGDG. Following incubation 3 ml hexane and 2 ml 50 mM KH_2PO_4 were added and mixed, the aqueous lower layer being discarded. A further 2 ml 50 mM KH_2PO_4 were added and mixed, the aqueous layer again being removed to waste. The hexane was removed by evaporation under OFN. The remaining residue was redissolved in 100 - 150 µl hexane and loaded as a 2 cm streak on a 20 x 20 cm TLC plate alongside a mixed isomer DAG standard. The chromatogram was developed in hexane / diethyl ether (1:1 v/v), the lipid class bands were visualised under U.V. light after spraying lightly with 2,7-DCF. The lower of the two DAG bands corresponding to *sn*-1,2-DAG was scraped from the plate and the DAG

recovered after elution with 3 x 5 ml $CHCl_3$. The DAG yielded was immediately used in the dinitrobenzoylchloride derivatisation procedure described below.

3.4.2.2 From phospholipid classes.

The procedure used for liberation of DAG from phospholipids was a modification of the procedure of Takamura *et al.* (1986) as described by Bell (1989). The procedure employed the use of phospholipase C purified from *Bacillus cereus* to cleave the phosphate head group from the *sn*-3 position of the glycerol backbone of the phospholipid (see Section 1) yielding *sn*-1,2-DAG which could be purified as detailed above and used in the dinitrobenzoylchloride derivatisation procedure described below.

Phospholipid purified using TLC was dried into a 5 ml Reactivial ™ and 1 ml of diethyl ether and 1 ml 0.01 M HEPES (pH 7.5) buffer were added. To this 100 - 300 μunits of phospholipase C (purchased from Boeringer Manheim ready purified from *B. cereus.*). The reacti-vial containing the mixture was flushed with OFN, sealed with a teflon-lined screw-cap and stirred vigorously for 6 hours using a magnetic stirrer. The heat from the stirred served to maintain the incubation mixture at *ca.* 37 °C. The digstion was stopped by transferring the mixture to a test tube, washing the reacti-vial with 8 ml $CHCl_3$ / MeOH + (0.01 % w/v) BHT (2:1 v/v) and adding the washings to the reaction mixture in the test tube. The mixture was washed with 1 ml 0.88 % KCl solution, shaken and centrifuged to aid separation of the organic and aqueous phases. The upper aqueous phase was discarded and the remaining organic phase dried under OFN prior to purification of the *sn*-1,2-DAG by TLC as described above.

3.4.3 3,5-Dinitrobenzoylchloride (DNBC) derivatisation.

To up to 5 mg DAG previously separated by TLC, 2 ml of pyridine dried over KOH pellets were added. The mixture was allowed to warm in a hot block at 60 °C for 5 min after which 150 mg of recrystallised 3,5-DNBC were added and allowed to dissolve. After a further 10 min at 60 °C a further 100 mg 3,5-DNBC were added. (A precipitate occasionally

formed at this stage but apparently did not affect the procedure). The mixture was incubated in the hot block for a further 20 min after flushing the tubes with OFN and stoppering. The tubes were cooled on ice after the incubation and 8 ml 0.1M HCl were added. 4 ml hexane were added and the mixture shaken and centrifuged to separate the two phases. The upper hexane layer was transferred and retained whilst the aqueous layer was extracted twice more with 4 ml hexane. All the hexane extracts were combined and dried down under OFN. The residue was redissolved in 5 ml hexane containing 0.01 % (w/v) BHT and sequentially washed with 3 x 5 ml 0.1 M HCl, 5 ml 0.1M $NaHCO_3$, 5 ml 0.1 M NaCl, 5 ml distilled H_20. Each time the aqueous layer was discarded. Following the final wash the organic layer was evaporated under OFN and the residue redissolved in a small volume (*ca.* 200 µl) of $CHCl_3$ prior to analysis by HPLC as described below. The yield efficiency of the derivatisation may be tested by chromatographing the products on a 10 x 10 cm HPTLC plate using the solvent mixture hexane / diethyl ether / glacial acetic acid (70:30:1 v/v/v).

3.4.4 HPLC separations of DNB-DAG derivatives.

The HPLC separations of the derivatised DAGs were based upon those of Bell (1989). The results presented in Section 8 of this thesis are a synthesis of the quantities obtained from the three separation protocols described below. Each solvent mixture and column packing combination has different separation properties which when combined act to complement the alternative systems. The result was that 'critical pairs' of molecular species which may co-elute in one solvent system were separated by at least one of the other solvent systems. Thus the relative contributions of the members of such critical pairs to the total composition were calculable by subtraction. No single solvent system was able to separate the entire range of molecular species commonly encountered. This makes molecular species separation and identification complex and time consuming.

The separation protocols employed are detailed below. The identification data, based upon the relative retention times (RRTs) of known standard molecular species used to spike the samples, for each of these protocols is held by Dr. M. V. Bell of the N.E.R.C. Unit of Aquatic Biochemistry Lipid Group.

Solvent 1: Methanol / *iso*-propanol (95:5 v/v),
25 cm x 0.46 cm ODS column
Flow rate 1.0 ml min^{-1}

Solvent 2: Acetonitrile / *iso*-propanol (80:20 v/v)
25 cm x 0.46 cm ODS column
Flow rate 1.0 ml min^{-1}

Solvent 3: Methanol / acetonitrile / water (93:5:2 v/v/v)
25 cm x 0.46 cm C8 column
Flow rate 1.2 ml min^{-1}

All the separation protocols employed isocratic elution using a Pye 4010 pump. Both HPLC columns (5 μm particle size) were obtained from Altex/Beckman (Beckman Instruments U.K. Ltd.) Detection was at 254 nm using a Pye Unicam 4020 detector. Quantification was achieved using a Shimadzu C-R6 recording integrator programmed using Shimadzu B.A.S.I.C. to calculate the relative retention time data required for interpolation on the identification graphs for each separation protocol. A sample trace showing the separation of the molecular species of MGDG, extracted from *N. oculata*, using the solvent 3 protocol is show in Figure 3.4.1.

Several authors (Safford & Nichols, 1970; Arao & Yamada, 1989) suggest that the long chain PUFA are preferentially located at the *sn*-1 position of the galactolipids of microalgae, in contrast to the phospholipids used to compose the RRT data. The RRTs of such reverse positional isomers may differ to an unknown extent. This may explain the ambiguities observed when attempting the identification of potentially reversed molecular species positional isomers (Section 8).

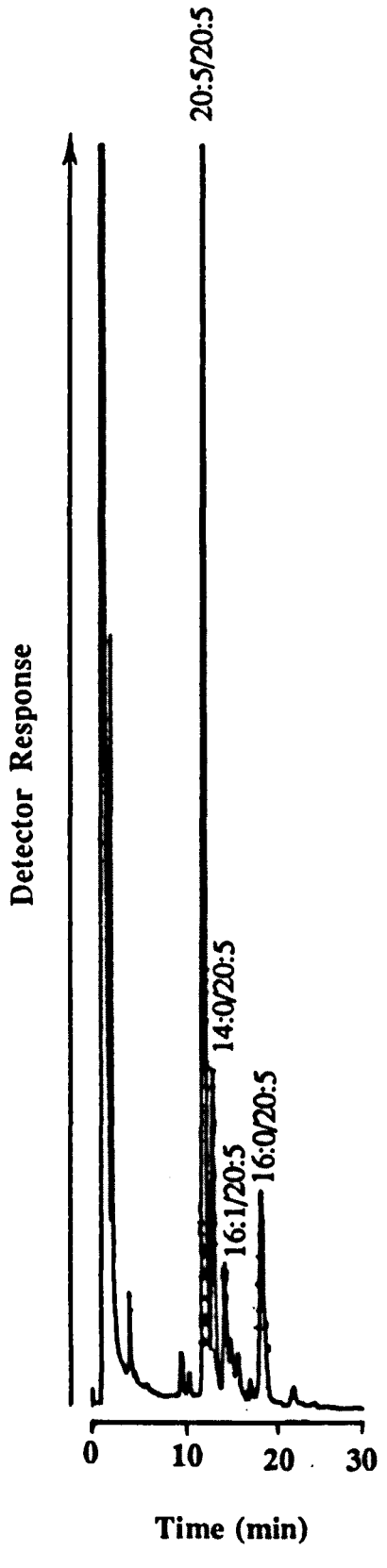


Figure 3.4.1 : A sample trace obtained from the separation of the molecular species from the MGDG of *N. oculata*†.

SECTION 4: ALGAL CULTURE METHODOLOGY.

4.1 INTRODUCTION (culture techniques & apparatus).

Several techniques are available for the axenic culture of unicellular microalgae in the laboratory. The most basic in terms of the apparatus required is batch culture. The culture vessel need only be a stoppered container, allowing transmission of light for photosynthetic culture and the facility for sparging with a gas (usually CO_2 enriched air) which is also useful for maintaining non-motile cells in suspension. This system is advantageous in that it is easy to maintain axenically, requiring little attention after inoculation. However, in the context of investigation of environmental effecters, batch culture has little real application except in maintaining inoculae cultures. Batch culture does have potential use in the study of phytoplanktonic blooms where the rapid local changes in water quality are analogous to those found in batch culture vessels.

Batch cultures have a limited lifespan, passing through a succession of phases from the time of inoculation to the eventual death of the culture (see Tempest, 1970). These phases are imposed upon the culture by the techniques used and are by no means fundamental to the growth of the organism (Droop, 1975). The unsuitability of batch cultures to this type of investigation where one variable is altered in a controlled manner whilst the others are required to be maintained as near constant as possible is, however, fundamental. At no time during the life of the culture is the composition of the cells steady, although in the stationary phase the change is very slow. The medium composition is constantly changing; unless the inoculum is impractically small *e.g.* 1000 cells ml^{-1} *Pavlova (Monochrysis) lutheri* (Droop), approximately 1000 times less than would normally be used (Droop, 1975). Under particularly favourable conditions the cell numbers would increase to such an extent that light limitation due to self-shading would occur adding yet another uncontrolled variable. In summary batch cultured cells are never in steady state, the medium composition is never in steady state and illumination is never constant.

Another technique involving slight improvement upon the basic batch culture is dialysis / cage culture. This technique, involving dialysis membrane tubes or Plexiglass™ cylinders the ends of which consist of Nuclepore™ membranes (also known as 'cages'), has been applied *in situ* (see Skoglund & Jensen, 1976; Sakshaug & Jensen, 1978; Ney *et al.*, 1981; Furnas, 1982; Vargo, 1984; Maestrini *et al.*, 1985.), but, as it requires a large medium reservoir, and has the same illumination drawbacks as batch culture, its laboratory use is limited. It is, however, relevant in that it provides the basic component for a more sophisticated apparatus; the cage culture turbidostat.

The commonly used alternative techniques all involve continuous culture apparatus although they differ significantly in mode of operation and versatility (see Tables 4.1.1 & 4.2.2). The classical continuous culture vessel is the chemostat (see Tempest, 1970; Evans *et al.*, 1970.). This device has many features to recommend it. In steady state it enables biomass, medium composition and physical conditions (especially illumination levels) to be held constant at any growth rate below maximum. This is achieved by control of limiting nutrient supply rate, thus controlling cell division rate or 'growth rate' when applied to the culture as a whole; and dilution rate, the rate at which new medium enters the culture vessel simultaneously removing cells from the vessel in the overflow. If the dilution rate exceeds the growth rate under the applied conditions cells are removed from the culture vessel faster than cell division can replenish them and 'washout' results in the loss of the culture. This also occurs when the maximum growth rate is approached, due mainly to the inability of the pumps to maintain a steady influx of culture medium resulting in a variable dilution rate. In the context of the present study another difficulty is encountered; under conditions of nitrogen limitation or starvation microalgae are known to accumulate lipid (see Section 2). However, Richardson *et al.* (1969) reported an inability to maintain chemostat cultures at a supply rate lower than 3 mM nitrate without washout. In batch culture however, growth does occur albeit at a slow rate with fully N-depleted medium (Richardson *et al.*, 1969) and lipid accumulates. It is for this reason that chemostat culture is not entirely suitable for this study.

The other commonly used continuously culture apparatus is the turbidostat, which exists in varying degrees of complexity. The 'original' designed by Myers & Clark (1944) and used in the continuous culture of *Chlorella* spp. has been modified and updated for use with micro-organisms other than microalgae (see Munson, 1970; Uno, 1971; Watson, 1972; Fenaux *et al.*, 1985.). Very broadly, the '-stat' device monitors a 'population effect', be it pH change, CO_2 production rate or, as the specific name here suggests, turbidity *via* transmitted / scattered light, (Watson, 1972). It is this effect that is used in triggering the addition of new medium to the culture vessel. This technique is very useful in studies where maximal growth rate and correspondingly high dilution rates are required (in contrast to the chemostat). Since it is the population itself that controls dilution rate washout is avoided. At low growth rates obviously little dilution occurs and no new medium enters the culture vessel, so, the culture resembles a pulsed batch culture, with periodic addition of new medium. In the light controlled system of the type described by Uno (1971) cell numbers are not the only factor involved in determining turbidity, cell size distribution also contributes along with chlorophyll concentration, it is the overall consistency of illumination within the culture that is maintained by such a device under a variety of culture conditions. A combination of this technique and dialysis / cage culture, modified to run on the laboratory bench constitute the final culture vessel to be discussed here; the cage-culture turbidostat originated by Skipnes *et al.* (1980).

The cage culture turbidostat (CCT) in the author's view represented the best apparatus to test the effects of environmental conditions upon the lipid metabolism of microalgae. However, since this was such a recent development there was much scope for improvement upon the basic design of Skipnes *et al.* (1980). To tailor the apparatus to metabolic, rather than growth rate investigation several modifications were made and incorporated into the CCT during the course of this study. The main advantage of this type of culture apparatus over standard chemostat and turbidostat set-ups was the possibility of maintaining a continuity of technique over a wide range of experimental conditions which

would normally require the use of several different types. A constant flow of new culture medium through the culture chamber eliminated variation in medium chemistry. A constant turbidity eliminated effects attributable to inconsistent illumination. This would normally occur within the cultures as a result of their differential growth rates under the conditions applied during a series of experiments.

Diagrammatic representation of the techniques described here are shown in Figure 4.1.1. A table of 'Modes of Operation' is presented with regard to the various culture techniques discussed (after Schultz and Gerhardt, 1969) (see Tables 4.1.1 and 4.2.2).

Table 4.1.1: Modes of operation of culture apparatus (After Schultz & Gerhardt, 1969; Sakshaug & Jensen, 1978).

Mode	Cells	Medium
1	Batch	Batch
2	Continuous	Batch
3	Batch	Continuous
4	Continuous	Continuous

Table 4.1.2 : Modes of operation possible with culture apparatus type

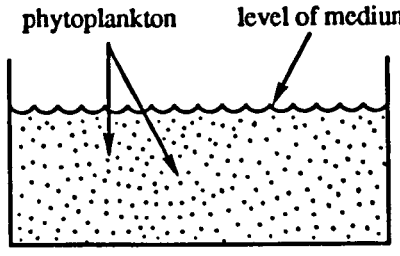
Culture apparatus	Modes			
	1	2	3	4
Batch	+	-	-	-
Dialysis / Cage	+	-	+	-
Chemostat	+	-	-	+
Turbidostat	+	*	-	+
CCT	+	*	+	+

+ possible.

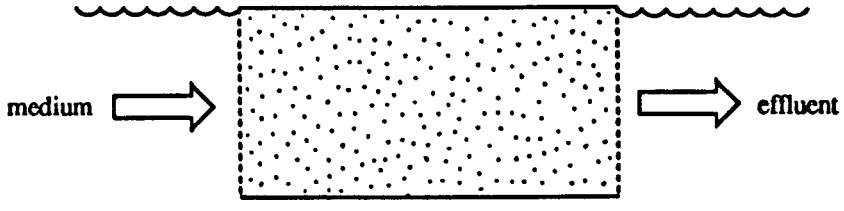
- not possible.

* possible over a limited period.

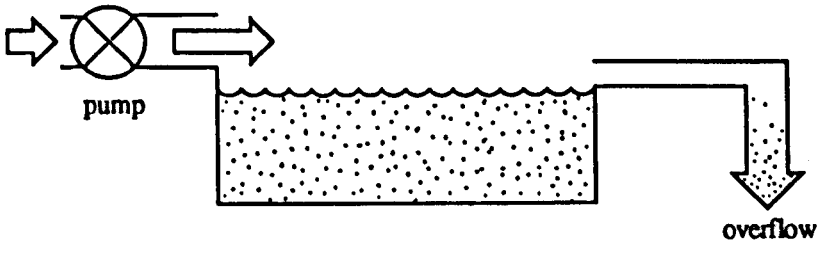
i) Batch



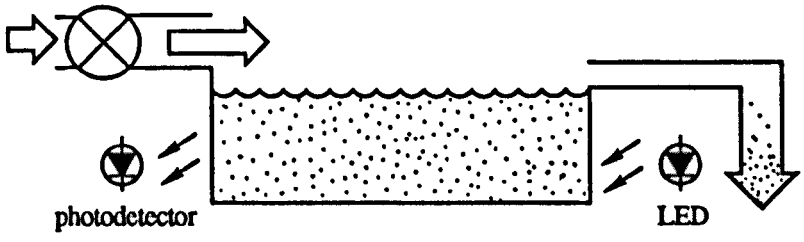
ii) Dialysis / Cage



iii) Chemostat



iv) Turbidostat



v) Cage culture turbidostat

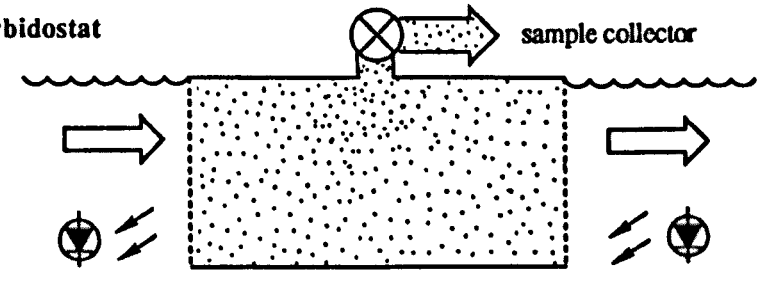


Figure 4.1.1: Schematic representations of phytoplankton culturing systems (redrawn from Parrish & Wangersky, 1987)[††].

4.2 BATCH CULTURE

4.2.1 Culture medium

The culture medium used throughout the experiments was a half seawater strength fully defined medium, S88 (Droop, 1968). The composition is provided as Appendix D. When making the bulk medium slurry, nitrate and glycine were omitted to allow for greater flexibility with respect to nitrogen source. Unless otherwise specified S88 refers to medium to which KNO_3 and glycine had been added prior to use.

4.2.2 Inoculum cultures

Axenic inoculum cultures were maintained in autoclave sterilised S88 under normal laboratory temperature and illumination in 250 ml Pyrex™ conical flasks. These were plugged with non absorbent cotton wool, facilitating gaseous exchange, and contained approximately 150 ml of algal suspension. Inoculum cultures were agitated by hand weekly rather than being aerated. Subculturing into fresh S88 was carried out on a monthly basis.

4.2.3 Batch culture vessels

Experimental culture vessels were Schott™ bottles varying in volume from 100 ml to 5 l dependent upon the amount of material required for analysis. The bottle screw tops were modified to allow the introduction of aeration and culture transfer tubes (Figure 4.2.1). The open ends of the tubing were sealed using aluminium foil and autoclave tape. The bottles were filled to the required volume with complete culturing medium and autoclaved with the tops in place at 121 °C, 15 psi for 30 min. After cooling to the experimental temperature inoculation to the required cell density was carried out by aseptic transfer, from an inoculum culture following cell counts to ascertain the volumes required, in a laminar flow cabinet. Aeration of the cultures, *via* a 0.22 μm Acrodisc™ CR filter, was from a diaphragm pump (Rena™, France).

4.2.4 Cell counting & sizing

Cell counts were carried out using an improved Neubauer haemacytometer. Means were calculated from 6 replicate full field counts. Early attempts were made at counting and sizing the cells using a Model Z_B Coulter Counter™ using the size window technique (Strickland & Parsons, 1968). These were discontinued as the resolution between debris and the smaller cells (*ca.* 1 μm) was limited by the smallest orifice tube available being 30 μm diameter. This resulted in an overestimation of the number of cells at the smaller end of the size range.

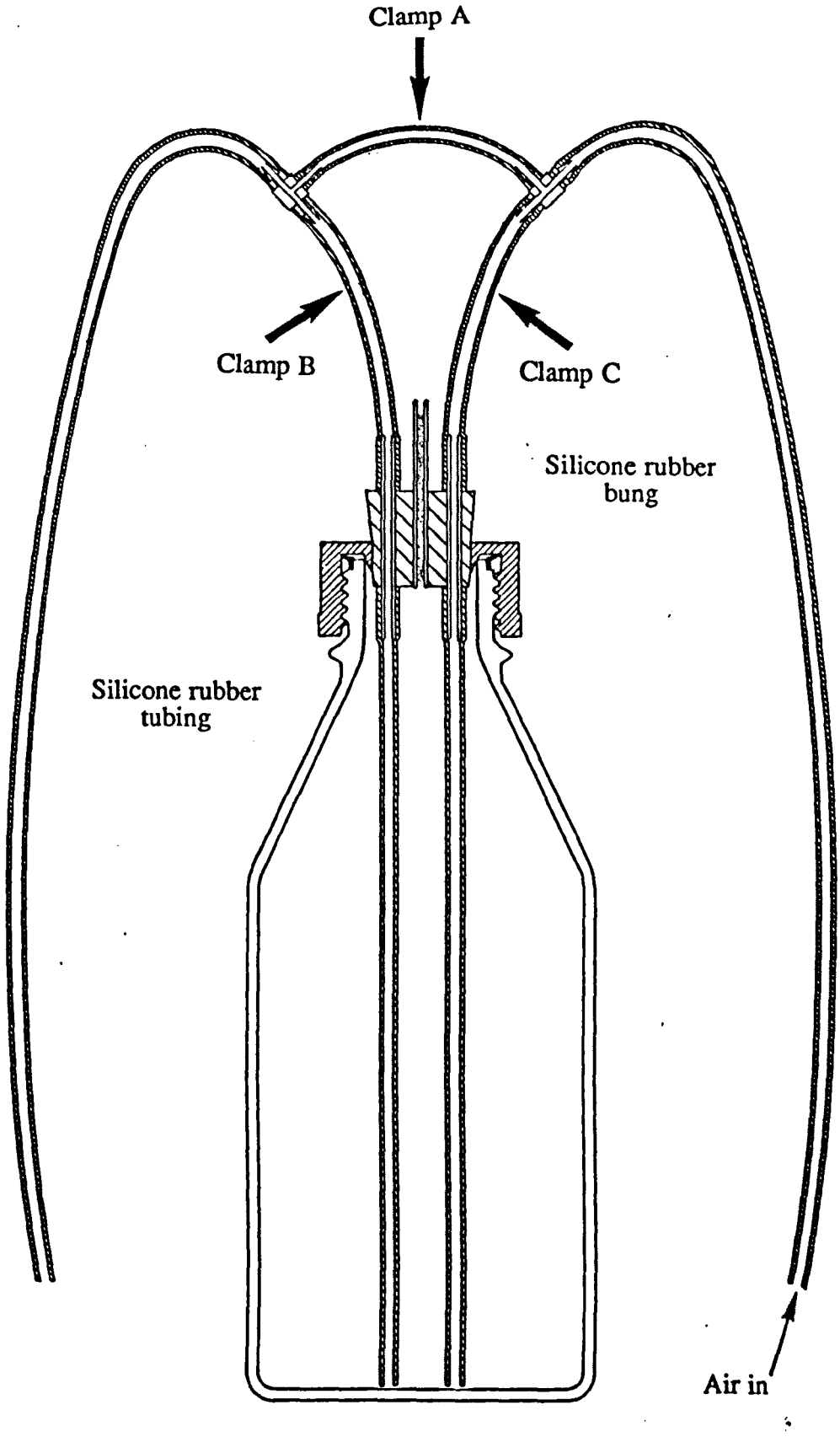


Figure 4.2.1 : The S.M.B.A. design of batch culture vessel based upon the Schott™ bottle.†§

4.3 THE CAGE CULTURE TURBIDOSTAT

4.3.1 Introduction

Because there is little construction detail for cage culture turbidostats (CCTs) the set up used here will be described in proper detail. The design was essentially that of Skipnes *et al.* (1980). Modification / improvements tailor the device to the requirements of the study. Since the design stage the use of CCTs in the culture of *Phaeodactylum tricornutum* for lipid class analysis has been described (Parrish & Wangersky, 1987).

4.3.2 Principle of operation

The algal cells are maintained in suspension between two membrane filter discs. This allows a continuous flow of culture medium through the vessel without the potential loss of cells through washout. Culture turbidity was maintained at a predetermined level by periodic withdrawal of medium plus suspended cells. This 'dilution' occurs *via* a peristaltic pump operating on cannula penetrating the culture vessel or 'cage'. Culture growth causes decrease in the intensity of light transmitted through the cage. This results in a decreased electrical signal from a photodiode. If the signal falls below a preset threshold the control unit (C-R unit) triggers the dilution pump. Suspended cells are removed until the turbidity falls to the threshold level.

4.3.3 Construction

The culture apparatus glassware was constructed from 76 mm i.d. Pyrex™ glass tubing (Mr. G. Reed, STS Glass-blowing, Stirling University). The vessel (see Figure 4.3.1) consists of two end-pieces shouldered recessed and flanged (to hold cushioning and sealing O-rings along with support screens and membranes in the original version, see Appendix B). Each end-piece is equipped with a single Quickfit™ 14/23 socket stem to accept the medium in / outflow tubing assemblies. The culture vessel or 'cage' is an open ended, double flanged cylinder equipped with a Quickfit 19/26 socket. The capacity of the cage was increased from 135 ml to 900 ml (500 ml in the original version, see Appendix B) to provide a larger mass of cell material for analysis.

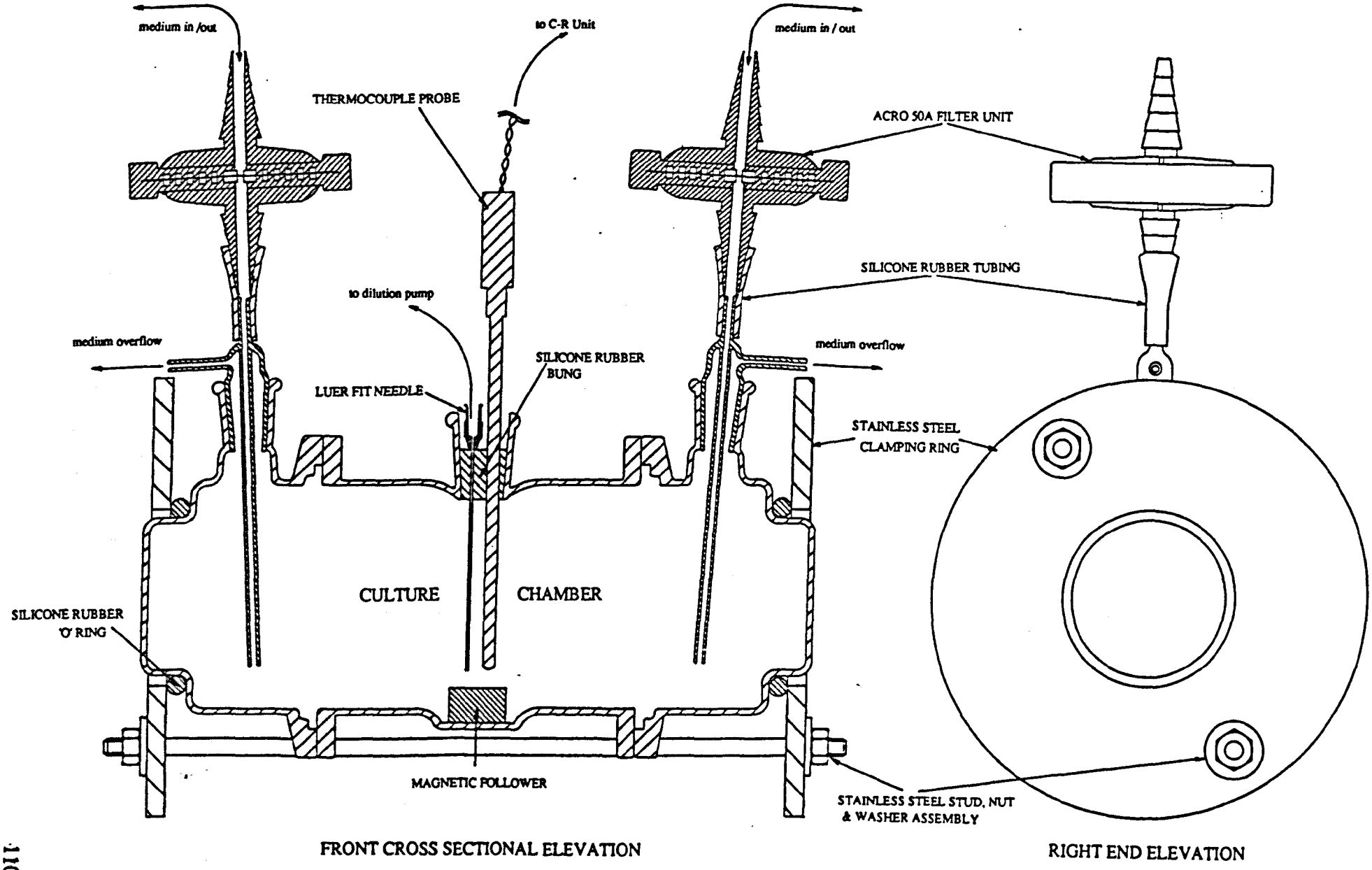


Figure 4.3.1 : Diagram showing the detail of the assembled culture vessel of the Cage Culture Turbidostat†.

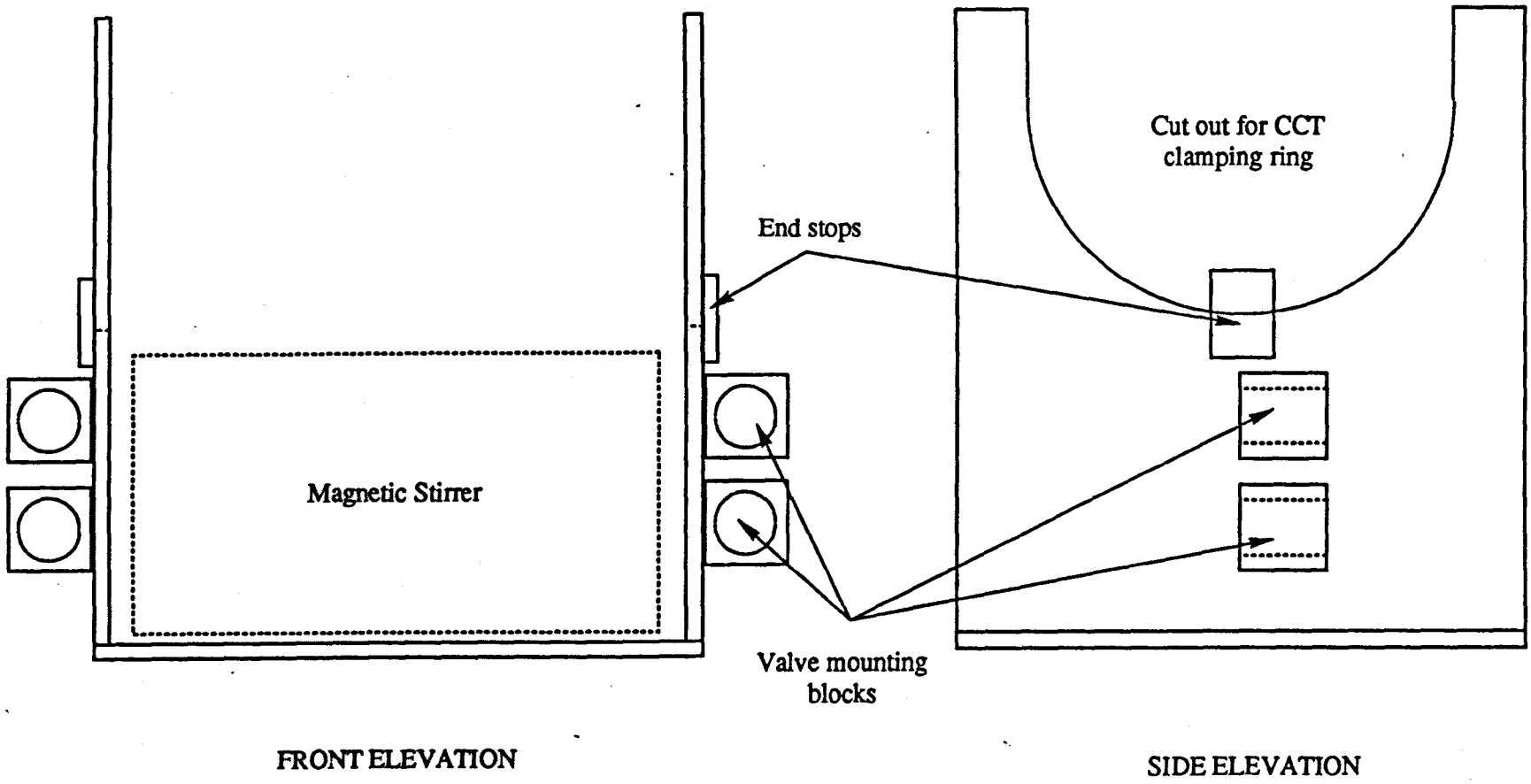


Figure 4.3.2: Diagram showing the detail of the CCT supporting cradle[1].

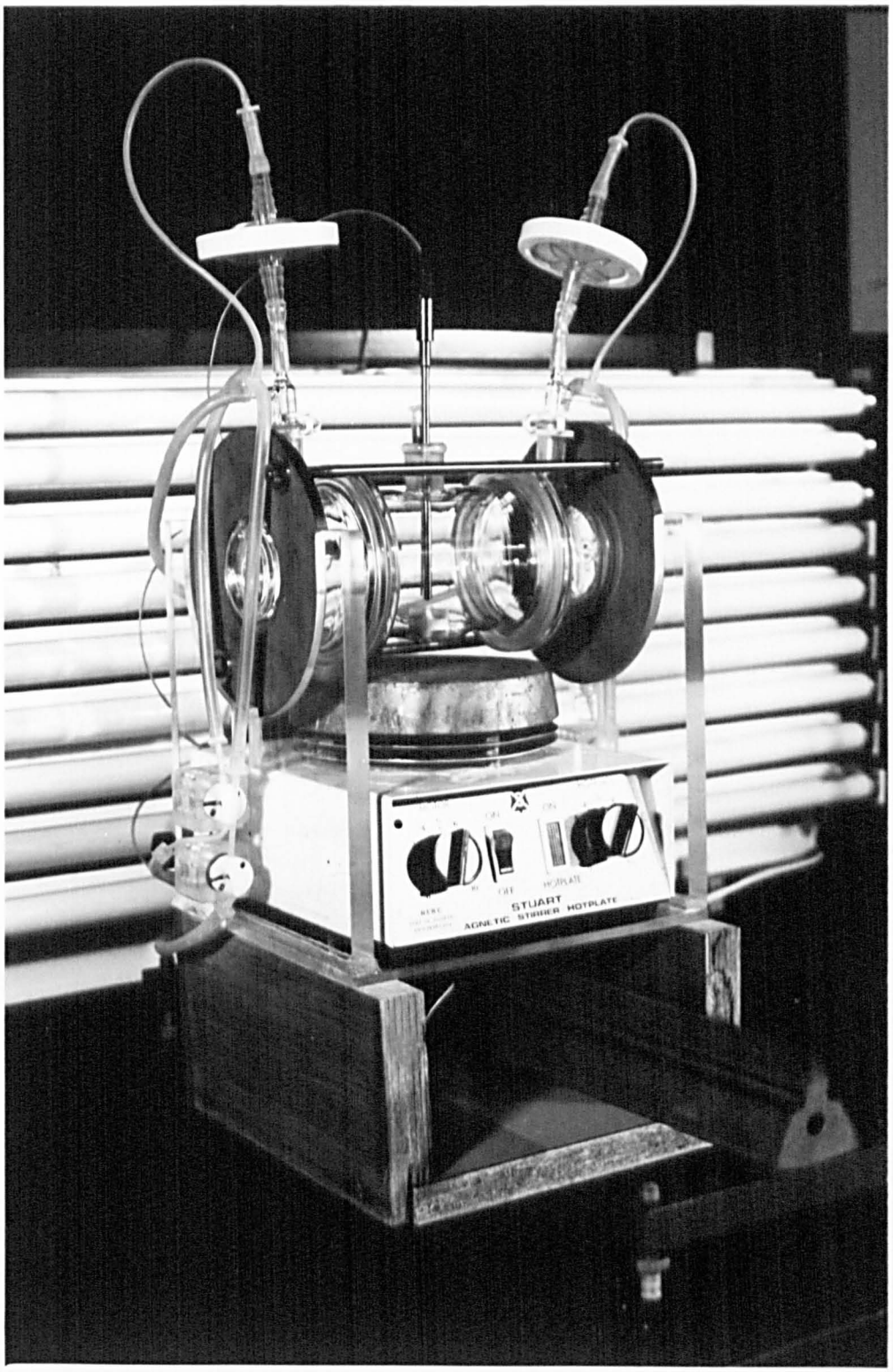


Plate 4.3.1 : A photograph of the assembled CCT culture vessel mounted on its supporting cradle.

The three major sections were clamped together by means of two stainless steel discs machined out to butt against the shoulders of the end-pieces. These were tightened together by means of two stainless steel studs plus nuts and washers (STS Engineering, Stirling University) rather than individual screw clamps (Skipnes *et al.*, 1980) (see Figure 4.3.1 and Plate 4.3.1).

The apparatus was designed to sit on a simple transparent Perspex™ cradle (see Figure 4.3.2 and Plates 4.3.1 & 4.3.2) (STS Engineering) during operation. This held the vessel in fixed position and supported it above a Stuart™ magnetic stirrer hotplate (BDH Ltd., Poole, U.K.. Cat.No. 333/0190/00). The cradle sides were a convenient mounting point for the solenoid pinch clamps (Model No. 390NO 2-way pinch Asco-Angar solenoid actuated valve, Platon Flowbits Ltd., Basingstoke, U.K.. Cat.No. 597100-11) used in flow diversion.

The C-R unit electronics (Mr. W. Stirling, STS Electronics, Stirling University) were based upon those of Skipnes *et al.* (1980) with additional flow reversal features and refined control of turbidity (see Figures 4.3.3 & 4.3.4 for block and circuit diagrams). A pulse generator was linked to a modified Spectra-Physics model 155A He-Ne laser (peak wavelength 632.8 nm) (Spectra-Physics Inc. 1250 West Middlfield Rd. Mt. View. California 94042.) so it emits 1 KHz square wave modulated light. Modulated and ambient light (50 Hz AC mains frequency) are received by a photodiode, converted to electrical impulses and amplified. Mains frequency ambient light signal is filtered out by an active filter. The remaining 1 KHz signal is converted to D.C. by a synchronous rectifier. The resultant signal is compared with an adjustable 'reference' voltage. This is monitored on a Liquid Crystal Display (LCD) and fed to a pen recorder.

If the illumination induced voltage fell below the reference voltage threshold a relay triggered the dilution pump. The operation of the pump was recordable *via* charge build up in a time ramp. Its regular discharge resulted in pulses on the pen recording corresponding to

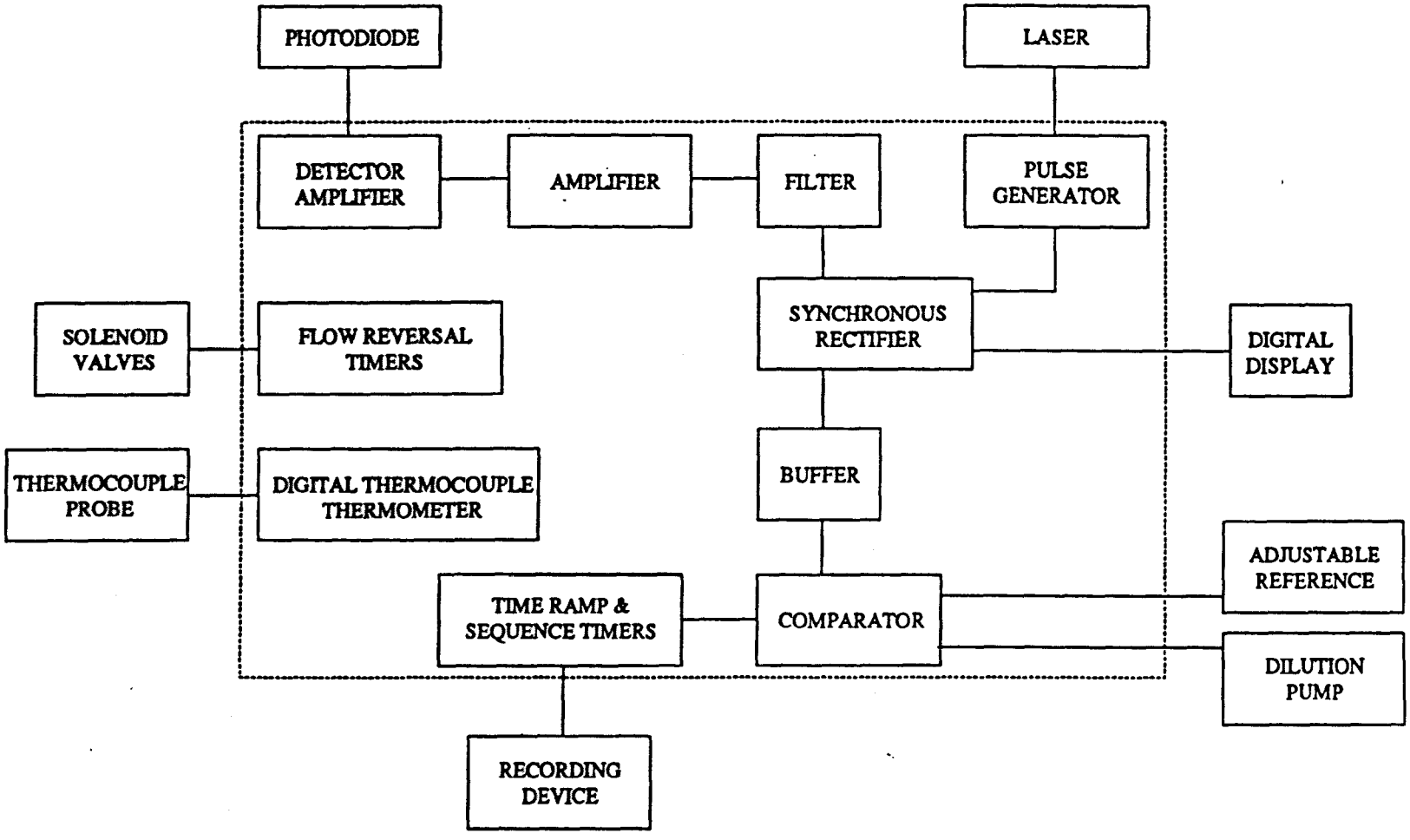


Figure 4.3.3: Block diagram for the C-R unit of the CCT showing peripheral apparatus[8]

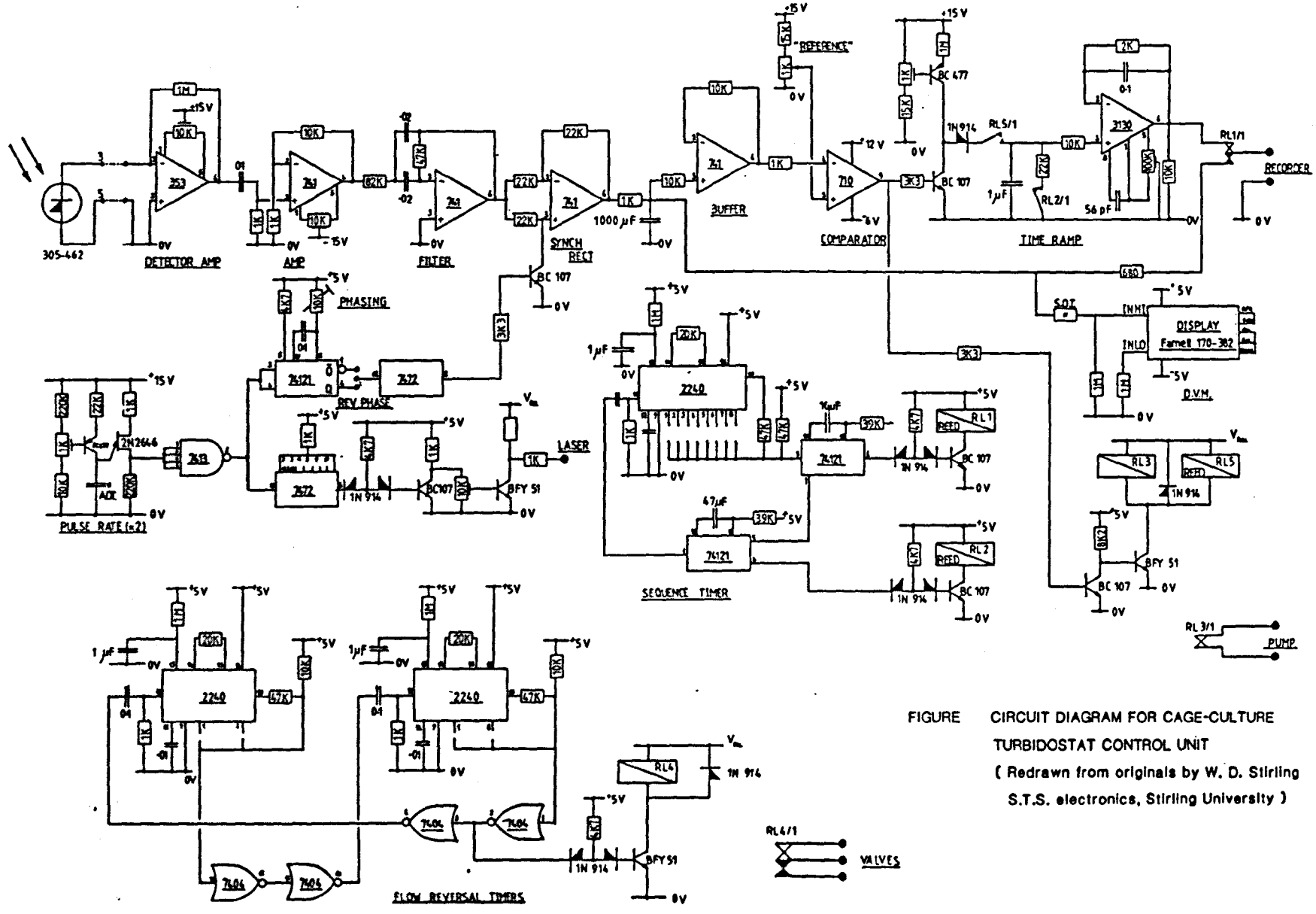


FIGURE CIRCUIT DIAGRAM FOR CAGE-CULTURE TURBIDOSTAT CONTROL UNIT

(Redrawn from originals by W. D. Stirling S.T.S. electronics, Stirling University)

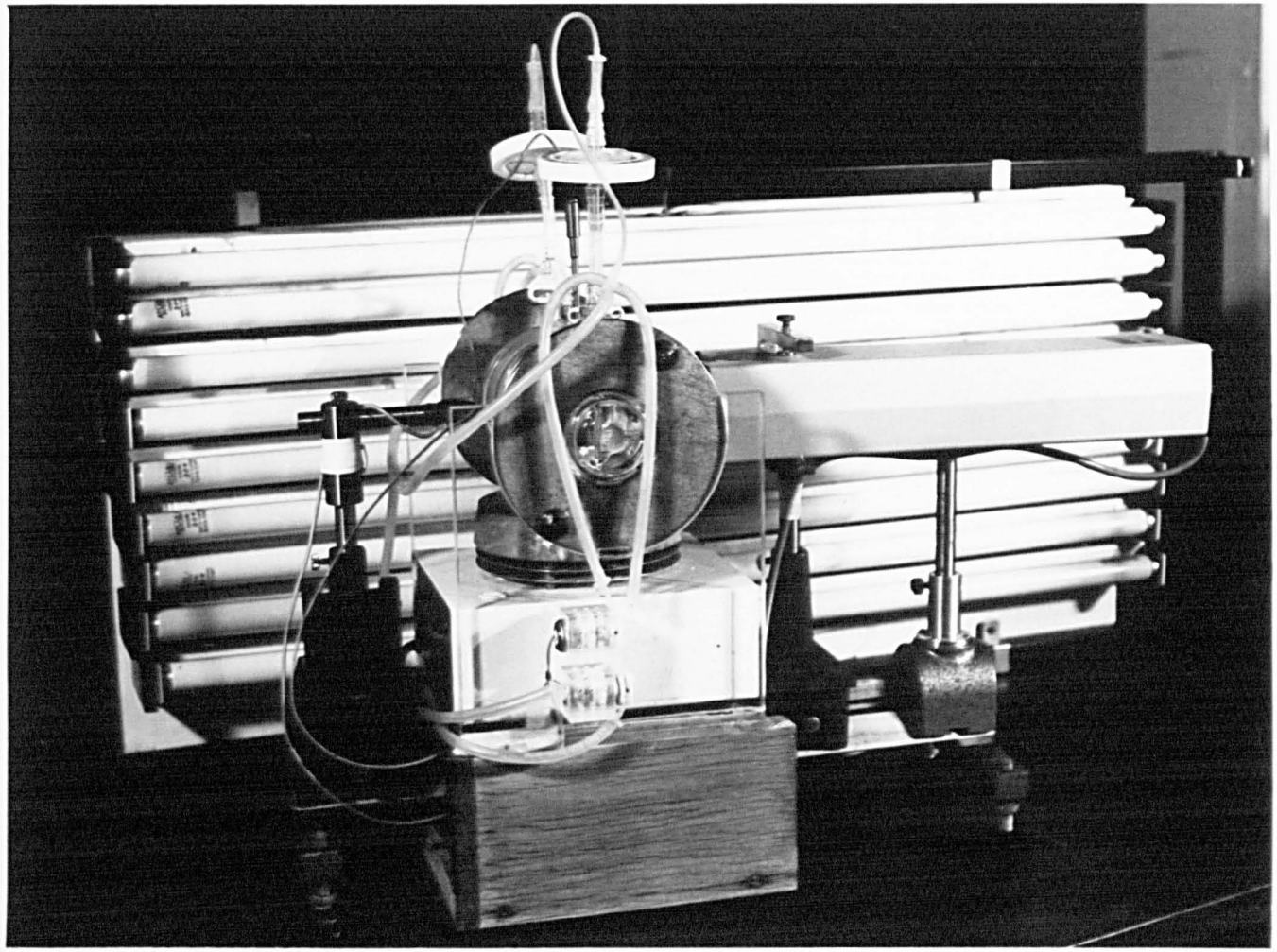


Plate 4.3.2 : A photograph of the CCT apparatus showing the alignment of the laser and photodiode in relation to the culture chamber.

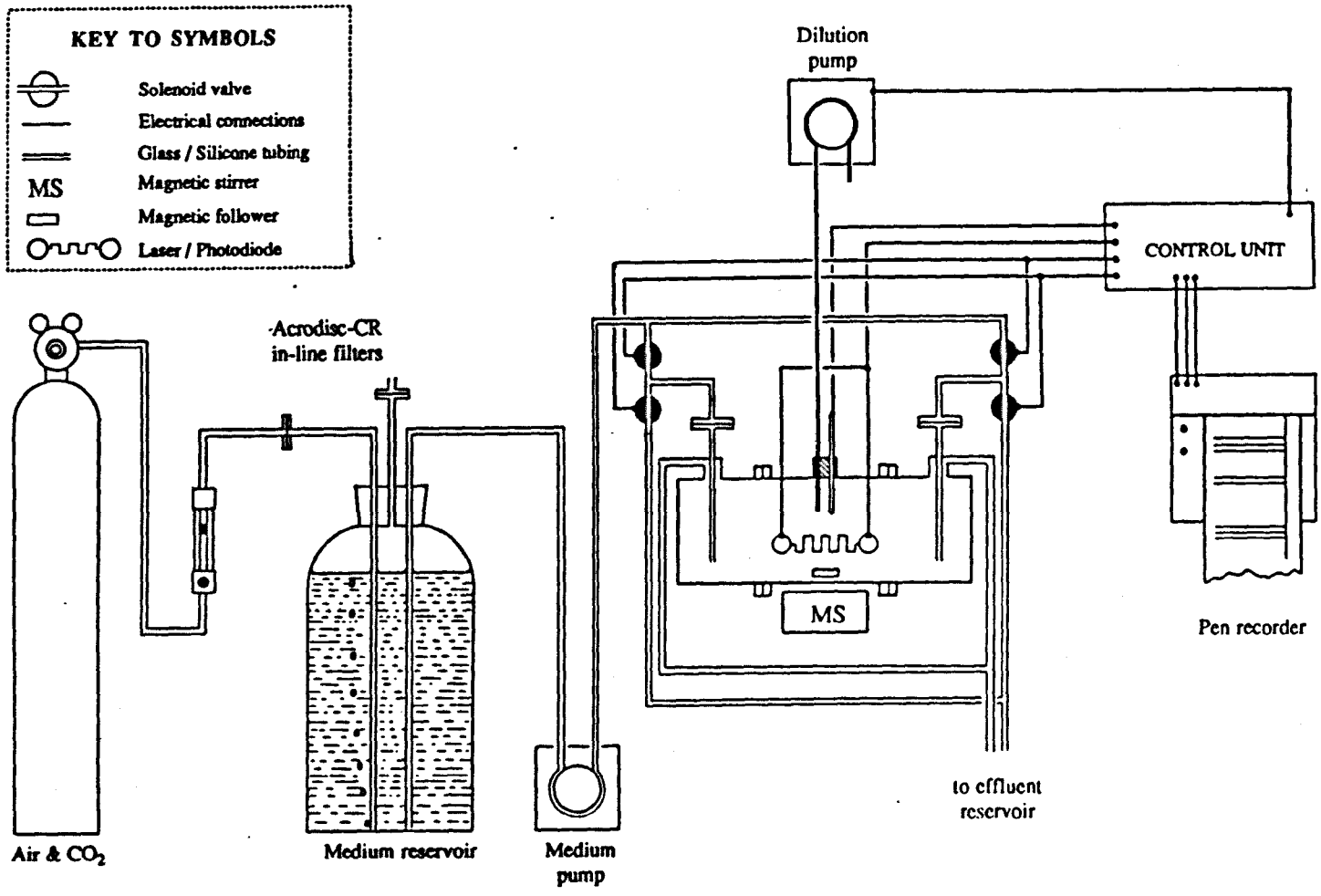


Figure 4.3.5 : Schematic diagram showing the relationship between the CCT and peripheral apparatus[†§].

the pump activation times (see Figure 4.3.5). The dilution pump ran at a known fixed speed. The dilution could be calculated from pumping times or accumulation of 'waste' in the collection reservoir over a time period. If cell numbers were known, growth rate could be determined (Skipnes *et al.*, 1980). The C-R unit also contained the timers for solenoid pinch clamp cycling. The clamps operated in complementary pairs on the in / outflow tubings. One pair opened permitting medium to enter at one end and exit at the opposite; whilst the other pair were closed. The direction of medium flow through the cage reciprocated, in effect back-washing the upstream filter to reduce clogging. This arrangement maintained a unidirectional flow - influent to effluent reservoirs. Skipnes *et al.* (1980) achieved a similar effect by reversing the medium pump periodically. This had the disadvantage of drawing spent medium back into the culture vessel.

Temperature inside the culture cage could be monitored after the inclusion of a thermocouple thermometer. The autoclave sterilisable thermocouple probe (Type 'T' Ref No. MN/9/88/1302) Labfacility was attached to a Farnell Electronics digital thermocouple thermometer DTT 2T using a Model D505A power supply (Farnell Electronics).

4.3.4 Assembly and Preparation

4.3.4.1 Glassware assembly

The flanges of the three main sections were lightly coated with a high melting point Molycote™ III compound molybdenum grease (N.V. Dow-Corning S.A., Belgium.). The sections were then clamped together with all socket stems aligned and containing a 22 mm diameter Nalgene™ stirbar star head™ TFE magnetic follower. The medium in / outflow tubing assemblies with the overflow tubing attached were inserted into their respective greased socket stems. 4 cm lengths of 3.5 mm i.d. silicone rubber tubing (Esco (Rubber) Ltd.) (into which the filter units were later inserted (see Section 4.2.5)) were slipped over the in / outflow tubes. The bung, penetrated by the cannula and the thermocouple probe, was inserted into its socket stem.

4.3.4.2 Sterilisation

The open ends of the silicone rubber tubings were wrapped in aluminium foil held in place with autoclave tape. The remaining tubings to connect between the CCT and the medium reservoirs also had their ends sealed with foil. The assembled components and tubes were autoclaved at 120 °C, 15 psi for 30 minutes.

4.3.4.3 Bulk medium preparation

Bulk medium was prepared and filtered serially through prefilter pads, 0.8 μm and 0.22 μm pore size 47 mm diameter membrane filter discs (Millipore (U.K.) Ltd., Cat.Nos. AP 25, AA WP and GS WP respectively) into a 20 l Pyrex aspirator bottle. The bottle was stoppered by a silicone rubber bung (Esco (Rubber) Ltd. Cat. No. BGE057) penetrated by three 4 mm o.d. Pyrex glass tubes. From two of these depended silicone rubber tubings to reach the base of the medium reservoir. Two 0.22 μm Acrodisc-CR (Gelman Sciences, MI, U.S.A.) hydrophobic venting filters were fitted, one to the remaining plain Pyrex tube and the other as an in-line filter in the aeration line (see Figure 4.3.6). Culture medium and reservoir are autoclaved at 121 °C, 15 psi for 45 minutes.

4.3.5 Setting up procedure

Autoclaved glassware was transferred to a laminar flow cabinet. The culture vessel was fitted with the Acro™ 50A 0.45 μm pore size, presterilised filter units (Gelman Sciences Inc.) and the remaining tubing pushed on. The inoculation flask containing the inoculum was attached to the free end of the cannula.

During operation the unit was located in a constant temperature (CT) room. Illumination was from a bank of up to ten, 30 W (36") 'daylight' balanced fluorescent tubes (Osram-GEC, U.K.) mounted horizontally along one wall. The laser and photodiode were held in vernier mountings on an optical bench. The clamped glassware was placed in its cradle between the two optical devices (see Plate 4.3.2). 3 mm i.d. silicone rubber tubing (Esco (Rubber) Ltd., Cat. No. TSR 0300 200) was connected between the medium input

tubes on the culture vessel and medium reservoir *via* two of the solenoid pinch clamps and the pumphead of a Gilson minipuls™ peristaltic pump. Several different tube types were used here; the pinch clamps used 1.6 mm i.d., 3.2 mm o.d. silicone rubber tubing (Platon Flowbits Ltd., Cat. No. 597101-03), and the Gilson pump used 3 mm i.d. Isoversinic™ flow tubing (Anachem Ltd. Cat.No. 817747). Outflow tubing was fed into the effluent reservoir *via* the remaining two solenoid pinch clamps. The overflow tubing was also fed into the effluent reservoir. Cannula tubing was attached to the pumphead of a variable speed LKB VarioPerpex™ peristaltic pump. The medium reservoir airline was attached *via* a 30 mm scale Gapmeter™ with needle valve (Type GSYV with 5 - 100 ml min^{-1} air calibrated tube and float, Platon Flowbits Ltd., Cat. Nos. 50123-100 and 01351 respectively) to a supply of 5 % CO_2, 95 % air (B.O.C. Ltd., U.K.).

The vessel was filled *via* the Gilson minipuls pump operating at maximum flow rate. The air displaced from the culture vessel was bled off into the waste reservoir by unclamping the 'overflow' tubes during filling (see Figures 4.3.1 & 4.3.5).

Once the culture vessel had been set up, and the possibility of medium splashing / spillage eliminated the electrical C-R unit was set up. Solenoid pinch clamps and LKB™ dilution pump were connected to their respective power outlets on the C-R unit. Photodiode and laser were connected to the pulse generator *via* a 5-pin DIN plug / socket. The laser was aligned so its beam passed through the diameter of the cage and struck the photodiode target positioned against the opposite glass. Their positions were adjusted until a known maximum reading (*ca.* 300) was obtained on the C-R unit LCD.

4.2.6 Inoculation

Inoculation was carried out in the CT room after aligning the laser and photodiode through cell free medium. A simple procedure for axenic inoculation with the apparatus assembled for operation was developed.

C-R unit and magnetic stirrer power supplies were switched on. The reference threshold dial (RTD) was set to a minimum (off) position. The LKB dilution pump was switched manually to the 'reverse' position. The RTD was then set to a maximum position, activating the LKB dilution pump. The inoculum was pumped from the flask (see Figure 4.3.7) into the cage *via* the cannula. Culture turbidity was monitored on the C-R unit LCD. Once the desired turbidity was attained the RTD was turned down until the pump cut out. This turbidity was maintained by returning the dilution pump to its 'forward' setting once the apparatus was up and running.

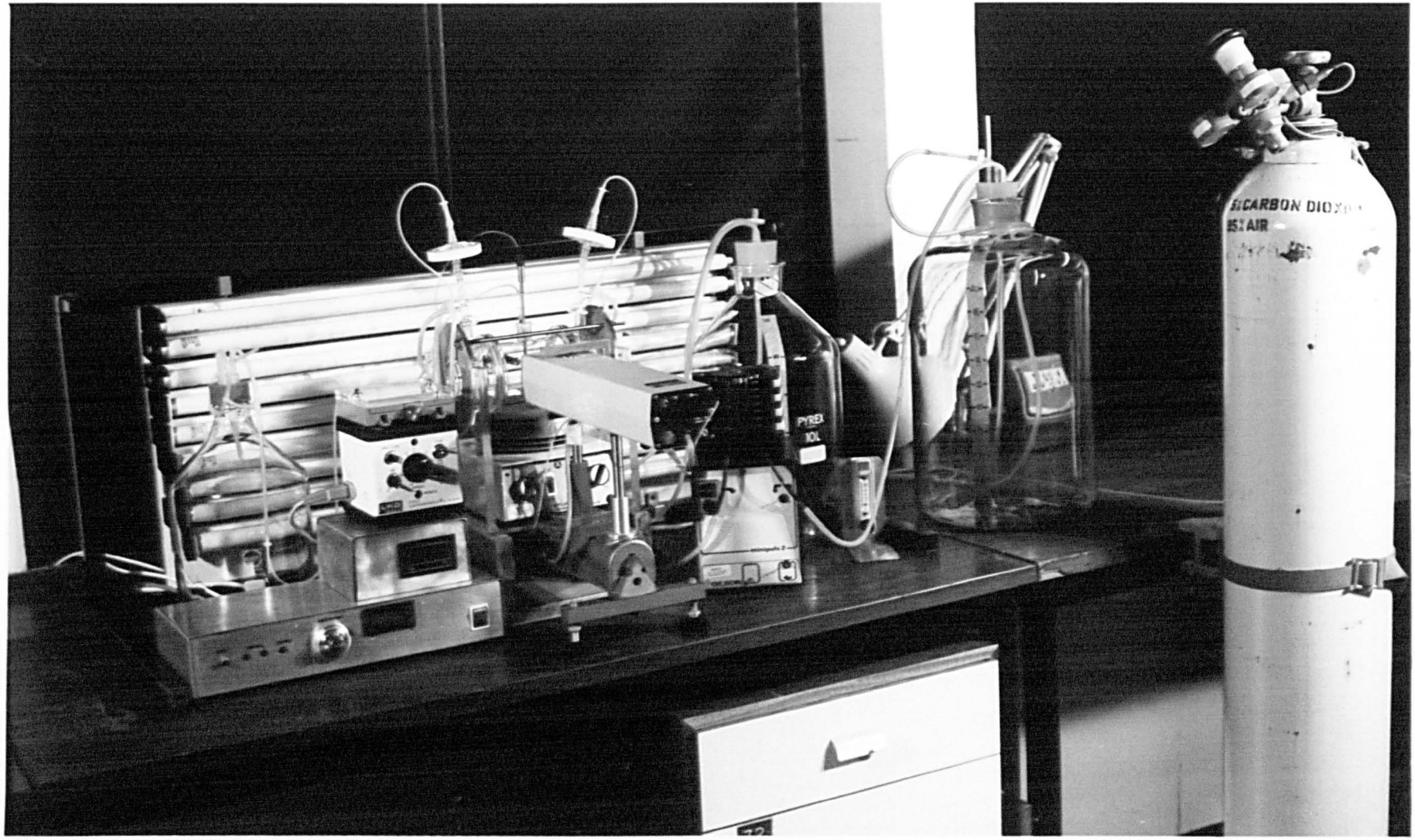


Plate 4.3.3 : A photograph of the CCT apparatus showing all the ancillary equipment required for its operation.

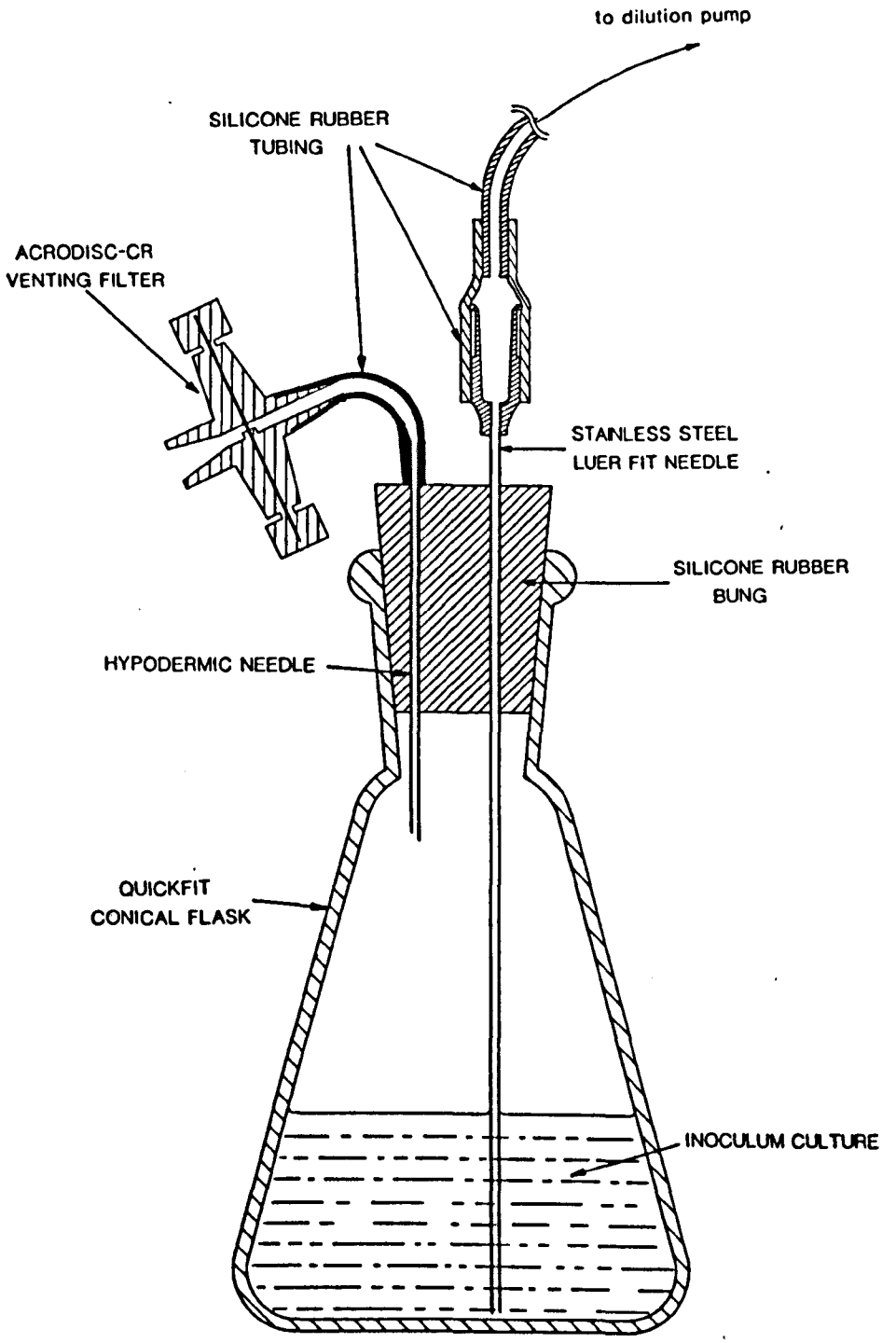


Figure 4.3.6 : Diagram of the inoculation flask for the Cage Culture Turbidostat†.

4.2.7 Operation

After inoculation, operation of the device was achieved as follows: Opening the CO_2 / air cylinder and adjusting the flow to 5 ml min^{-1} using the Gapmeter. Medium pumping is started by switching on the Gilson minipuls peristaltic pump (*n.b.* air bubbles could be eliminated by selective clamping of medium in / outflow tubes). Cycling of flow reversal and time-ramp operation were initiated by pushing the 'start' button on the C-R unit. Once the unit was up and running control was automatic. The only maintenance required is the provision of fresh medium and the emptying of effluent reservoirs for bulk and dilution pumps. The necessary disconnections and reconnections were carried out under ethanol.

Should the filter discs begin to clog it was found possible to free them by switching the medium pump briefly to maximum flow and rotating the filter housings between thumb and forefinger. Air bubbles trapped within the filter housings and the pulsed medium flow dislodged any algal build up which was then washed into the culture vessel.

4.4 HARVESTING & LYOPHILISATION OF ALGAE

Algal suspensions were centrifuged at 12,000 rpm for 45 min in a Sorvall GSA 6 x 250 ml rotor (Du Pont (U.K.) Ltd., Herts, U.K.) using a Sorvall RC-5B refrigerated superspeed centrifuge™ (Du Pont (U.K.) Ltd.) operating at algal sample culture temperature. Pellets were combined and resuspended in 30 ml 0.4 M ammonium formate $_{(aq)}$ (w/v) (Pillsbury, 1980) and recentrifuged in a Sorvall SS-34 8 x 40 ml rotor (Du Pont (U.K.) Ltd.) at 16,000 rpm for 30 min. The pellet was then resuspended, spun down again and resuspended in *ca.* 1.5 ml 0.4 M ammonium formate and stored at $^{-}$80 ^{o}C overnight. The frozen suspension was then lyophilised for 48 hrs in a tared vial prior to reweighing..

SECTION 5: VARIATION IN LIPID COMPOSITION WITH GROWTH PHASE IN BATCH CULTURES.

5.1 INTRODUCTION.

The aim of the project in its early stages was to find at least one algal species which contained a high proportion of long chain (*n*-3) PUFA in its total lipid and was available in axenic culture. More specifically the bias was towards the fatty acids characteristic of marine oils in general, especially 20:5(*n*-3) (eicosapentaenoic acid, EPA) and / or 22:6(*n*-3) (docosahexaenoic acid, DHA) (see Sections 1 & 2).

Epidemiological research conducted by several groups (see Lands, 1986; Simopoulos *et al.*, 1986) upon populations consuming diets with a high marine oil content (*e.g.* Greenland Innuit and Japanese fishing communities) showed negative correlations between dietary intake of marine oils and the incidences of coronary heart disease and atherosclerosis. Growing interest in the beneficial effects of dietary (*n*-3) and (*n*-6) PUFA amongst the medical fraternity has consequently spread to the commercial sector, notably the pharmaceutical and healthfood industries. The current trend of a more health-concious U.K. general public towards a 'natural' diet has contributed to recent marketing strategies for seafood and marine oil products (*e.g.* MaxEPA™, and cod liver oil) based upon public awareness of the importance of dietary PUFA. The (*n*-3) family of PUFA in which 20:5(*n*-3), more often called EPA in the biomedical literature, appears to hold a position of prime importance and is the subject of a recent report (Burr *et al.*, 1989). The report extols the virtues of increased consumption of oily fish (*e.g.* mackerel, herring & salmon) in decreasing the probability of fatal heart attacks. Media interest outside the general scientific press was such that slots in national television newscasts and the national press were devoted to it.

Such publicity has increased the willingness of fish producers to investigate the fatty acid profiles of their products and the feed they use as part of their marketing campaigns.

This reinforces the traditional concern of fish producers with the essential fatty acid requirements, particularly the dietary (*n*-3) PUFA requirements of the fish themselves from the post-larval stages (*cf.* Section 1). To increase yield / cost efficiency it is necessary to decrease larval mortality during the critical stage of metamorphosis. When the larval yolk sac has been absorbed the juvenile fishes become dependent upon their diet for their nutrition. In commercial culture this is supplied by the fish farmer. Recent research (Cowey & Sargent, 1977; Scott & Middleton, 1979; Watanabe, 1982; Bell *et al.*, 1985a; 1985b; Henderson & Sargent, 1985; Bell *et al.*, 1986; Sargent *et al.*, 1989) has shown a critical dietary requirement for specific PUFA. These essential fatty acids are generally the members of the (*n*-3) PUFA series.

Obviously, it was not within the scope of this thesis to become too deeply involved with the practical details and problems associated with juvenile fish nutrition and development. However, not to consider them, even briefly, would be to omit an important factor in the relevance of the present project. Major problems are encountered with formulated or 'artificial' post-larval feeds due to the size of the dietary particles required and the preservation of the PUFA component from auto-oxidation during large scale manufacture.

Apart from problems of production, inert feeds often have associated presentation problems, movement being an important cue for initiation of the larval feeding response. Leaching of water soluble components once the diet is suspended in rearing tanks is a further difficulty which is often encountered. Currently, one of the most widely used first food sources for commercially farmed post-larval fishes is the rotifer *Brachionus plicatilis* (Muller) (see review by Watanabe *et al.*, 1983). Although the use of living organisms, such as rotifers, avoids many of the stumbling blocks mentioned above it does create new ones.

Rotifers, mass cultured on mixtures of yeast and algae are fed to the juvenile fishes until they grow large enough to feed upon the nauplii of the brine shrimp *Artemia salina*. *B. plicatilis* has been shown to incorporate dietary fatty acids unmodified (Ben-Amotz *et al.*,

1987; Minkoff, 1987). The rotifer cultures therefore assume the fatty acid profile of their food particles. In this way the rotifer may be used as a vector to introduce essential fatty acids into the juvenile fishes. As a consequence of such a technique, the food organism on which the rotifers are fed is important. Yeasts reportedly supported the growth of rotifers to approximately ten times the density of *Chlorella* sp. (Watanabe *et al.*, 1983). Being heterotrophic organisms they do not require costly illumination for growth and therefore are cheap and widely available in a convenient dried and packaged form.

Although they are excellent sources of protein, the yeasts are deficient in (*n*-3) PUFA as are many chemoheterotrophic microorganisms. Both Ben-Amotz *et al.* (1987) and Minkoff, (1987) contrast the difference in composition of *B. plicatilis* grown on yeasts and on different algal species. Minkoff (1987) studied the effects of a secondary enrichment period of 24 to 48 hours, during which yeast-grown mass cultures of *B. plicatilis* were supplied with *Isochrysis galbana* or *Nannochloropsis oculata*, on the fatty acid profiles of the rotifers. The production of commercially prepared diets for rotifers from lyophilised *Chlorella* has been attempted (Hirayama & Nakamura, 1976) and some projects are currently investigating the potential of lyophilised, heterotrophically grown microalgae to produce cheap, (*n*-3) rich diets for shellfish and rotifers. This has obvious, but potentially surmountable, drawbacks (*cf.* Nichols *et al.* (1967) (see Section 2)).

Because much of the compositional type research is conducted in the context of aquacultural and maricultural nutrition studies, the emphasis is on the total lipid component and its FAME composition. The analyses are, therefore, more often than not conducted upon samples from a single point on the growth curve of a batch culture, and so lack detail. With hindsight this situation is understandable. Detailed lipid class and fatty acid analyses are time consuming, particularly when one realises the numbers of samples involved in characterising compositional variations, even within a single species. These analyses also require specialised equipment which is not always readily available. However, considering the relatively small number of algal species commonly used in aquaculture it would be practical

to consider a cursory characterisation of the ten most widespread species in batch culture (*cf.* Volkman *et al.*, 1989).

The technique most widely employed to provide microalgal material for analysis of lipid and fatty acyl composition is that of batch culture. This has the obvious advantages of economy and simplicity, particularly when employed upon a laboratory scale (see Section 4). However, beyond these factors it seems that many workers pay little attention to the effects upon the physiological behaviour of the microalgal cells during the various phases of growth that are the characteristic hallmark of batch cultures.

The growth phases result from the expression of changing physiology within the cultured cells. The original culture medium and growth conditions define *limits* for certain parameters such as total nutrient pool, temperature and photon flux. The growth of the culture produces changes which are both chemical and, in a narrower sense, physical. Within the confines of a closed vessel the cell is in effect exposed to the consequences of the growth of previous generations (*e.g.* nutrient depletion, extracellular product accumulation *etc.*). It is important to remember that the initiative for the pattern of physiological changes originates both from the cells themselves and from the constraints of the closed vessel batch culture technique. Provided that the cultures are set up identically, the chain of events should follow the same predictable pattern. This is indeed the case at a general level. However, it is almost impossible to determine the influence of an individual factor within these patterns (see Section 2).

The growth curves of experimental cultures are rarely described and the point of harvesting of the cells is often rather glibly referred to as 'during the exponential phase of growth'. The lengths of the phases are very much dependent upon inoculum size, initial nutrient concentration and other factors, such as temperature and illumination, which affect growth rates in addition to the algal species or strain itself (Fogg & Thake, 1987). The literature shows a set pattern of approach to the culturing of microalgae; the harvesting points

generally being 'the exponential phase' and 'the stationary phase'. The implication here is that these two points represent the extremes of a spectrum of lipid compositional variation. However, the detail of the progression through one phase into another has only recently begun to receive attention (Emdadi & Berland, 1988) and until the potential variations have been observed it seems unlikely that the metabolic control mechanisms or functional changes will be elucidated.

Because batch culture is such a common technique, it seemed appropriate to illustrate the types of variation observed during preliminary experimentation with several more formal series of investigations. Experiments were, therefore, designed whereby the changes in lipid class and fatty acyl composition occurring over the growth cycle of microalgal batch cultures could be monitored under a variety of environmental conditions. Initially these were based upon arbitrarily designated 'standard' conditions (*i.e.* the conditions under which the alga *N. oculata* had been maintained, for the 30 years following its isolation, at the S.M.B.A. culture collection based at Oban.)

In the first instance, gross analytical approaches were adopted with respect to fatty acyl compositional changes. The aim of this approach was to determine the point on the growth curve where maximum unsaturation of the total lipid occurred and whether or not this could be correlated with changes in lipid classes or growth rate. The depth of analysis was later increased to take into consideration the potential for variation of the acyl compositions of the individual lipid classes.

Having defined both the qualitative and quantitative lipid class and fatty acyl variation under 'standard' culture conditions, this information would provide the locus around which future experimentation and data interpretation was to revolve. The range of compositional data obtained was to be contrasted with a series of continuous culture experiments (see Section 7) to illustrate that batch culture may not necessarily be the best technique to employ in investigating the effects of environmental factors upon microalgal

biochemistry. The culture strategies employed by the various workers during the course of such investigations may, in fact, be contributory to the confusion which surrounds this topic.

The results presented later, in Section 5.3, precipitated a follow up experiment to construct a more detailed picture of the changes occurring in the lipid composition of *N. oculata* during what is conventionally known as the lag phase of batch culture. Of particular interest were the changes in individual cell mass, the proportion of TAG in the total lipid extract and how these related to the changes in unsaturation observed in the total FAME. From the data it was obvious that *N. oculata* had the ability to change the partitioning of its photosynthetically fixed carbon in rapid response to changes in both its chemical and physical environments.

When subjected to an environmental shock, such as inoculation into fresh culture medium, it appeared that a mechanism operated whereby further cell division was suspended and photosynthetically fixed carbon was shunted into TAG biosynthesis. This resulted in a short term accumulation of TAG. During the same period a fall in the relative proportion of cellular PUFA occurred. The obvious question was, did the decrease in the proportion of PUFA result from decreasing PUFA proportions in all lipid classes, or did the decrease occur as a consequence of the accumulation of a major, relatively saturated lipid class such as TAG ?

To answer the above question a more detailed analytical approach was taken when compared with previous experimentation. The total lipid extracts were separated into their component classes using preparative scale double development TLC. The classes were then transmethylated for analysis of their FAME derivatives by GLC. Later this was linked with a $^{14}C\text{-}HCO_3$ incorporation time course experiment designed to follow the progression of the partitioning of photosynthetically fixed carbon between total lipid, individual lipid classes and the individual fatty acids of the major storage and membrane acyl lipid classes. The

results fron this series of experiments are not included in the present thesis. The incorporation of radioactivity into both lipid classes and FAME was always in direct proportion to their mass.

5.2 PRELIMINARY SCREENING OF ALGAL SPECIES.

5.2.1 Experimental.

Several algal species were grown (either in the N.E.R.C.-U.A.B. by the author or at the Dunstaffnage Marine Laboratories, Oban by Dr. J. W. Leftley) in small scale (*ca.* 800 ml to 1000 ml) batch cultures of unmodified S88 medium at 20 °C and continuous illumination of 150 $\mu E\ m^{-2}\ sec^{-1}$ with continuous air sparging. The cultures were harvested and lyophilised and the total lipid extracted. Aliquots of the lipid extracts were transesterified and analysed by GLC to ascertain their total fatty acid compositions.

The algal strains were selected from the S.M.B.A. culture collection (now incorporated into the C.C.A.P., Oban.) on the basis of their relevance to current research projects and data available in the literature. *Brachiomonas submarina* var. *pulsifera* (Droop) (Droop, 1953; 1955) (C.C.A.P. strain 7/2A) has been extensively used in food chain energetics studies (Droop & Scott, 1978; Scott, 1980). Both *Nannochloropsis oculata* (Droop) (Droop, 1955) Hibberd (C.C.A.P. strain 849/1) and *Isochrysis galbana* (Parke) (S.M.B.A. Strain No. 58; C.C.A.P. strain 927/1 was not available in axenic culture) were being used as a secondary enrichment diet for the rotifer *Brachionus plicatilis* (Muller) prior to feeding to larval herring, *Clupea harengus* (L.), in a growth study (Minkoff, 1987). *Chlorella minutissima* had a reportedly high 20:5 (*n*-3) content (Seto *et al.*, 1984) as did *Chlorella salina* (C.C.A.P. strain 211/25), *Nannochloris atomus* and a *Tetraselmis* sp. (tentatively *T. suecica* personal communication, Dr. J. W. Leftley).

5.2.2 Results & Discussion.

Four of the seven species screened were from the Chlorophyceae although within this class *B. submarina* was from the order Volvocales whilst both the *Chlorella* spp. and *N. atomus* were from the order Chlorococcales. *Tetraselmis* sp. was classified in the Prasinophyceae division of the phylum Chlorophyta. *Nannochloropsis oculata* and *I. galbana* were both members of the phylum Chrysophyta but in the classes

Eustigmatophyceae and Prymnesiophyceae respectively. *Nannochloropsis oculata*, formerly *Nannochloris oculata*, was reclassified by Hibberd (1981) as the type of the new Eustigmatophycean genus *Nannochloropsis* on the basis of its lack of chlorophyll b, a distinguishing feature between Eustigmatophyceans and Chlorophytceans, and cytological observations by Antia *et al.* (1975), (Turner & Gowen, 1984).

The results of GLC analyses of the total FAME composition of the screened species are arranged in groups according to their taxonomic classification in Table 5.2.1. Comparing the various strains of the same phylum, there did not seem to be an overall pattern of fatty acyl composition. *Brachiomonas submarina* and *C. salina* showed similar fatty acid profiles with differences in quantitative rather than qualitative distribution. Both strains possessed significant proportions of 16:4, 18:1(*n*-7) and 18:3(*n*-3) whilst lacking C_{20} fatty acids. Similar was true of *Tetraselmis* sp. and *N. atomus*. Although both of these species possessed high proportions of the monounsaturated fatty acid 18:1(*n*-9) and lesser proportions of 16:4 and 18:3(*n*-3), they also had higher proportions of 20:5(*n*-3) and some C_{22} fatty acids. *Chlorella minutissima* had a quite different fatty acid profile with relatively little C_{16} PUFA and a higher proportion of the monounsaturate 16:1. Similarly, with the C_{18} fatty acids moderate proportions of 18:1(*n*-9) were present but there were little or no C_{18} PUFA. However, the amount of total PUFA was increased by a significant proportion (10.7 %) of the fatty acid 20:5(*n*-3). Interestingly, *C. minutissima* possessed a fatty acyl profile which resembled that of *N. oculata* very closely, but was significantly different from the more closely related species *C. salina*, particularly with respect to the presence of 20:5(*n*-3) and absence of 18:3(*n*-3) (*cf.* Section 1). This may have reflected a taxonomic difference as was originally the case with *N. oculata* which was classified in the same order as the *Nannochloris* and *Chlorella* sp. (*cf.* Menzel & Wild, 1989).

Amongst the species screened *N. oculata* had the highest proportion of C_{16} and C_{20} fatty acids and the lowest proportion of C_{18} fatty acids. However, *Isochrysis galbana* had the highest proportion of PUFA and shared the highest proportion of total (*n*-3) jointly with

TABLE 5.2.1: Total FAME composition of several marine microalgal species cultured under similar conditions during the preliminary selection stages.

	ALGAL STRAIN*						
	Chlorophyta					Chrysophyta	
FATTY ACID	*B. sub.*	*C. sal.*	*C.min.*	*T.sp.*	*N. ato.*	*N. ocu.*	*I. gal.*
14:0	0.5	6.7	10.0	4.8	0.9	4.2	12.2
15:0	0.6	1.7	0.4	0.9	.	0.2	.
16:0	21.9	28.2	29.1	29.1	19.7	30.3	9.3
16:1(*n*-7)	11.9	7.9	32.4	8.9	3.3	24.0	2.9
16:1(*n*-13)t.	.	.	.	.	.	.	.
16:2	1.5	1.0	0.2	2.2	4.8	0.7	0.9
17:0	0.2	0.5	0.4	1.2	.	.	.
16:3	0.8	1.5	0.2	3.0	7.7	1.0	.
16:4(*n*-3)	12.3	4.2	.	.	.	.	0.7
18:0	1.0	2.7	1.2	4.1	1.2	1.2	0.7
18:1(*n*-9)	1.6	4.2	8.6	21.7	32.6	6.6	13.6
18:1(*n*-7)	8.8	5.0	.	.	.	.	2.7
18:2(*n*-6)	4.5	6.2	2.5	2.5	.	6.1	13.7
18:3(*n*-6)	1.2	.	.	0.5	.	.	.
18:3(*n*-3)	20.2	15.8	0.1	2.1	9.1	.	9.7
18:4(*n*-3)	2.3	1.6	.	0.6	0.8	.	9.1
20:0	.	0.4	.	0.4	.	.	5.3
20:1	.	.	.	4.5	.	.	.
20:3(*n*-6)	.	.	.	.	.	.	.
20:4(*n*-6)	1.5	0.6	1.6	0.9	0.7	3.7	.
20:3(*n*-3)	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	0.1	.	.	.
20:5(*n*-3)	0.6	0.7	10.7	4.2	2.0	18.2	0.7
22:4	.	.	.	.	.	.	.
22:6(*n*-3)	.	.	.	1.8	.	.	15.2
Unidentified:	8.6	10.9	2.6	6.5	17.1	3.7	3.2
Total sats :	24.2	40.2	41.1	40.5	21.8	36.0	27.5
Total monos :	22.3	17.1	41.0	35.1	35.9	30.6	19.2
Total PUFA :	44.9	31.6	15.3	17.9	25.2	27.7	50.0
Total (*n*-9) :	1.6	4.2	8.6	21.7	32.6	6.6	13.6
Total (*n*-6) :	7.2	6.8	4.1	3.9	0.7	9.7	13.7
Total (*n*-3) :	35.4	22.3	10.8	8.1	12.0	18.2	35.4
(*n*-3)/(*n*-6) :	4.9	3.3	2.6	2.1	17.6	1.9	2.6
16:1/16:0 :	0.5	0.3	1.1	0.3	0.2	0.8	0.3
20:5/16:0 :	0.0	0.0	0.4	0.1	0.1	0.6	0.1

tr.,<0.1% ., not detected

*Abbreviations used : *B. sub, Brachiomonas submarina; C. sal, Chlorella salina; C.min, Chlorella minutissima; T.sp, Tetraselmis sp., N. ato, Nannochloris atomus; N. ocu, Nannochloropsis oculata; I. gal, Isochrysis galbana.*

B. submarina. *Isochrysis galbana* was also the only species to contain more than trace quantities of 22:6 (*n* -3) but contained only traces of 20:5(*n*-3). It was also one of only two species, along with *N. atomus*, to contain a proportion of C_{18} fatty acids higher than its proportion of C_{16} fatty acids. In contrast, the species *C. minutissima* and *N. oculata* both contained C_{20} fatty acids at the expense of C_{18} fatty acids, whereas *B.submarina, C. salina, Tetraselmis* sp. and even *N. atomus* all contained C_{18} fatty acids at the expense of C_{20} fatty acids.

Despite their high (*n*-3) PUFA content both *B. submarina* and *I. galbana* were discarded in favour of *N. oculata* as the alga for study. Although *I. galbana* had a wider range of unsaturated fatty acids it did not grow well in either the stock or the experimental cultures. The poor growth of this strain of *Isochrysis* was also independently noted during its growth in chemostat culture. This was attributed to deterioration of the strain's viability during long term isolation (Personal communication, 1988, J. M. Scott, Oban). Both *B. submarina* and *N. oculata* proved to be more robust.

Brachiomonas submarina was not used in the study due to its lack of $C_{20\,/\,22}$ PUFA, notably 20:5(*n*-3), although in other respects the strain was a suitable candidate for the present study, possessing both 18:3 and 18:4(*n*-3) acids instead.

Nannochloropsis oculata was finally selected on the basis of several criteria apart from its high 20:5(*n*-3) content. It was undemanding in its maintenance requirements. Viable axenic parent stock cultures (*ca.* 100 ml) were retained in cotton wool plugged conical flasks on the laboratory bench for several months without requiring attention. Turner & Gowen (1984) had previously defined the nutritional requirements and capabilities of *N. oculata* as far as regarded carbon and nitrogen sources utilised in both autotrophic (illuminated) and heterotrophic (non-illuminated) nutrition during batch culture. Nitrate was the preferred N source (*i.e.* that which supported the greatest increase in biomass measured by following the increasing O.D. of cultures) followed by nitrite and ammonium then urea. Glycine was not

utilised to any significant extent. Dissolved amino acids found to support significant growth were as follows: *L*-cysteine, *L*-cystine, *L*-glutamine and *DL*- tryptophan. Neither sodium acetate, nor glucose, were utilised during incubation in darkness, although sodium [^{14}C] acetate was incorporated into the lipids of *N. oculata* during illuminated incubation (Henderson *et al.*, 1990a/b). The culture medium, S88 (Droop, 1968; Turner, 1979), used throughout this study was fully defined and had no available organic carbon source provided (see Appendix D). Any net increase in carbon therefore, must be due to photosynthetic fixation of inorganic carbon. Of the three potential nitrogen sources in the medium recipe, KNO_3, glycine and glycylglycine (see Appendix D), only the added KNO_3 was available to *N. oculata*. This restricted field of nutrient utilisation was useful in the context of the present study because it allowed unrestricted glycylglycine buffering to be used in the fully defined S88 medium. *Nannochloropsis oculata* did not require vitamins B_1 or B_{12} to be supplied in the culture medium.

5.3 VARIATIONS IN THE LIPID COMPOSITION DURING THE BATCH CULTURE GROWTH CYCLE.

5.3.1 Experimental.

Cultures of *N. oculata* were grown (using the techniques described in Section 4.2) in order to characterise the behaviour of this alga under the proposed standard reference conditions. These conditions were as follows: S88 medium (Droop, 1968; Turner 1979), pH adjusted to 8.0 using 2 M NaOH; temperature 20 °C and an illumination of 70 $\mu E\ m^{-2}\ sec^{-1}$, measured at the culture vessel surface parallel to the light source. Culture vessels were aseptically inoculated to an initial cell density of 2×10^6 cells ml^{-1} and transferred to a continuously illuminated constant temperature room where they were maintained throughout the duration of the experiment. Cell counts were conducted upon aliquots removed aseptically from the main cultures during the course of sampling and cell harvesting. Following centrifugal cell harvesting supernatant samples were taken and frozen at ¯20 °C prior to nitrate analyses using a Technicon™ autoanalyser (see Strickland & Parsons, 1967. Nitrate analyses were carried out at the Dunstaffnage Marine Laboratory).

In this initial experiment the lipid was extracted from the lyophilised cells and the total fatty acid composition analysed by GLC. The treatment of time course samples followed the protocol described in Figure 5.3.1 with the exceptions that at this stage 17:0 internal standard was omitted and individual lipid class fatty acid compositions were not analysed. The total lipid extracts were however analysed using double development TLC followed by staining with cupric acetate reagent (Fewster *et al.*, 1967). The developed, stained chromatograms were scanned using a Shimadzu CS-930 scanning densitometer (later upgraded to a Shimadzu CS9000 scanning densitometer) operating in the zig-zag mode (*cf.* Olsen & Henderson, 1989).

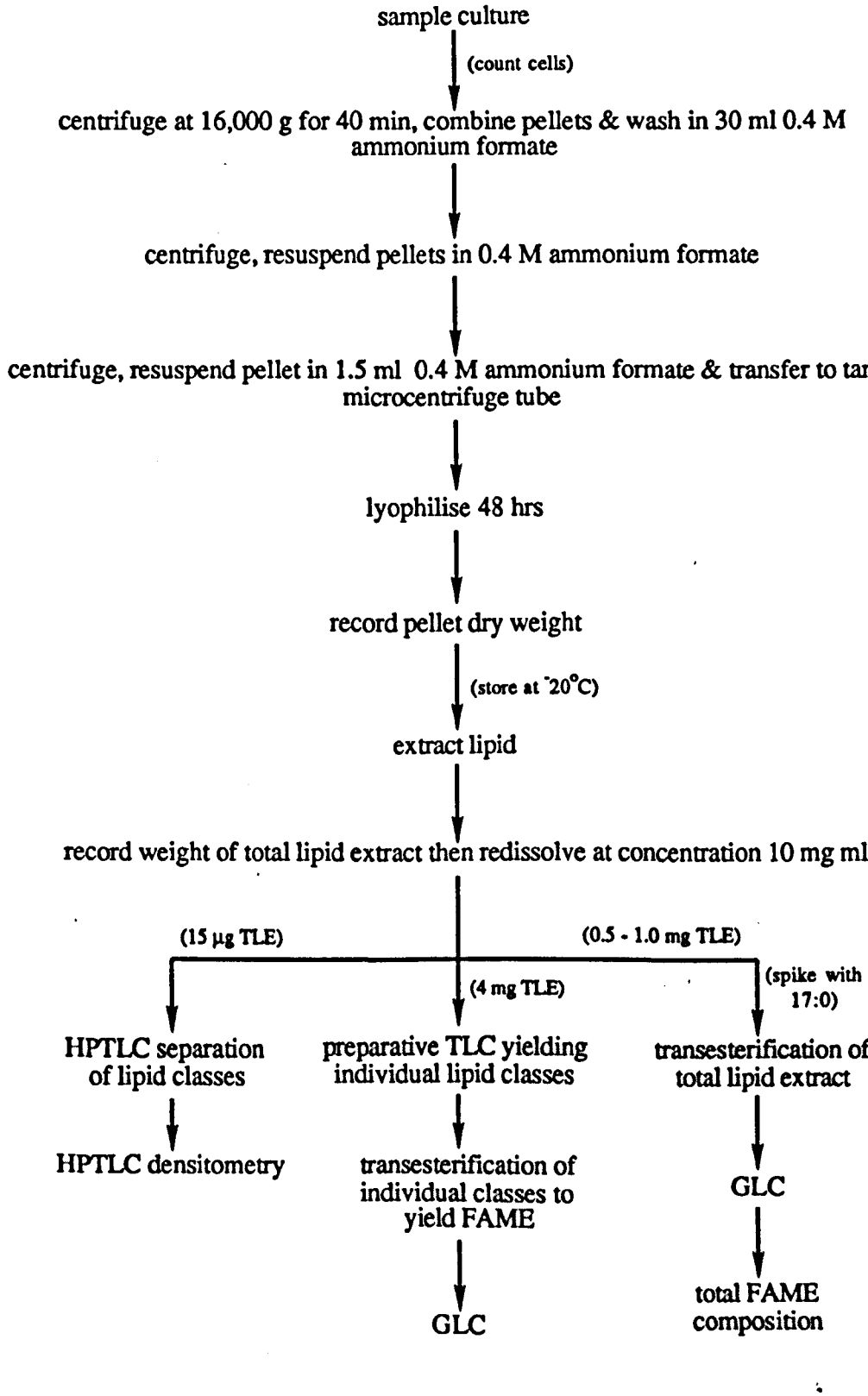


Figure 5.3.1: Sample protocol for time course experiments[1].

5.3.2 Results & Discussion.

The growth curve of *N. oculata* in batch culture under 'standard' conditions is shown as Figure 5.3.2. A characteristic pattern of lag, exponential and stationary phases was clearly displayed although in this case the lag phase was relatively short at less than 20 hours. This was most probably due to the culture being grown at close to optimal temperature (*cf.* cultures grown at lower temperatures, Section 6.2) and being inoculated from an actively dividing stock culture. The exponential phase was also short, being limited to a duration *ca.* 140 hours, or 4 doubling periods when expressed in terms of cell division. The most probable explanation for this was the exhaustion of medium nitrate which resulted in a steady decline in cell division rate leading into the stationary phase. The decline of the culture medium nitrate concentration was plotted with the growth curve as Figure 5.3.3. Nitrate concentration fell linearly during the exponential growth phase from 100 mM at 48 hours post inoculation to the limit of detection at 144 hours post inoculation. Nitrate exhaustion was correlated with the fall in cell division rate and the trough of the individual cell mass curve (*cf.* Figures 5.3.2 & 5.3.3).

Striking changes in individual cell mass occurred at specific points during the culture time course. These showed a close negative correlation with changes in the cell division rate, low division rates being associated with high cell mass values and *vice versa*. This was also confirmed by microscopic examination, the cells being visibly smaller in diameter during the exponential phase when compared with those cells in the lag and stationary phases. The magnitude of the short term increase in individual cell mass, from *ca.* 5 to 7 pg cell^{-1} (see Figure 5.3.2) during the hours immediately following inoculation, was unexpected. This was particularly the case because the omission of sampling during this early period evidently masked the true extent of the phenomenon. However, a more representative value for the peak individual cell mass may have been as high as 8 to 9 pg cell^{-1}. This range was estimated by back-extrapolating the slope of the line joining the mass value points for the 48 and 96 hour samples. This change in mass is the equivalent of a 50 to 60 % mass increase in less than 24 hours. This estimate was confirmed using interpolation on the growth curve

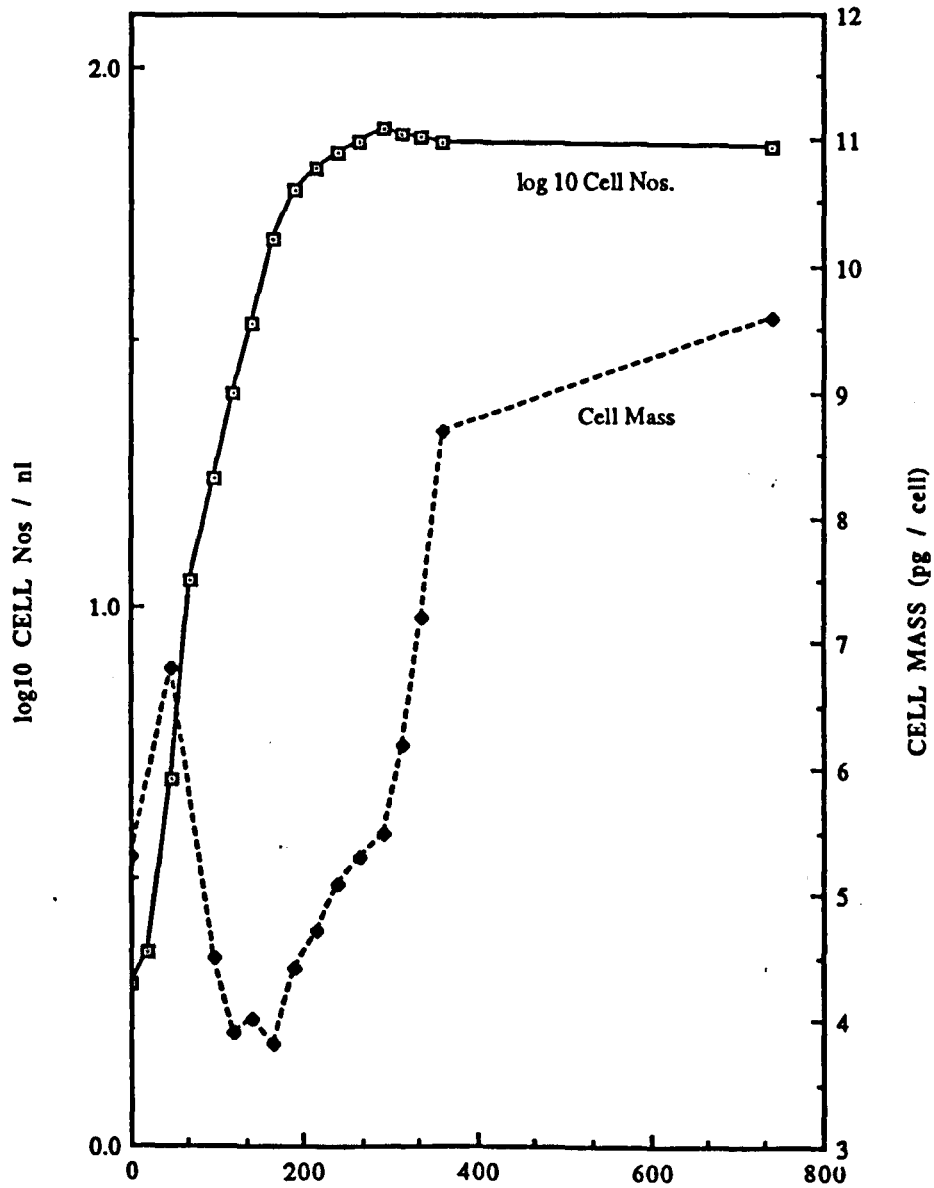


Figure 5.3.2 : Growth curve of *N. oculata* in batch culture under 'standard' conditions showing the variation in cell mass with culture age.

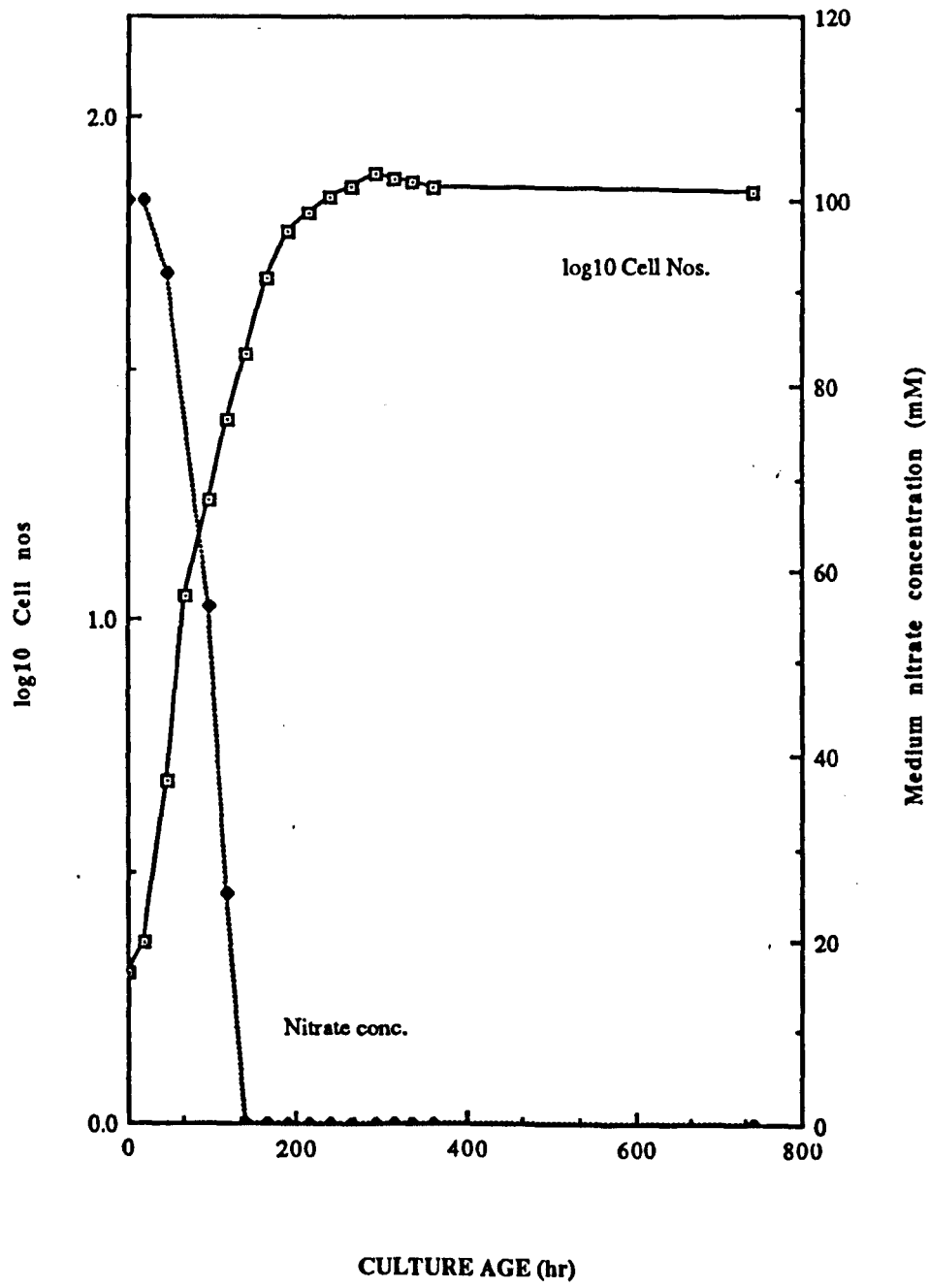


Figure 5.3.3 : Growth curve of *N.oculata* in batch culture under 'standard' conditions showing the decline of medium nitrate concentration with culture age¶.

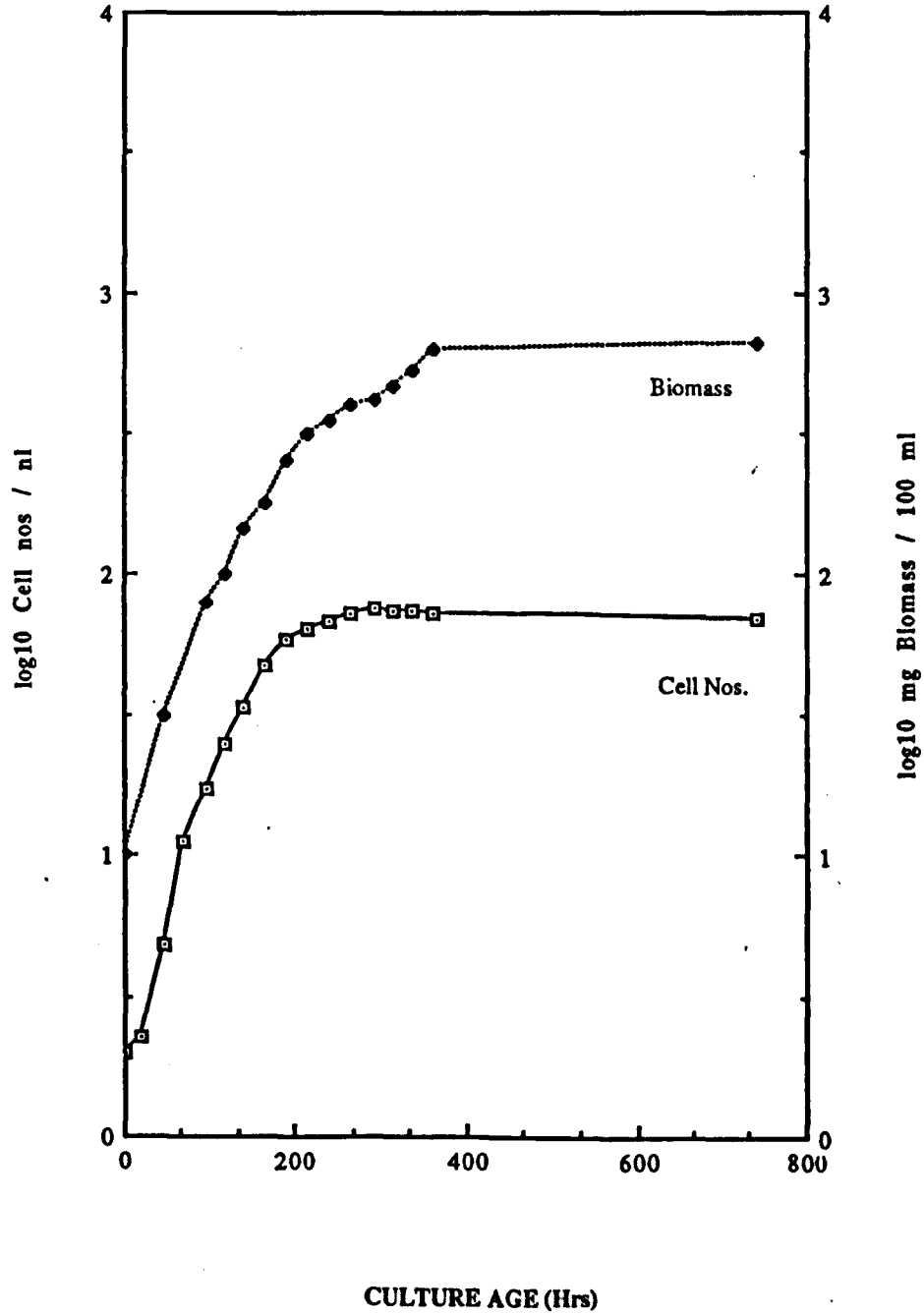


Figure 5.3.4 : Growth curves of *N. oculata* under standard conditions. Data is expressed in terms of cell numbers and biomass per unit volume.

expressed in terms of biomass accumulation (see Figure 5.3.4). Using the ratio of cells to estimated sample biomass at 20 hours post inoculation, a peak cell mass of 8.7 pg cell^{-1} was predicted for the lag phase.

The inference from the above is that when growth is expressed in terms of the biomass accumulation rate (see Figure 5.3.4), the lag phase becomes much less evident (the difference is much more pronounced in Section 6.2). Therefore, during the brief cessation of cell division, otherwise known as the lag phase, the rate of biomass accumulation as a result of photosynthetic carbon fixation is relatively unaffected. This in turn leads to the firm conclusion that the two non-division phases, *i.e.* the lag and stationary phases, were distinctly different in their pattern of progression.

A close relationship appeared to exist between the changes in individual cell mass and the calculated lipid yield per cell. As the cell mass increased during the lag phase, both the total lipid yielded per cell (see Figures 5.3.5 & 5.3.6) and the relative proportion of its TAG component increased. As stated above these changes correlated with the decreases in cell division rate. During the exponential phase, between 48 and 192 hours post inoculation, cell mass decreased to a minimum of 3.8 pg cell^{-1}. Lipid yield per cell and its percentage of TAG also decreased to minimum values over the same time period. However, when the medium nitrate source was exhausted, after 144 hours post inoculation (see Figure 5.3.3), the cell division rate declined and individual cell mass, lipid yield per cell and its percentage of TAG all began to increase. The mass increase progressed rapidly following a rough linear trend (see Figure 5.3.4) as the cell division rate decreased further. At 360 hours post inoculation an individual cell mass of 8.7 pg was attained. This implied that photosynthetic carbon fixation was minimally affected up to this stage. The culture thereafter entered the stationary phase during which cell mass continued to increase, but at a very much lower rate, (apparently negligible in Figure 5.3.4 & 5.3.5). The peak recorded values of cell mass (9.6 pg cell^{-1}) and lipid yield per cell (7.7 pg cell^{-1}) were after 740 hours in culture, *ca.* 400 hours after cell division had ceased. At this point the yield of lipid as a proportion of cell mass was 80 % as compared with a value *ca.* 35 % in the growth phase.

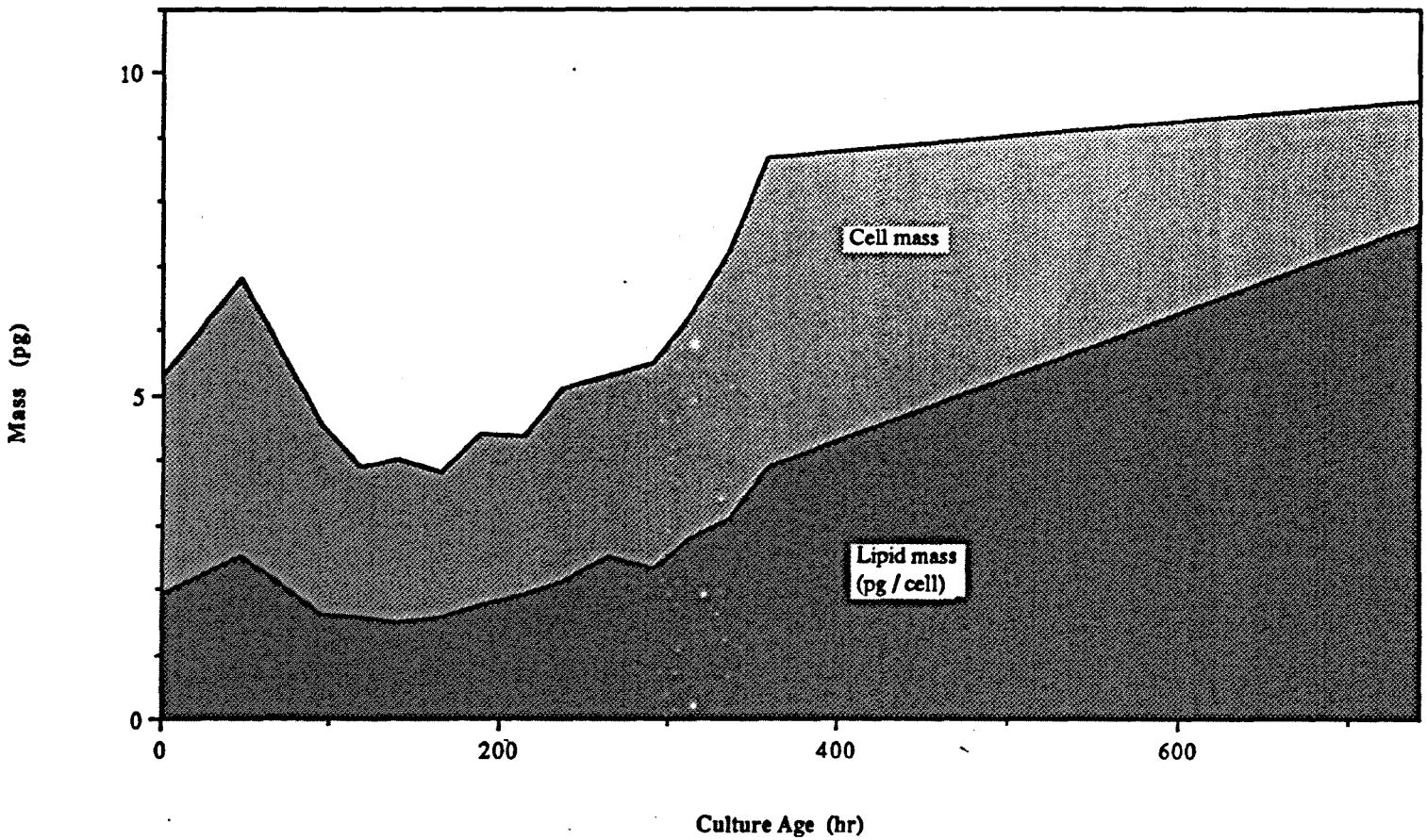


Figure 5.3.5 : The relatioship between individual cell mass and cellular proportion of lipid over 742 hours in batch culture under 'standard' conditions[1].

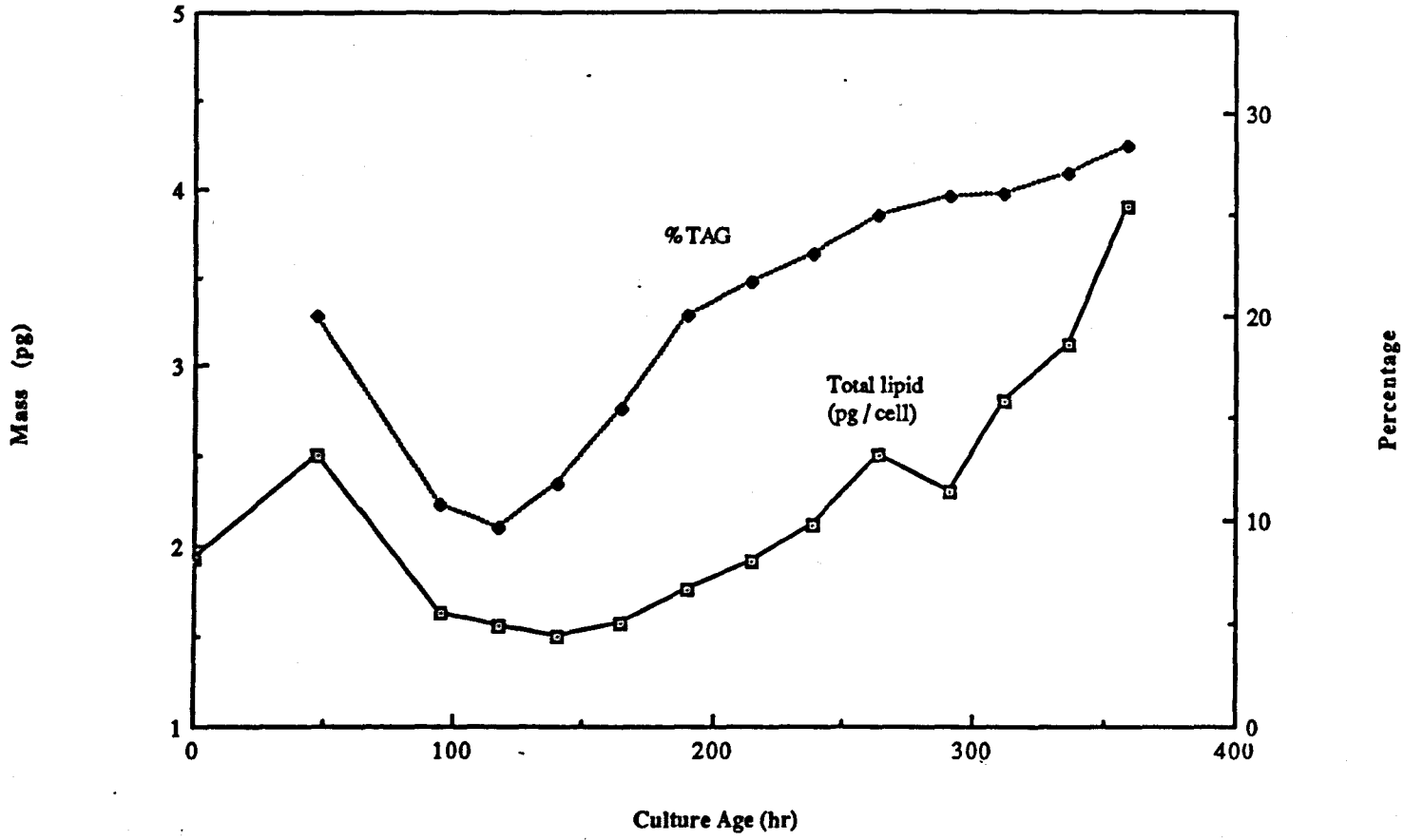


Figure 5.3.6 : The relationship between changes in cellular proportion of total lipid and its proportion present as TAG[1].

A significant change in the partitioning of carbon in the microalgal cells occurred during the period from 360 hours through to 740 hours post inoculation. Cell mass increased by 9.7 % whereas the lipid yield increased by 96 % (*i.e.* almost doubled) (see Figure 5.3.5). The increase in mass was too low to be accounted for by simple accumulation of lipid. Had this been the case the final cell mass would have been *ca.* 12 pg. During the latter stages of the stationary phase biomass accumulation virtually ceased indicating a drastically decreased net carbon fixation rate. However, the increasing proportion of lipid may be accounted for in two ways. 1) Cellular polymeric carbohydrate reserves were being catabolised whilst the bulk of carbon fixed was being shunted into storage lipid biosynthesis, or, as was more likely, 2) Carbohydrate was being directly converted to storage lipid within the cell as was suggested for *Chlorella ellipsoidea* by Iwamoto & Nagahashi (1955) (see Section 2).

Lipid classes other than TAG, specifically the phospholipids and glycolipids, showed a positive correlation with increased cellular division rate. Whether the observed increases (see Figure 5.3.7) in the proportions of these classes were real or artifacts of the form of expression of the data is difficult to ascertain. The restrictions upon translating this data into absolute mass were those described earlier (see Section 3.2.2). Although such translations could readily be performed, the assumptions and errors introduced would have been so great as to disqualify any benefits such manipulation of the data may have given.

Table 5.3.1 shows the changes which occurred in the total FAME of *N. oculata* during the culture period from inoculation to 360 hours post-inoculation. The changes in overall unsaturation correlate closely with the changes in lipid class composition presented in Figure 5.3.7. The peak in the proportion of TAG during the lag-phase (48 hours post-inoculation) coincides with the trough in 20:5(*n*-3) and corresponding peak in the proportion of saturates and monounsaturates. This phenomenon was summarised by the use of the ratios 16:1/16:0 and 20:5/16:0. Because the proportion of 16:1 remained comparatively constant the former ratio reflected the variation in 16:0 whereas the latter ratio could be regarded as a rough guide to the PUFA / saturate ratio in total FAME.

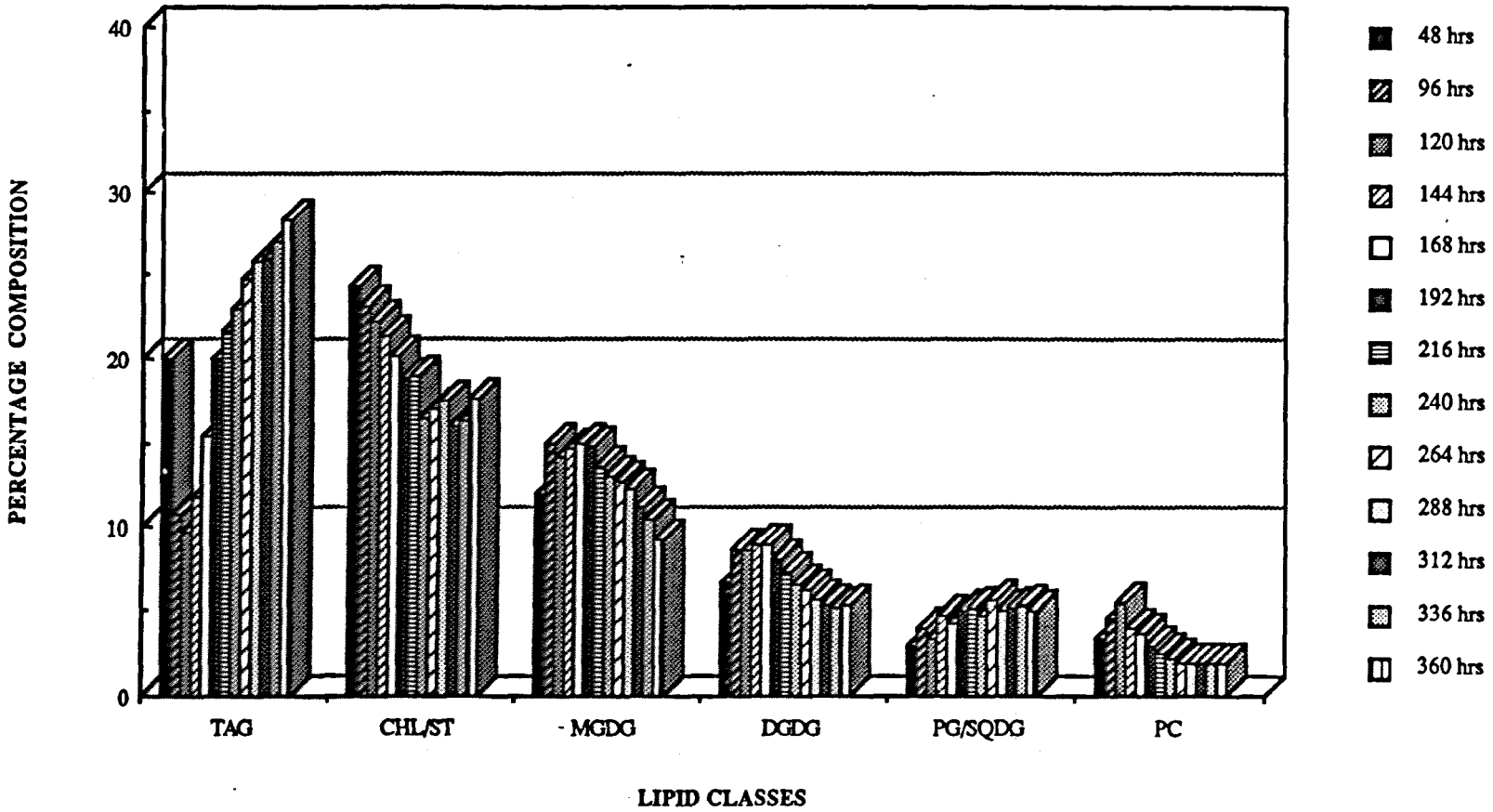


Figure 5.3.7 : Changes in the relative proportions of the major lipid classes of *N. oculata* during batch culture under 'standard' conditions[1].

TABLE 5.3.1: Total FAME composition of *N. oculata* with culture age over the batch culture growth cycle in unmodified S88 medium.

FATTY ACID	SAMPLE TIME (Hrs)											
	0	48	95	120	144	168	192	216	240	291	336	360
14:0	4.1	3.2	4.1	4.6	4.3	4.4	4.2	4.0	3.7	4.0	3.7	3.6
15:0	0.3	0.6	0.3	0.3	0.4	0.4	0.3	0.4	0.3	0.4	0.3	0.3
16:0	17.1	27.0	17.8	19.0	17.2	22.4	26.0	27.0	27.5	26.8	26.9	27.4
16:1(*n*-7)	26.1	30.8	29.0	28.3	26.1	26.1	26.7	28.4	28.0	29.4	29.7	29.9
16:1(*n*-13)t.		.	.	.	.	.	.	.	.	.	.	.
16:2	0.4	0.4	0.4	0.3	0.4	0.3	0.2	0.4	0.2	0.2	0.2	0.2
17:0	.	.	.	.	.	.	.	.	.	.	.	.
16:3	0.2	0.3	0.1	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1
16:4	.	.	.	.	.	.	.	.	.	.	.	.
18:0	0.7	1.6	0.8	0.9	1.2	1.0	1.1	1.1	1.0	1.1	1.3	1.4
18:1(*n*-9)	3.8	3.9	3.4	4.0	4.2	4.5	4.2	4.6	4.9	5.7	6.7	7.5
18:1(*n*-7)	0.3	0.7	0.4	0.3	0.4	0.3	0.3	0.4	0.4	0.4	0.5	0.5
18:2(*n*-6)	2.8	1.5	1.8	1.8	1.9	1.6	1.7	1.9	2.1	2.7	3.2	3.4
18:3(*n*-6)	.	.	.	.	.	.	.	.	.	.	.	.
18:3(*n*-3)	0.4	0.1	0.2	0.2	0.3	0.2	0.2	0.3	0.2	0.3	0.4	0.4
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.
20:0	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
20:3(*n*-6)	0.6	1.3	0.7	0.7	0.6	1.2	1.9	1.9	2.0	1.5	1.5	1.5
20:4(*n*-6)	5.1	3.9	4.2	4.4	4.1	4.0	4.1	4.1	4.3	4.4	3.9	3.4
20:3(*n*-3)	.	.	0.2	0.2	0.2	0.2	0.3	0.2	0.2	0.1	0.2	0.2
20:4(*n*-3)	0.1	0.2	0.2	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2
20:5(*n*-3)	30.6	18.4	29.0	28.2	29.8	26.4	22.8	20.6	19.5	18.6	17.4	15.4
Unidentified :	7.2	5.9	7.1	6.3	8.3	6.5	5.5	4.2	5.2	3.9	3.6	4.5
Total sats :	22.4	32.6	23.3	25.1	23.4	28.4	31.8	32.7	32.7	32.5	32.4	32.9
Total monos :	30.2	35.4	32.8	32.6	30.7	30.9	31.2	33.4	33.3	35.5	36.9	37.9
Total PUFA :	40.2	26.1	36.8	36.0	37.6	34.2	31.5	29.7	28.8	28.1	27.1	24.7
Total (*n*-9) :	3.8	3.9	3.4	4.0	4.2	4.5	4.2	4.6	4.9	5.7	6.7	7.5
Total (*n*-6) :	8.5	6.7	6.7	6.9	6.6	6.8	7.7	7.9	8.4	8.6	8.6	8.3
Total (*n*-3) :	31.1	18.7	29.6	28.7	30.4	27.0	23.5	21.3	20.1	19.2	18.2	16.1
(*n*-3)/(*n*-6) :	3.6	2.8	4.4	4.2	4.6	4.0	3.1	2.7	2.4	2.2	2.1	1.9
16:1/16:0 :	1.5	1.1	1.6	1.5	1.5	1.2	1.1	1.1	1.0	1.1	1.1	1.1
20:5/16:0 :	1.8	0.7	1.6	1.5	1.7	1.8	0.9	0.8	0.7	0.7	0.6	0.6
ΣC_{16} :	43.8	58.5	47.3	47.7	43.9	48.9	53.0	55.9	55.8	56.5	56.9	57.6
ΣC_{18} :	8.0	4.8	6.6	7.2	8.0	7.6	7.5	8.3	8.6	10.2	12.1	13.2
ΣC_{20} :	36.6	24.0	34.6	33.9	35.1	32.2	29.5	27.2	26.4	25.0	23.4	20.8

Data expressed in as percentages of total FAME mass.

tr.,<0.1%

., not detected

Data from samples taken at 264 & 312 hrs post inoculation omitted due to space limitations. FAME compositions were almost identical to those at 291 & 336 hrs respectively.

During the lag-phase significant changes occurred in the fatty acyl chain length distribution profile of the total lipid FAME. The proportion of C_{16} fatty acyl chain lengths increased from 43.8 % to 58.5 %, C_{18} chain lengths decreased from 8.0 % to 4.8 % and C_{20} chain lengths from 36.6 % to 24.0 % in a 48 hour time period. The overall trend was toward shorter chain length fatty acids of increased saturation and monounsaturation.

By 95 hours post-inoculation, the lag-phase had passed, active cell division was again evident and the proportions of 16:0 and 20:5(*n*-3) had reverted to levels similar to those in the inoculum culture (*i.e.* at 0 hours). The chain length distribution profile was also similar to that observed at the time of inoculation, having an increased proportion of C_{20} PUFA in comparison to the cells during the lag-phase. The exponential phase of growth was foreshortened, this being most probably due to the use of S88 without supplemental nitrate and phosphate (see Figure 5.3.3). Thus the cultures growth rate began to decline and this was not only evident from the growth curve (Figure 5.3.2). The increasing proportion of TAG and the increasing saturation and carbon chain shortening observed in the total FAME composition after 144 hours post-inoculation, also pointed towards declining cell division rates. This trend continued; the proportion of 16:0 increased from a minimum of 17.2 % to a maximum of 27.4 % by 360 hours post-inoculation and that of 20:5(*n*-3) decreased from 29.8 % to 15.4 % during he same time period. The final acyl chain length distribution profile differed from that which was observed during the lag-phase. There was an increased proportion of saturated and monounsaturated fatty acids but there was a greater proportion of the C_{18} acyl chains than observed previously. The overall range of values observed for the proportions of the acyl chain lengths at 144 hours and 360 hours post-inoculation were as follows: 43.9 % to 57.6 % C_{16}, 8.0 % to 13.2 % C_{18} and 35.1 % to 20.8 % C_{20}.

The results recorded above suggested that the S88 medium used to culture the algal cells should be modified to extend the exponential growth phase. The overwhelming evidence shown in Figure 5.3.3 pointed to increasing the starting concentration of nitrate in the medium. Because it was possible that phosphate may have then become limiting its

concentration was also increased in future cultures. The projected effect of these changes was to extend the time period of exponential growth and therefore enable more samples to be taken during the dynamic period of growth prior to the stationary phase. This aim was pursued and the results recorded in the later sections of this thesis.

5.4 SHORT-TERM CHANGES IN THE LIPID COMPOSITION FOLLOWING INOCULATION.

5.4.1 Experimental.

Cultures of *N. oculata* were grown as described in Section 5.3.1 except that the medium used was modified with respect to its nitrate and phosphate components. The concentration of KNO_3 was increased from 100 mg l^{-1} to 300 mg l^{-1} (*i.e.* 100 mM to 300 mM) and that of KH_2PO_4 increased from 10 mg l^{-1} to 20 mg l^{-1} (*i.e.* 73.5 μM to 147 μM). This modification was used in all subsequent batch culture experiments, so increasing the length of the exponential phase from 3 to 5 doublings (*c.f.* Figure 5.3.2). The modified medium is referred to henceforth as S88 plus excess nitrate & phosphate.

A 5 litre culture vessel was aseptically inoculated to a cell density of *ca.* 4 x 10^6 cells ml^{-1} using a stock culture 'in the exponential phase of growth'. The culture was maintained at a constant temperature of 20 °C, an illumination intensity of 70 μE m^{-2} sec^{-1} and continuously aerated at a rate of 100 ml min^{-1}. An aliquot of the inoculum was harvested as the 'time-zero' sample. Samples of 650 ml were withdrawn aseptically at 90 min and 3 hours post inoculation and at intervals of 3 hours thereafter up to 18 hours post inoculation. Sampling procedures followed the protocol established earlier (see Figure 5.3.1). This required an increased amount of total lipid, *ca.* 3 - 5 mg, to be yielded by each sample in the time course. Having established earlier (see Section 5.3) that the lipid yield per cell was *ca.* 1 - 2 pg (see Figure 5.3.4), a sample of 2 - 3 x 10^9 cells was sufficient.

Having calculated the required sample size, several constraints immediately became obvious. 1) The largest culture vessel available was 5 litres in volume. 2) The inoculation density of the cultures had to be as low as was practicably possible to maintain the desired comparability with the proposed time course studies. 3) Sampling was to be designed to cover as much of the first 24 hours following the inoculation of the culture as possible. The final solution was a compromise between the final two factors. The inoculation density was increased from 1.5 x 10^6 to 4 x 10^6 cells ml^{-1} which allowed a sample of 650 ml to be

removed, yielding *ca.* 2.6 x 10^9 cells in total. This in turn allowed sufficient material for 7 samples to cover the period from 90 min to 18 hours post inoculation.

The sample treatment protocol applied was that employed in the previous section (see Figure 5.3.1) but samples were withdrawn at 90 min, 3 hours and subsequently at intervals of three hours post-inoculation. The major difference between the two series of samples, other than the frequency at which they were taken, was the detail of the analyses employed. The total lipid extracted from each sample on the current time course was separated into its major component lipid classes using preparative scale TLC. A procedure scaled up from the HPTLC version of the double development - single dimensional procedure detailed in Section 3.2.2 was employed. The classes were separated in sufficient quantity on a single 20 x 20 cm TLC plate so as to allow preparation of their FAME derivatives for analysis by capillary column GLC. Such individual lipid class FAME analyses complemented the total FAME and HPTLC densitometric analyses performed upon every sample.

5.4.2 Results & Discussion.

The aims of the work presented in the earlier sub-sections were pitched at a relatively general level, covering the whole of the batch culture cycle. Those of the work encompassed in this sub-section however, addressed the changes occurring over a specific period during the growth phase of a culture, namely the 'lag phase'. From the data of Section 5.3 it was evident that significant physiological changes were occurring within the cell during the period immediately following inoculation into a fresh batch of culture medium. This had implications for the use of the 'pulse-chase' type of experimentation when tracing the incorporation and distribution of ^{14}C radiolabelled fatty acids through elongation and desaturation pathways.

The changes in the FAME composition of the total lipid extracted from *N. oculata* over a period of 18 hours are shown in Table 5.4.1. This period was roughly equivalent to half the doubling period of this alga during its exponential growth phase at 20 °C, as

TABLE 5.4.1: Total FAME composition of *N. oculata* with culture age over the first 18 hr in batch culture in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)							
FATTY ACID	0	1.5	3	6	9	12	15	18
14:0	3.2	3.1	3.3	3.3	3.6	3.2	3.2	3.4
15:0	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
16:0	15.9	14.8	14.0	15.3	17.5	19.3	23.0	24.2
16:1(*n*-7)	25.7	25.8	25.6	24.8	26.0	25.1	25.4	25.4
16:1(*n*-13)t.	0.9	1.0	0.9	1.0	1.0	0.9	0.8	0.8
16:2	0.6	0.5	0.5	0.5	0.4	0.4	0.3	0.4
17:0	.	.	.	.	.	.	.	.
16:3	0.4	0.3	0.3	0.2	0.2	0.2	0.2	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.4	0.8	0.7	0.7	0.8	0.9	0.9	0.8
18:1(*n*-9)	2.9	2.3	2.4	2.4	2.7	3.2	3.0	2.5
18:1(*n*-7)	0.5	0.5	0.5	0.4	0.4	0.4	0.4	0.4
18:2(*n*-6)	2.6	2.4	2.4	2.0	1.8	1.9	2.0	1.9
18:3(*n*-6)	0.4	0.4	0.4	0.6	0.5	0.6	0.7	0.8
18:3(*n*-3)	0.2	0.2	0.2	0.2	0.1	0.1	0.3	0.1
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.4	0.5	0.5	0.6	0.7	0.9	1.0	1.2
20:4(*n*-6)	4.1	4.2	4.1	4.3	4.0	4.0	3.8	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	30.8	32.9	33.8	32.7	31.0	29.9	28.1	27.8
Unidentified :	9.5	9.8	9.9	10.5	8.8	8.5	6.4	5.8
Total sats :	21.0	19.2	18.5	19.8	22.4	23.9	27.6	28.9
Total monos :	30.0	29.6	29.4	28.6	30.1	29.6	29.6	29.1
Total PUFA :	39.5	41.4	42.2	41.1	38.7	38.0	36.4	36.2
Total (*n*-9) :	2.9	2.3	2.4	2.4	2.7	3.2	2.3	2.5
Total (*n*-6) :	7.5	7.5	7.4	7.5	7.0	7.4	7.5	7.7
Total (*n*-3) :	31.0	33.1	34.0	32.9	31.1	30.0	28.4	27.9
(*n*-3)/(*n*-6) :	4.1	4.4	4.6	4.4	4.4	4.1	3.8	3.6
16:1/16:0 :	1.6	1.7	1.8	1.6	1.5	1.3	1.1	1.0
20:5/16:0 :	1.9	2.2	2.4	2.1	1.8	1.5	1.2	1.1
ΣC_{16}:	43.5	42.4	41.3	41.3	45.1	45.9	49.7	51.0
ΣC_{18}:	8.0	6.6	6.6	6.3	6.3	7.1	7.3	6.5
ΣC_{20}:	35.5	37.8	38.6	37.8	35.9	35.0	33.1	33.0

Data expressed as a percentage of the total FAME mass.
tr.,<0.1%
., not detected

determined for previous batch cultures. As can be seen lipid synthesis continued following its normal pattern for the 3 hours immediately following inoculation of the cells into new medium. The proportion of 16:0 decreased from 15.9 % to a minimum of 14.0 % and that of 20:5(*n*-3) increased from 30.8 % to a maximum of 33.8 %. The proportion of 16:1 remained almost constant throughout the 18 hour time course. The changes in the two other major fatty acids were manifest in the continued increase in the ratios of 16:1/16:0 from 1.6 to 1.8 and 20:5/16:0 from 1.9 to 2.4 during this time. However, by 6 hours post inoculation it was evident that the previous trend of changes had become reversed. The proportion of 16:0 had increased from 14.0 % to 15.3 % and that of 20:5(*n*-3) had decreased from 33.8 % to 32.7 % between 3 and 6 hours post inoculation. This was a trend that continued resulting in a proportion for 16:0 of 24.2 % and for 20:5(*n*-3) of 27.8 % at 18 hours post inoculation. The changes in these two fatty acids were not entirely complementary. The proportion of 16:0 increased at the expense of 20:5(*n*-3) and an unidentified component eluted from the capillary column immediately before 16:0. This accounted for the steady fall in the proportion of the total unidentified component of each sample.

Analysis of the samples using HPTLC-densitometry showed a significant increase in the proportion of the total lipid extract present as TAG. The remaining lipid classes generally showed complementary but limited decreases in proportion. It can be seen from Figure 5.4.1 that the increase in TAG occurred in the period between 6 and 9 hours post inoculation and could be correlated with changing growth phase. This corresponded to the early stages of the decrease in unsaturation of total FAME (see Table 5.4.1). This may have implied that the accumulation of the reportedly saturate rich TAG class was solely responsible for the decline in unsaturation of the total FAME. The next stage in analysis showed this was not entirely the case.

Analysis of the individual lipid class FAME showed that each had a characteristic fatty acyl profile (see Tables 5.4.2 to 5.4.8). As was expected the galactolipids were highly unsaturated. MGDG in particular contained the highest proportion of 20:5(*n*-3) and 14:0. Correspondingly, this lipid class displayed the lowest proportions of C_{16} and C_{18} acids.

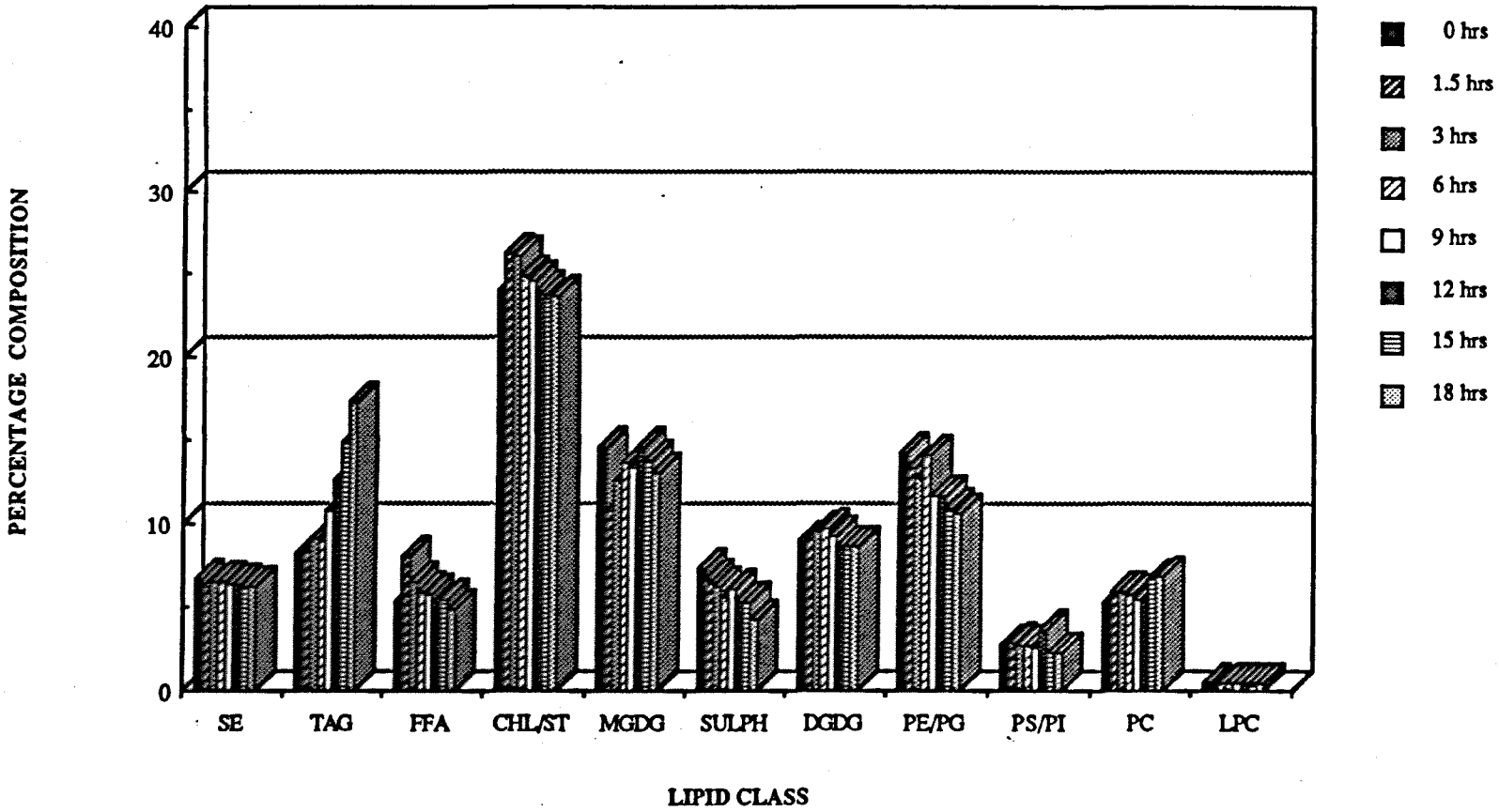


Figure 5.4.1 : Changes in the relative proportions of lipid classes of *N. oculata* over the first 18 hours in batch culture at 20 °C.

Digalactosyldiacylglycerol also showed a high proportion of 20:5(*n*-3) but possessed higher proportions of 16:0 and 16:1 than MGDG. The fraction containing PG, PE and SQDG possessed roughly equivalent proportions of the three major fatty acids and generally had a lower unsaturation than the other chloroplast lipid classes MGDG and DGDG. This fraction was characterised by the presence of the marker fatty acid *trans* 16:1(*n*-13), the distribution of which is limited to PG (although it is known to occur in the biosynthetically related lipid class cardiolipin, (Harwood, 1980)). The fraction containing both PI and PS was characterised by its high proportion of arachidonic acid or 20:4(*n*-6) which often became the dominant PUFA present in this fraction. The only class in which the C_{18} fatty acids made a significant contribution to mass was PC, the major non-chloroplast phospholipid component. In this class the total C_{18} acids contributed up to 26 %, as against less than 10 % in the other classes. However, the major C_{18} acids were 18:1(*n*-9) and 18:2(*n*-6), and neither of the 18:3 isomers was present in proportions higher than 5 %. Phosphatidylcholine had a relatively high 16:1/16:0 ratio in comparison with the other classes. This was due to a comparatively low percentage of 16:0 and high percentage of 16:1.

The neutral lipid class TAG showed a composition similar to the majority of the other lipid classes. It possessed 16:0, 16:1 and 20:5(*n*-3) as its major fatty acids although the quantitative proportions of these were subject to considerable variation; as will be discussed below.

At the level of total FAME the effects of changes in the fatty acyl profile of TAG were counteracted during the earliest stages of the time course by concomitant changes in the polar classes. Whilst C_{16} saturates and monosaturates were accumulated in TAG, the galactolipids (MGDG to a greater extent than DGDG) were still showing increasing proportions of PUFA, notably 20:5(*n*-3). The pattern observed in the total FAME profile was therefore more conservative until the galactolipid classes became affected. This occurred at around 6 hours post-inoculation when the galactolipids attained their peak unsaturation

TABLE 5.4.2: Individual lipid class FAME composition of *N. oculata* 90 minutes after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.4	3.3	7.8	3.8	1.9	.	1.1	3.1
15:0	0.3	0.6	0.7	0.5	0.5	.	0.3	0.3
16:0	16.2	9.0	9.1	19.5	28.5	.	6.8	14.8
16:1(*n*-7)	23.5	17.9	8.6	23.0	25.3	.	37.8	25.8
16:1(*n*-13)‡	.	.	.	.	4.5	.	.	.
16:2	0.5	0.3	0.1	0.7	0.3	.	1.3	0.5
17:0	0.2	0.4	0.2	.	.	.	.	.
16:3	0.5	.	0.2	.	0.4	.	0.8	0.3
16:4	.	.	.	.	.	.	0.5	.
18:0	1.7	4.0	3.7	1.5	1.9	.	2.2	0.8
18:1(*n*-9)	3.2	2.1	1.4	0.8	1.2	.	9.0	2.3
18:1(*n*-7)	.	.	.	.	.	.	.	.
18:2(*n*-6)	1.2	1.0	1.4	1.1	0.7	.	10.5	2.4
18:3(*n*-6)	0.3	0.2	0.4	.	0.2	.	2.6	0.4
18:3(*n*-3)	0.3	0.3	0.3	.	0.3	.	0.5	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	.	.	2.2	.	.	.
20:3(*n*-6)	1.1	.	1.0	1.1	0.3	.	0.7	0.5
20:4(*n*-6)	6.2	4.9	2.2	.	4.0	.	5.6	4.2
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	37.3	51.6	58.4	45.1	24.9	.	12.5	32.9
Unidentified :	4.1	4.4	4.5	2.9	2.9	.	7.8	10.3
Total sats :	21.8	17.3	21.5	25.3	32.8	.	10.4	19.2
Total monos :	26.7	20.0	10.0	23.8	31.0	.	46.8	29.1
Total PUFA :	47.4	58.3	64.0	48.0	33.3	.	35.0	41.4
Total (*n*-9) :	3.2	2.1	1.4	0.8	1.2	.	9.0	2.3
Total (*n*-6) :	8.8	6.1	5.0	2.2	5.2	.	19.4	7.5
Total (*n*-3) :	37.6	51.9	58.7	45.1	25.2	.	13.0	33.1
(*n*-3)/(*n*-6) :	4.3	8.6	11.7	20.5	4.8	.	0.7	4.4
16:1/16:0 :	1.5	2.0	0.9	1.2	0.9	.	5.6	1.7
20:5/16:0 :	2.3	5.7	6.4	2.3	0.9	.	1.8	2.2
ΣC_{16}:	40.7	27.2	18.0	43.2	59.0	.	47.2	42.4
ΣC_{18}:	6.7	7.6	7.2	3.4	4.3	.	24.8	6.6
ΣC_{20}:	44.6	56.6	61.6	46.2	31.4	.	18.8	37.8

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, „ not detected

‡, including SQDG, Insufficient PS/PI for analysis

TABLE 5.4.3 : Individual lipid class FAME composition of *N. oculata* 3 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.7	3.2	7.9	4.3	1.6	1.0	1.0	3.3
15:0	0.4	.	0.2	0.5	0.4	0.4	0.3	0.3
16:0	17.7	9.7	7.7	19.3	24.8	20.6	6.5	14.0
16:1(*n*-7)	26.6	19.5	6.0	22.6	21.5	21.3	39.1	25.6
16:1(*n*-13)t	.	.	.	.	4.9	.	.	0.9
16:2	0.4	0.3	0.2	0.7	0.1	.	1.6	0.5
17:0	0.2	0.3	0.2	.	.	.	.	.
16:3	0.3	0.4	0.4	0.3	0.3	.	0.6	0.3
16:4	.	.	.	.	.	.	.	.
18:0	1.5	4.7	2.3	1.3	1.7	3.1	2.2	0.7
18:1(*n*-9)	3.3	2.1	0.6	0.8	1.3	8.7	10.6	2.4
18:1(*n*-7)	.	.	.	.	.	.	.	0.5
18:2(*n*-6)	1.1	1.1	0.6	1.1	0.8	1.9	10.5	2.4
18:3(*n*-6)	0.2	.	0.1	.	0.1	.	2.7	0.4
18:3(*n*-3)	0.3	.	0.3	0.5	0.3	.	0.6	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	1.1	.	0.2	.	1.1	.	0.8	0.5
20:4(*n*-6)	5.9	5.2	2.1	1.2	5.6	20.4	4.2	4.1
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	34.3	49.9	69.8	45.5	28.9	16.2	12.6	33.8
Unidentified :	3.0	3.1	1.4	2.0	6.4	6.4	6.2	9.9
Total sats :	23.5	17.9	18.3	25.4	28.7	25.1	9.9	18.5
Total monos :	29.9	21.6	6.6	23.4	27.7	30.0	49.7	29.4
Total PUFA :	43.6	56.9	73.7	49.3	37.2	38.5	34.2	42.2
Total (*n*-9) :	3.3	2.1	0.6	0.8	1.3	8.7	10.6	2.4
Total (*n*-6) :	8.3	6.3	3.0	2.3	7.6	22.3	18.2	7.4
Total (*n*-3) :	34.6	49.9	70.1	46.0	29.2	16.2	13.2	34.0
(*n*-3)/(*n*-6) :	4.8	7.9	23.4	20.4	3.8	0.7	0.7	4.6
16:1/16:0 :	1.5	2.0	0.8	1.2	0.9	1.0	6.0	1.8
20:5/16:0 :	1.9	5.1	9.1	2.4	1.2	0.8	1.9	2.4
ΣC_{16} :	45.0	29.8	14.3	24.9	51.6	41.9	47.8	41.3
ΣC_{18} :	6.4	7.9	3.9	3.7	4.2	13.7	26.6	6.6
ΣC_{20} :	41.3	55.1	72.1	46.7	35.6	36.6	17.6	38.6

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, -, not detected
‡, including SQDG

TABLE 5.4.4 : Individual lipid class FAME composition of *N. oculata* 6 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.1	2.9	6.8	4.2	2.0	1.0	1.1	3.3
15:0	0.4	0.4	0.5	.	1.2	1.2	0.2	0.3
16:0	20.0	9.9	7.3	20.1	26.7	25.0	7.0	15.3
16:1(*n*-7)	28.3	21.5	5.8	25.1	24.2	27.9	35.5	24.8
16:1(*n*-13)‡	.	.	.	.	5.2	.	.	.
16:2	0.4	0.4	0.1	0.9	0.3	.	1.4	0.5
17:0	0.2	0.4	.	.	.	.	.	.
16:3	0.4	0.3	0.4	0.3	0.2	0.4	0.6	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.3	4.6	1.4	0.8	0.8	2.2	1.2	0.7
18:1(*n*-9)	2.9	2.5	0.4	0.6	1.0	3.5	10.5	2.4
18:1(*n*-7)	.	.	.	.	.	.	.	0.4
18:2(*n*-6)	1.0	0.4	0.4	1.1	0.6	1.2	9.0	2.0
18:3(*n*-6)	0.3	0.4	0.1	0.1	.	0.5	3.1	0.6
18:3(*n*-3)	0.3	0.4	0.3	0.3	.	0.2	0.5	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	0.2	.	0.4	.	2.5	.	.	.
20:3(*n*-6)	1.0	0.3	0.2	.	.	.	1.0	0.6
20:4(*n*-6)	5.4	4.8	2.1	1.4	4.1	17.7	5.1	4.3
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	29.9	43.9	71.5	43.5	27.5	13.8	13.2	33.7
Unidentified :	3.9	5.9	2.3	1.6	3.7	5.3	10.6	9.5
Total sats :	26.0	18.2	16.0	25.1	30.7	29.4	9.5	19.8
Total monos :	31.2	24.0	6.2	25.7	30.4	31.4	46.0	28.6
Total PUFA :	38.9	51.9	75.5	47.6	35.2	33.9	33.9	42.1
Total (*n*-9) :	2.9	2.5	0.4	0.6	0.1	3.5	10.5	2.4
Total (*n*-6) :	7.7	6.9	2.8	2.6	4.7	19.5	18.2	7.5
Total (*n*-3) :	30.2	44.3	71.8	43.8	27.5	14.0	13.7	33.9
(*n*-3)/(*n*-6) :	3.9	6.4	25.6	16.8	5.9	0.7	0.8	4.5
16:1/16:0 :	1.4	2.2	0.8	1.2	0.9	1.1	5.1	1.6
20:5/16:0 :	1.5	4.4	9.8	2.2	1.0	0.6	1.9	2.2
ΣC_{16}:	49.1	32.1	136	46.4	56.6	53.3	44.5	40.8
ΣC_{18}:	5.8	8.3	2.6	2.9	2.4	7.6	24.3	4.5
ΣC_{20}:	36.5	49.0	74.2	44.9	34.1	31.5	19.3	38.8

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, ., not detected
‡, including SQDG

TABLE 5.4.5: Individual lipid class FAME composition of *N. oculata* 9 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	1.5	2.6	8.0	4.4	1.5	2.2	0.4	3.6
15:0	0.4	0.2	0.3	0.4	0.4	0.7	0.2	0.3
16:0	26.4	11.5	9.7	22.8	30.1	24.0	8.2	17.5
16:1(*n*-7)	28.8	22.2	7.4	25.6	23.1	23.9	34.7	26.0
16:1(*n*-13)t	.	.	.	.	6.0	.	.	.
16:2	0.5	0.4	0.1	0.7	0.1	0.6	1.2	0.4
17:0	0.3	0.4	.	.	.	.	0.2	.
16:3	0.3	0.4	0.5	0.4	0.2	.	0.2	0.2
16:4	.	.	.	.	.	.	0.3	.
18:0	1.3	3.3	1.7	0.6	0.6	2.5	1.3	0.8
18:1(*n*-9)	3.5	2.2	0.8	0.7	1.0	3.4	13.1	2.7
18:1(*n*-7)	.	.	.	.	.	.	.	.
18:2(*n*-6)	0.9	1.0	0.4	1.1	0.5	1.0	8.3	1.8
18:3(*n*-6)	0.3	0.2	0.2	0.1	0.2	0.4	3.6	0.5
18:3(*n*-3)	0.3	0.4	0.3	0.3	0.2	.	0.5	0.1
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	.	.	2.7	.	.	.
20:3(*n*-6)	1.0	0.3	0.3	.	.	.	1.1	0.7
20:4(*n*-6)	4.8	5.0	2.0	1.0	4.2	17.0	5.2	4.0
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	25.2	43.3	65.3	39.0	25.2	13.3	12.4	31.0
Unidentified :	4.5	6.1	3.0	2.9	4.0	11.1	9.1	9.2
Total sats :	29.9	18.0	19.7	28.2	32.6	29.4	10.3	22.4
Total monos :	32.3	24.4	8.2	26.3	30.1	27.3	47.8	29.7
Total PUFA :	33.3	51.0	69.1	42.6	33.3	32.2	32.8	38.7
Total (*n*-9) :	3.5	2.2	0.8	0.7	1.0	3.4	13.1	2.7
Total (*n*-6) :	7.0	6.5	2.9	2.2	4.9	8.4	8.2	7.0
Total (*n*-3) :	25.5	43.7	65.6	39.3	25.4	13.3	12.9	31.1
(*n*-3)/(*n*-6) :	3.6	6.7	22.6	17.9	5.2	0.7	0.7	4.4
16:1/16:0 :	1.1	1.9	0.8	1.1	0.8	1.0	4.2	1.5
20:5/16:0 :	1.0	3.8	6.7	1.7	0.8	0.6	1.5	1.8
ΣC_{16}:	56.0	34.5	17.7	49.5	59.5	48.5	44.6	45.1
ΣC_{18}:	6.3	7.1	3.4	2.8	2.5	7.3	26.8	6.3
ΣC_{20}:	31.0	48.6	67.6	40.0	32.1	30.3	18.7	35.9

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, ., not detected
‡, including SQDG

TABLE 5.4.6: Individual lipid class FAME composition of *N. oculata* 12 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.9	1.7	7.2	1.0	1.4	1.6	1.0	3.2
15:0	0.4	0.5	0.4	0.5	0.4	0.6	0.3	0.3
16:0	31.8	15.5	8.2	23.3	26.4	24.2	9.8	19.3
16:1(*n*-7)	27.7	22.2	5.9	24.9	21.9	24.0	31.2	25.1
16:1(*n*-13)‡	.	.	.	.	5.0	.	.	.
16:2	0.4	.	0.7	0.7	0.3	.	1.1	0.4
17:0	0.3	.	0.1	.	0.1	.	0.1	.
16:3	0.3	.	0.2	0.3	0.3	.	0.6	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.4	5.8	1.0	0.8	1.0	2.5	1.8	0.9
18:1(*n*-9)	4.2	3.5	0.7	0.7	1.3	3.4	12.4	3.2
18:1(*n*-7)	.	.	.	.	.	.	.	0.4
18:2(*n*-6)	1.1	0.4	1.1	1.3	0.7	0.9	8.4	1.9
18:3(*n*-6)	0.3	.	.	0.1	0.3	0.6	3.4	0.6
18:3(*n*-3)	0.2	.	.	0.5	0.3	.	0.4	0.1
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	.	.	2.7	.	.	.
20:3(*n*-6)	1.1	.	0.2	.	0.5	.	1.2	0.9
20:4(*n*-6)	4.2	5.7	2.1	1.1	5.7	22.0	5.6	4.0
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	19.4	35.6	70.8	42.7	27.7	13.7	11.7	29.9
Unidentified :	3.3	9.1	1.4	2.1	4.0	6.2	11.1	9.4
Total sats :	37.8	23.5	16.9	25.6	29.3	28.9	12.9	23.9
Total monos :	31.9	25.7	6.6	25.6	28.2	27.7	43.6	28.7
Total PUFA :	27.0	41.7	75.1	46.7	38.5	37.2	32.4	38.0
Total (*n*-9) :	4.2	3.5	0.7	0.7	1.3	3.4	12.4	3.2
Total (*n*-6) :	6.7	6.1	3.4	2.5	7.2	23.5	18.6	7.4
Total (*n*-3) :	19.6	35.6	70.8	43.2	28.0	13.7	12.1	30.0
(*n*-3)/(*n*-6) :	2.9	5.8	20.8	17.3	3.9	0.6	0.7	4.1
16:1/16:0 :	0.9	1.4	0.7	1.1	0.8	1.0	3.2	1.3
20:5/16:0 :	0.6	2.3	8.6	1.8	1.0	0.6	1.2	1.5
ΣC_{16}:	60.2	37.7	15.0	49.2	53.9	48.2	42.7	45.9
ΣC_{18}:	7.2	9.7	2.8	3.4	3.6	7.4	26.4	7.1
ΣC_{20}:	24.7	41.3	73.1	43.8	36.6	45.7	18.5	35.0

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, ., not detected

‡, including SQDG

TABLE 5.4.7: Individual lipid class FAME composition of *N. oculata* 15 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.9	2.1	8.4	4.7	1.3	1.7	0.8	3.2
15:0	0.4	0.5	0.2	0.4	0.5	.	0.4	0.3
16:0	33.7	16.1	8.8	23.8	27.5	22.6	11.1	23.0
16:1(*n*-7)	29.6	20.9	5.6	23.9	20.1	20.3	30.1	25.4
16:1(*n*-13)*t*	.	.	.	.	5.8	.	.	.
16:2	0.3	0.2	.	0.6	.	.	1.0	0.3
17:0	0.2	0.4	.	.	0.3	.	0.2	.
16:3	0.2	0.3	0.4	0.2	0.2	.	0.4	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.3	4.5	3.4	0.8	1.2	5.6	1.7	1.0
18:1(*n*-9)	4.2	2.7	0.2	0.8	2.1	2.4	10.8	3.0
18:1(*n*-7)	.	.	.	.	.	.	.	0.4
18:2(*n*-6)	1.3	1.1	0.3	1.0	0.7	0.8	8.4	2.0
18:3(*n*-6)	0.4	0.6	0.1	0.1	0.7	.	4.0	0.7
18:3(*n*-3)	0.2	0.3	0.3	0.4	0.2	.	0.4	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	0.5	.	2.8	.	.	.
20:3(*n*-6)	1.4	0.5	0.1	.	0.4	0.3	1.6	1.0
20:4(*n*-6)	3.8	5.6	1.9	1.0	4.4	22.1	5.3	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	16.9	38.2	66.5	39.9	26.4	15.7	13.1	28.1
Unidentified :	2.2	6.0	3.3	2.3	5.8	6.5	10.7	6.4
Total sats :	39.5	23.6	20.8	29.7	30.4	31.9	14.2	27.6
Total monos :	33.8	23.6	5.8	24.7	28.0	22.7	40.9	29.6
Total PUFA :	24.5	46.8	70.1	43.3	35.8	38.9	34.2	36.4
Total (*n*-9) :	4.2	2.7	0.2	0.8	2.1	2.4	10.8	3.0
Total (*n*-6) :	6.9	7.8	2.4	2.1	6.2	23.2	9.3	7.5
Total (*n*-3) :	17.1	38.5	66.8	40.4	26.6	15.7	13.5	28.4
(*n*-3)/(*n*-6) :	2.5	4.9	27.8	19.2	4.3	0.7	0.7	3.8
16:1/16:0 :	0.9	1.3	0.6	1.0	0.7	0.7	2.7	1.1
20:5/16:0 :	0.5	2.4	7.6	1.7	1.0	0.7	1.2	1.2
ΣC_{16}:	63.8	37.5	14.8	48.5	53.6	42.9	41.7	49.7
ΣC_{18}:	7.4	9.2	4.3	3.1	4.9	8.8	25.3	7.3
ΣC_{20}:	22.1	44.3	49.0	40.9	34.0	38.1	20.0	33.1

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, „ not detected

‡, including SQDG

TABLE 5.4.8: Individual lipid class FAME composition of *N. oculata* 18 hours after transfer to a fresh batch of S88 medium modified to contain excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.6	2.8	9.9	5.2	1.6	2.8	1.1	3.4
15:0	0.4	0.5	0.3	0.4	0.4	2.7	3.3	0.3
16:0	33.8	16.7	8.5	23.7	28.2	33.4	12.0	24.2
16:1(*n*-7)	28.7	22.2	5.4	22.4	21.1	31.0	27.6	25.4
16:1(*n*-13)‡	.	.	.	.	5.9	.	.	.
16:2	0.5	.	.	0.6	.	.	1.2	0.4
17:0	0.3	0.3	.	.	0.3	.	.	.
16:3	0.3	0.3	0.7	0.3	0.2	.	0.5	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.3	3.8	2.4	0.7	1.2	3.1	1.6	0.8
18:1(*n*-9)	4.1	2.9	0.5	0.7	1.0	3.9	6.6	2.5
18:1(*n*-7)	.	.	.	.	.	.	.	0.4
18:2(*n*-6)	1.8	1.2	0.4	1.0	0.6	1.2	7.8	1.9
18:3(*n*-6)	0.5	0.4	0.2	0.1	0.4	.	4.9	0.8
18:3(*n*-3)	0.2	0.4	0.2	0.4	0.1	.	0.4	0.1
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	0.2
20:2	.	.	0.6	0.2	3.2	.	.	.
20:3(*n*-6)	1.6	0.5	.	.	0.5	.	2.0	1.2
20:4(*n*-6)	3.8	4.7	1.8	0.9	4.3	9.3	6.9	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	16.1	36.8	64.8	40.3	26.2	9.5	16.2	27.8
Unidentified :	3.0	6.4	4.3	3.1	4.8	4.1	7.9	5.8
Total sats :	39.4	24.1	21.1	30.0	31.7	42.0	18.0	28.9
Total monos :	32.8	25.1	5.9	23.1	28.0	34.9	34.2	29.1
Total PUFA :	24.8	44.4	68.7	43.8	35.5	19.0	39.9	36.2
Total (*n*-9) :	4.1	2.9	0.5	0.7	1.0	3.9	6.6	2.5
Total (*n*-6) :	7.7	6.8	2.4	2.0	5.8	9.4	1.6	7.7
Total (*n*-3) :	16.3	37.3	65.0	40.7	26.3	9.5	16.6	27.9
(*n*-3)/(*n*-6) :	2.1	5.5	27.1	20.4	4.5	1.0	0.8	3.6
16:1/16:0 :	0.8	1.3	0.6	0.9	0.7	0.9	2.3	1.0
20:5/16:0 :	0.5	2.2	7.6	1.7	0.9	0.3	1.4	1.1
ΣC_{16}:	63.3	39.2	14.6	47.0	55.4	64.4	41.3	51.0
ΣC_{18}:	7.9	8.7	3.7	2.9	3.3	8.2	21.3	6.5
ΣC_{20}:	21.5	42.0	67.2	41.4	34.2	18.8	25.1	33.0

Data expressed as a percentage of the individual lipid class FAME mass.
tr.,<0.1%, ., not detected

‡, including SQDG

(see Tables 5.4.2, 5.4.3 and 5.4.4). This point was also correlated with the sharp accumulation of TAG characteristic of the batch culture lag phase (Figure 5.4.1). The trend of decrease in the acyl chain length and unsaturation observed in TAG began to show in other classes at the 6 hour time point. This phenomenon pointed towards the rapid response time of the algal cells in terms of TAG biosynthesis. The effects of the changed environment required a longer time before being expressed in the acyl component of the polar lipid classes.

As mentioned briefly above, the proportions of the acyl components underwent considerable variation in a short time period. The distributions of both acyl chain length and unsaturation changed indicating a modification in the pathways of biosynthesis leading to the TAG component. These can be summarised at two levels; the increase in the proportion of TAG in the total lipid extract and the change in acyl distribution. The TAG component has not been implicated in the elongation and desaturation pathways for acyl moieties. Therefore, the alternative implication was that modifications had occurred in other pathways supplying the component fatty acids. From the pattern of change two putative conclusions were drawn. Firstly, that the elongation mechanism was 'switched off' resulting in an accumulation of C_{16} saturates and monounsaturates. Or secondly, that there was selective incorporation of the C_{16} acyl chain lengths into the TAG component. The latter explanation is the most likely, based upon the evidence obtained from higher plant studies. How this fits into the scheme of lipid class-specific elongation and desaturation pathways is by no means clear.

The acyl moieties in higher plant TAGs are derived from the same precursor as is required in polar lipid synthesis, namely phosphatidic acid *via* the Kennedy or Kornberg-Pricer pathway. The observed changes in TAG acyl composition were difficult to explain in terms of PC or MGDG being the substrates for the putative lipid-linked desaturation and elongation pathways (see Section 1). The primary reason for this was that TAG showed a rapid response to the environmental change whereas the phospholipid and galactolipid classes maintained a short term increase in proportions of PUFA. This may have reflected

the presence of a pool of intermediates specific to phosphoglyceride rather than triacylglycerol synthesis (see Gurr, 1980). However, data from radio-incorporation and turnover studies using higher plant seeds have shown that acyl accumulation in TAG is slow by comparison to that observed in the phospholipids and galactolipids (see Gurr, 1980). This indicated that the TAG component had a comparatively low turnover rate. In contrast, Roehm & Privett (1970) showed the rapid replacement of polyunsaturated molecular species in the early stages of TAG accumulation in soybeans.

If the data showing the changes in TAG acyl composition (see Tables 5.4.2 to 5.4.8) were correlated with those showing the changes in the proportion of TAG in the total lipid extracts, the accumulation of TAG could be accounted for by the increasing proportion of C_{16} acyl moieties. The C_{16} monounsaturated acids were accumulated proportionately as the quantitative increase progressed. Thus, in contrast with 16:0 and 20:5(*n*-3), 16:1 did not show a major change in proportion. The assumption based upon this data was that, during the lag-phase, 16:0 was incorporated into TAG in preference to 20:5(*n*-3). Whether a change in the overall rate of TAG synthesis occurred is open to debate. However, the evidence supports this as being the case.

Assuming that the doubling period for *N. oculata* was approximately 36 hours, to maintain an equilibrated lipid composition would require a similar doubling period for all lipid classes. However, for the preferential accumulation of a single lipid class an increase in its synthesis rate, either in absolute or in relative terms, is obviously required. If cell division ceased, as was the case in the lag-phase, one might predict one of two outcomes. 1) The quantity of fatty acid per cell would increase in proportion to the time period during which cell division was suspended. If the relative proportions of the lipid classes remained the same then one would surmise that lipid class synthesis was unaffected and the increase in lipid mass per cell was due to cessation of cell division and the consequent accumulation of biomass. Individual cells would simply become larger whilst their chemical composition remained essentially similar. 2) A possible alternative was that the rate of TAG synthesis

was unaffected, or more probably increased. The remaining structurally important lipid classes had undergone a decrease in synthesis rate. This was more likely to be an effect of the cessation of cell division rather than its cause. The data does not allow a firm conclusion to be drawn regarding changes in the overall rate of fatty acyl synthesis. Therefore it is not possible to comment upon whether this remained constant or otherwise.

The latter of the scenarios described above may be used to explain the experimental data obtained from several levels of analysis. This also provides a framework within which one can express the potential implications of the inter-relationships amongst acyl flux, the biosynthetic pathways of the lipid classes and the growth kinetics of the culture considered as a whole and as individual cells. A reduction in the rate of *de novo* synthesis of the polar lipid classes possibly precipitated an increase in the flux of diacylglycerol moieties from the common Kornberg-Pricer pathway into the committed step of the TAG biosynthetic pathway. However, it is suggested that there is a link between the structural complex lipid classes and TAG. This was envisaged as taking the form of a positionally specific acyl exchange *via* an acyltransferase following biosynthesis, possibly through a common free fatty acid pool.

TAG extracted from *N. oculata* showed a more saturated, shorter chain length acyl composition than those of the terrestrial seed oils commonly used as model systems (*e.g.* sunflower, safflower & soybean). This may demonstrate that the higher plants and algae show a different bias in the pathways *via* which the acyl moieties of TAG are derived. Much of the data pertaining to higher plants stresses the involvement of fatty acyl exchange between TAG and both the phosphoglycerides and glycosylglycerides. Since these classes are also implicated in the desaturation pathways and probably in the acyl elongation pathways it seems obvious that acyl moieties from these sources should show some similarity.

In this case the present results support the operation of such a phenomenon. Figure 5.4.2 illustrates the significant points of interaction. The two situations represented show the

proposed changes in the fluxes of fatty acids between TAG and the polar lipid classes. The width of the arrows is a rough guide to the magnitude of the fluxes. For the purposes of the hypothesis it is assumed that the rate of acyl synthesis is similar but it is the relative fluxes through the committed steps of each pathway that are important. *De novo* synthesis yielded 16:0 which was desaturated to 16:1 *prior* to the committed steps - thus accounting for the constancy of its proportion in the TAG component. All further elongation and desaturation steps are attributed to the right hand pathway *via* complex lipid-linked desaturation and elongation. Diacylglycerols entering the left hand pathway are incorporated into TAG without further modification.

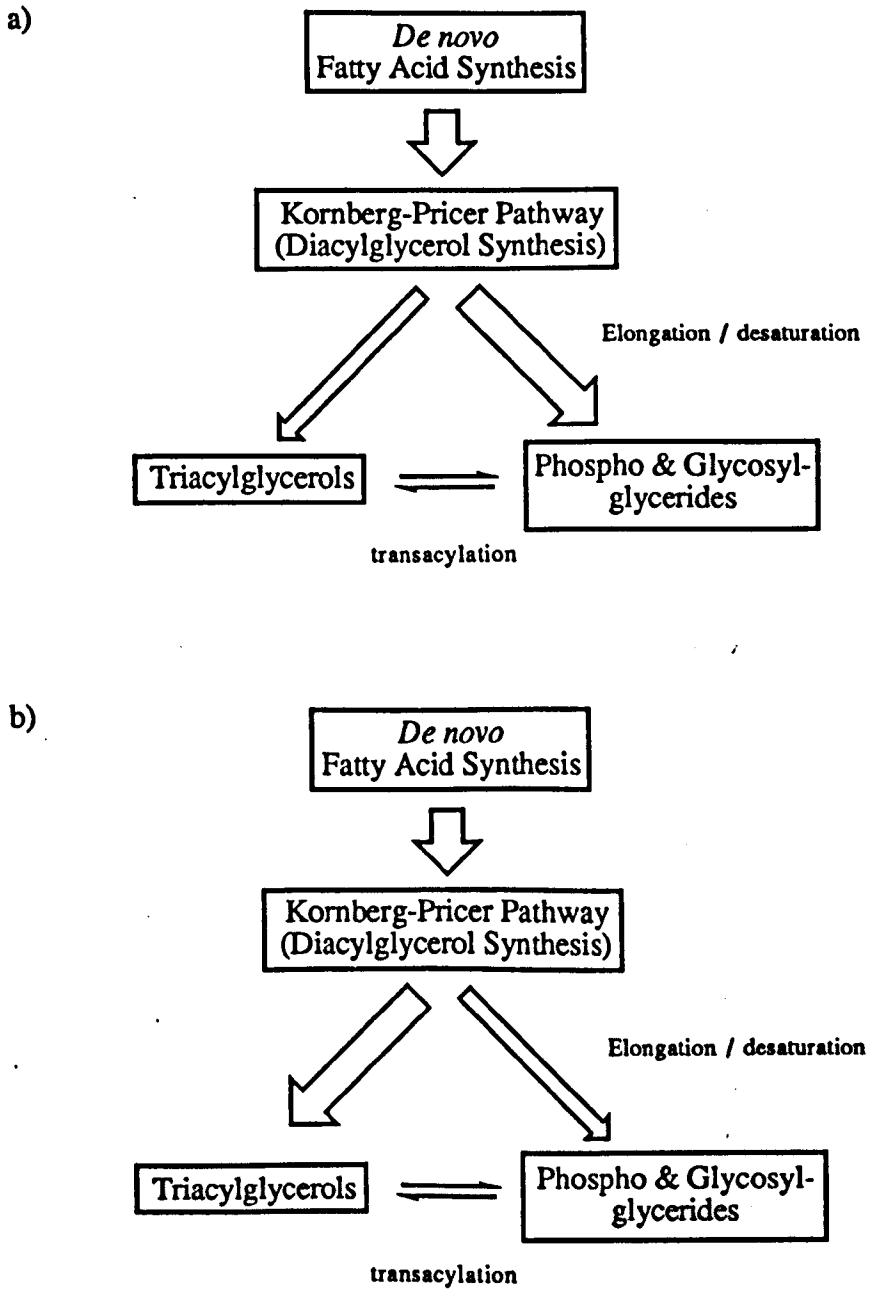


Figure 5.4.2 : A general scheme illustrating the possible changes occurring in the partitioning of fatty acids between : a) the exponential phase of growth and b) the lag-phase of growth[1].

In the exponential phase of growth the major acyl flux is into the right hand path. Along this path the C_{16} acids are elongated and desaturated further to yield up to 20:5(*n*-3). Acyl turnover between TAG and polar lipid classes results in a net flux of unsaturated fatty acids into TAG where they are accumulated due to its low turnover rate. Because TAG synthesis is occurring at a relatively low rate when compared to the complex lipid classes, the flux of unsaturated acids from the right to the left hand side of Figure 5.4.2.a. is quantitatively comparable with the contribution from the Kornberg-Pricer pathway. Consequentially TAG decreases as a proportion of the total lipids and shows a high proportion of 20:5(*n*-3) as do the complex lipid classes. When the cells enter the lag-phase fatty acyl synthesis continues but the partitioning of diacylglycerol flux from the Kornberg-Pricer pathway is shifted from left to right as shown in Figure 5.4.2.b.. The result of this is an increase in the TAG synthesis rate and a net flux of C_{16} saturates and monounsaturates into this component. As a consequence the relative proportion of 20:5(*n*-3), originating from the right hand branch as a consequence of acyl transfer is reduced. The overall effect is an increased proportion of more saturated, shorter chain length TAG. The change does not affect the polar lipid classes immediately due to the time required for the acyl flux between the neutral and polar lipid pools to equilibrate.

The FFA pool seemingly acts as an intermediary buffer in the equilibration stage. Evidence in support of this can be found in Tables 5.4.2 to 5.4.8. During the exponential phase of growth the FFA component is highly unsaturated and shows statistically high compositional correlation with all other lipid classes. This indicates that the other lipid classes are derived from it, or that FFA is derived from these classes. The FFA show an initially high proportion of 20:5(*n*-3) at 90 min post-inoculation, after 3 to 6 hours this was reduced slightly with increases in the proportions of both C_{16} and C_{18} monounsaturates. When the proportion of 20:5(*n*-3) in FFA had fallen to *ca.* 35 % the changes in this acid's proportion in the polar lipid classes became marked. The proportion of 20:5(*n*-3) in the galactolipids began to fall and their proportion of saturates rose slightly.

Phosphatidylcholine showed an initial, short term decrease in its proportion of 16:0 and an increase in its proportion of 16:1 over the first three hours post inoculation. After 6 hours post-inoculation the trend had reversed with the proportion of 16:0 rising from 6.5 % to 12.0 % and that of 16:1 falling from 39.1 % to 27.6 % at the end of the culture period (*i.e.* 18 hours post-inoculation). Variation in 20:5(*n*-3) was more complex but limited by comparison to C_{16} and C_{18} fatty acids. Its proportions rose slightly from 12.5 % to 13.4 % during the first 6 hours and then fell to 11.7 % during the period 6 to 12 hours post-inoculation before finally rising again to 16.2 % after 18 hours. These fluctuations are reflected in the data yielded by HPTLC-densitometry (see Figure 5.4.1). The densitometry data indicated that an increase in the proportion of PC occurred between 12 and 18 hours post-inoculation. This was consistent with the increase in the proportion of 20:5(*n*-3) during the same time period.

These events may be explained in terms of the model proposed above and summarised in Figure 5.4.2. By turning attention away from the three quantitatively major fatty acids a further pattern begins to emerge. During the first 9 hours following inoculation the proportion of 16:0 in PC increased slightly. At the same time the proportion of 16:1 fell and that of 18:1 rose by a comparable proportion (*ca.* 3 %). However, the proportions of the (*n*-6) series fatty acids 18:2, 18:3, 20:3 and 20:4 increased during the period 90 min to 9 hours post-inoculation. During this period the proportion of PC had undergone a slight decrease indicating a lack of net synthesis of PC. Such a decrease was arguably a relative effect due to the increasing proportions of TAG. After 12 hours the C_{18} monounsaturated fatty acids showed a decrease in proportion whereas the (*n*-6) fatty acids showed increased proportions, as did 20:5(*n*-3). Correlated with the increase in polyunsaturation were increases in the proportions of saturated fatty acids and that of PC (even though TAG was *still* increasing in proportion !). This suggested that the lag-phase was ending and the observations made between 12 and 18 hours were of a transition period when elongation and desaturation resumed in PC and the proportions of fatty acids were beginning to re-equilibrate. Increased proportions of 16:0 observed in PC during this period also suggested

that the flux of fatty acids synthesised *de novo* was beginning to re-enter the right hand pathway of Figure 5.4.2, the acyl partitioning becoming more characteristic of exponential-phase growth. The proportions of the fatty acids in the TAG component also stabilised at this time, again indicating a transition away from the flux of saturates and monounsaturates into this class.

SECTION 6: VARIATION IN LIPID COMPOSITION AS A CONSEQUENCE OF GROWTH PHASE INDEPENDENT EFFECTERS IN BATCH CULTURE.

6.1 INTRODUCTION.

In the previous sub-sections (Sections 5.2, 5.3 & 5.4) the major concern was with the patterns of variation observed in the lipid extract of the alga *N. oculata* grown in batch culture. The findings presented in these sections defined characteristic patterns of variation in both growth and lipid composition. These occurred as a result of the growth of the cells in culture and the consequent changes in the chemistry of the surrounding culture medium (*e.g.* nitrate depletion) and the physical environment of the cells (as a consequence of self shading). The logical progression from this point was to conduct a comprehensive comparative study of the effects of the factors which varied least during the growth of the cells in cultures. In this way the effects of variation in the tested environmental factor were observed being superimposed upon the characteristic batch culture variation pattern of each culture as it progressed through the phases of growth.

The obvious first choice for a test variable was temperature. Being a physical variable this was unaffected by the growth phase of the culture and was well suited to test using a batch culture strategy for the reasons discussed previously in Section 4.1. It has been suggested in the literature that culture incubation temperature may have a significant effect upon the lipids of algae, as well as those from other sources such as higher plants and animals, especially fishes (see Section 2). The effect of decreasing culture incubation temperature, within physiological boundaries, is reported to increase both the proportion of PUFA and their mean acyl chain length. One theoretical possibility for this is the effects that these two parameters have upon the phase transition temperature, and therefore fluidity at a given temperature, of the biomembranes of which the fatty acids are components (Lewis, 1962; Marr & Ingerham, 1962; Brown & Rose, 1963; Knipprath & Mead, 1966).

Nannochloropsis oculata provided an unusual case for study due to its high proportion of 20:5(*n*-3) at a temperature of 20 °C. This is well above the mean seawater temperature around the strain's isolation site on the West Coast of Scotland. However, because this particular strain of *N. oculata* was isolated from a low volume (*ca.* 10 litres) supra-littoral pool on the Isle of Cumbrae one would expect it to have been subjected to a range of temperatures wider than that of the local seawater. During the summer months when *N. oculata* was a dominant species in the pool the water temperature must have been relatively high, possibly approaching equilibrium with ambient air temperature at *ca.* 20 °C. Therefore the S.M.B.A. strain of this alga must not be regarded as typical of wild, mid-latitude oceanic phytoplankton populations. Having been maintained in laboratory culture under the same conditions for *ca.* 36 years a high degree of selection may also have occurred. Quite how this accounts for the high proportions of PUFA present at what is a high temperature for the temperate marine environment remains open to conjecture. It seems unlikely that the purpose of such a high proportion of C_{20} PUFA in this particular case should be to increase membrane fluidity.

In continuation of the theme for in-depth investigation of the factors least affected by the growth phase of the cultures, salinity (or more accurately sodium chloride concentration) was chosen as an additional factor for investigation. The reasons for this choice were several-fold, and are outlined as follows. Firstly, and perhaps most importantly for this series of experiments, was the amenability of this variable to controlled manipulation. A batch of S88 medium slurry was made up (as per Section 4) without the addition of the major bulk ingredient NaCl (see Appendix D). Crude manipulation of 'salinity' through the addition or omission of NaCl was possible at the rehydration stage. Although NaCl concentration is strictly speaking only one factor amongst several responsible for determining true salinity (see Harvey, 1957; Riley & Chester, 1971), it is the major contributor. For the purposes of the present study, however, the use of grammes of NaCl per litre sufficed, and at the same time eliminated the requirement for a definition of salinity with respect to what was, after all, a wholly artificial growth medium. Modifying the NaCl

concentration in proportion to the original S88 recipe therefore gave approximate salinities only.

Secondly, although NaCl concentration is a chemical variable it is nutritionally unimportant to the algae in that they do not accumulate it to any great extent as part of the particulate biomass of the culture. Therefore NaCl concentration in the culture medium remained stable during the experiments and did not decline to become a growth limiting component. This is in contrast to nutritionally important molecules such as nitrate and phosphate which were abstracted from solution and incorporated into the biomass of the growing cells.

Thirdly, salinity variation was particularly relevant with respect to the natural habitat of wild populations of *N. oculata*. As was mentioned briefly above, the original isolation site of the S.M.B.A. strain of *N. oculata* used in this series of experiments was a supra-littoral pool at Skate Point on the Isle of Cumbrae off the West Coast of Scotland (Droop, 1955; Thompson *et al.*, 1988). Pools such as those on the high shore are dynamic environments by comparison to those tidal pools on the lower shore which are regularly inundated by seawater. The supra-littoral pools are subject to wide variation in both salinity and alkalinity as well as temperature. One would, therefore, expect that any algal species surviving in them would be tolerant to the range of environmental conditions encountered. The pools of the supra-littoral zone, that is to say the zone on the shore above the mean high water mark, are formed by a combination of freshwater from precipitation and seawater spray. Those familiar with the climate of Western Scotland will realise that the bias in such an environment is from precipitation. Direct freshwater input from rainfall and run off water from the surrounding bare basalt caused dilution of the seawater resulting in small brackish pools (*ca.* 10 litres in volume in the Skate Point locale, as described by Droop, 1955) of relatively high temperature (*i.e.* above ambient seawater in the spring and summer months). Droop (1955) recorded the salinities of the pools in which *N. oculata* was a dominant species to be in the range 0.4 ‰ to 25 ‰. The range of salinities tolerated in laboratory culture was found to be

between 2 ‰ and 54 ‰ but maximal growth was sustained in the salinity range 4 ‰ to 36 ‰ (approximating to a salinity range between 1/9 th that of normal seawater and normal seawater itself).

In the following series of experiments the basic protocol established for time course experiments was maintained (see Figure 5.3.1). Because of the high numbers of low biomass and low lipid yield samples generated, detailed analyses of individual lipid classes over the complete time course was impossible. As an alternative, and to allow comparisons to be drawn between different culture temperatures, several 'equivalence points' were designated for each respective culture's growth curve. Detailed analyses for inter-culture comparison were conducted at samples falling closest to these points. The data were also subject to several transformations to allow direct graphical comparison of their growth in terms of both cell numbers and biomass per unit culture volume over the time course.

6.2 VARIATIONS IN LIPID COMPOSITION WITH TEMPERATURE IN BATCH CULTURE.

6.2.1 Experimental.

Cultures of *N. oculata* were grown as detailed previously (see Section 5.2.2) using S88 culture medium modified to provide excess nitrate and phosphate as described in Section 5.3.2. The specific growth conditions were as follows: S88 plus excess NO_3^- & PO_4^{2-}, pH adjusted to 8.0 using 2 M NaOH and illumination of 70 $\mu E\ m^{-2}\ sec^{-1}$. Temperature was varied between 25 and 5 °C in 5 degree intervals. The volume of the culture vessels was 2000 ml in all cases except that of the culture grown at 5 °C, this culture requiring the maximum 5000 ml vessel to compensate for the low growth rate of *N. oculata* at this temperature.

The culture vessels (see Section 4.2.3), equilibrated at the appropriate experimental temperature overnight, were inoculated aseptically to an initial cell density *ca.* 1.5×10^6 cells ml^{-1}. The vessels were then transferred to a continuously illuminated, thermostatically controlled incubator (LMS Ltd., Sevenoaks, Kent) maintained at experimental temperature. Cultures were aerated using an external diaphragm pump (Rena) *via* the sparging port in the wall of the incubator. At experimental culture temperatures lower than the external ambient temperature it was necessary to dry the air by passing it through a column of silica gel (coarse, self indicating, Fisons Ltd.). This prevented condensation formation within the airlines which otherwise caused wetting of the filters and resulted in stoppage of airflow to the cultures.

Cell counts were conducted on a daily basis and the mean values of 6 full field replicate counts were plotted as the growth curves for the cultures. Sampling was conducted as previously described in Figure 5.3.1. The lipid was extracted and the total FAME analysed for every sample. The extracts were also analysed using single dimensional double development HPTLC densitometry. Having plotted the growth curves for the cultures in the series it proved possible to identify several cross-representative sampling points. These were

marked on the growth curves of the cultures by the horizontal lines linking points where the cultures had reached equivalent stages of growth when expressed in terms of cell division (see Figure 6.2.1). Due to the increased quantities of total lipid and, therefore, cell material required for complete analysis, the 'equivalence points' were by necessity in the upper regions of each growth curve. This also ensured that the cultures were in the exponential phase of growth when sampled. The individual lipid classes of the total lipid extracts from the samples falling close to the equivalence lines were analysed with respect to their FAME compositions after separation. Apart from adding greater detail to the analytical data, these analyses also allowed inter-culture comparison between the lipid class FAME compositions as a test for culture environment effects at the alternative level of acyl composition.

6.2.2 Results & Discussion.

The growth kinetics data for the cultures grown at the five experimental temperatures were summarised in several presentation formats in Figures 6.2.1, 6.2.2, 6.2.3, 6.2.4 & 6.2.5. Figure 6.2.1 displays the classical semi-log plot of the cell numbers per unit culture volume at the various sampling points for all five cultures *i.e.* culture growth expressed in terms of cell division. When the five curves were compared several features were noted regarding the effects of incubation temperature upon cell division kinetics in batch culture. In the temperature range between 25 °C and 15 °C, which encompassed the optimal growth temperature, the variation in cell division rate was low. In comparison, at temperatures below 15 °C the variation of growth rate with temperature became much more distinct. This was evident from the decreasing gradients of the exponential phase portions of the cultures growth curves. Another general pattern which emerged was the effect of decreasing temperature upon the duration of the lag phases of the cultures. At temperatures 25 °C to 20 °C the lag phases were so short as to be almost indiscernible. However, with decreasing temperature the lag phase duration period increased from *ca.* 48 hours at 15 °C to *ca.* 200 hours at 5 °C.

When the data for biomass accumulation were plotted (see Figure 6.2.2) the lag phases became much less evident. The semi-log plot showed a linear trend of biomass

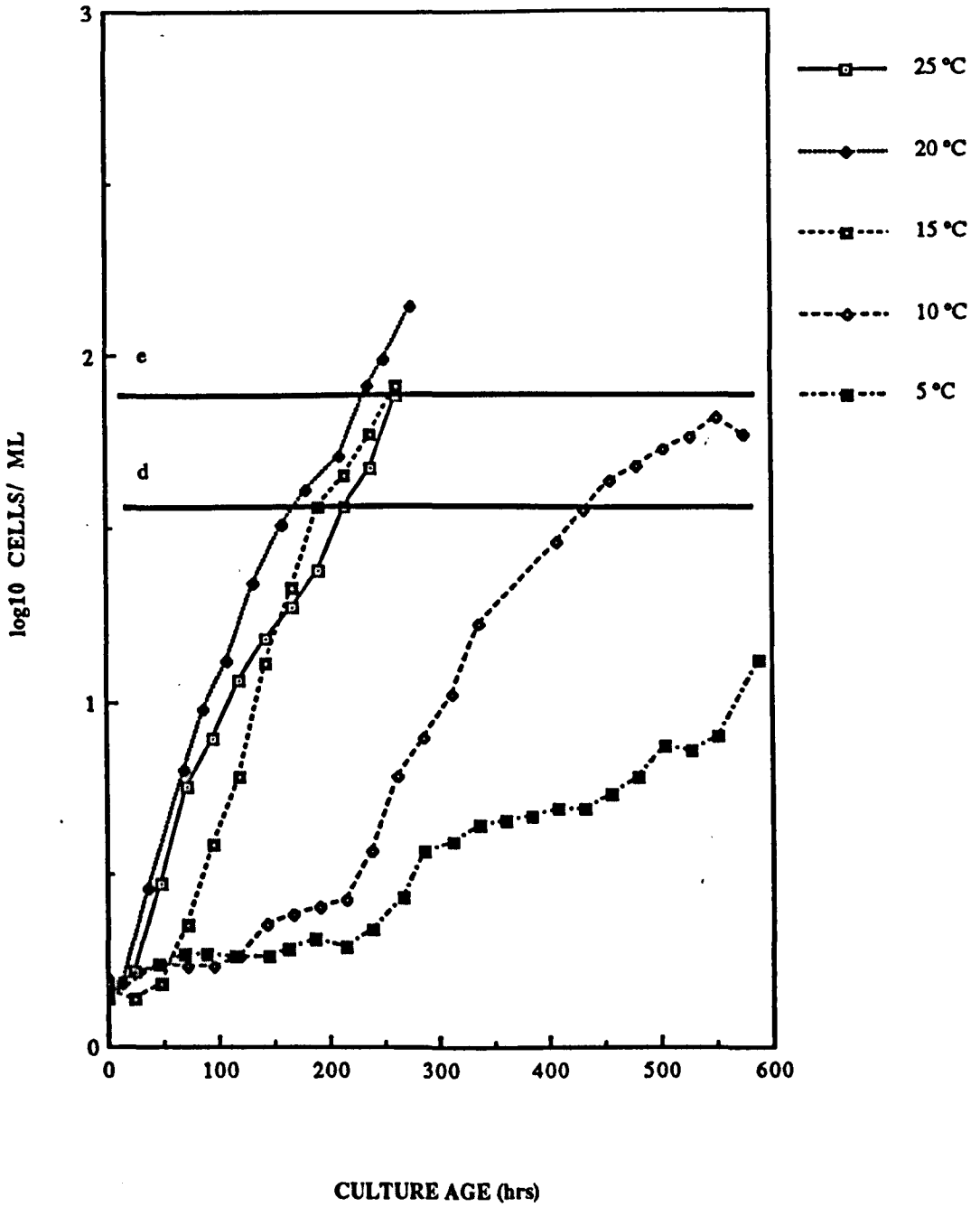


Figure 6.2.1 : Growth curves of *N. oculata* over the temperature range 25 °C to 5 °C. The lines 'd' and 'e' mark the equivalence points detailed in the text[1].

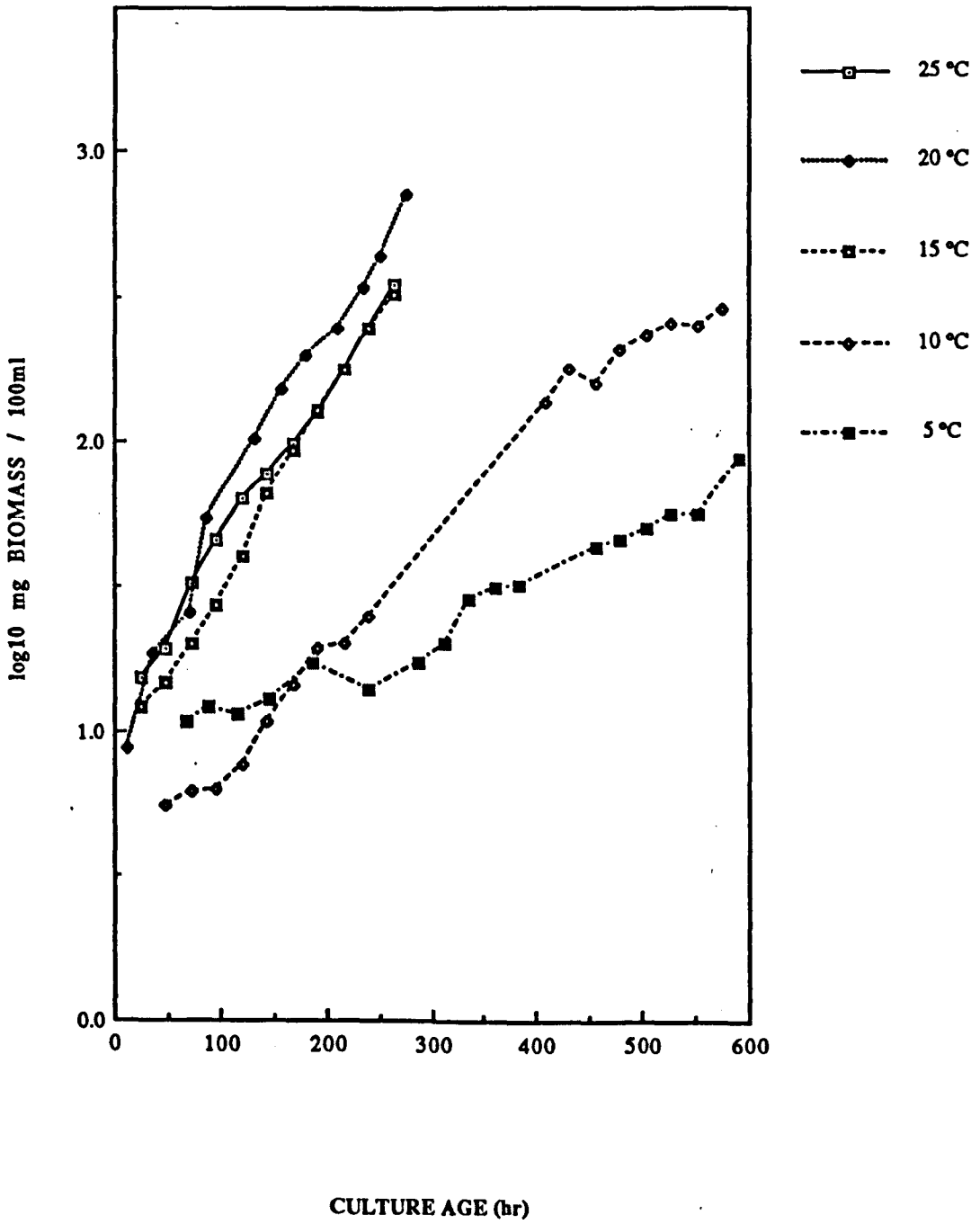


Figure 6.2.2 : Growth curves of *N. oculata* over the temperature range 25 °C to 5 °C. Data expressed in terms of biomass[1].

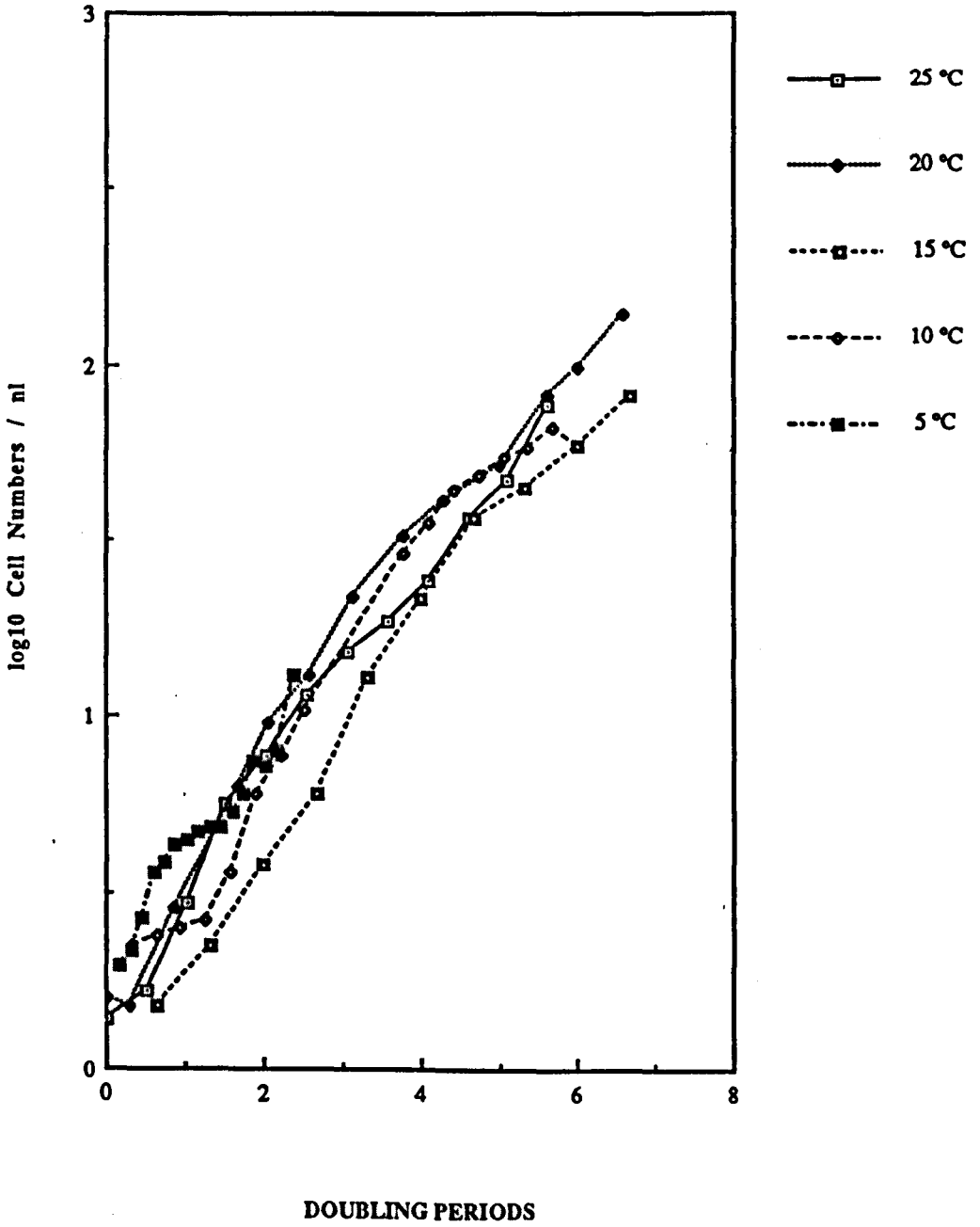


Figure 6.2.3 : Transformation of culture age data into doubling periods. (see text for details)¶.

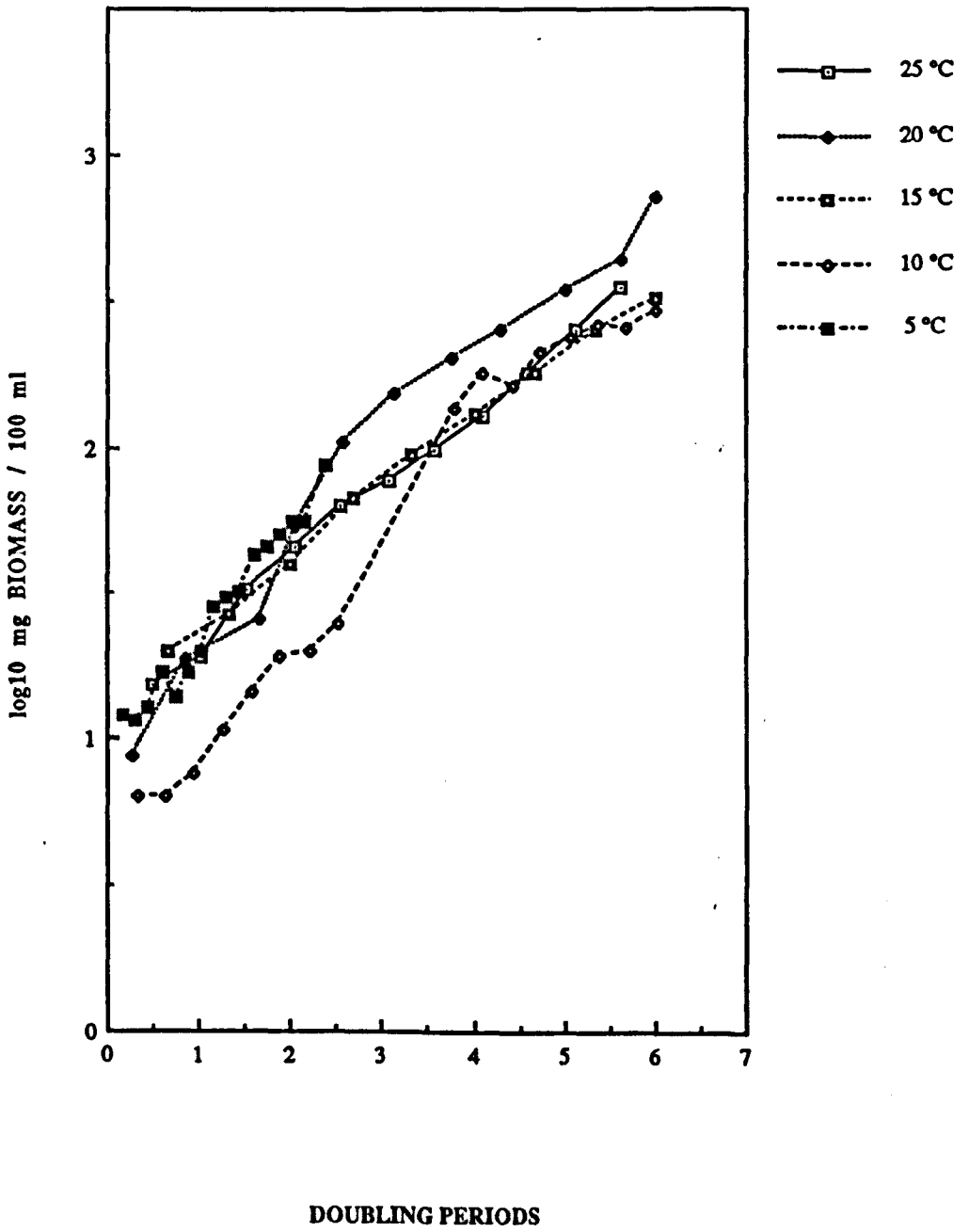


Figure 6.2.4 : Graph showing the growth curves expressed in terms of biomass following transformation to account for the differential growth rates of *N. oculata* grown in the temperature range 25 °C to 5 °C[1].

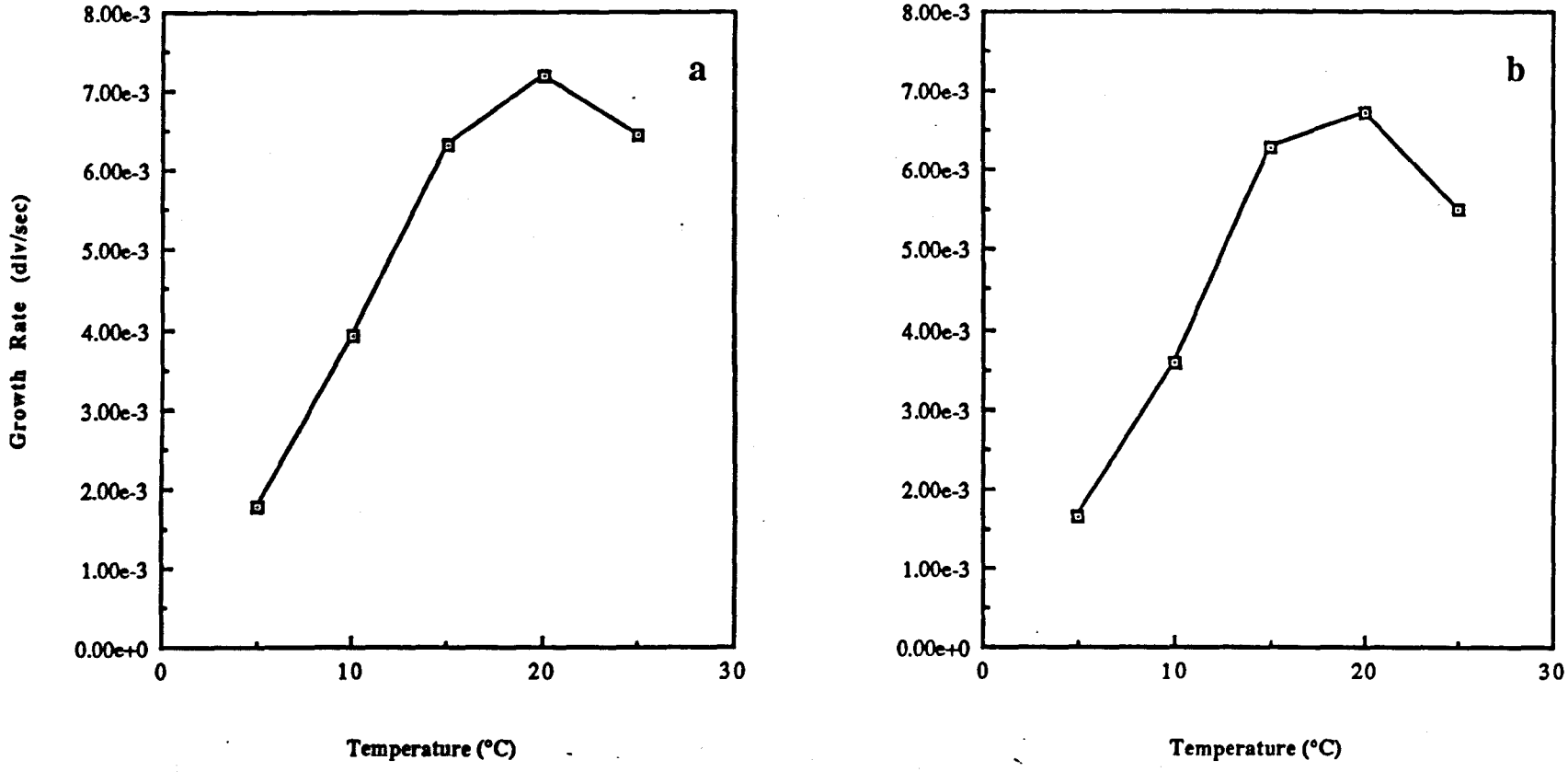


Figure 6.2.5 : The temperature dependent changes in the growth rate of *N. oculata* in batch culture in the temperature range 25 °C to 5 °C. Data expressed in terms of a) cell numbers or, b) biomass[1].

accumulation from the time of inoculation onwards. This was significant in that the growth rate measured in these terms was closer to a measure of the 'instantaneous growth rate' of the culture.

$$K = \frac{\log Nt - \log No}{t}$$

Where No = cell numbers at inoculation, Nt = final cell numbers, t = incubation period.

The growth rate measured solely during the exponential phase was more subjective, due to its dependence upon the accuracy of judgment involved in the determination of the beginning of the exponential phase. These findings were in agreement with observations recorded in Sections 5.2 & 5.3. Although the lag phase was negligible on all the biomass plots, the grouping of the curves still remained similar to those of the cell number plots. The curves representing the cultures grown at 25 °C to 15 °C were closely spaced, having similar gradients, whereas those of 10 °C & 5 °C were of much shallower gradients and more widely spaced. Both these measures of the cultures growth rates showed non-linear relationships between temperature and growth rate over the measured range (see Figure 6.2.5).

Figure 6.2.3 presents data which was transformed to support the previous statements. The lag phases, when present, were eliminated and timing was rescaled, the estimated start of the exponential phase being set as time zero. The amended data were transformed into doubling period equivalents using values for the specific growth rates calculated from the gradients of the growth curves in each of the cultures' respective exponential phase. A measure of the accuracy of estimation of the lag phase lengths and growth rates is given by the coincidence of the lines from different cultures. A similar exercise conducted using the biomass data (see Figure 6.2.4) does not show as close a relationship but does approximate to the previous Figure 6.2.3. The spread of variation amongst the data reflected that which originated in the harvesting and washing stages of sample concentration. Inevitably, this influenced the estimation of individual cell mass which

was used later in this section (see Figure 6.2.7). An important consideration at this point was that biomass accumulation over a cellular division period was relatively consistent. Only the absolute rate of accumulation, and not its magnitude, varied with temperature. The temperature dependence of the two measures of growth rate is summarised in Figure 6.2.5. This figure illustrates the difference between the two, each curve displaying a slightly different optimal growth rate.

The variation in the mass yield of lipid per cell at each temperature is shown in Figure 6.2.6. Because this figure only displays the period from the start of cell division, and not the preceding lag-phase, the true extent of lipid accumulation is not obvious for the cultures grown at 10 °C and 5 °C. The higher growth rate cultures (25 °C to 15 °C) showed a rapid peak in cellular lipid content, the first measurement being at the head of an exponentially declining trend in all three cases. The culture grown at 10 °C showed the characteristic pattern elucidated in the earlier Sections 5.2 & 5.3, but spread over a longer period of time. The trend of increasing lipid yield per cell at 10 °C progressed into the culture's division phase and only began to decline after the completion of the first doubling period. A similar observation was predicted for the culture grown at 5 °C. Although the slow growth rate of the cells at this temperature did not allow this to be fully confirmed, a very general decline in the mass of lipid yielded per cell may be noted after *ca.* 1.5 doubling periods (see Figure 6.2.6). The differences in the lipid yield per cell observed between the high and low growth rate cultures (25 °C to 15 °C and 10 °C to 5 °C respectively) may be attributed, in part, to the necessity for the expression of data in terms of the *average* cell. Clearly, in a non-synchronous culture there are cells representative of all growth stages in the cell cycle. It is apparent that cellular division requires a short time period by comparison to that required for the accumulation of biomass prior to cellular division. We propose that at higher incubation temperatures the cells undergo a form of synchronisation induced by inoculation into a new batch of culture medium.

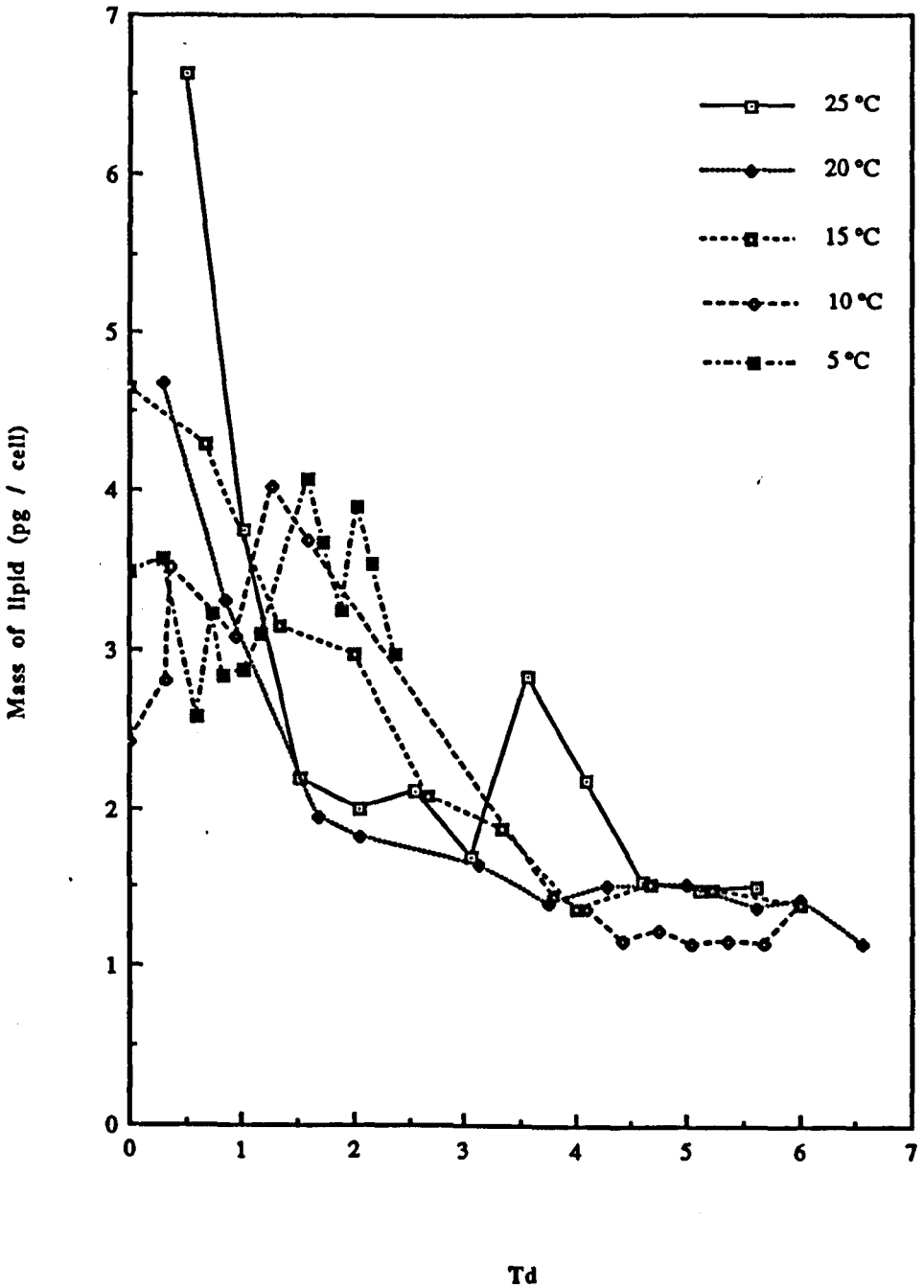
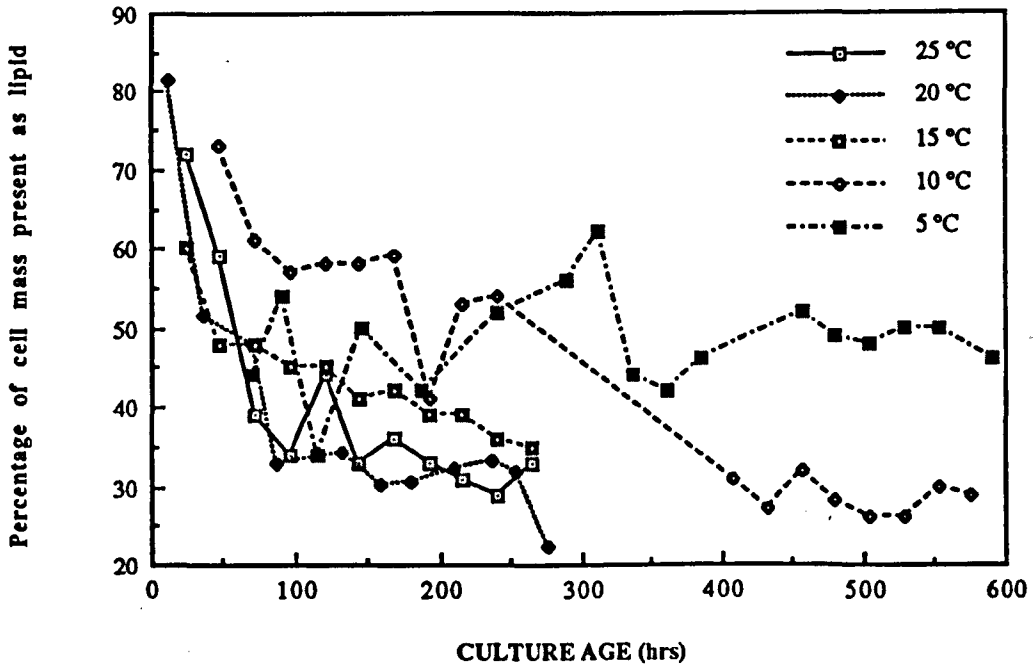


Figure 6.2.6 : The changes in the mass of lipid yielded by the cells of *N. oculata* grown in the temperature range 25 °C to 5 °C plotted against the respective doubling period (T_d) for each culture.

The cells increase in mass rapidly during a short 'lag-phase' and then divide, in effect simultaneously as a consequence of the frequency of observations. Because the frequency of sampling was similar to the doubling period for the cells at this temperature, the high growth rate cultures were only occasionally sampled more than once per division cycle. In contrast the cultures incubated at lower temperatures were sampled several times per division cycle. This was particularly true for the culture grown at 5 °C which was sampled 7 to 8 times per division cycle due to its long doubling period. With a sampling frequency many times that of the division frequency the effects of the cell cycle were superimposed on to those of the culture cycle, therefore, more low level peaks and troughs in the cell and lipid masses were observed. Then one begins to obtain results similar to those presented earlier, in Section 5. Had it been possible to measure cell volume the mass fluctuations may also have showed as differences in the size distribution of the cells. From the data we would predict a greater spread of mass over the potential range (*i.e. ca.* 4 to 10 pg cell^{-1}) with decreasing temperature. The low temperature cultures would contrast with those at higher temperature, particularly during the lag-phase when the majority of the cells attained their peak mass during a short time period.

Figure 6.2.7 illustrates the differences between two forms of expression of the data pertaining to the variation in the mass of lipid yielded as a percentage of the *average* cell mass. Figure 6.2.7.a shows the data plotted against time and apparently indicates an obviously higher proportion of total lipid for both the low temperature cultures. When the same data are expressed in terms of cell division (Figure 6.2.7.b) one can see that in actual fact the percentage of the cell mass present as lipid was similar at equivalent points on the horizontal axis once the cells had undergone a single cell division. When the cells had undergone between five and six division cycles the difference in the proportion of the individual cell mass as lipid was even less accentuated. Similarly in the case of the absolute mass of lipid per cell (Figure 6.2.6) the greatest variation occurred during the early stages of cell division. Once the cells had undergone five to six divisions they yielded similar masses of lipid regardless of culture temperature.

a



b

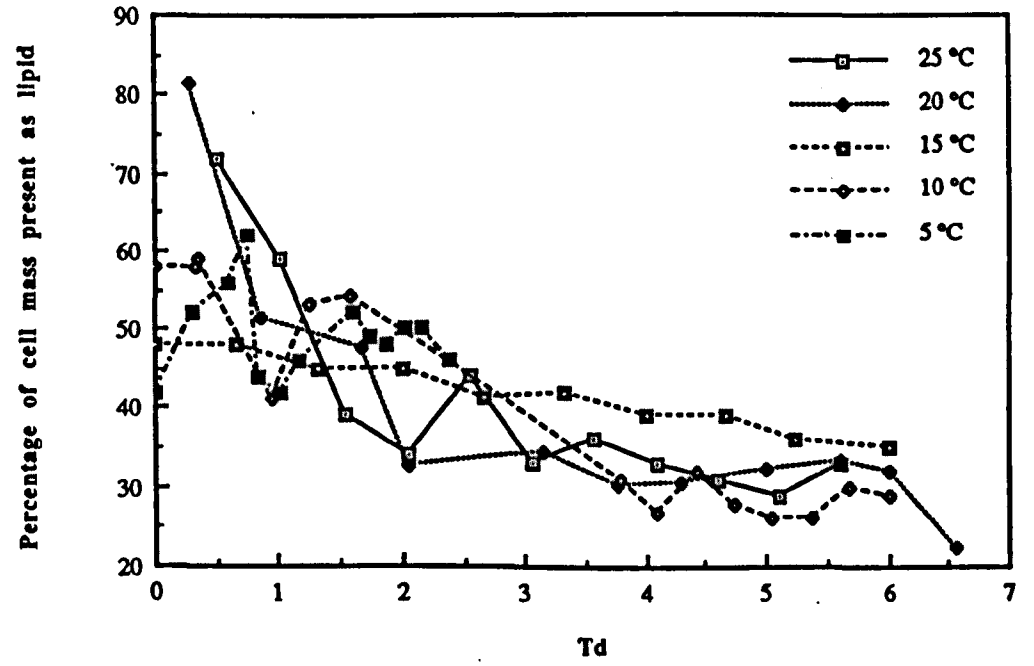


Figure 6.2.7 : The variation in the percentage of cell mass present as total lipid extract for *N. oculata* grown in the temperature range 25 °C to 5 °C. Data expressed on a time-scale of a) Hours, and b) Doubling periods[1].

In summary the cells grown at different temperature showed the greatest inter-culture variation in the early stages of the cultures lifespan, *i.e.* during the first two doubling periods. When the cells had entered the exponential phase proper, after *at least* five doubling periods, they could be seen to be almost equivalent in their mass yield of total lipid extract in both percentage and absolute terms (Figures 6.2.6 & 6.2.7). This suggested that the effect of temperature was one which modified the *rate* of events for individual cells. The use of non-synchronous cultures in this case meant that the data obtained from a sample series was compounded by the proportions of the cells within a culture at the various different points in the cell cycle. This was particularly noticeable during the early stages of the culture lifespan when division halted resulting in a lag-phase of temperature dependent duration.

The separation of total lipid into its component classes showed a pattern of variation similar to that recorded in Sections 5.3 and 5.4. The time scale of the changes was again modified by both temperature and the increases made in the culture medium's nitrate and phosphate concentrations. These nutrients may otherwise have become limiting. In the case of the cultures included in this section the addition of extra nitrate and phosphate delayed the onset of the stationary phase. However, it was not possible to monitor the medium nitrate concentration in this series of experiments and so, correlations of growth phase events were based upon assumptions derived from observations originating from the data in Section 5.3.

Figures 6.2.8 to 6.2.12 show data derived from double development TLC densitometric analyses (Section 3.2.2 & Appendix C). Because of the limitations of the TLC-photodensitometry technique, a quantitative comparison between the data sets was not realistically possible. Within each of the data sets any changes in the percentage values for the lipid classes may be interpreted as being representative of the general trends displayed by each of the respective lipid classes. These were very similar across the range of incubation temperatures between 25 ^{o}C and 10 ^{o}C. The growth rates of the cultures were the most significant factor in determining the differences in the lipid composition at a given time point. The observations detailed above still hold true for the individual lipid class profiles of the total lipid extracts from each of the cultures.

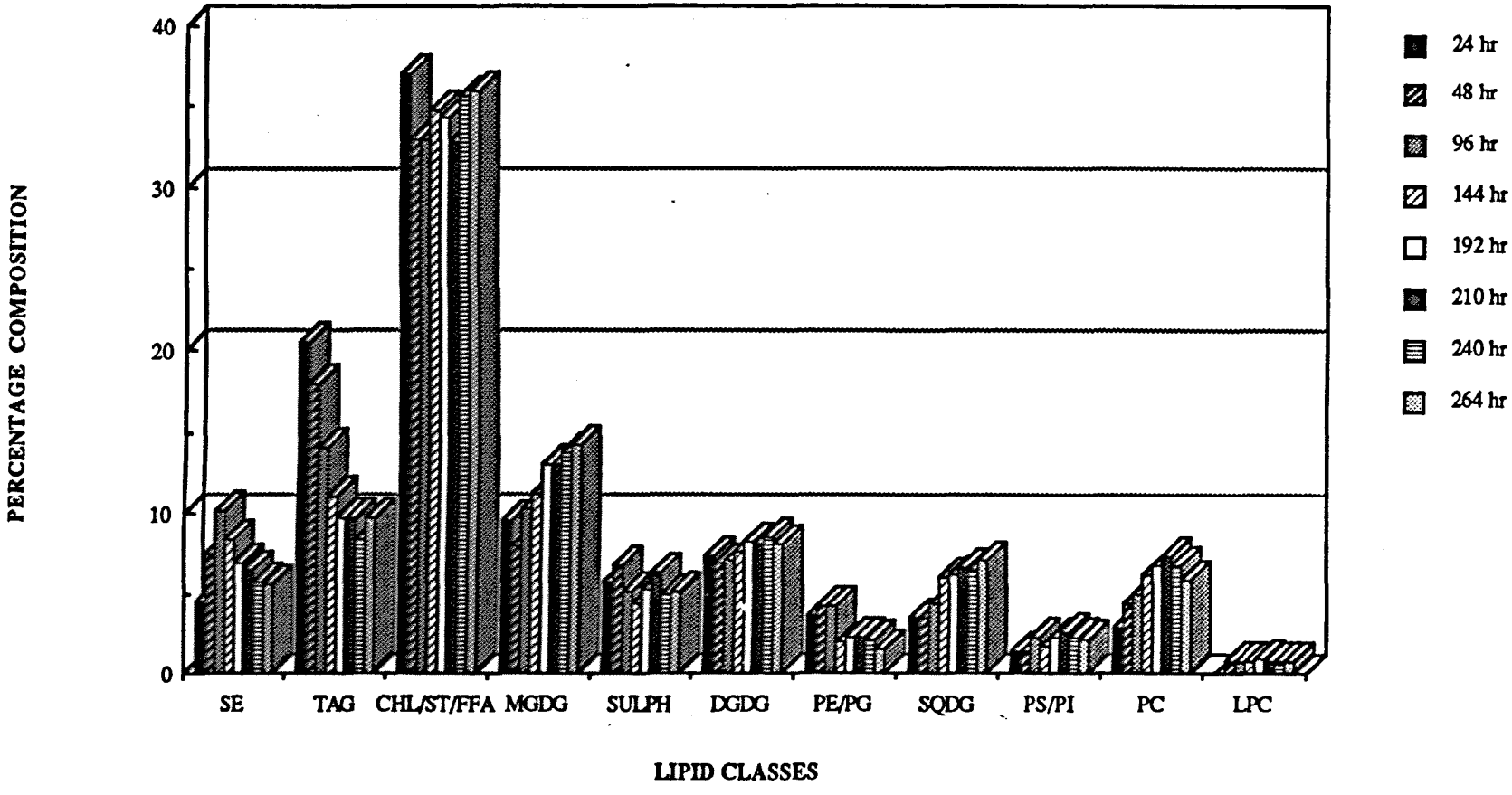


Figure 6.2.8 : Changes in the relative proportions of the lipid classes of *N. oculata* over 24 to 264 hours in batch culture in S88 medium at 25 °C.

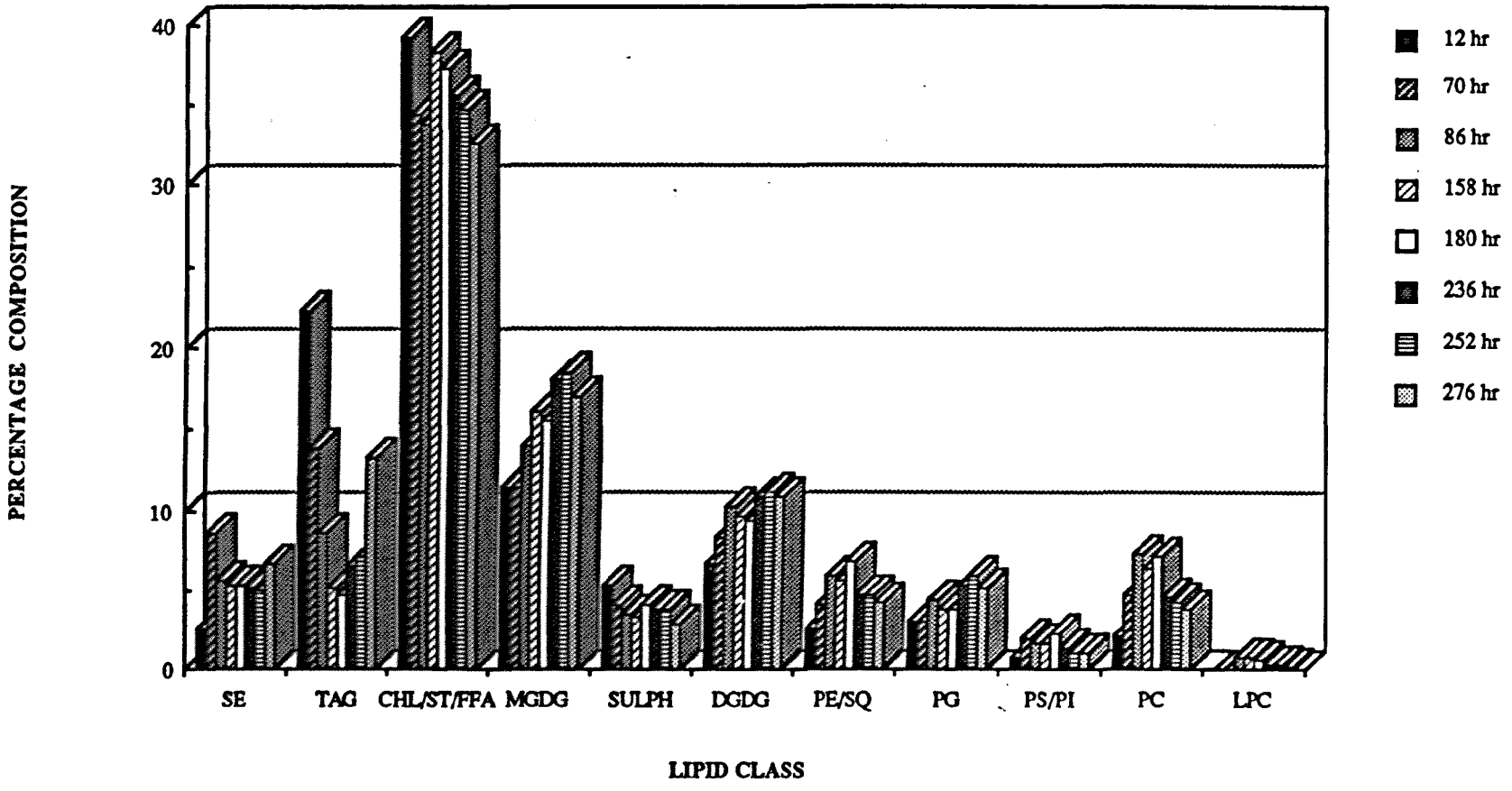


Figure 6.2.9 : Changes in the relative proportions of the lipid classes of *N. oculata* over 12 to 276 hours in batch culture in S88 medium at 20 °C1.

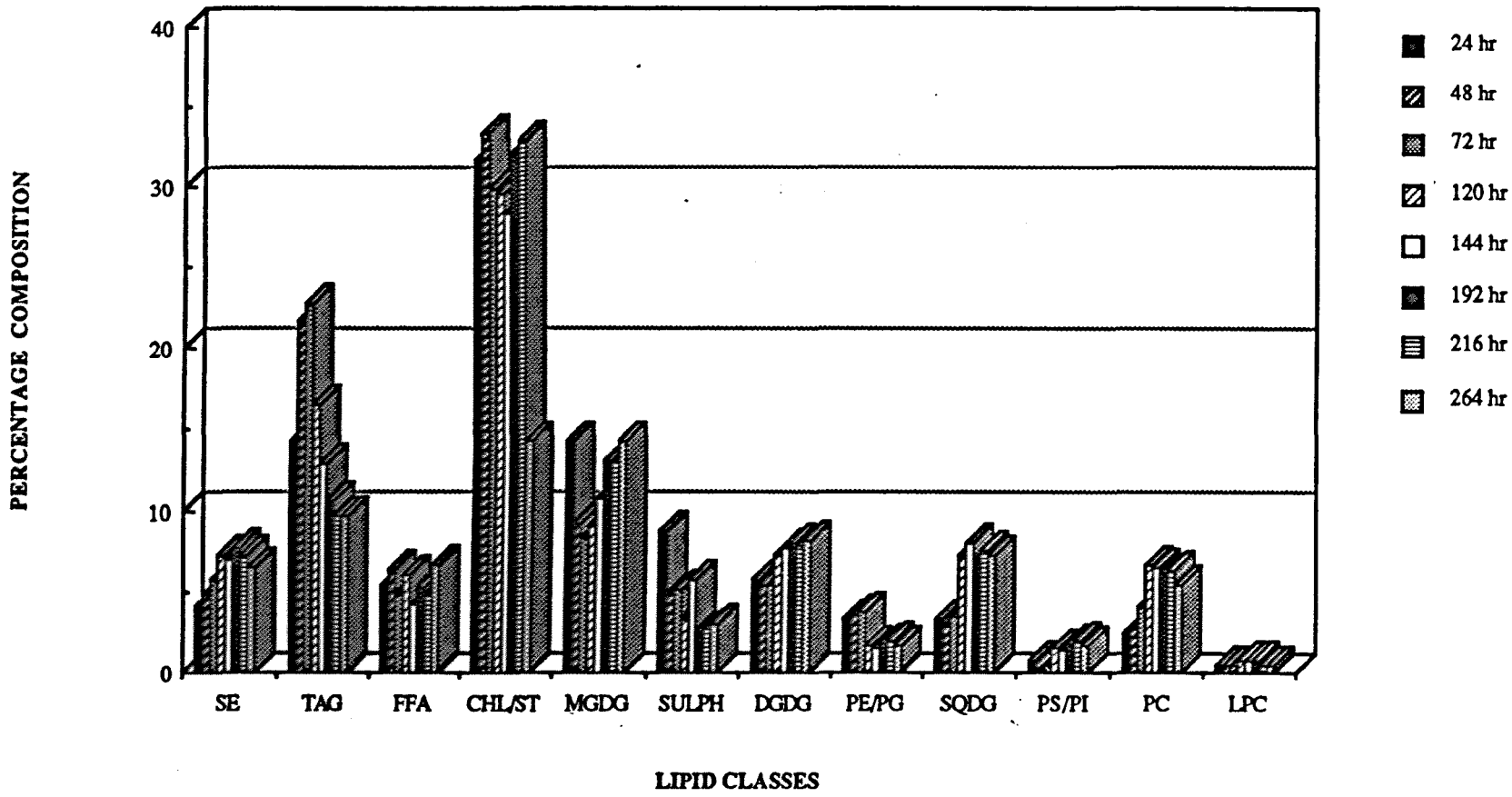


Figure 6.2.10 : Changes in the relative proportions of the lipid classes of *N. oculata* over 24 to 264 hours in batch culture in S88 medium at 15 °C1.

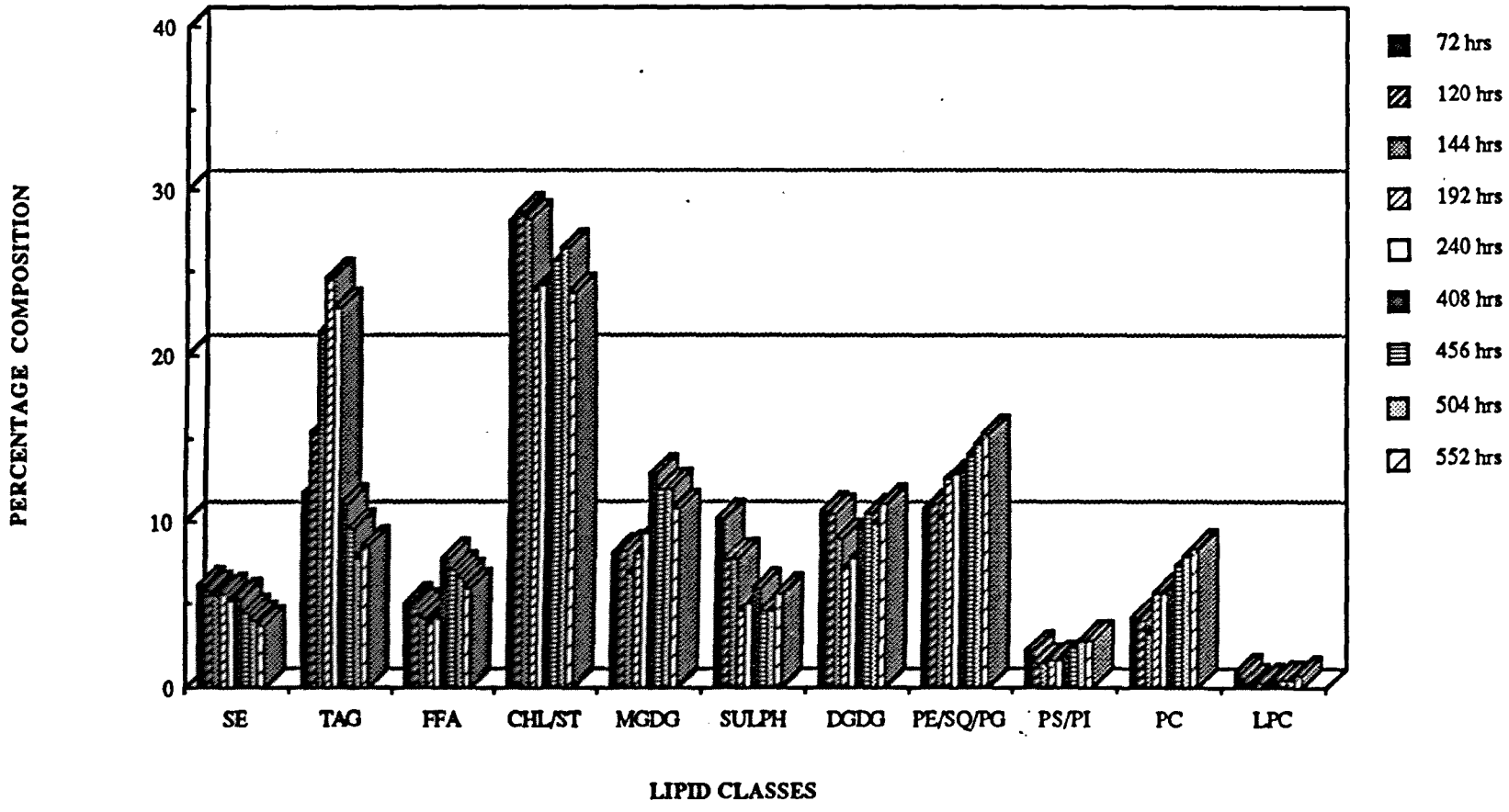


Figure 6.2.11 : Changes in the relative proportions of the lipid classes of *N. oculata* over 72 to 540 hours in batch culture in S88 medium at 10 °C[1].

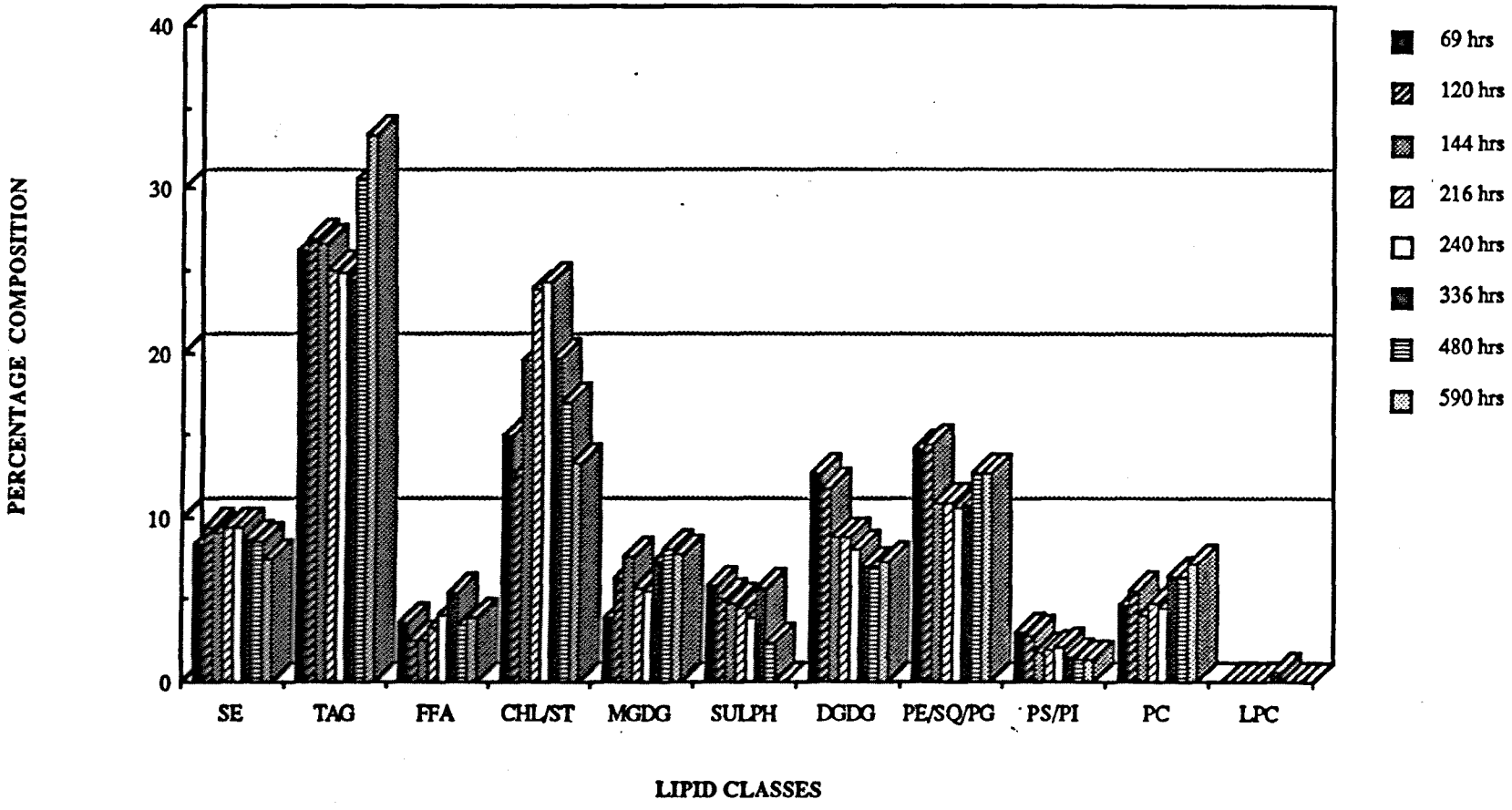


Figure 6.2.12 : Changes in the relative proportions of the lipid classes of *N. oculata* over 69 to 590 hours in batch culture in S88 medium at 5 °C[1].

The single most obvious features which can be seen in Figures 6.2.8 to 6.2.12 are the changes in the relative proportions of the TAG component of the total lipid. Although these figures are plotted using a reference scale in terms of hours, it is also helpful to imagine them in terms of doubling periods as used in the earlier figures. At first sight it seemed that decreased incubation temperature resulted in increased proportions of TAG. These increases were well correlated with those of the proportion of the cell mass present as total lipid extract (see Figures 6.2.6 & 6.2.7). As cell division progressed the proportion of TAG decreased accordingly and the apparent increase in TAG was found to be an artifact of the form of data expression. By the same token it was concluded that the observed lesser changes in the *relative* proportions of the polar lipids were not real.

The observed negative correlation resulted from the major changes occurring in the proportions of the TAG and pigment / sterol components. This was borne out by analyses of data which omitted the neutral lipid component. In these cases the relative proportions of the polar lipid classes over the time-courses remained similar, the small variations noted were attributed to local variability in TLC separations.

Similar patterns of lipid class variation were observed for all the incubation temperatures. The now familiar pattern first described in Section 5 was modified by the effect of temperature upon the division rates of the cultures. For the cultures incubated at 25 ^{o}C and 20 ^{o}C the rapid fall in the proportion of the cell's mass accounted for by lipid was echoed by a rapid decrease in the proportion of TAG. The culture incubated at 20 ^{o}C, close to the optimal growth temperature, progressed rapidly and showed indications of entering the stationary phase with an increasing proportion of TAG in the final two samples. At 15 ^{o}C the first signs of the lag-phase associated rise and fall in proportion of TAG could be seen. These signs were even more evident in the cells grown at 10 ^{o}C - as were the effects of sharp increases in the TAG and pigment / sterol upon the proportions of the polar lipid classes. It was apparent that the cells in the culture incubated at 5 ^{o}C were accumulating significant quantities of TAG. This contrasted with those cultures incubated at higher

temperatures which rapidly disseminated the TAG they synthesised amongst daughter cells. This evidence tended to support the conclusions made earlier in Section 5.4. These were; that low cell division rate, in an environment which did not restrict photosynthetic carbon fixation, initiated preferential partitioning of the carbon into fatty acyl synthesis. The fatty acids were then shunted into TAG biosynthesis prior to elongation (see Figure 5.4.2)

At equivalent points on the growth curves of these cultures, TAG was either being consumed at a rate higher than that, *or* being synthesised at a rate lower than that, required to maintain a constant pool within the cell at the specific cell division rate. It is this which points towards a primitive but effective system for uncoupling fatty acyl synthesis from the polar lipid synthesis required by cell division. The effect of this seems to be to reduce the dependence of the photosynthetic fixation of carbon upon the availability of cell division limiting nutrients, such as nitrogen and phosphate. By accumulating the molecular building blocks for the resumption of cellular division, should nutrient availability increase, or for catabolism during unfavourable conditions the cells seem to be suited to an opportunistic life strategy.

Analysis of the total FAME from each of the cultures yielded data in support of the previous conclusions. Each of the cultures incubated at different temperatures could be seen to exhibit overlapping, recognisable features which formed a more detailed picture of the observations in Section 5.3. The data sets presented in this section were complementary to those presented earlier in Section 5.4. The higher growth rate cultures, incubated at 25 °C and 20 °C, may be regarded as a continuation of that section and illustrate the culture's progression into the exponential and, in the case of the 20 °C culture, into the early stationary phase. By virtue of their lower growth rate, the cultures grown at 15 °C and 10 °C confirmed the details regarding the progression cultures through the lag-phase and into the exponential phase.

Tables 6.2.1 to 6.2.5 contain the total FAME compositional data gathered over the time course of each culture incubated at its respective temperature. For the purposes of inter-

comparison of the data sets, the columns of each table are marked with superscripted letters. Columns with the same letter are approximately equivalent in terms of cell division. The columns marked 'd' and 'e' correspond to the lines marked on the original growth curve plots in Figure 6.2.1. These letters also correspond to the samples chosen for more detailed analysis of their individual lipid class FAME compositions; the results of which are included later in this section.

The pattern of the changes in the total FAME compositions were all very similar within the experimental temperature range. After inoculation the FAME became less unsaturated as a consequence of the increase in their proportion of saturates and monounsaturates, particularly 16:0 and 16:1. The overall acyl chain length distribution was modified, becoming shorter as the proportions of the major fatty acids (16:0, 16:1 and 20:5) changed. The initial data presented in Tables 6.2.1 and 6.2.2 show that the cultures incubated at 25 ^{o}C and 20 ^{o}C were already at this stage when sampling commenced.

TABLE 6.2.1: Variation of total FAME composition with culture age for *N. oculata* grown at 25 °C in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)										
FATTY ACID	24	48[a]	72[b]	92	120	144	168[c]	192	216[d]	240	264[e]
14:0	2.3	2.6	3.6	3.6	3.5	5.5	5.6	6.1	5.8	6.2	6.1
15:0	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.3
16:0	31.1	32.7	27.7	24.3	24.9	23.1	22.5	21.4	19.2	18.5	18.8
16:1(*n*-7)	30.4	31.3	30.0	26.6	25.9	24.4	24.2	24.3	23.0	24.4	23.2
16:1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.
16:2	0.5	0.3	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.5
17:0	0.4	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
16:3	0.4	0.2	0.2	.	0.2	0.1	0.1	0.2	0.2	0.3	0.2
16:4	.	.	.	.	.	.	.	.	.	.	.
18:0	1.4	1.2	1.0	0.9	1.0	0.9	0.9	1.0	2.2	0.7	1.8
18:1(*n*-9)	3.6	2.8	2.5	2.6	2.9	3.1	3.2	3.6	4.8	2.8	4.0
18:1(*n*-7)	1.5	1.1	0.7	0.5	0.4	0.4	0.3	0.4	0.7	0.3	0.5
18:2(*n*-6)	1.8	1.4	1.5	1.7	2.0	2.2	2.5	2.7	2.7	2.7	2.1
18:3(*n*-6)	0.8	0.6	0.5	0.4	0.4	0.4	0.3	0.3	0.3	0.3	0.4
18:3(*n*-3)	0.2	0.2	0.3	0.1	0.3	0.3	0.3	0.2	0.3	0.3	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:0	0.2	0.2	0.2	0.3	0.3	.	0.3	0.3	0.3	0.3	.
20:3(*n*-6)	1.2	0.9	0.7	0.6	0.5	0.4	0.4	0.3	0.3	0.3	0.4
20:4(*n*-6)	3.5	3.3	3.6	4.0	3.6	3.2	3.5	3.4	3.8	3.4	4.0
20:3(*n*-3)	0.2	0.4	0.5	0.4	0.3	0.5	0.3	0.4	0.3	0.3	0.2
20:4(*n*-3)	0.1	0.1	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	13.3	13.7	18.9	23.7	24.2	23.4	26.2	26.1	27.3	30.0	30.2
Unidentified :	6.7	6.4	7.1	9.3	8.6	11.1	8.4	8.3	8.4	8.5	6.7
Total sats :	35.8	37.3	33.1	29.7	30.3	30.1	29.9	29.4	28.0	26.3	27.2
Total monos :	35.5	35.2	33.2	29.7	29.2	27.9	27.7	28.3	28.6	27.4	27.8
Total PUFA :	22.0	21.1	26.6	31.3	31.9	30.9	34.0	34.0	35.1	37.8	38.3
Total (*n*-9) :	3.6	2.8	2.5	2.6	2.9	3.1	3.2	3.6	4.8	2.8	4.0
Total (*n*-6) :	7.3	6.2	6.3	6.7	6.5	6.2	6.7	6.7	7.1	6.7	7.0
Total (*n*-3) :	13.8	14.4	19.7	24.3	24.8	24.2	26.8	26.7	27.3	30.3	30.5
(*n*-3)/(*n*-6) :	1.9	2.3	3.1	3.6	3.8	3.9	4.0	4.0	3.8	4.5	4.4
16:1/16:0 :	1.0	1.0	1.1	1.1	1.0	1.1	1.1	1.1	1.2	1.3	1.2
20:5/16:0 :	0.4	0.4	0.7	1.0	1.0	1.0	1.2	1.2	1.4	1.6	1.6
ΣC_{16}:	62.4	64.5	58.3	51.3	51.4	48.0	47.2	46.3	42.8	43.7	42.7
ΣC_{18}:	9.3	7.3	6.5	6.2	7.0	7.3	7.5	8.2	10.7	7.0	9.1
ΣC_{20}:	18.5	18.6	23.9	29.0	28.9	27.5	30.7	30.5	31.8	34.0	34.6

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

[a], [b], [c], [d], [e], inter-data set equivalence points in terms of cell division (see text for details).

TABLE 6.2.2 : Variation in total FAME composition with culture age for *N. oculata* grown at 20 °C in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)										
FATTY ACID	12	36[a]	50	78[b]	132[c]	158[d]	178	202	236[e]	252	276
14:0	3.1	2.5	2.6	3.4	4.3	4.2	3.8	4.2	4.2	4.5	4.4
15:0	.	.	.	.	.	.	.	.	.	.	.
16:0	33.3	31.1	27.1	21.3	15.4	13.4	13.6	14.2	14.6	15.5	17.3
16:1(*n*-7)	25.6	31.7	32.0	32.7	28.2	27.0	25.8	24.4	24.0	25.4	24.9
16.1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.
16:2	.	0.2	0.1	0.4	0.6	0.8	0.7	0.9	0.9	0.8	0.5
17:0	.	.	.	.	.	.	.	.	.	.	.
16:3	0.4	0.3	0.2	.	0.2	0.3	0.3	.	0.3	0.3	0.2
16:4	.	.	.	.	.	.	.	.	.	.	.
18:0	2.2	1.8	1.6	0.9	0.6	0.5	0.4	0.5	0.8	0.7	0.7
18:1(*n*-9)	3.7	4.3	3.9	2.7	2.9	2.2	2.3	2.0	1.9	2.4	2.3
18:1(*n*-7)	.	1.3	1.2	0.7	0.5	0.4	0.3	0.6	0.6	0.5	0.5
18:2(*n*-6)	2.4	2.4	2.7	1.7	2.2	2.1	2.2	1.9	1.5	1.7	1.6
18:3(*n*-6)	0.4	0.8	0.6	0.5	0.4	0.5	0.4	0.4	0.3	0.4	0.4
18:3(*n*-3)	0.5	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:0	0.5	0.2	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.4
20:3(*n*-6)	1.3	1.3	1.3	0.9	0.5	0.4	0.4	0.4	0.3	0.4	0.7
20:4(*n*-6)	2.0	3.4	3.2	3.8	4.1	4.2	4.5	4.0	3.8	4.0	4.0
20:3(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	15.8	15.9	19.5	27.1	32.6	36.1	36.4	37.0	39.2	37.6	35.7
Unidentified :	7.9	2.6	3.5	3.4	7.0	7.4	8.4	8.9	6.9	4.9	6.1
Total sats :	39.6	35.6	31.6	25.9	20.6	18.4	18.1	19.3	20.0	21.2	22.8
Total monos :	29.3	37.3	37.1	36.1	31.6	29.6	28.4	27.0	26.5	26.9	27.7
Total PUFA :	23.2	24.5	27.8	34.6	40.8	44.6	45.1	44.8	46.6	45.6	43.4
Total (*n*-9) :	3.7	4.3	3.9	2.7	2.9	2.2	2.3	2.0	1.9	2.4	2.3
Total (*n*-6) :	6.1	7.9	7.8	6.9	7.2	7.2	7.5	6.7	5.9	6.5	6.7
Total (*n*-3) :	16.8	16.1	19.7	27.3	32.8	36.3	36.6	37.2	39.5	37.9	36.0
(*n*-3)/(*n*-6) :	2.8	2.0	2.5	4.0	4.6	5.0	4.9	5.6	6.7	5.8	5.4
16:1/16:0 :	0.8	1.0	1.2	1.5	1.8	2.0	1.9	1.7	1.6	1.6	1.4
20:5/16:0 :	0.5	0.5	0.7	1.3	2.1	2.7	2.7	2.6	2.7	2.4	2.1
ΣC_{16}:	59.3	63.3	59.4	54.4	44.4	41.5	40.4	39.5	39.8	42.0	42.9
ΣC_{18}:	9.6	10.8	10.2	6.7	6.8	5.9	5.8	5.6	5.4	6.1	5.8
ΣC_{20}:	20.1	20.8	24.3	32.1	37.5	41.0	41.6	41.8	43.7	42.4	40.8

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

[a], [b], [c], [d], [e], inter-data set equivalence points in terms of cell division (see text for details).

TABLE 6.2.3 : Variation in total FAME composition with culture age for *N. oculata* grown at 15 °C in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)										
FATTY ACID	24	48	72[a]	96	120[b]	144	168[c]	192[d]	216	240	264[e]
14:0	2.3	1.9	1.6	2.0	2.0	3.1	2.9	3.5	4.0	3.5	3.8
15:0	0.4	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3
16:0	27.4	30.7	31.9	32.3	31.3	29.1	23.4	20.9	19.8	17.0	17.5
16:1(*n*-7)	27.4	33.3	33.0	32.5	30.5	31.0	30.0	30.0	29.3	27.5	23.6
16:1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.
16:2	0.3	0.2	.	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.4
17:0	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2	0.2
16:3	0.3	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2	.	.
16:4	.	.	.	.	.	.	.	.	.	.	.
18:0	2.6	1.4	1.3	1.1	1.2	1.1	0.8	0.7	0.6	0.8	0.7
18:1(*n*-9)	7.4	4.3	4.0	3.5	3.6	3.3	3.6	4.2	3.7	3.3	2.5
18:1(*n*-7)	1.9	1.6	1.5	1.9	0.9	0.7	0.6	0.6	0.3	0.4	0.3
18:2(*n*-6)	1.9	2.0	1.8	1.3	1.2	1.2	1.4	1.6	1.6	1.7	1.5
18:3(*n*-6)	1.4	1.8	1.6	1.1	0.9	0.6	0.5	0.4	0.4	0.4	0.4
18:3(*n*-3)	0.2	0.1	0.1	.	.	0.1	0.1	0.2	0.1	0.1	0.1
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.2	0.2
20:3(*n*-6)	1.2	1.6	1.7	1.6	1.6	1.1	0.8	0.7	0.7	0.6	0.5
20:4(*n*-6)	3.0	3.4	3.2	3.0	3.0	2.8	3.5	3.5	4.1	4.1	4.2
20:3(*n*-3)	0.2	0.3	0.2	0.2	0.2	0.3	0.3	0.3	0.2	0.2	0.3
20:4(*n*-3)	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.1	0.1
20:5(*n*-3)	14.0	12.8	13.6	15.7	19.2	19.5	27.0	26.3	32.2	36.0	39.6
Unidentified :	7.5	3.4	3.4	2.6	3.1	4.7	3.9	5.8	1.7	3.3	4.0
Total sats :	33.2	34.8	35.5	36.1	35.2	34.1	27.8	25.8	25.1	22.1	22.7
Total monos :	36.7	39.2	38.5	37.9	35.0	35.0	34.2	34.8	33.3	31.2	26.5
Total PUFA :	22.6	22.6	22.6	23.4	26.7	26.2	34.1	33.6	39.9	43.4	46.8
Total (*n*-9) :	7.4	4.3	4.0	3.5	3.6	3.3	3.6	4.2	3.7	3.3	2.5
Total (*n*-6) :	7.5	8.8	8.3	6.9	6.7	5.7	6.2	6.2	6.8	6.8	6.7
Total (*n*-3) :	14.5	13.4	14.1	16.1	19.6	20.1	27.6	27.0	32.6	36.4	39.7
(*n*-3)/(*n*-6) :	1.9	1.5	1.7	2.3	2.9	3.5	4.5	4.4	4.8	5.4	5.9
16:1/16:0 :	1.0	1.1	1.1	1.0	1.0	1.1	1.3	1.4	1.5	1.6	1.6
20:5/16:0 :	0.5	0.4	0.4	0.5	0.6	0.7	1.2	1.3	1.6	2.1	2.3
ΣC_{16}:	55.4	64.4	65.1	65.2	62.2	60.5	53.7	51.3	49.6	44.8	41.5
ΣC_{18}:	15.4	11.2	10.3	8.9	7.8	7.0	7.0	7.7	6.7	6.7	5.6
ΣC_{20}:	18.7	18.5	19.1	20.9	24.4	24.1	32.0	31.2	37.4	41.2	44.5

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

[a], [b], [c], [d], [e], inter-data set equivalence points in terms of cell division (see text for details).

TABLE 6.2.4 : Variation in total FAME composition with culture age for *N.oculata* grown at 10 °C in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)								
FATTY ACID	72	120	144[a]	192	240[b]	408	456	504	552[c]
14:0	3.6	4.3	3.9	3.8	4.0	4.6	4.7	4.3	4.2
15:0	.	.	.	0.4	.	.	.	.	.
16:0	15.4	17.7	28.5	29.1	29.7	15.7	14.3	13.3	12.9
16:1(*n*-7)	27.5	28.5	33.0	32.1	29.5	28.3	28.0	26.8	26.2
16:1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.
16:2	.	0.2	.	.	.	.	0.3	0.4	0.4
17:0	.	.	.	.	.	.	.	.	.
16:3	0.4	0.4	0.3	0.2	0.1	0.2	0.3	0.3	0.3
16:4	.	.	.	.	.	.	.	.	.
18:0	1.7	1.7	1.3	0.9	0.8	0.4	0.3	0.3	0.3
18:1(*n*-9)	9.0	8.0	5.5	4.3	4.2	4.0	3.5	3.3	2.9
18:1(*n*-7)	.	.	0.7	0.5	0.4	0.2	0.2	.	0.2
18:2(*n*-6)	3.4	4.1	1.9	1.5	1.3	1.6	1.6	1.7	1.8
18:3(*n*-6)	0.4	0.5	1.0	1.1	0.7	0.3	0.3	0.3	0.3
18:3(*n*-3)	0.2	0.2	0.2	0.1	0.2	0.2	0.2	0.2	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:0	.	0.2	0.1	0.1	.	.	.	.	.
20:3(*n*-6)	0.4	0.6	0.8	1.2	1.3	0.6	0.6	0.4	0.6
20:4(*n*-6)	4.0	3.4	2.5	2.5	2.4	4.4	4.6	4.0	4.5
20:3(*n*-3)	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	26.2	23.6	16.4	18.0	19.3	31.8	33.7	37.0	37.8
Unidentified :	7.8	6.6	3.9	4.2	6.1	7.7	7.4	7.7	7.4
Total sats :	20.7	23.9	33.8	34.4	34.5	20.7	19.3	17.9	17.4
Total monos :	36.5	36.5	39.2	36.9	34.1	32.5	31.7	30.1	29.3
Total PUFA :	35.0	33.0	23.1	24.6	25.3	39.1	41.6	44.3	45.9
Total (*n*-9) :	9.0	8.0	5.5	4.3	4.2	4.0	3.5	3.3	2.9
Total (*n*-6) :	8.2	8.6	6.2	6.3	5.7	6.9	7.1	6.4	7.2
Total (*n*-3) :	26.4	23.8	16.6	18.1	19.5	32.0	33.9	37.2	38.0
(*n*-3)/(*n*-6) :	3.2	2.8	2.7	2.9	3.4	4.6	4.8	5.8	5.3
16:1/16:0 :	1.8	1.6	1.2	1.1	1.0	1.8	2.0	2.0	2.0
20:5/16:0 :	1.7	1.3	0.6	0.6	0.6	2.0	2.4	2.8	2.9
ΣC_{16}:	43.3	46.8	61.8	61.4	59.3	44.2	42.9	40.8	39.8
ΣC_{18}:	14.7	14.5	10.6	8.4	7.6	6.1	6.1	5.8	5.7
ΣC_{20}:	30.6	27.8	19.8	21.8	23.0	36.8	38.9	41.4	42.9

Abbreviations & Symbols.
Data expressed in terms of % mass of FAME, tr.,<0.1%, ., not detected.
[a,b,c,d,e], inter-data set equivalence points in terms of cell dividion (see text for details).

TABLE 6.2.5 : Variation in total FAME composition with culture age for *N. oculata* grown at 5 °C in S88 medium modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)								
FATTY ACID	69	120	144	216	240[a]	336[b]	384	480	590
14:0	6.0	3.7	6.6	6.3	7.4	6.7	6.7	6.0	5.8
15:0	0.5	0.4	0.5	0.4	0.5	0.4	0.4	0.4	0.4
16:0	21.5	22.3	22.2	22.8	22.7	25.2	25.1	27.7	27.6
16:1(*n*-7)	34.0	37.0	35.7	35.4	35.3	33.8	33.1	32.5	31.7
16:1(*n*-13).	.	.	.	.	.	.	.	.	.
16:2	0.1	0.1	0.1	.	.	.	.	.	.
17:0	.	.	.	.	.	.	.	.	.
16:3	0.3	0.1	0.3	0.3	0.2	0.2	0.2	0.2	0.2
16:4	.	.	.	.	.	.	.	.	.
18:0	1.1	0.7	0.8	0.7	0.6	0.8	0.6	0.5	0.5
18:1(*n*-9)	8.5	8.5	9.3	10.8	9.6	11.3	11.8	11.7	11.5
18:1(*n*-7)	.	.	.	.	.	.	.	.	.
18:2(*n*-6)	1.8	1.3	1.9	1.1	1.3	1.2	1.2	1.4	1.2
18:3(*n*-6)	0.5	0.4	0.4	0.3	0.3	0.4	0.3	0.4	0.3
18:3(*n*-3)	0.1	.	.	.	.	.	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:0	0.1	0.1	.	0.1	0.1	.	.	.	.
20:3(*n*-6)	1.1	1.0	0.9	0.9	0.7	0.7	0.6	0.9	0.7
20:4(*n*-6)	2.4	2.3	2.1	1.9	2.0	2.0	1.8	1.8	1.8
20:3(*n*-3)	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	19.4	19.0	17.2	17.6	16.8	15.1	16.2	15.4	16.0
Unidentified :	2.6	3.1	2.0	1.4	2.5	2.2	2.0	1.2	2.3
Total sats :	29.2	27.2	30.1	30.3	31.3	33.1	32.8	34.6	34.3
Total monos :	42.5	45.5	45.0	46.2	44.9	45.1	44.9	44.2	43.2
Total PUFA :	25.7	24.2	22.9	22.1	21.3	19.6	20.3	20.0	20.2
Total (*n*-9) :	8.5	8.5	9.3	10.8	9.6	11.3	11.8	11.7	11.5
Total (*n*-6) :	5.8	5.0	5.3	4.2	4.3	4.3	3.9	4.4	4.0
Total (*n*-3) :	19.5	19.0	17.2	17.6	16.8	15.1	16.2	15.4	16.0
(*n*-3)/(*n*-6) :	3.4	3.8	3.2	4.2	3.9	3.5	4.1	3.5	4.0
16:1/16:0 :	1.6	1.7	1.6	1.6	1.6	1.3	1.3	1.2	1.1
20:5/16:0 :	0.9	0.9	0.8	0.8	0.7	0.6	0.6	0.6	0.6
ΣC_{16} :	55.9	59.5	58.3	58.5	58.2	59.2	58.4	60.4	59.5
ΣC_{18} :	12.0	10.9	12.4	12.9	11.8	13.7	13.9	14.0	13.5
ΣC_{20} :	23.0	22.4	20.2	20.5	19.6	17.8	18.6	18.1	18.5

Abbreviations & Symbols.
Data expressed in terms of % mass of FAME, tr.,<0.1%, ., not detected.
[a,b,c,d,e], inter-data set equivalence points in terms of cell dividion (see text for details).

However, the data for the remaining cultures, incubated at 15 °C, 10 °C and 5 °C (see Tables 6.2.3, 6.2.4 & 6.2.5), show the progression of changes described above which was assumed to have taken place in the higher temperature cultures. Because exponential-phase cultures were used as inoculae they were at a stage in their cycle growth when they had their peak unsaturation. This was reflected in the starting compositions of the low growth rate cultures. The culture incubated at 10 °C was a good illustration of this point. The FAME from this culture showed high proportions of 20:5(*n*-3) and low proportions of 16:0. As time progressed the culture entered the lag-phase the chain length distribution of the fatty acids changed with the proportions of both C_{18} and C_{20} chain lengths decreasing. The culture passed through the lag-phase and began its period of exponential-phase growth during which time the proportions of the longer chain length fatty acids increased, as did the proportions of PUFA. At all the incubation temperatures the proportions of monounsaturates did not vary to the same extent as saturates and PUFA during each time course. This was a function of the complementary variation in the saturated acid 16:0 and the PUFA 20:5.

The head of each of the Tables 6.2.1 to 6.2.5 is marked with letters 'a' to 'e'. These correspond to selected equivalence points chosen on the basis of the number of doubling periods each culture had undergone. The earlier proposal that temperature only affected the rate of growth of a culture required that inter-culture comparison should be made upon the basis of a unit of measure which reflected the metabolic rate of each specific culture. The explanation of this approach requires the omission of factors such as the effect of temperature on the solubility of gases such as oxygen, which are thought to have effects upon the desaturation reactions, although this has not been proven.

At each temperature in the experimental range the cells would be dividing at their maximum rate (for the specific culture conditions). The effects of temperature upon the cells were due to changes in metabolic rate. The rate of the changes the cells in turn produced in their environment (*e.g.* depletion of dissolved nutrients and decreasing photon flux through the culture) would also be growth rate dependent. Therefore, the prevailing culture

conditions after a specific number of cell doubling periods should be the same regardless of culture temperature. Figures 6.2.6 and 6.2.7 showed that, at the whole cell and total lipid extract levels, this indeed seemed to be the case. Because of the limitations of HPTLC-densitometry of the individual lipid classes, it was not possible to carry these conclusions through to that level of the analysis. However, conclusions may be drawn from the analyses of the FAME derived from both the total lipid and its component classes.

By defining 'equivalence points' inter-culture comparison across the experimental temperature range was facilitated. If all the columns labelled with the letter 'a' in Tables 6.2.1 to 6.2.5 are compared then one is able to see that the values for the percentage mass compositions of the total FAME were approximately similar across the temperature range 25 °C to 10 °C. As the cultures aged, further comparisons were made at the time points marked 'b', 'c', 'd' and 'e'. Unfortunately the sampling frequencies of the 5 °C and 10 °C cultures were sacrificed in order to prolong the time period that sampling was able to cover. As a result several of the equivalence points for these two cultures had to be omitted.

At equivalence point 'b' some differences in the total FAME compositions of the cultures incubated in the range 25 °C to 15 °C were observed. These differences in the proportions of 16:0 and 20:5 were attributed to slight differences in the proportions of TAG as the cells began dividing in the early exponential-phase. The inference from earlier data in this section was that, once the cultures had undergone four to five doubling periods, they would settle into less variable regions of their growth and their biochemical compositions would stabilise. When the cultures had reached this stage of growth, (*i.e.* the region between the lines 'd' and 'e' on Figure 6.2.1) the inter-culture differences in total FAME composition were very much reduced. Within the accuracy of the experimental work it is probable that they were for all intents and purposes 'identical'. Because the culture incubated at 5 °C did not undergo the required number of doubling periods it was excluded from the following discussion.

The culture incubated at 25 °C attained a maximum PUFA composition of 38.3 % and a 16:1/16:0 ratio of 1.2, its maximum 20:5/16:0 ratio being 1.6. For this culture the maximum proportion of C_{20} fatty acids was 34.6 %, 264 hours post-inoculation or after *ca.* 5 doubling periods. Of this proportion, the fatty acid 20:5(*n*-3) accounted for 30.2 %, the remainder being made up of 20:4(*n*-6). The distribution of the other major acyl chain lengths was 42.7 % C_{16} and 9.1 % C_{18} (see Table 6.2.1). The culture incubated at 20 °C attained a higher proportion of PUFA than that of the 25 °C culture, having a composition of 46.6 % PUFA, a 16:1/16:0 ratio of 1.6 and a 20:5/16:0 ratio of 2.7. The signs of the imminent early stationary-phase were obvious in the declining proportions of PUFA and C_{20} fatty acids as well as the increasing proportions of C_{16} fatty acids. After six doubling periods the cells showed the following acyl chain length distribution; 39.8 % C_{16}, 5.4 % C_{18} and 43.7 % C_{20}.The proportion of the C_{20} acyl chains accounted for by the fatty acid 20:5(*n*-3) was 39.2 %. The acyl chain length distribution had shifted from that observed in the 25 °C culture. The 20 °C culture showed lower proportions of C_{16} and C_{18} fatty acids and a concomitantly higher proportion of C_{20} acyl chain length fatty acids (see Table 6.2.2). Standing alone, these data would support the argument for increasing unsaturation and acyl chain length with decreasing temperature. However, the trend in the rest of the data does not.

The culture grown at 15 °C showed a maximum PUFA composition of 46.8 % and had a 16:1/16:0 ratio of 1.6 and a 20:5/16:0 ratio of 2.3. In this culture the signs of the onset of the stationary-phase were still absent at 264 hours post-inoculation. The acyl chain length distribution for these cells was 41.5 % C_{16}, 5.6 % C_{18} and 44.5 % C_{20}. The proportion of 20:5(*n*-3) attained after six doubling periods was 39.6 %. Compared with the cultures discussed above it can be seen that the differences between the 15 °C and 20 °C cultures were very minor, amounting to no greater than 1.2 % amongst the various column totals. The slight discrepancy between the percentages of 16:0 may be accounted for by the appearance of a spurious peak eluting immediately prior to 16:0 during the GLC analysis of the 20 °C samples. Thus also accounted for the higher unidentified peak total in Table 6.2.2.

At 10 °C the PUFA composition of *N.oculata* was similar to those described for the other cultures after 6 doubling periods. The cells showed a maximum PUFA composition of 45.9 % but had a 16:1/16:0 ratio of 2.0 and a 20:5/16:0 ratio of 2.9. The main reason for this difference from the previous cultures was the relatively lower proportion of 16:0. This was complemented by an increased proportion of 16:1 thus, the overall fatty acyl chain length distribution was apparently unaffected. The acyl chain length distributions were as follows: 39.8 % C_{16}, 5.7 % C_{18} and 42.9 % C_{20}. These figures compared very closely to those of the previous cultures, the differences in percentage terms again being accounted for by the higher proportion of 'unidentified' components which were always potential artifacts. At 37.8 % the proportion of 20:5(*n*-3) attained after six doubling periods was also on a level comparable with the cultures grown at higher temperatures.

Following the analyses of the total FAME compositions of the cells grown at each temperature, the FAME compositions of the individual lipid classes were determined for the samples falling closest to equivalence-point 'e' on Figure 6.2.1. At these points the FAME compositions of the lipid classes should have been at their most stable, reflecting the similarities in both lipid class and fatty acyl distribution between the four cultures grown in the temperature range 25 °C to 10 °C and noted above. The data shown in Tables 6.2.6 to 6.2.9 demonstrated that, in practical terms, this was the case as far as the polar lipid classes were concerned. There was no trend in the data which could be correlated with the changing incubation temperatures of the cultures.

The fatty acyl composition of each of the individual lipid classes fluctuated in a culture-dependent manner. The three cultures incubated in the temperature range 20 °C to 10 °C showed the closest similarities in the FAME compositions of each of their respective individual lipid classes, as was the case for their total FAME compositions. The exception to this, as was expected from its total FAME composition, was the culture incubated at 25 °C. This culture showed considerably lower degrees of fatty acyl unsaturation amongst the lipid classes TAG, FFA and MGDG. The remaining lipid classes, most notably DGDG and PC,

were affected to a lesser extent. This phenomenon is also relevant with respect to the later sections of this thesis.

The lipid class which underwent the greatest variation was TAG. The variation did not occur in a temperature-dependent manner as may be seen from the saturation and chain length summaries for the individual lipid classes recorded for 'equivalence-point' 'e' (see Tables 6.2.6 to 6.2.9). The acyl composition of the TAG was possibly determined by both the growth rate of the cells in culture and the flux of photosynthetically fixed carbon into the pathways of fatty acyl synthesis, elongation and desaturation. The TAGs have the potential to act as a buffering mechanism between carbon fixation and polar lipid synthesis. This would explain the dependence of the cellular proportion of this lipid class on the cellular division rate. In this case the HPTLC densitometric analysis data did not provide evidence for a definite conclusion. The variation in the acyl composition of TAG may have been due to variations in its quantity of TAG as observed in Section 5.4.

To summarise, it seemed that the total PUFA composition of *N. oculata* was not affected by the culture incubation temperature *per se*. The variation in the PUFA composition observed between cultures incubated at different temperature was more of an effect of temperature upon the metabolic rate of the cells rather than active compensation by the cell for the change in its environment. Setting the PUFA aside, there was a general but rather ill-defined trend for the proportions of total saturates and total monounsaturates to decrease and increase respectively with decreasing temperature. This observation corresponds, in part, to the current belief that fatty acyl unsaturation increases with decreasing incubation temperature. The true explanation is apparently more complex than that which is proposed in this thesis, otherwise the cultures with similar growth rates (*i.e.* those grown at 25 ^{o}C and 15 ^{o}C) would have had more closely matching lipid compositions. Therefore the influence of other factors, acting in concert with temperature, cannot be ruled out.

As can be seen from the fluctuations in total FAME compositions observed due to the growth phase of the culture, any effect of temperature over the 20 ^{o}C experimental range

TABLE 6.2.6 : Individual lipid class FAME composition of *N. oculata* grown in batch culture at 25 °C for 264 hours using S88 medium modified to provide excess nitrate and phosphate.

	LIPID CLASSES								
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG	SQDG	PS/PI	PC	TOTAL
14:0	3.6	3.8	15.8	10.1	4.5	7.1	5.6	2.6	5.7
15:0	.	.	0.3	0.4	0.7	0.7	0.8	0.4	0.3
16:0	22.2	11.5	8.1	21.1	38.6	18.9	33.6	11.5	17.7
16:1(*n*-7)	24.0	22.4	6.6	20.8	31.8	23.1	32.6	25.9	18.2
16:1(*n*-13)ɣ	.	.	.	.	1.6	.	.	.	.
16:2	.	0.4	0.3	1.0	0.7	0.8	.	1.0	0.5
17:0	.	.	.	.	.	0.3	.	.	0.2
16:3	.	.	.	.	.	0.3	.	0.9	0.2
16:4	.	.	.	.	.	.	.	0.3	.
18:0	1.6	2.2	0.7	0.3	0.3	0.6	0.9	0.9	1.7
18:1(*n*-9)	4.3	2.4	0.6	0.5	0.9	1.3	6.0	8.2	3.8
18:1(*n*-7)	0.7	0.3	0.2	0.2	0.2	0.4	.	0.5	0.5
18:2(*n*-6)	1.7	1.2	0.6	1.1	0.5	1.6	1.7	8.6	2.0
18:3(*n*-6)	0.3	.	.	.	.	0.5	.	2.0	0.4
18:3(*n*-3)	.	.	0.4	0.2	.	0.6	.	.	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	0.4	.
20:0	0.4	0.4	0.3	0.3	.	.	.	.	.
20:3(*n*-6)	0.7	0.3	.	.	.	.	.	.	0.4
20:4(*n*-6)	3.5	3.7	1.3	0.7	2.6	3.4	5.5	7.6	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.	0.2
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	27.7	35.3	60.7	38.3	11.2	30.8	5.2	15.8	28.4
Unidentified :	9.3	16.1	3.5	5.0	6.4	9.6	8.1	13.4	12.2
Total sats :	27.8	17.9	25.2	32.2	44.1	27.6	40.9	15.4	25.6
Total monos :	29.0	25.1	7.8	21.5	34.5	24.8	38.6	34.6	26.0
Total PUFA :	33.9	40.9	63.5	41.3	15.0	38.0	12.4	36.6	36.2
Total (*n*-9) :	4.3	2.4	0.6	0.5	0.9	1.3	6.0	8.2	3.8
Total (*n*-6) :	6.2	5.2	2.1	1.8	3.1	5.5	7.2	18.2	3.8
Total (*n*-3) :	27.7	35.3	61.1	38.5	11.2	31.4	5.2	16.2	28.9
(*n*-3)/(*n*-6) :	4.5	6.8	29.1	21.4	3.6	5.7	0.7	0.9	4.4
16:1/16:0 :	1.1	1.9	0.8	1.0	0.9	1.3	1.0	2.3	1.2
20:5/16:0 :	1.2	3.1	7.5	1.8	0.3	1.6	0.2	1.4	1.6
ΣC_{16} :	46.2	33.9	15.0	42.9	72.7	43.1	66.2	39.6	42.7
ΣC_{18} :	8.6	6.1	2.5	2.3	1.9	5.0	8.6	20.6	9.1
ΣC_{20} :	32.3	39.7	62.3	39.3	13.8	34.2	10.7	23.4	34.6

Abbreviations & Symbols
Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

TABLE 6.2.7 : Individual lipid class FAME composition of *N. oculata* grown in batch culture at 20 °C for 232 hours using S88 medium modified to provide excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.8	3.7	8.2	6.0	2.7	3.3	1.2	4.2
15:0	.	.	.	.	.	.	.	.
16:0	14.7	7.8	7.0	21.5	26.6	26.5	6.6	14.6
16:1(*n*-7)	21.5	15.6	4.9	23.4	26.4	34.1	30.8	24.0
16:1(*n*-13)*t*	.	.	.	.	3.2	.	.	.
16:2	0.2	0.4	.	.	.	.	0.6	0.9
17:0	0.3	0.2	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	0.3
16:4	.	.	.	.	.	.	.	.
18:0	1.2	1.9	0.6	0.8	0.4	1.4	0.9	0.8
18:1(*n*-9)	3.1	3.3	0.3	0.5	0.7	4.3	7.7	1.9
18:1(*n*-7)	0.7	0.8	0.1	0.3	0.5	.	.	0.6
18:2(*n*-6)	1.4	2.4	0.4	0.8	0.7	1.5	7.7	1.5
18:3(*n*-6)	0.4	0.3	.	.	0.2	.	2.1	0.3
18:3(*n*-3)	0.2	0.6	.	.	0.1	.	0.2	0.3
18:4(*n*-3)	.	.	.	.	.	.	0.4	.
20:0	0.4	0.6	0.4	0.4	0.3	.	0.5	0.4
20:3(*n*-6)	0.9	0.3	.	.	0.2	.	0.7	0.3
20:4(*n*-6)	4.3	4.3	1.2	1.0	3.6	8.1	7.5	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	37.5	44.7	73.6	41.0	24.1	9.3	16.2	39.2
Unidentified :	9.4	13.1	3.1	4.3	10.3	11.5	16.9	6.9
Total sats :	20.4	14.2	16.3	28.7	30.0	31.2	9.2	20.0
Total monos :	25.3	19.7	5.4	24.3	30.8	38.4	38.5	26.5
Total PUFA :	44.9	53.0	75.3	42.8	28.9	18.9	35.4	46.6
Total (*n*-9) :	3.1	3.3	0.3	0.5	0.7	4.3	7.7	1.9
Total (*n*-6) :	7.0	7.3	1.6	1.7	4.7	9.6	18.0	5.9
Total (*n*-3) :	37.7	45.3	73.6	41.0	24.2	9.3	16.8	39.5
(*n*-3)/(*n*-6) :	5.4	6.2	46.0	24.1	5.1	1.0	0.9	6.7
16:1/16:0 :	1.5	2.0	0.7	1.1	1.0	1.3	4.7	1.6
20:5/16:0 :	2.6	5.7	10.5	1.9	0.9	0.4	2.5	2.7
ΣC_{16} :	36.4	23.8	11.9	44.9	56.2	60.6	38.0	39.8
ΣC_{18} :	7.0	9.3	1.4	2.4	2.6	7.2	19.0	5.4
ΣC_{20} :	43.1	49.3	74.8	42.4	28.2	20.8	24.9	43.7

Abbreviations & Symbols
Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected
‡, including SQDG

TABLE 6.2.8 : Individual lipid class FAME composition of *N. oculata* grown in batch culture at 15 °C for 264 hours using S88 medium modified to provide excess nitrate and phosphate.

	LIPID CLASSES								
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG	SQDG	PS/PI	PC	TOTAL
14:0	4.6	3.7	10.5	8.8	2.0	6.1	3.5	1.6	3.5
15:0	0.4	0.4	0.3	0.4	0.4	0.5	.	0.2	0.2
16:0	19.7	10.8	5.6	21.1	33.4	12.8	32.5	8.7	15.9
16:1(*n*-7)	35.6	24.1	4.1	20.7	28.4	23.0	34.8	25.4	21.5
16:1(*n*-13)*t*	.	.	.	.	1.3	.	.	.	.
16:2	0.5	0.5	.	0.4	0.4	0.6	.	1.4	0.4
17:0	0.4	0.3	.	.	.	.	.	0.2	0.2
16:3	0.2	.	.	.	.	.	.	0.5	.
16:4	.	.	.	.	.	.	.	.	.
18:0	0.9	1.9	0.6	0.4	0.6	0.6	1.1	0.6	0.6
18:1(*n*-9)	3.0	2.0	0.7	0.5	1.0	0.9	3.9	6.3	2.3
18:1(*n*-7)	0.6	0.4	.	0.2	0.2	0.4	.	0.5	0.3
18:2(*n*-6)	0.8	0.8	0.3	0.4	0.4	1.0	0.9	6.8	1.4
18:3(*n*-6)	0.4	.	.	.	0.2	0.3	.	1.8	0.4
18:3(*n*-3)	0.3	.	.	.	.	.	.	0.2	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	0.3	.
20:0	0.2	0.4	.	0.2	.	.	.	0.4	0.2
20:2	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.8	.	.	.	1.7	.	.	0.8	0.5
20:4(*n*-6)	3.0	4.0	0.8	0.4	.	3.7	6.9	9.8	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.	0.3
20:4(*n*-3)	.	.	.	.	2.9	.	.	.	0.1
20:5(*n*-3)	23.4	40.4	74.3	43.1	18.3	40.9	4.3	22.8	36.0
Unidentified :	5.2	10.3	2.9	3.4	8.9	9.2	12.1	11.7	7.8
Total sats :	26.2	17.5	17.0	30.9	36.4	20.1	37.1	11.7	20.6
Total monos :	39.2	26.5	4.7	21.4	30.9	24.3	38.7	32.2	28.5
Total PUFA :	29.4	45.7	75.4	44.3	23.9	46.5	12.1	44.4	43.1
Total (*n*-9) :	3.0	2.0	0.7	0.5	1.0	0.9	3.9	6.3	2.3
Total (*n*-6) :	5.0	4.8	1.1	0.8	2.3	5.0	7.8	19.2	6.1
Total (*n*-3) :	23.7	40.4	74.3	43.1	21.2	40.9	4.3	23.3	36.6
(*n*-3)/(*n*-6) :	4.7	8.4	67.5	53.9	9.2	8.2	0.6	1.2	6.0
16:1/16:0 :	1.8	2.2	0.7	1.0	0.9	1.8	1.1	2.9	1.6
20:5/16:0 :	1.2	3.7	13.3	2.0	0.5	3.2	0.1	2.6	2.3
ΣC_{16}:	56.0	35.4	9.7	42.4	63.5	36.4	67.0	36.0	41.5
ΣC_{18}:	6.0	5.1	1.6	1.5	2.4	3.2	5.0	16.5	5.6
ΣC_{20}:	27.4	44.8	75.1	43.5	22.9	44.6	11.2	33.8	44.5

Abbreviations & Symbols
Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

TABLE 6.2.9 : Individual lipid class FAME composition of *N. oculata* grown in batch culture at 10 °C for 552 hours using S88 medium modified to provide excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.8	2.9	9.4	8.8	2.7	2.6	1.5	4.2
15:0	.	.	.	.	.	.	.	.
16:0	15.4	6.7	6.8	19.8	24.6	19.2	7.2	12.9
16:1(*n*-7)	33.1	25.2	4.6	23.2	23.2	23.7	28.3	26.2
16:1(*n*-13)‡	.	.	.	.	.	.	.	.
16:2	.	.	.	.	.	.	0.7	0.4
17:0	0.2	.	.	.	.	.	0.2	.
16:3	0.3	.	.	.	.	.	0.9	0.3
16:4	.	.	.	.	.	.	0.3	.
18:0	0.5	1.1	0.2	0.3	0.2	0.8	0.3	0.3
18:1(*n*-9)	2.9	2.5	0.4	0.4	0.7	3.3	12.9	2.9
18:1(*n*-7)	0.4	0.5	0.8	0.6	0.5	1.8	1.2	0.2
18:2(*n*-6)	0.6	1.6	0.2	0.4	0.5	1.2	8.1	1.8
18:3(*n*-6)	0.2	.	.	.	0.1	0.4	1.7	0.3
18:3(*n*-3)	0.1	.	.	0.1	.	.	0.1	0.2
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	0.1	.	.	0.1	.	.	0.2	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	1.0	.	.	.	.	1.3	0.8	0.6
20:4(*n*-6)	3.4	4.7	0.6	0.8	2.5	23.7	6.6	4.5
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	33.0	44.4	73.8	42.5	32.3	9.7	19.1	37.8
Unidentified :	4.0	10.4	3.2	3.0	12.7	12.3	9.9	7.4
Total sats :	21.0	10.7	16.4	29.0	27.5	22.6	9.4	17.4
Total monos :	36.4	28.2	5.8	24.2	24.4	28.8	42.4	29.3
Total PUFA :	38.6	50.7	74.6	43.8	35.4	36.3	38.3	45.9
Total (*n*-9) :	2.9	2.5	0.4	0.4	0.7	3.3	12.9	2.9
Total (*n*-6) :	5.2	6.3	0.8	1.2	3.1	26.6	17.2	7.2
Total (*n*-3) :	33.0	44.4	73.8	42.6	32.3	9.7	19.2	38.0
(*n*-3)/(*n*-6) :	6.4	7.0	92.3	35.5	10.4	0.4	1.1	5.3
16:1/16:0 :	2.1	3.8	0.7	1.2	0.9	1.2	3.9	2.0
20:5/16:0 :	2.1	2.6	10.9	2.1	1.3	0.5	2.7	2.9
ΣC_{16}:	48.8	31.9	11.4	43.0	47.8	42.9	37.4	39.8
ΣC_{18}:	4.7	5.7	1.6	1.8	2.0	7.5	24.3	5.7
ΣC_{20}:	37.5	49.1	74.4	43.4	34.8	25.0	26.5	42.9

Abbreviations & Symbols
Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected
‡, including SQDG

was comparatively insignificant. These generally involved the simple, or neutral, lipid class TAG. It was probable that the fluctuations occurred as a result of the shifts in the rate of photosynthesis *and* the pattern of partitioning of the resulting fixed carbon. The picture built up from the analysis of the changing proportions and acyl compositions of the lipid component may have been augmented by concurrent analyses of both the carbohydrate and protein components of the cells. It was clear that to consider the lipid component of the cell in a context which abstracted it from the other major cellular components was not an ideal approach. However, a more complete analysis was not possible due to considerations of time, equipment availability and the small amount of cell material available in the case of many of the samples. This was also the case for the following section, in which the variations in fatty acyl and lipid class composition of *N. oculata* resulting from growth in media containing different concentrations of sodium chloride were examined.

6.3 VARIATION IN LIPID COMPOSITION WITH MEDIUM SODIUM CHLORIDE CONCENTRATION OR "SALINITY" IN BATCH CULTURE.

6.3.1 Experimental.

Cultures of *N. oculata* were grown as described in the previous sub-section. The S88 culture medium itself was further modified with respect to its sodium chloride concentration creating the salinity gradient used for this series of cultures. A bulk batch of S88 medium was made with the omission of sodium chloride, nitrate and glycine. Glycine was added to the rehydrated medium base in accordance with the recipe as described in Appendix D. Nitrate was added to three times the normal S88 concentration and the phosphate was supplemented to give a concentration twice that in normal S88.

Because *N. oculata* was isolated from a brackish environment, the medium used in its artificial maintenance, S88, was a half salinity medium. As a rough guide, normal salinity may be regarded to be *ca.* 32 - 35 g NaCl l^{-1}. In the following series of cultures the sodium chloride concentration of the medium was assumed to be equivalent to salinity. It must be borne in mind, however, that this is not strictly the case. The range of sodium chloride concentrations used was 64 g l^{-1}, 48 g l^{-1}, 32 g l^{-1}, 16 g l^{-1}, 8 g l^{-1} and 0 g l^{-1}. This range approximated to salinities of double, one and a half, normal, half and one quarter those of natural seawater, with the final medium lacking NaCl completely and corresponding to near freshwater. Salinity was varied in this way to avoid complications which may have arisen from changes in trace component concentration or medium buffering capacity had all the medium components been changed by similar proportions.

The cultures were sampled and harvested in accordance with the routine detailed in Figure 5.3.1. An inter-culture comparison strategy similar to that described in Section 6.2 was applied. 'Equivalence points' were established in relation to growth expressed as a function of cell numbers per unit volume. Again, the lipid extracts from the samples falling closest to these points were used for the more detailed analysis of the acyl composition of

their individual lipid classes (detailed in Section 5.4). The lipid extracts of all samples were analysed by HPTLC densitometry and total FAME were prepared and analysed by GLC.

6.3.2 Results & Discussion.

The cultures inoculated into the S88 medium modified to contain 64 g and 0 g NaCl l^{-1} showed no growth. Over a period of several days immediately following inoculation, both of these cultures had bleached and shown steady decreases in cell numbers per unit volume. These cultures were aborted and data regarding them are not included in the following section.

Examination of the growth curve data for the cultures, shown in Figures 6.3.1 and 6.3.2, showed that culture salinity had an obvious effect upon the growth of *N. oculata*, regardless of whether it was expressed in terms of cell numbers or biomass per unit culture volume. The growth rate data were plotted in terms of the instantaneous cell division rate (μ) *vs.* salinity (see Figure 6.3.3) (*i.e.* the straight line gradient of the line between the first and last sampling points). The line connecting the points could be extrapolated, knowing that no growth occurred at the two experimental extremes of salinity. Peak growth rate was measured at a sodium chloride concentration of 16 g l^{-1}. This corresponded to a salinity approximately half that of normal seawater (*i.e.* that of unmodified S88). As can be see from Figure 6.3.3 the growth rate decreased sharply at both sub-optimal and supra-optimal salinities. A significant difference between the growth of cultures where salinity was the experimental variable and the previous cultures, in which temperature was the variable factor, was found. Comparison of Figures 6.2.1 and 6.3.1 shows that, whilst temperature affected the culture's exponential growth rates, it also affected the length of duration of their lag phases. Variation in salinity, however, affected only the growth rate of the cultures. Any lag-phase was indiscernible across the range of salinity variation. In the light of the data in Section 6.2 this would be expected at the near optimal growth temperature of 20 °C. An explanation for the observed difference could be the previously discussed shift in the partitioning of fixed carbon which was thought to have occurred with temperature. As will

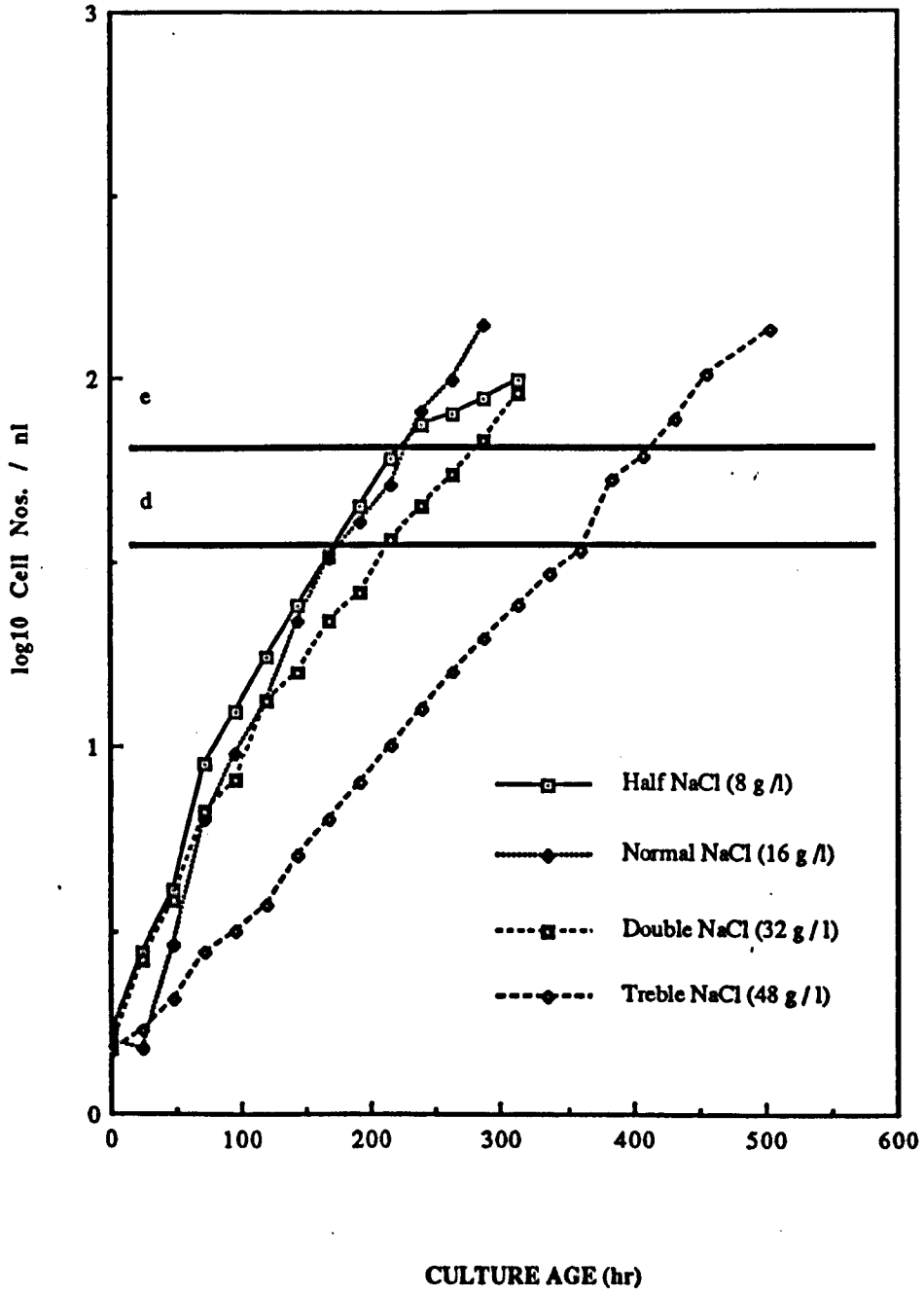


Figure 6.3.1 : Growth curves for *N. oculata* grown in batch culture at 20 °C using S88 medium modified with respect to its sodium chloride concentration. Lines 'd' & 'e' mark the equivalence points detailed in the text¶.

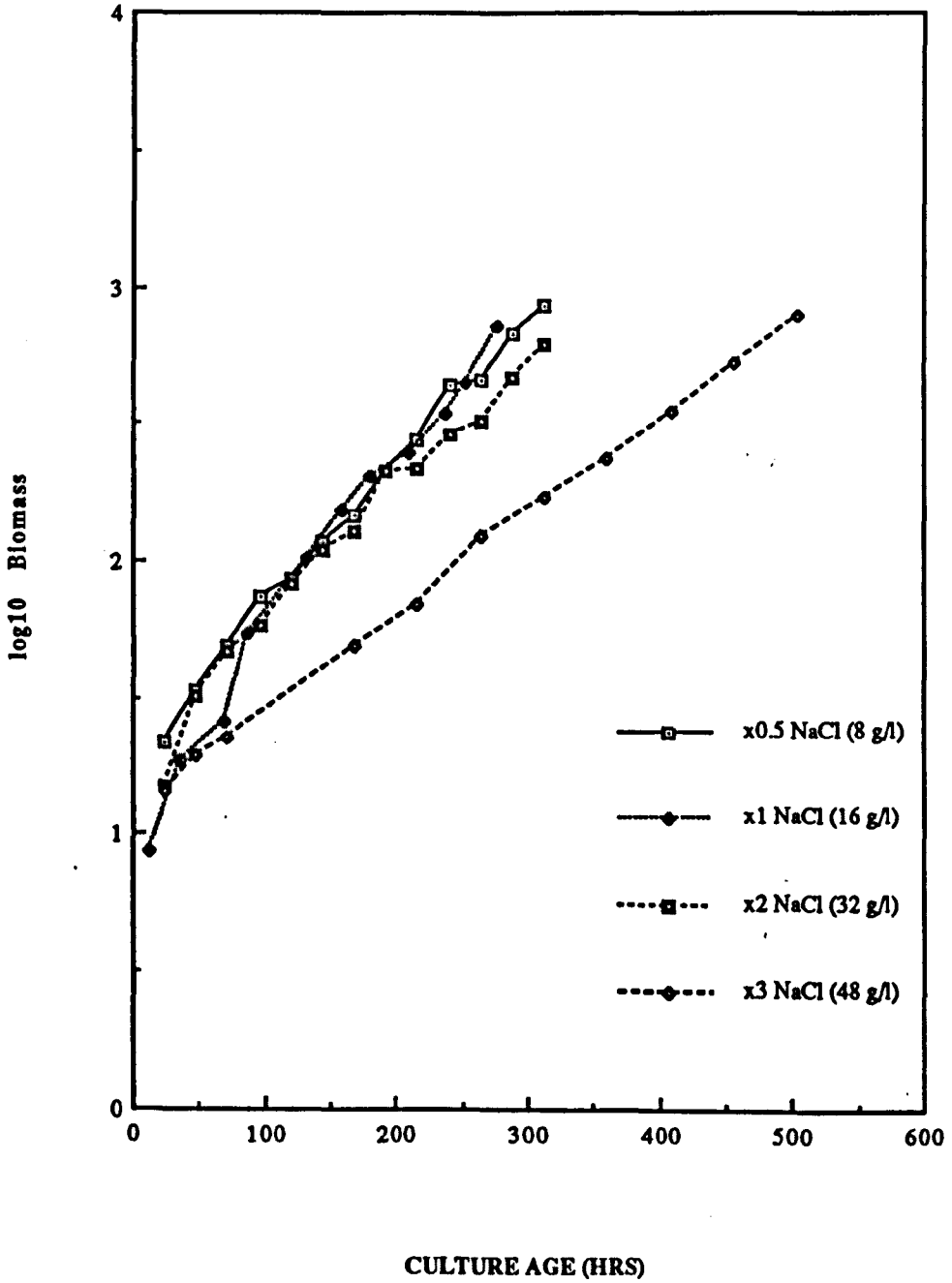


Figure 6.3.2 : Growth curves for *N. oculata* grown in batch culture at 20 °C using S88 medium modified with respect to its sodium chloride concentration. Data expressed in terms of biomass per unit volume[1].

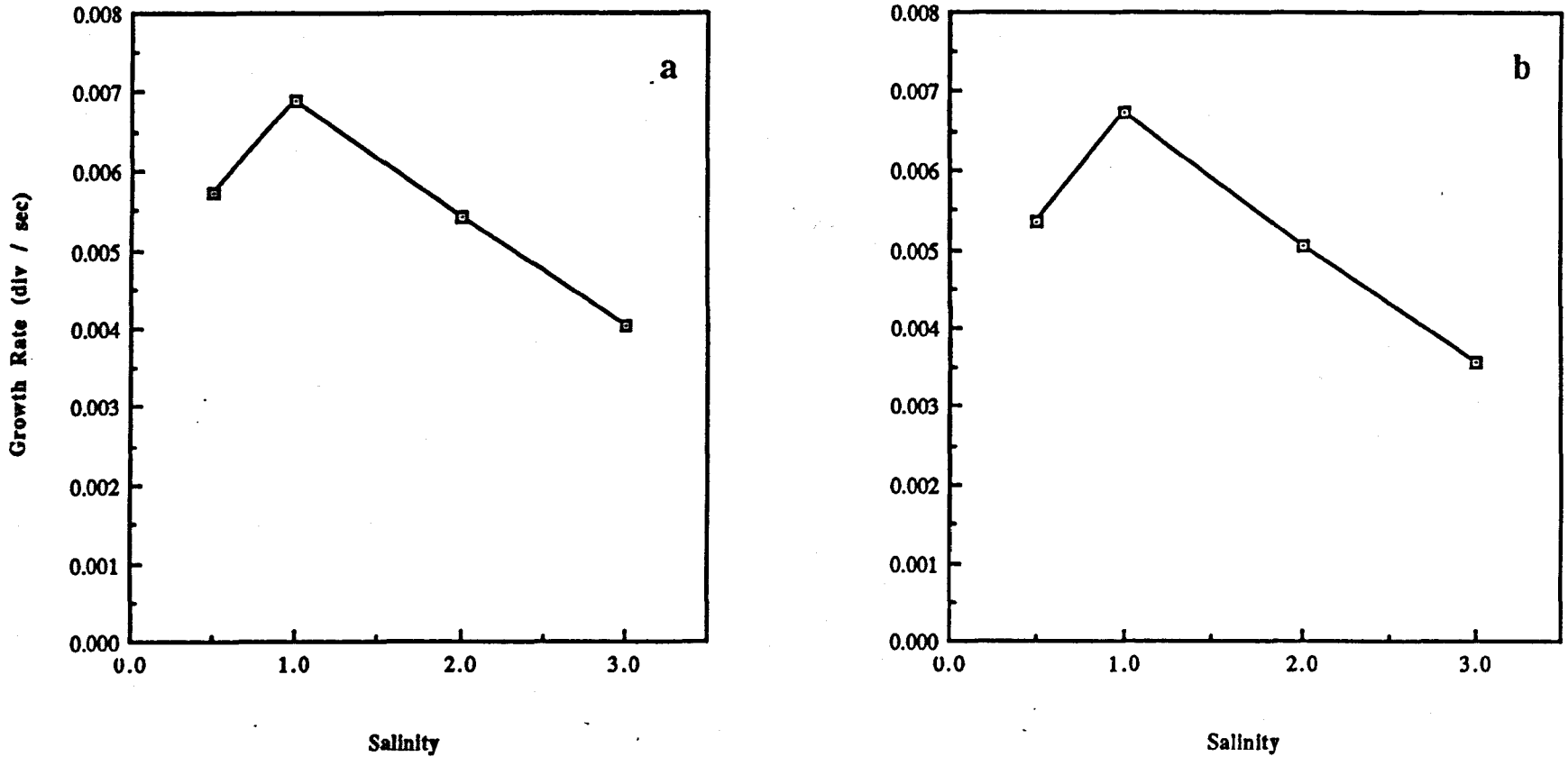


Figure 6.3.3 : Changes in the exponential phase growth rate of *N.oculata* with salinity. Data expressed in terms of a) cell numbers or, b) biomass[1].

be discussed below signs of a lag phase were visible at the total FAME level as they were for the previous cultures.

By expressing growth as the accumulation of biomass per unit culture volume several similar phenomena were noted. The lag phase did not become any more or less evident and the progression of growth of all four cultures followed patterns of exponential increases in mass (Figure 6.3.2). The rates of growth expressed in these terms are plotted in Figure 6.3.3.b.

Tables 6.3.1, 6.3.2, 6.3.3 and 6.3.4 show the changes in the total FAME composition of the cultures of *N. oculata* under the four different salinity regimes for which full data were available. The data contained within these tables showed that the progression of the cultures through the characteristic changes in acyl composition was similar to that expected for a batch culture, corroborating the findings from previous experiments. Over the first sampling periods, the total FAME compositions of the cultures generally became more saturated and the mean acyl chain length became shorter with increased proportions of the C_{16} fatty acids 16:0 and 16:1 and decreased proportions of 20:5(*n*-3). This trend was reversed after the lag-phase. Although a lag-phase was not immediately obvious from the growth curves expressed in terms of cell numbers, it was evident that the cells from all the cultures were affected in a similar way in terms of their acyl composition.

The data for the culture grown at a salinity half that of normal S88 medium (or *one quarter* that of normal seawater) are shown in Table 6.3.1. This culture demonstrated a typical pattern in its total FAME composition which was predictable in the light of the data obtained from previous batch cultures and outlined above. At inoculation the fatty acyl profile consisted of the three major fatty acids of *N. oculata* in the proportions of 16:0 at 14.0 %, 16:1 at 27.3 % and 20:5(*n*-3) at 38.1 %. During the first period of 24 hours post inoculation the proportions of these acids changed to 26.2 %, 29.7 % and 27.1% respectively. Concomitant changes in the proportion of total saturates from 18.3 % to 31.1

TABLE 6.3.1: Total FAME composition of *N. oculata* with culture age grown in S88 medium modified to contain half its normal NaCl concentration and excess nitrate & phosphate.

FATTY ACID	SAMPLE TIME (Hrs)												
	0	24[a]	48	72[b]	96	120[c]	144	168[d]	192	240[e]	264	288	312[f]
14:0	3.3	3.5	3.9	4.7	4.8	5.2	4.8	4.5	4.5	4.3	4.5	4.2	3.8
15:0	0.6	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3
16:0	14.0	26.2	21.6	18.4	18.3	18.0	17.6	16.1	15.1	14.8	14.9	17.5	20.8
16:1(*n*-7)	27.3	29.7	26.4	25.9	26.3	27.5	27.4	27.6	25.1	24.3	23.4	23.9	23.9
16:1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.	.	.
16:2	0.7	0.4	0.5	0.4	0.4	0.3	0.4	0.4	0.5	0.5	0.5	0.4	0.3
17:0	.	.	.	.	.	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	.	.	.	.	.	.
16:4	.	.	.	.	.	.	.	.	.	.	.	.	.
18:0	0.4	1.0	0.8	0.4	0.6	0.6	0.6	0.5	0.5	0.5	0.5	0.8	1.2
18:1(*n*-9)	1.7	2.6	2.1	2.5	2.2	3.2	2.3	2.0	2.5	2.1	2.9	5.1	6.4
18:1(*n*-7)	1.1	.	.	.	.	.	.	.	0.8	.	.	.	.
18:2(*n*-6)	2.2	1.3	1.4	1.7	1.8	1.8	1.7	1.7	1.4	1.4	1.8	2.2	2.5
18:3(*n*-6)	0.5	0.3	0.2	0.2	0.4	0.2	0.3	0.3	0.4	0.3	0.3	0.4	0.6
18:3(*n*-3)	0.6	0.4	0.5	0.5	0.4	0.4	0.4	0.3	0.4	0.5	0.4	0.3	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.1	0.8	0.6	0.4	0.5	0.4	0.4	0.4	0.2	0.3	0.5	0.7	1.8
20:4(*n*-6)	4.1	3.8	4.4	4.4	4.1	4.2	4.1	4.1	3.3	3.7	4.0	3.9	3.3
20:3(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	38.1	27.1	33.4	35.6	35.5	31.9	34.9	37.1	37.7	42.1	41.5	38.1	30.7
Unidentified:	5.3	2.5	3.9	4.6	4.3	5.9	4.8	4.7	7.3	4.9	4.5	2.2	4.1
Total sats :	18.3	31.1	26.6	23.8	24.0	24.2	23.3	21.4	20.4	19.1	20.2	22.8	26.1
Total monos :	30.1	32.3	28.5	28.4	28.5	30.7	29.7	29.6	28.4	26.4	26.3	29.0	30.3
Total PUFA :	46.3	34.1	41.0	43.2	43.2	39.2	42.2	44.3	43.9	48.8	49.0	45.6	39.5
Total (*n*-9) :	1.7	2.6	2.1	2.5	2.2	3.2	2.3	2.0	2.5	2.9	5.1	6.4	
Total (*n*-6) :	6.9	6.2	6.6	6.7	6.8	6.6	6.5	6.5	5.3	5.7	6.6	7.2	8.2
Total (*n*-3) :	38.7	27.5	33.9	36.1	36.0	32.3	35.3	37.4	38.1	42.6	41.9	38.4	31.0
(*n*-3)/(*n*-6) :	5.6	4.4	5.1	5.4	5.3	4.9	5.4	5.8	7.2	7.5	6.3	5.3	3.8
16:1/16:0 :	2.0	1.1	1.2	1.4	1.4	1.5	1.6	1.7	1.7	1.6	1.6	1.4	1.1
20:5/16:0 :	2.7	1.0	1.5	1.9	1.9	1.8	2.0	2.3	2.5	2.8	2.8	2.2	1.5
ΣC_{16}:	42.0	56.3	48.5	44.7	45.0	45.8	45.4	44.1	40.7	39.6	38.8	41.8	45.0
ΣC_{18}:	6.5	5.6	5.0	5.3	5.4	6.2	5.3	4.8	6.0	4.8	5.9	8.8	11.0
ΣC_{20}:	42.3	31.7	38.4	40.4	40.1	36.5	39.4	41.6	41.2	46.1	46.0	42.7	35.8

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected

[a], [b], [c], [d], [e], and [f], inter-data set equivalence points in terms of cell division (see text for details).

TABLE 6.3.2 : Total FAME composition of *N. oculata* grown in S88 medium unmodified with respect to its NaCl concentration but modified to provide excess nitrate & phosphate.

	CULTURE AGE (Hrs)										
FATTY ACID	12	36	50[a]	78[b]	132	158	178[d]	202	236[e]	252[f]	276
14:0	1.7	2.5	2.6	3.4	4.3	4.2	3.8	4.2	4.2	4.3	4.4
15:0	.	.	.	.	.	.	.	.	.	.	.
16:0	18.1	31.1	27.1	21.3	15.4	13.4	13.6	14.2	14.6	14.7	17.3
16:1(*n*-7)	13.9	31.7	32.0	32.7	28.2	27.0	25.8	24.4	24.0	24.1	24.9
16.1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.
16:2	.	0.2	0.1	0.4	0.6	0.8	0.7	0.9	0.9	0.8	0.5
17:0	.	.	.	.	.	.	.	.	.	.	.
16:3	0.2	0.3	0.2	.	0.2	0.3	0.3	.	0.3	0.3	0.2
16:4	.	.	.	.	.	.	.	.	.	.	.
18:0	3.1	1.8	1.6	0.9	0.6	0.5	0.4	0.5	0.8	0.5	0.7
18:1(*n*-9)	18.6	4.3	3.9	2.7	2.9	2.2	2.3	2.0	1.9	1.4	2.3
18:1(*n*-7)	.	1.3	1.2	0.7	0.5	0.4	0.3	0.6	0.6	0.4	0.5
18:2(*n*-6)	28.5	2.4	2.7	1.7	2.2	2.1	2.2	1.9	1.5	1.3	1.6
18:3(*n*-6)	0.2	0.8	0.6	0.5	0.4	0.5	0.4	0.4	0.3	0.3	0.4
18:3(*n*-3)	0.5	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:0	0.5	0.2	0.3	0.3	0.3	0.3	0.3	0.4	0.4	0.4	0.4
20:3(*n*-6)	0.7	1.3	1.3	0.9	0.5	0.4	0.4	0.4	0.3	0.4	0.7
20:4(*n*-6)	1.1	3.4	3.2	3.8	4.1	4.2	4.5	4.0	3.8	3.8	4.0
20:3(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	8.6	15.9	19.5	27.1	32.6	36.1	36.4	37.0	39.2	35.7	35.7
Unidentified :	4.3	2.6	3.5	3.4	7.0	7.4	8.4	8.9	6.9	11.3	6.1
Total sats :	23.4	35.6	31.6	25.9	20.6	18.4	18.1	19.3	20.0	19.9	22.8
Total monos :	32.5	37.3	37.1	36.1	31.6	29.6	28.4	27.0	26.5	25.9	27.7
Total PUFA :	39.8	24.5	27.8	34.6	40.8	44.6	45.1	44.8	46.6	42.9	43.4
Total (*n*-9) :	18.6	4.3	3.9	2.7	2.9	2.2	2.3	2.0	1.9	1.4	2.3
Total (*n*-6) :	30.5	7.9	7.8	6.9	7.2	7.2	7.5	6.7	5.9	5.8	6.7
Total (*n*-3) :	9.1	16.1	19.7	27.3	32.8	36.3	36.6	37.2	39.5	36.0	36.0
(*n*-3)/(*n*-6) :	0.3	2.0	2.5	4.0	4.6	5.0	4.9	5.6	6.7	6.2	5.4
16:1/16:0 :	0.8	1.0	1.2	1.5	1.8	2.0	1.9	1.7	1.6	1.6	1.4
20:5/16:0 :	0.5	0.5	0.7	1.3	2.1	2.7	2.7	2.6	2.7	2.4	2.1
ΣC_{16}:	59.3	63.3	59.4	54.4	44.4	41.5	40.4	39.5	39.8	42.0	42.9
ΣC_{18}:	9.6	10.8	10.2	6.7	6.8	5.9	5.8	5.6	5.4	6.1	5.8
ΣC_{20}:	20.1	20.8	24.3	32.1	37.5	41.0	41.6	41.8	43.7	42.4	40.8

Abbreviations & Symbols
Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected.
[a], [b], [c], [d], [e], and [f], inter-data set equivalence points in terms of cell division (see text for details)

TABLE 6.3.3 : Total FAME composition of *N. oculata* with culture age grown in S88 medium modified to contain double its normal NaCl concentration and excess nitrate & phosphate.

FATTY ACID	SAMPLE TIME (Hrs)												
	0	24[a]	48	72[b]	120	144[c]	168	192	216[d]	240	264	288[e]	312[f]
14:0	3.3	4.6	4.6	4.6	4.4	4.2	4.2	4.7	4.0	3.7	4.0	4.1	4.2
15:0	0.6	0.7	0.4	0.4	0.4	0.4	0.3	0.4	0.5	0.4	0.4	0.3	0.3
16:0	14.0	30.9	33.1	30.8	30.9	30.4	27.9	27.8	21.7	17.0	16.2	17.9	19.7
16:1(*n*-7)	27.3	28.5	28.0	26.7	31.6	31.2	31.7	30.7	31.4	31.8	30.4	30.4	32.1
16:1(*n*-13)t.	.	.	.	.	.	.	.	.	.	.	.	.	.
16:2	0.7	0.3	0.2	0.1	0.1	.	.	.	0.2	0.2	0.2	0.2	0.2
17:0	.	.	.	.	.	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	.	.	.	.	.	.
16:4	.	.	.	.	.	.	.	.	.	.	.	.	.
18:0	0.4	2.0	1.2	1.0	1.0	1.0	1.4	1.4	0.7	0.5	0.5	0.5	0.7
18:1(*n*-9)	1.7	5.4	4.6	4.8	5.6	6.0	6.5	4.6	4.7	3.9	2.0	0.8	3.2
18:1(*n*-7)	1.1	.	.	.	.	.	.	1.8	.	.	0.9	.	1.1
18:2(*n*-6)	2.2	2.0	1.9	2.1	1.8	1.9	1.4	2.2	1.9	2.6	2.1	1.7	1.9
18:3(*n*-6)	0.5	0.4	0.4	0.5	0.2	0.2	0.2	.	0.4	0.3	0.5	0.3	0.5
18:3(*n*-3)	0.6	0.2	0.2	0.2	.	.	.	.	.	.	.	0.3	.
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.1	1.2	1.6	1.7	1.1	0.9	0.8	0.7	0.5	0.4	0.5	0.5	1.6
20:4(*n*-6)	4.1	3.5	3.7	4.5	4.1	4.3	4.5	4.1	5.3	5.6	5.8	5.5	4.8
20:3(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	38.1	19.8	17.5	20.4	17.7	18.5	20.1	19.3	26.7	31.6	34.1	35.9	27.3
Unidentified:	5.3	0.5	2.6	2.2	1.1	1.0	1.0	2.3	2.4	2.0	2.4	1.6	2.5
Total sats :	18.3	38.2	39.3	36.8	36.7	36.0	33.3	34.3	26.9	21.6	21.1	22.8	24.9
Total monos :	30.1	33.9	32.6	31.5	37.2	37.2	38.3	37.1	36.1	35.7	33.3	31.2	36.4
Total PUFA :	46.3	27.3	25.5	29.5	25.0	25.8	26.9	26.3	34.6	40.7	43.2	44.4	36.3
Total (*n*-9) :	1.7	5.4	4.6	4.8	5.6	6.0	6.5	4.6	4.7	3.9	2.0	0.8	3.2
Total (*n*-6) :	6.9	7.1	7.6	8.8	7.2	7.3	5.5	7.0	8.1	8.9	8.9	8.0	8.8
Total (*n*-3) :	38.7	20.0	17.7	20.6	17.7	18.5	20.1	19.3	26.3	31.6	34.1	36.2	27.3
(*n*-3)/(*n*-6) :	5.6	2.8	2.3	2.3	2.5	2.5	2.9	2.8	3.2	3.6	3.8	4.5	3.1
16:1/16:0 :	2.0	0.9	0.8	0.9	1.0	1.0	1.1	1.1	1.4	1.9	1.9	1.7	1.6
20:5/16:0 :	2.7	0.6	0.5	0.7	0.6	0.6	0.7	0.7	1.2	1.9	2.1	2.0	1.4
ΣC_{16}:	42.0	59.7	61.3	57.6	62.6	61.6	59.6	58.5	53.3	49.0	46.8	48.5	52.0
ΣC_{18}:	6.5	10.0	8.3	8.6	8.6	9.1	9.5	10.0	7.7	7.3	6.0	3.6	7.4
ΣC_{20}:	42.3	24.5	22.8	26.6	22.9	23.7	25.4	24.1	32.5	37.6	40.4	41.9	33.7

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected.

a, b, c, d, e, and f, inter-data set equivalence points in terms of cellular division (see text for details)

TABLE 6.3.4 : Total FAME composition of *N. oculata* with culture age grown in S88 medium modified to contain treble its normal NaCl concentration and excess nitrate & phosphate.

FATTY ACID	SAMPLE TIME (Hrs) 0	24	48	72[a]	120	168[b]	216	264[c]	312	360[d]	408[e]	456[f]	504
14:0	3.3	3.6	4.7	4.0	4.0	3.4	3.6	3.6	3.9	3.5	3.7	3.1	3.2
15:0	0.6	0.7	0.6	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.3	0.3	0.3
16:0	14.0	22.4	26.7	29.1	31.1	32.1	30.0	29.1	28.7	27.1	25.9	24.0	25.4
16:1(*n*-7)	27.3	25.6	29.2	30.2	31.2	30.6	30.8	30.0	30.4	30.0	29.7	29.6	30.6
16:1(*n*-13)*t*.	.	.	.	.	.	.	.	.	.	.	.	.	.
16:2	0.7	0.4	0.3	0.2	0.2	.	.	.	.	.	.	.	.
17:0	.	.	.	.	.	.	.	.	.	.	.	.	.
16:3	.	.	.	0.3	.	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.2
16:4	.	.	.	.	.	.	.	.	.	.	.	.	.
18:0	0.4	1.9	1.4	1.1	1.2	1.1	0.9	0.9	1.0	0.9	0.9	0.8	0.7
18:1(*n*-9)	1.7	10.2	9.3	8.4	9.8	9.7	9.1	8.5	10.5	10.4	10.4	9.3	10.9
18:1(*n*-7)	1.1	.	0.5	0.6	0.7	.	.	0.5	.	.	.	0.5	0.9
18:2(*n*-6)	2.2	1.5	1.9	2.2	2.0	2.1	2.2	2.1	2.0	2.3	2.2	2.3	2.2
18:3(*n*-6)	0.5	0.2	0.2	0.3	0.2	0.3	0.3	0.3	0.2	0.3	0.3	0.4	0.5
18:3(*n*-3)	0.6	0.3	0.2	0.2	.	.	.	.	.	.	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.1	0.3	0.4	0.6	0.6	0.7	0.9	0.9	0.7	0.8	0.7	1.0	1.1
20:4(*n*-6)	4.1	2.6	3.0	3.2	2.9	3.0	3.7	4.0	4.0	4.5	4.4	5.7	5.4
20:3(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	38.1	23.0	18.2	16.0	12.9	13.6	16.1	17.0	16.5	17.9	18.4	21.0	16.9
Unidentified:	5.3	7.3	3.4	3.1	2.8	2.8	1.8	2.5	1.5	1.8	2.9	1.7	1.7
Total sats :	18.3	28.6	33.4	34.7	36.7	37.0	34.9	34.0	34.0	31.8	30.8	28.2	29.6
Total monos :	30.1	35.8	39.0	39.2	41.7	40.3	39.9	39.0	40.9	40.4	40.1	39.4	42.4
Total PUFA :	46.3	28.3	24.2	23.0	18.8	19.9	23.4	24.5	23.6	26.0	26.2	30.7	26.3
Total (*n*-9) :	1.7	10.2	9.3	8.4	9.8	9.7	9.1	8.5	10.5	10.4	10.4	9.3	10.9
Total (*n*-6) :	6.9	4.6	5.5	6.3	5.7	6.1	6.1	7.3	6.7	7.9	7.6	9.4	9.2
Total (*n*-3) :	38.7	23.3	18.4	16.2	12.9	13.6	16.1	17.0	16.7	17.9	18.4	21.0	16.9
(*n*-3)/(*n*-6) :	5.6	5.1	3.3	2.6	2.3	3.2	2.3	2.3	2.5	2.3	2.4	2.2	1.8
16:1/16:0 :	2.0	1.1	1.1	1.0	1.0	1.0	1.0	1.0	1.1	1.1	1.1	1.2	1.2
20:5/16:0 :	2.7	1.0	0.7	0.5	0.4	0.4	0.5	0.6	0.6	0.7	0.7	0.9	0.7
ΣC_{16}:	42.0	48.4	56.8	59.8	62.5	62.9	61.0	59.3	59.3	57.3	55.8	53.9	56.2
ΣC_{18}:	6.5	14.1	13.5	12.8	13.9	13.7	12.5	12.3	13.7	14.1	14.0	13.3	15.2
ΣC_{20}:	42.3	25.9	21.6	19.8	16.4	17.3	20.7	21.9	21.2	23.2	23.5	27.7	23.4

Abbreviations & Symbols

Data expressed in terms of % mass of FAME; tr.,<0.1%; ., not detected.

[a], [b], [c], [d], [e] and [f], inter-data set equivalence points in terms of cellular division (see text for details).

%, total monounsaturates from 30.1 % to 32.3 % and total PUFA from 46.3 % to 34.1 % also occurred. The ratio of 20:5/16:0 underwent a decrease from a value of 2.7 to 1.0 whilst that of 16:1/16:0 decreased in value, to a lesser extent, from 2.0 to 1.1. The change in the acyl chain length distribution of the total FAME showed the switch in the proportions of C_{16} fatty acids from 42.0 % to 56.3 % and in C_{20} fatty acids from 42.3 % to 31.7 % . In previous experiments these observations were correlated with the non-dividing phases of growth, the lag- and stationary-phases. During the next 24 hour period, up to 48 hours post-inoculation, the proportions of the fatty acids reverted to a pattern more characteristic of a divisional phase. The proportion of 16:0 decreased to 21.6 %, that of 16:1 also decreased to 26.4 % whilst the proportion of 20:5(*n*-3) increased to 33.4 %. As a result of these changes the acyl chain length distribution profile of the total FAME was modified having proportions of 48.5 % C_{16}, 5.0 % C_{18} and 38.4 % C_{20}. The trend continued as the culture entered the exponential phase and the cells were dividing at their optimum rate for the applied conditions.

Maximum polyunsaturation occurred between 240 and 264 hours post inoculation after 5 doubling periods. The proportions of the major fatty acids were similar to those in the inoculum culture, although in this case unsaturation was further increased to give an observed maximum proportion of total PUFA between 48.8 % and 49.0 %. Of this, 42.1 % to 41.5 % was accounted for by 20:5(*n*-3). The proportions of the C_{16} fatty acids had fallen to 14.8 % of 16:0 and 24.3 % of 16:1. These proportions were again reflected in the values for the total saturates, at *ca.* 20 % and total monounsaturates, at *ca.* 26 %. The ratios of 20:5/16:0 peaked at a value of 2.9 at 216 hours post inoculation. The acyl chain length distribution of the cells at 240 hours post-inoculation was 39.6 % C_{16}, 4.8 % C_{18} and 46.1 % C_{20} Due to a decrease in the proportion of 16:1 during growth at half normal salinity, the peak recorded value for the ratio of 16:1/16:0 was in the inoculum. However, the highest value, after a substantial time in culture was 1.7, recorded over the period between 144 hours and 216 hours post-inoculation. During the remainder of the sampling period this culture showed signs of entering the non-dividing, stationary-phase. By 288 hours post-

inoculation the proportions of C_{16} and C_{18} saturates and monounsaturates had begun to rise at the expense of C_{20} fatty acids which had decreased in proportion to 35.8 %. The final sample, at 312 hours post inoculation, showed the early accumulation of 18:1(*n*-9) and 18:2(*n*-6) associated with the cessation of cellular division. The cellular proportion of C_{18} fatty acids had risen to 11.0 % at 312 hours post-inoculation.

The culture grown in medium modified to give a salinity double that of normal S88 (or one approximating 'normal' seawater) took longer to reverse the trend of decreasing unsaturation in its total FAME composition. The decline began sharply, being more abrupt than the similar effects observed in the cultures grown at lower salinities. In the first 24 hours post-inoculation the proportion of 16:0 increased from 14.0 % to 30.9 % and that of 20:5(*n*-3) decreased from 38.1 % to 19.8 %. The acyl chain length distribution and saturation was modified resulting in shorter, more saturated fatty acids. At *ca.* 6 to 10 % the proportions of C_{18} fatty acids in this culture were generally higher than those recorded for the culture grown in half salinity and normal S88. The proportions of the C_{16} chain length fatty acids were generally higher than those for cultures grown at lower salinities. On the other hand, at *ca.* 42 % maximum the proportions of C_{20} fatty acids were generally several percent lower than those observed for cultures grown at lower salinity.

The ratios of 16:1/16:0 and 20:5/16:0 fell from 2.0 to 0.9 and 2.7 to 0.6 respectively within 24 hours of transfer to the new double-salinity culture medium. The proportion of 16:1 was relatively unaffected but the proportion of 18:1(*n*-9) increased from 1.7 % to 5.4 %. This may have been due, in part, to a loss of resolution between the (*n*-9) and (*n*-7) isomers of 18:1. However, an increase in the C_{18} monounsaturates *was* evident. By 48 hours post-inoculation the proportions of 16:0 and 20:5(*n*-3) had attained their respective peak and trough values of 33.1 % and 17.5 %, as had the proportions of C_{16} and C_{20} fatty acid chain lengths (61.3 % and 22.8 % respectively). During the next period, 72 to 96 hours post inoculation, the quantitative trends reversed. The proportions of 16:0 gradually decreased from 33.1 % to 16.2 % by 264 hours post-inoculation. Complementary increases

in the proportion of 20:5(*n*-3) resulted in a peak proportion of 35.9 % recorded 288 hours after inoculation of the culture. The timing of the two extremes of 16:0 and 20:5 proportions were slightly asynchronous. This was due to a slight decrease in the proportions of both 18:1(*n*-9) and 18:2(*n*-6) between the samples taken at 264 hours and 288 hours post-inoculation. This resulted in complementary increases in the proportion of 20:5(*n*-3). However, it may also be noted that the 288 hour sample was significant in that it was also the start of the decline of unsaturation and acyl chain length, and the concurrent increase in saturation and monounsaturation, characteristic of nutrient limitation at the end of a batch culture's lifespan. The proportion of the acyl chain lengths changed from this time point onwards to display an increase in C_{16} fatty acids (from 46.8 % to 52.0 %) and decrease in C_{20} fatty acids (from 40.4 % to 33.7 %) commensurate with the decline of the cellular division rate.

The culture grown in the S88 medium modified to contain treble the NaCl concentration of normal S88 (*i.e.* one and a half times that of 'normal' seawater) showed a continuation of the observed trends in acyl composition. At a superficial level this was manifested in a general decrease in the unsaturation and acyl chain length of its total FAME composition over the culture period. The trend towards an extended lag-phase, in terms of acyl composition rather than cellular division, was also perpetuated. The data presented in Table 6.3.4 showed a change in the pattern of acyl distribution that differed from that recorded in the previous tables of this sub-section (see Tables 6.3.1, 6.3.2 & 6.3.3). In addition to the characteristic changes occurring in the proportions of 16:0 and 20:5(*n*-3) during the first 24 hours post-inoculation, there was a substantial increase in the proportion of 18:1(*n*-9). This phenomenon had also been observed in the data obtained from the culture grown at double normal NaCl concentration (see Table 6.3.3), but to a lesser extent, the increase in that case being in the order of 3 %. The phenomenon could also be correlated with the decreases observed in the growth rates of cultures grown at NaCl concentrations of 16 g l^{-1}, 32 g l^{-1} and 48 g l^{-1} following inoculation. Increased proportions of one or both 18:1 isomers and 18:2(*n*-6) were offset more by acyl chain shortening rather than

lengthening (*i.e.* C_{18} to C_{20} elongation was reduced rather than that from C_{16} to C_{18} being increased). This was evident from the decline in the proportion of 20:5(*n*-3) throughout this series of cultures. The fatty acyl chain length distribution profile for the culture grown in S88 containing treble its normal NaCl concentration at the time point 456 hours post-inoculation, when the maximum proportions of PUFA were recorded was as follows:- 53.9 % C_{16}, 13.3 % C_{18} and 27.7 % C_{20}. After this time point the culture began to show the signs of entering the stationary-phase. The proportions of C_{16} fatty acids rose, as did the proportion of C_{18} acids. In contrast to this, the proportion of C_{20} fatty acids in the total FAME fell.

At the level of the total FAME composition, the effects of increasing the culture medium salinity were to shorten the mean acyl chain length and decrease the proportion of PUFA. In contrast to the effects of the growth phase of the culture on lipid composition which, when based upon the statement above, were broadly similar, the effect of increased salinity was to increase the proportions of C_{16} and C_{18} chain length fatty acids whilst concurrently decreasing the proportion of C_{20} fatty acids. In terms of acyl unsaturation, the effect of increasing the culture medium salinity was to decrease the proportion of 20:5(*n*-3), the major C_{20} fatty acid. In the case of *N. oculata* a decrease in the proportion of C_{20} fatty acids inherently produced a decrease in the proportion of PUFA.

Analyses of the total lipid extracts using HPTLC densitometry again showed the now familiar pattern of variation in the separated lipid classes. The progression of the pattern was dictated by the growth rate of the cells as observed in the previous sub-section. As can be seen from Figures 6.3.4 to 6.3.7 the characteristic trough in the proportion of TAG associated with the exponential-phase of cellular division shifts in time. The shift occurred in such a manner that it correlated with the changes in the growth rates of the cultures with medium salinity as plotted in Figure 6.3.1. The TAG minima, when interpolated upon their respective growth curves coincided with the points corresponding to five doubling periods on Figure 6.3.1. This seemed to indicate that the culture medium salinity was influencing the

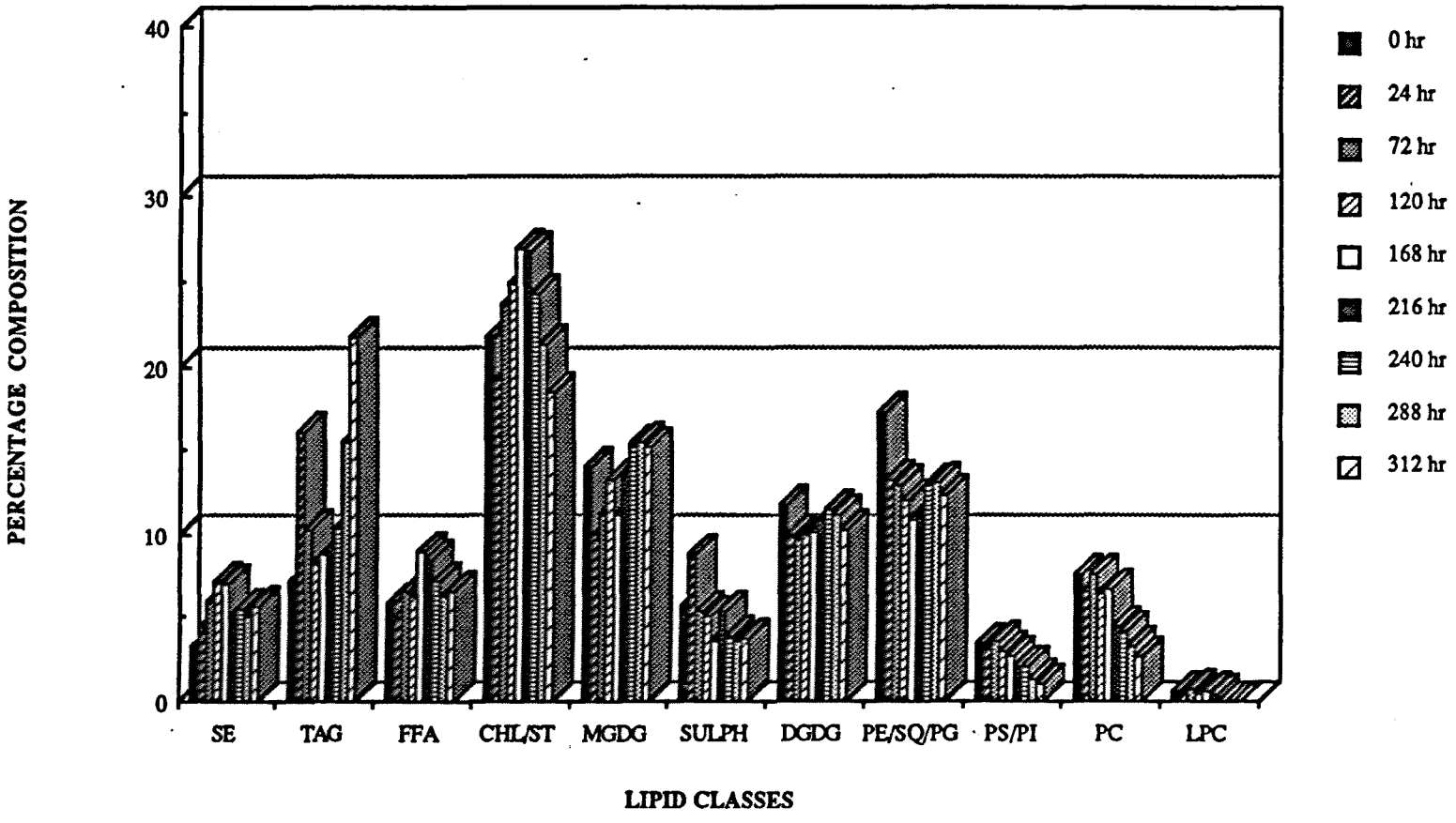


Figure 6.3.4 : Changes in the relative proportions of the lipid classes of *N. oculata* grown in batch culture in S88 medium modified to contain half its normal NaCl concentration[1].

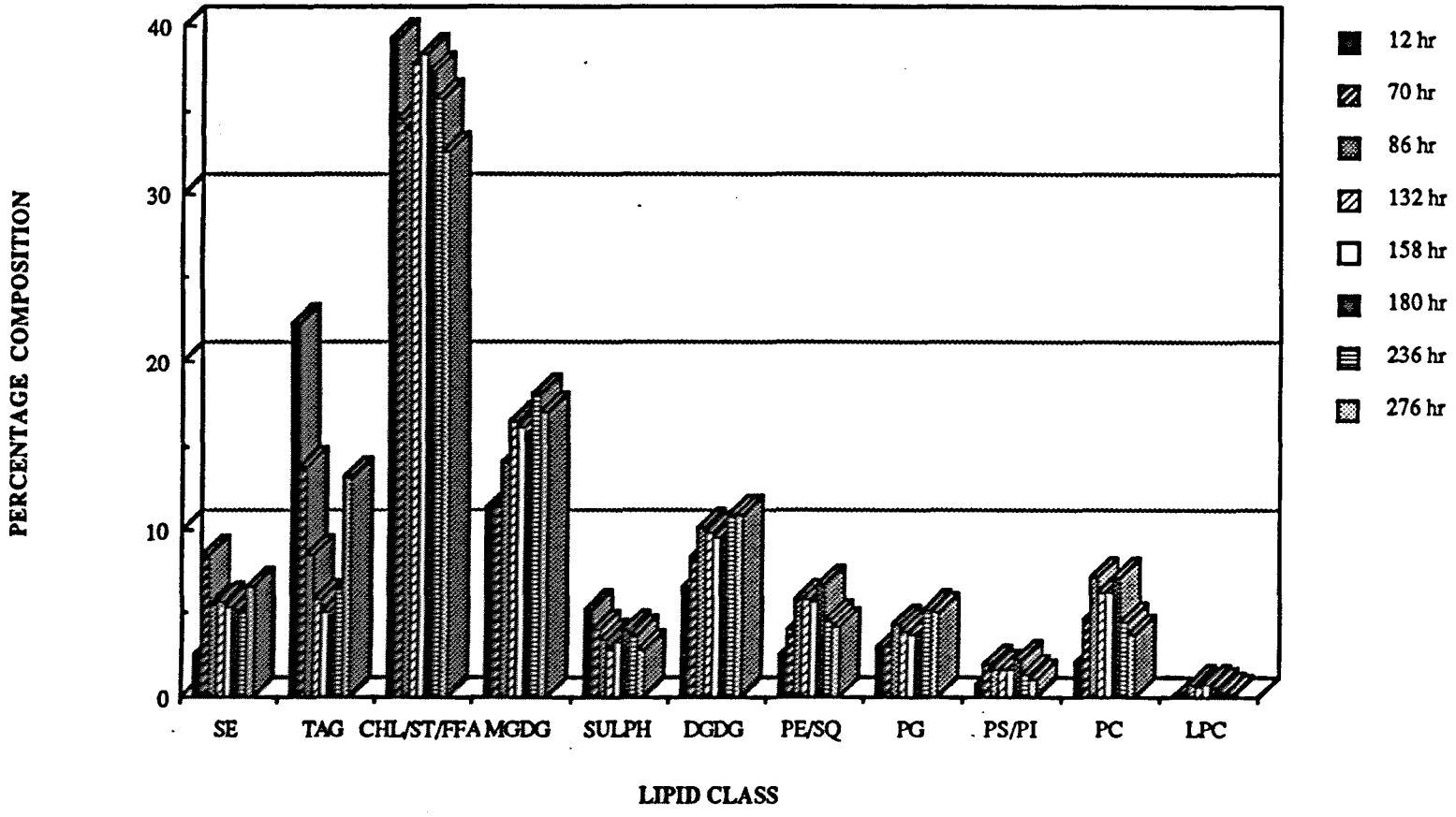


Figure 6.3.5 : Changes in the relative proportions of the lipid classes of *N. oculata* grown in batch culture in S88 medium ummodified with respect to its NaCl concentration[1].

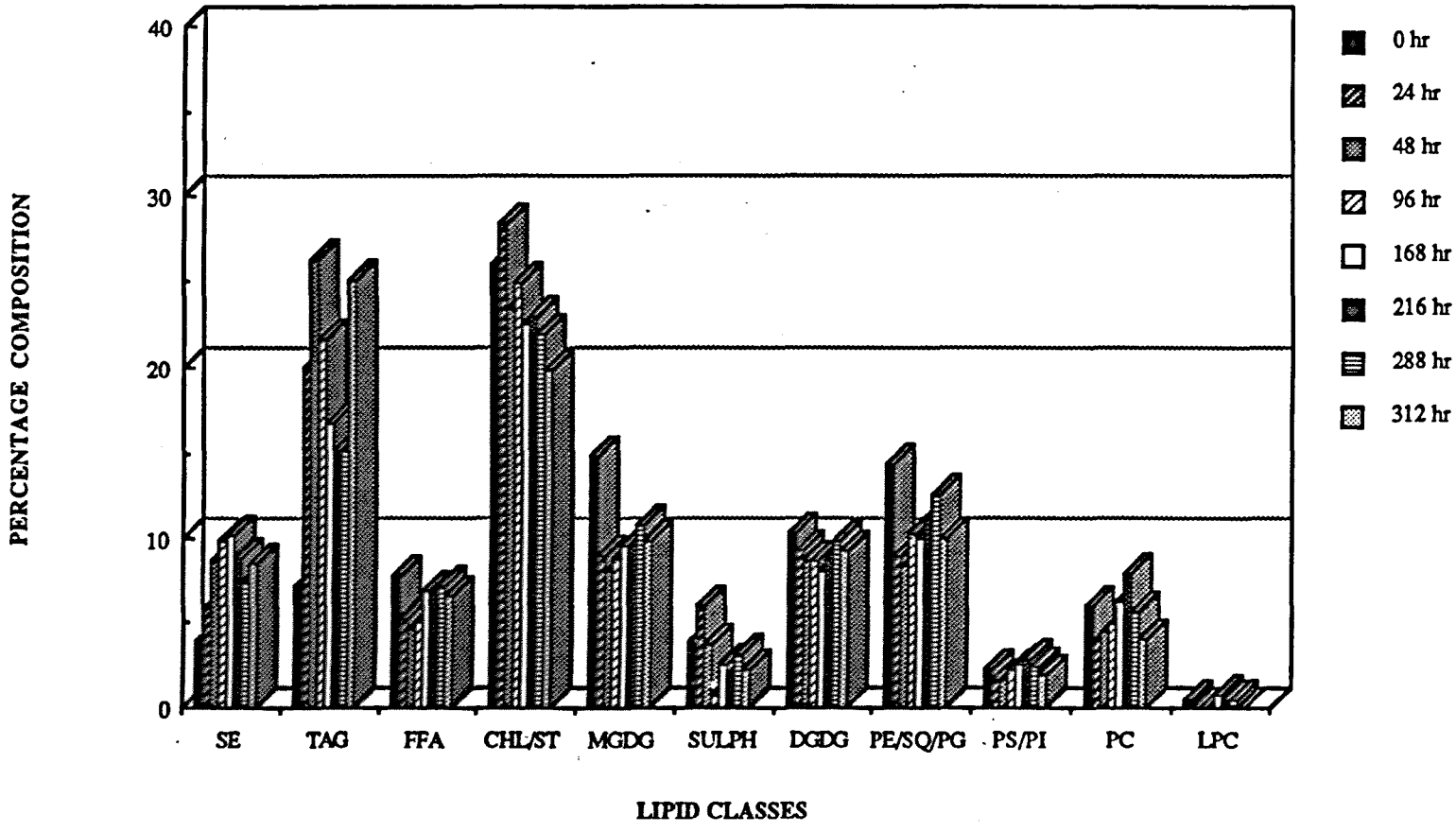


Figure 6.3.6 : Changes in the relative proportions of the lipid classes of *N. oculata* grown in batch culture in S88 medium modified to contain double its normal NaCl concentration[1].

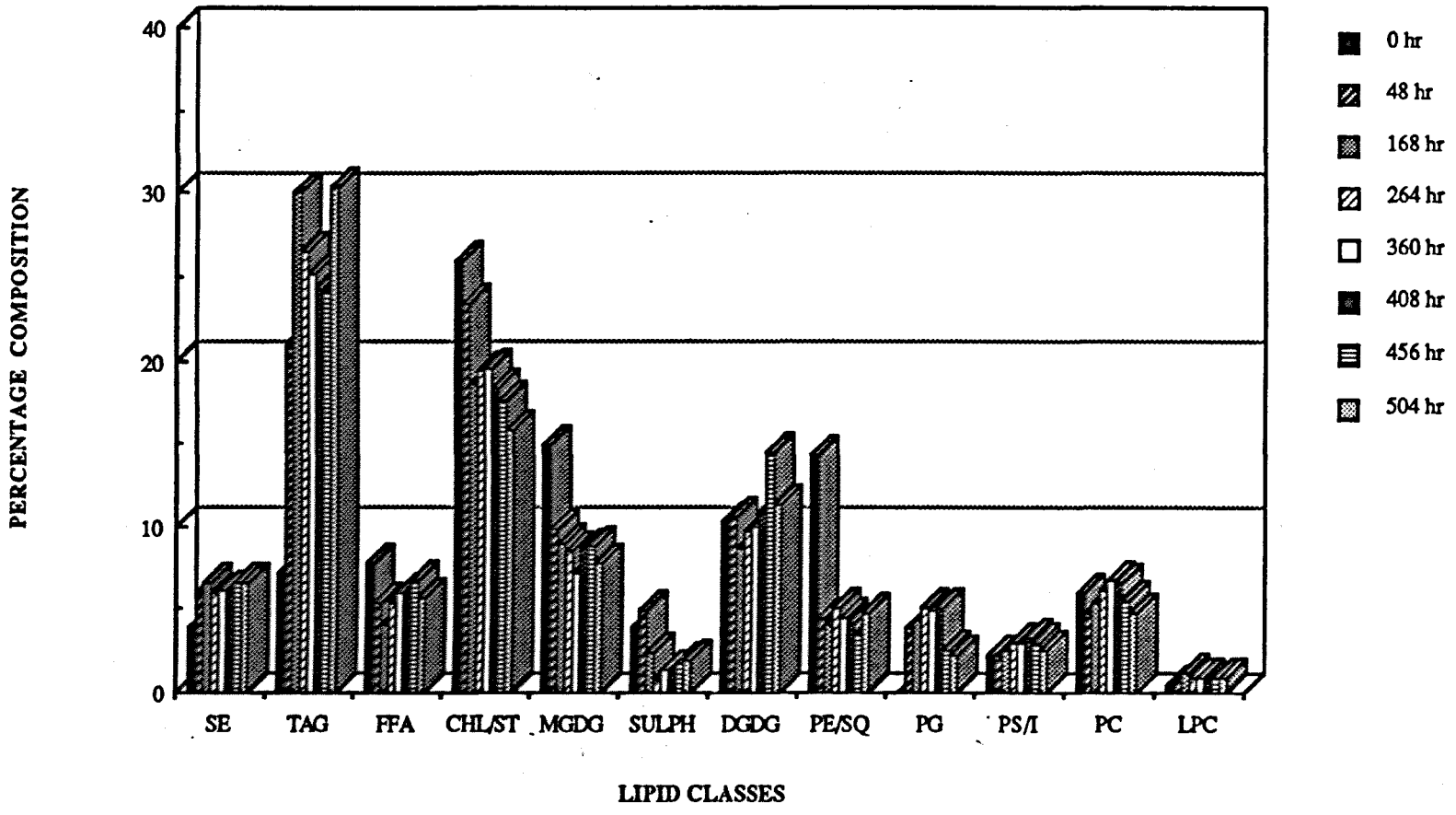


Figure 6.3.7 : Changes in the relative proportions of the lipid classes of *N. oculata* grown in batch culture in S88 medium modified to contain treble its normal NaCl concentration[1].

division rate of the cells in a uniform manner and that in quantitative terms the lipid classes were equivalent at equivalent points on their growth curves.

The data presented in Figures 6.3.4 to 6.3.7 show the characteristic variation in the proportion of TAG during the progression of the culture through the lag-phase, into the exponential phase and then into the early stationary-phase. The polar lipid classes showed a pattern of variation in their proportions which was complementary to that of TAG. The explanation for this may be attributed, as previously in Sections 5 and 6, to the expression of the data in relative terms, the variation in the proportion of TAG causing concomitant, complementary changes in the proportions of the other quantitatively less variable lipid classes.

From the total FAME composition data presented in Tables 6.3.1 to 6.3.4 it seemed likely that significant variation had occurred in the individual lipid class FAME compositions with salinity. This was in contrast to the experiments in which culture incubation temperature was varied. In these cultures the observed 'variations' were attributed to the effect of temperature upon the overall metabolic rate of the cells in batch culture. The individual lipid class FAME were analysed for the samples from each culture falling closest to the 'equivalence-point'. Tables 6.3.5 to 6.3.8 cover the period around four doubling periods whilst Tables 6.3.9 to 6.3.12 cover the period around the five to six doubling periods 'equivalence-point' (see Figure 6.3.1).

When inter-culture comparisons were made amongst the tables in each of the two data sets several salinity-dependent differences were immediately obvious. At an overall level there was a clear general trend towards increasing polyunsaturation of the fatty acids with decreasing salinity. The culture grown in half salinity S88 medium showed a high proportion of PUFA (37.1 %) at 168 hours post-inoculation, a stage in its growth when this value could be expected to increase further still. The analysis of the individual lipid class FAME of the culture showed that all the lipid classes of the total lipid extract contained

TABLE 6.3.5 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 168 hrs in S88 medium modified to contain half normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.4	2.4	3.0	5.9	2.9	3.5	1.0	4.5
15:0	0.3	0.3	0.1	0.3	0.4	0.5	0.2	0.3
16:0	15.9	9.7	9.7	22.6	30.0	30.8	8.6	16.1
16:1(*n*-7)	21.3	14.4	5.0	23.1	28.3	35.3	30.6	27.6
16:1(*n*-13)‡	.	.	.	.	4.6	.	.	.
16:2	0.8	0.6	.	0.1	.	.	2.5	0.4
17:0	0.3	0.4	.	.	.	.	.	.
16:3	0.4	0.4	0.2	0.6	.	.	1.1	.
16:4	.	.	.	.	.	.	.	.
18:0	0.9	1.9	0.9	0.4	0.5	1.5	0.5	0.5
18:1(*n*-9)	1.7	1.5	1.0	0.5	0.9	3.7	8.4	2.0
18:1(*n*-7)	0.3	0.3	.	0.2	0.2	.	.	.
18:2(*n*-6)	0.6	0.9	0.4	0.6	0.5	0.9	9.6	1.7
18:3(*n*-6)	0.2	0.1	.	.	.	.	2.4	0.3
18:3(*n*-3)	0.3	0.4	0.3	0.3	0.3	.	0.6	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	1.0	0.3	.	.	.	.	0.9	0.4
20:4(*n*-6)	5.7	6.2	1.2	0.7	0.4	10.7	7.9	4.1
20:3(*n*-3)	..	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	43.0	56.7	75.8	42.9	25.8	10.8	20.0	37.1
Unidentified :	3.9	3.6	2.5	1.9	5.3	2.4	5.7	4.7
Total sats :	20.8	14.7	13.7	29.2	33.8	36.2	10.3	21.4
Total monos :	23.4	16.1	5.9	23.7	34.0	39.0	48.6	29.6
Total PUFA :	51.9	65.6	77.9	45.2	26.9	22.4	35.4	44.3
Total (*n*-9) :	1.7	1.5	1.0	0.5	0.9	3.7	8.4	2.0
Total (*n*-6) :	6.9	6.7	1.2	6.7	0.4	10.7	11.1	6.5
Total (*n*-3) :	43.4	57.1	76.1	43.2	26.0	10.8	20.7	37.4
(*n*-3)/(*n*-6) :	6.3	8.5	63.4	6.4	65.0	1.0	1.9	5.8
16:1/16:0 :	1.3	1.5	0.5	1.0	0.9	1.1	3.6	1.7
20:5/16:0 :	2.7	5.9	7.8	1.9	0.9	0.4	2.3	2.3
ΣC_{16}:	38.4	15.4	14.9	46.4	62.9	66.1	42.8	44.1
ΣC_{18}:	4.0	5.1	2.6	2.0	2.4	6.1	21.5	4.8
ΣC_{20}:	49.7	63.2	77.0	43.6	26.3	21.5	28.8	41.6

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME.
tr.,<0.1%;., not detected; ‡, including SQDG.

TABLE 6.3.6 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 158 hours in S88 medium unmodified with respect to its NaCl concentration but modified to provide excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.4	1.8	9.9	6.5	2.6	2.3	1.2	4.2
15:0	.	.	.	.	.	.	.	.
16:0	15.1	6.5	7.0	20.8	24.0	16.9	6.7	13.4
16:1(*n*-7)	28.4	21.4	5.3	24.8	22.4	20.1	35.4	27.0
16:1(*n*-13)ɣ	.	.	.	.	.	.	.	.
16:2	0.3	.	0.2	0.7	0.6	.	2.1	0.8
17:0	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	0.3
16:4	.	.	.	.	.	.	.	.
18:0	1.3	1.8	0.8	0.5	0.4	2.1	0.7	0.5
18:1(*n*-9)	3.5	1.8	0.4	0.4	0.6	3.2	5.8	2.2
18:1(*n*-7)	0.6	0.4	.	0.2	0.2	.	1.3	0.4
18:2(*n*-6)	1.3	1.3	0.4	0.9	0.7	1.3	9.0	2.1
18:3(*n*-6)	0.3	.	.	.	.	.	2.7	0.5
18:3(*n*-3)	0.1	.	.	0.2	.	.	0.9	0.2
18:4(*n*-3)	.	.	.	.	.	.	0.3	.
20:0	0.4	0.6	0.4	0.4	0.3	.	0.5	0.3
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.9	.	.	.	.	.	0.7	0.4
20:4(*n*-6)	4.0	4.7	0.9	1.1	4.9	21.9	4.6	4.2
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	34.9	48.3	71.3	40.9	26.3	15.4	15.8	36.1
Unidentified :	5.6	11.4	3.4	2.6	17.0	16.8	12.3	7.4
Total sats :	20.2	10.7	18.1	28.2	27.3	21.3	9.1	18.4
Total monos :	32.4	23.6	5.7	25.4	23.2	23.3	42.5	29.6
Total PUFA :	41.0	54.3	72.8	43.8	32.5	38.6	36.1	44.6
Total (*n*-9) :	3.5	1.8	0.4	0.4	0.6	3.2	5.8	2.2
Total (*n*-6) :	6.5	6.0	1.3	2.0	5.6	23.2	17.0	7.2
Total (*n*-3) :	35.0	48.3	71.3	41.1	26.3	15.4	17.0	36.3
(*n*-3)/(*n*-6) :	5.4	8.1	54.8	20.6	4.7	0.7	1.0	5.0
16:1/16:0 :	1.9	3.3	0.8	1.2	0.9	1.2	5.3	2.0
20:5/16:0 :	2.3	7.4	10.2	2.0	1.1	0.9	2.4	2.7
ΣC_{16}:	43.8	27.9	12.5	46.3	47.0	37.0	44.2	41.5
ΣC_{18}:	7.1	5.3	1.6	2.2	1.9	6.6	20.7	5.9
ΣC_{20}:	39.8	53.6	72.2	42.4	31.5	37.3	21.6	41.0

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

TABLE 6.3.7 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 216 hrs in S88 medium modified to contain double normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.6	3.0	7.3	4.8	2.1	3.2	1.3	4.0
15:0	0.5	0.4	.	0.3	0.3	0.4	.	0.5
16:0	25.2	13.8	11.8	23.3	26.8	30.5	9.1	21.7
16:1(*n*-7)	34.2	22.6	6.5	23.9	26.6	32.1	26.8	31.4
16:1(*n*-13)ɣ	.	.	.	.	3.8	.	.	.
16:2	0.8	.	.	.	.	.	0.7	0.2
17:0	0.3	.	.	.	.	.	.	.
16:3	0.3	0.5	.	.	.	.	0.4	.
16:4	.	.	.	.	.	.	.	.
18:0	1.2	2.6	1.9	0.5	0.5	1.7	0.4	0.7
18:1(*n*-9)	5.8	3.4	1.7	0.8	1.3	4.7	11.2	4.7
18:1(*n*-7)	0.9	0.4	0.3	0.2	0.2	.	.	.
18:2(*n*-6)	.	1.2	0.5	0.8	0.7	1.0	12.8	1.9
18:3(*n*-6)	.	.	.	.	.	.	1.9	0.4
18:3(*n*-3)	.	.	.	.	.	.	0.2	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	.	.	.	.	0.5	.	0.9	0.5
20:4(*n*-6)	3.4	5.6	2.1	1.0	7.7	15.3	10.8	5.3
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	19.4	43.4	65.3	43.9	27.9	9.6	19.5	26.7
Unidentified :	2.7	2.6	2.5	0.6	1.8	1.5	3.8	2.4
Total sats :	31.8	19.9	21.0	28.9	29.6	35.8	10.8	26.9
Total monos :	39.9	26.4	8.5	24.9	36.9	36.8	38.1	36.1
Total PUFA :	25.6	51.2	68.0	45.6	36.7	25.9	47.3	34.6
Total (*n*-9) :	5.8	3.4	1.7	0.8	1.3	4.7	11.2	4.7
Total (*n*-6) :	4.1	6.0	2.1	1.0	8.2	15.3	13.6	8.1
Total (*n*-3) :	19.4	43.4	65.3	43.9	27.9	9.6	19.7	26.3
(*n*-3)/(*n*-6) :	4.7	7.2	31.1	43.9	3.4	1.6	1.4	3.2
16:1/16:0 :	1.4	1.6	0.6	1.0	1.0	1.1	3.0	1.4
20:5/16:0 :	0.8	3.2	5.5	1.9	1.0	0.3	2.2	1.2
ΣC_{16}:	60.5	36.9	18.3	47.2	57.2	62.6	37.0	53.3
ΣC_{18}:	7.9	7.6	4.4	2.3	2.7	7.4	30.1	7.7
ΣC_{20}:	22.8	49.0	67.4	44.9	36.1	24.9	31.2	32.5

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

TABLE 6.3.8 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 360 hrs in S88 medium modified to contain treble normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.9	3.0	8.2	5.3	2.4	3.8	6.0	3.5
15:0	0.4	0.5	0.3	0.3	0.3	0.4	0.1	0.3
16:0	29.8	18.5	14.6	24.0	26.2	35.0	11.6	27.1
16:1(*n*-7)	40.6	20.0	8.2	22.7	23.2	33.9	20.5	30.0
16:1(*n*-13)x	.	.	.	.	3.2	.	.	.
16:2	.	.	.	.	.	.	.	.
17:0	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	0.2
16:4	.	.	.	.	.	.	.	.
18:0	0.9	3.7	2.6	0.5	0.6	2.2	0.6	0.9
18:1(*n*-9)	10.2	6.5	3.5	0.9	2.5	7.0	24.3	10.4
18:1(*n*-7)	1.3	.	.	0.2	0.3	.	.	.
18:2(*n*-6)	.	1.4	0.7	0.7	0.8	0.8	10.5	2.3
18:3(*n*-6)	.	.	0.3	.	.	.	1.4	0.3
18:3(*n*-3)	.	.	.	.	.	.	0.3	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.8	0.4	.	.	0.6	.	0.5	0.8
20:4(*n*-6)	2.0	6.7	3.1	0.9	9.6	8.1	9.6	4.5
20:3(*n*-3)	..	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	8.0	36.9	53.3	44.3	28.5	6.7	15.0	17.9
Unidentified :	1.1	2.4	5.1	0.2	1.8	2.0	4.8	1.8
Total sats :	36.0	25.6	25.8	30.0	29.5	41.4	13.3	31.8
Total monos :	52.1	26.5	11.7	23.8	29.2	40.9	44.8	40.4
Total PUFA :	10.8	45.4	57.5	46.0	39.5	15.6	37.2	26.0
Total (*n*-9) :	10.2	6.5	3.5	0.9	2.5	7.0	24.3	10.4
Total (*n*-6) :	2.9	7.1	3.4	0.9	10.3	8.1	11.5	7.9
Total (*n*-3) :	8.0	36.9	53.3	44.3	28.5	6.7	15.2	17.9
(*n*-3)/(*n*-6) :	2.8	5.2	15.7	49.2	2.8	0.8	1.3	2.3
16:1/16:0 :	1.4	1.1	0.6	0.9	0.9	1.0	1.8	1.1
20:5/16:0 :	0.3	2.0	3.7	1.8	1.1	0.2	1.3	0.7
ΣC_{16} :	70.4	38.5	22.8	46.7	52.6	68.9	32.1	57.3
ΣC_{18} :	12.4	11.6	7.1	2.3	4.2	10.0	37.1	14.1
ΣC_{20} :	10.8	44.0	56.4	45.2	38.7	14.8	25.1	23.2

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME
tr.,<0.1%; ., not detected; ‡, including SQDG.

relatively high proportions of 20:5(*n*-3) when compared to the cultures grown at higher salinities.

The neutral lipid classes, which were associated with relatively low proportions of PUFA in previous cultures, also showed comparatively high proportions of 20:5. TAG had 43.0 % of its mass of its FAME present as 20:5 whilst FFA had almost 57 % of its FAME as 20:5. These two classes contrasted markedly with those of the culture grown in the highest salinity medium where the proportions of 20:5 were 8.0 % for TAG and 36.9 % for FFA. The proportions of 20:5 for the neutral lipid classes in the intervening cultures on the salinity gradient were intermediate between those for the two extremes of salinity (see Tables 6.3.6 and 6.3.7). The pattern of variation for the culture's acyl chain length and unsaturation distribution profiles for TAG was one in which there was a trend towards increasing proportions of both C_{16} and C_{18} chain length saturates and monounsaturates. There was a particular accumulation of C_{18} monounsaturated fatty acids with increasing salinity which had not been observed in the previous investigations of the effects of culture growth phase or temperature upon lipid composition. This was coupled with increased proportions of 16:1 to yield a change in the total proportion of monounsaturated fatty acids of 23.4 % in half salinity S88 to 52.1 % in treble salinity S88. The proportion of saturates increased with salinity from 20.8 % to 36.0 % and the proportion of PUFA decreased from 51.9 % to 10.2 %. Observations in a similar vein were made for the FFA fractions (Tables 6.3.5 to 6.3.8).

The polar lipid classes followed the same trend as the neutral classes, with one exception. The fractions corresponding to PE/PG displayed an opposing trend, their proportions of 20:5 increasing from 25.8 % at the lowest salinity to 28.5 % at the highest salinity. This change was also accompanied by an increase in the proportion of 20:4(*n*-6) from 0.5 % up to 9.6 %. In this case, the trend was one in which acyl chain length and unsaturation increased with culture medium salinity. The other major polar lipid classes either displayed decreased unsaturation and acyl chain length with increased salinity, or remained almost unchanged. The galactolipid MGDG was of the former category, its

proportion of 20:5 decreasing from 75.0 % in half salinity S88 to 53.3 % in treble salinity S88. The other galactolipid, DGDG, had a relatively stable acyl composition across the experimental salinity range. Neither of the galactolipid classes contained a high proportion of C_{18} fatty acids, but the cultures grown in double and treble salinity S88 showed elevated proportions of 18:0 and 18:1 in comparison the lower salinity cultures. The major structural phospholipid class, PC, showed a general decline in its proportion of PUFA, although the data suggested a more complex pattern that could not be accounted for solely in terms of either changes in unsaturation or acyl chain length distribution profile. The trend appeared to involve a progression from 20:5(*n*-3) which decreased with salinity with a concomitant increase in the proportion of 18:1, 16:1 and 16:0. In the culture grown in double salinity S88, the proportion of 20:4(*n*-6) was elevated along with the proportions of 18:2 and 18:1, whilst the proportion of 16:1 had decreased in comparison to the normal salinity S88 culture. The culture grown in treble salinity S88 medium showed an increase in the proportion of 18:1 and 16:0 and decreased proportions of C_{20} PUFA. The underlying trend was one of increasing proportions of C_{18} fatty acids with increasing salinity, particularly 18:2 and 18:1, whilst the distribution amongst the C_{16} fatty acids shifted to give increased proportions of 16:0.

The following tables (Tables 6.3.9 to 6.3.12) contain data from the same cultures as the previous four tables (Tables 6.3.5 to 6.3.8) but from samples taken one doubling period later in the lifespan of each of the respective cultures (corresponding approximately to five doubling periods, or equivalence-point 'e' on Figure 6.3.1). These data show the increase in the proportions of PUFA in the individual lipid class FAME with culture growth and illustrated the effect of the growth phase of the culture which underlies all batch culture experimentation.

Firstly, it is necessary to point out that the same trends which existed amongst the previous data set were still in evidence as the culture aged. The neutral lipid classes showed a high proportion of C_{20} PUFA. This was mainly in the form of 20:5(*n*-3) but the

proportions of 20:4(*n*-6) were elevated slightly amongst the equivalence-point 'e' samples (Tables 6.3.9 to 6.3.12) by comparison with those from equivalence-point 'd' (Tables 6.3.5 to 6.3.8). Although there was little variation in the proportions of C_{18} fatty acids in either TAG or FFA with the increasing number of cellular divisions, there was a very slight general trend for the proportion of C_{18} acyl chain lengths to increase with time in each culture. The trend for the C_{16} acyl chain lengths was the opposite of the above. The proportion of both major C_{16} fatty acids decreased although the decline of 16:1 was generally greater than that of 16:0. This was a major contributory factor to the declining proportions of mono-unsaturates and saturates with culture age. Again, the trend was one of increasing chain length and unsaturation with culture age underlying the effects of culture medium salinity. This was maintained so long as the culture was undergoing active cell division.

TABLE 6.3.9 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 216 hrs in S88 medium modified to contain half normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.7	2.6	6.5	6.6	2.2	3.4	1.7	4.1
15:0	0.2	0.2	.	0.3	0.4	.	.	0.3
16:0	11.0	6.4	5.5	20.6	31.6	33.0	8.6	14.4
16:1(*n*-7)	15.8	9.6	3.6	21.3	32.6	32.5	22.8	24.3
16:1(*n*-13)t	.	.	.	.	3.4	.	.	.
16:2	1.5	0.8	.	0.4	1.0	.	3.5	0.6
17:0	0.6	0.4	.	.	0.2	.	.	.
16:3	0.3	0.3	.	1.2	0.3	.	1.5	.
16:4	.	.	.	.	.	.	.	.
18:0	0.9	1.2	0.3	0.3	0.3	2.6	0.8	0.5
18:1(*n*-9)	2.4	1.1	0.4	0.5	0.7	6.2	4.3	1.7
18:1(*n*-7)	0.5	0.3	0.6	0.2	0.3	.	0.6	0.6
18:2(*n*-6)	1.0	0.8	0.5	1.0	1.0	2.0	5.9	1.3
18:3(*n*-6)	.	0.3	.	.	0.3	.	1.6	0.3
18:3(*n*-3)	0.5	0.7	0.5	0.3	0.5	.	1.1	0.5
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.6	0.2	0.5	.	.	.	.	0.2
20:4(*n*-6)	5.6	5.3	2.2	0.9	2.5	6.8	1.3	3.7
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	52.9	66.7	77.4	44.0	20.2	12.7	28.6	41.9
Unidentified :	2.3	2.9	2.0	2.3	2.5	0.7	8.7	5.6
Total sats :	16.4	10.9	12.3	27.8	34.7	39.1	11.1	19.3
Total monos :	18.7	11.1	4.7	22.0	37.1	38.7	27.7	26.6
Total PUFA :	62.5	75.1	81.0	47.9	25.7	21.5	52.5	48.5
Total (*n*-9) :	2.4	1.1	0.4	0.5	0.7	6.2	4.3	1.7
Total (*n*-6) :	6.3	5.7	2.7	0.9	2.8	6.8	11.9	5.5
Total (*n*-3) :	53.4	67.4	77.8	44.3	20.7	12.7	29.8	42.4
(*n*-3)/(*n*-6) :	8.5	11.8	28.8	49.2	7.4	1.7	2.5	7.7
16:1/16:0 :	1.4	1.5	0.7	1.0	1.0	1.0	2.6	1.7
20:5/16:0 :	4.8	10.4	14.0	2.1	0.6	0.4	3.3	2.9
ΣC_{16}:	28.6	17.1	9.1	43.5	68.9	65.5	36.4	39.3
ΣC_{18}:	5.3	4.4	2.3	2.3	3.1	10.8	14.3	4.9
ΣC_{20}:	59.1	72.2	80.1	44.9	22.7	19.5	29.9	45.8

Abbreviations & Symbols
Data expressed in terms of % mass of individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

TABLE 6.3.10 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 232 hours in S88 medium unmodified with respect to its NaCl concentration but modified to provide excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.8	3.7	8.0	5.6	2.7	3.3	1.2	4.2
15:0	.	.	.	.	.	.	.	.
16:0	14.7	7.8	6.8	20.2	26.6	26.5	6.6	14.6
16:1(*n*-7)	21.5	15.6	4.8	22.2	26.4	34.1	30.8	24.0
16:1(*n*-13)t	.	.	.	.	3.2	.	.	.
16:2	0.2	0.4	.	.	.	.	0.6	0.9
17:0	0.3	0.2	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	0.3
16:4	.	.	.	.	.	.	.	.
18:0	1.2	1.9	0.6	0.8	0.4	1.4	0.9	0.8
18:1(*n*-9)	3.1	3.3	0.3	0.5	0.7	4.3	7.7	1.9
18:1(*n*-7)	0.7	0.8	0.1	0.3	0.5	.	.	0.6
18:2(*n*-6)	1.4	2.4	0.4	0.8	0.7	1.5	7.7	1.5
18:3(*n*-6)	0.4	0.3	.	.	0.2	.	2.1	0.3
18:3(*n*-3)	0.2	0.6	.	.	0.1	.	0.2	0.3
18:4(*n*-3)	.	.	.	.	.	.	0.4	.
20:0	0.4	0.6	0.4	0.4	0.3	.	0.5	0.4
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.9	0.3	.	.	0.2	.	0.7	0.3
20:4(*n*-6)	4.3	4.3	1.2	0.9	3.6	8.1	7.5	3.8
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	37.5	44.7	71.4	38.5	24.1	9.3	16.2	39.2
Unidentified :	9.4	13.1	6.0	9.8	10.3	11.5	16.9	10.6
Total sats :	20.4	14.2	15.8	27.0	30.0	31.2	9.2	20.0
Total monos :	25.3	19.7	5.2	23.0	30.8	38.4	38.5	26.5
Total PUFA :	44.9	53.0	73.0	40.2	28.9	18.9	35.4	46.6
Total (*n*-9) :	3.1	3.3	0.3	0.5	0.7	4.3	7.7	1.9
Total (*n*-6) :	7.0	7.3	1.6	1.7	4.7	9.6	18.0	5.9
Total (*n*-3) :	37.7	45.3	71.4	38.5	24.2	9.3	16.8	39.5
(*n*-3)/(*n*-6) :	5.4	6.2	44.6	22.6	5.1	1.0	0.9	6.7
16:1/16:0 :	1.5	2.0	0.7	1.1	1.0	1.3	4.7	1.6
20:5/16:0 :	2.6	5.7	10.5	1.9	0.9	0.4	2.5	2.7
ΣC_{16}:	36.4	23.8	11.9	44.9	56.2	60.6	38.0	39.8
ΣC_{18}:	7.0	9.3	1.4	2.4	2.6	7.2	19.0	5.4
ΣC_{20}:	43.1	49.3	74.8	42.4	28.2	20.8	24.9	43.7

Abbreviations & Symbols
Data expressed in terms of % mass of individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

TABLE 6.3.11 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 288 hrs in S88 medium modified to contain double normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	3.7	2.6	7.3	4.5	3.4	2.2	1.1	4.1
15:0	0.3	.	.	0.3	0.4	0.4	.	0.3
16:0	17.8	8.6	9.2	22.2	27.5	32.5	7.4	17.9
16:1(*n*-7)	30.2	14.3	6.4	22.8	35.2	29.5	25.3	30.4
16:1(*n*-13)t	.	.	.	.	1.0	.	.	.
16:2	.	.	.	.	0.4	.	1.8	0.2
17:0	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	0.8	.
16:4	.	.	.	.	.	.	.	.
18:0	1.0	1.9	0.9	0.4	0.3	2.5	0.5	0.5
18:1(*n*-9)	2.7	1.7	1.0	0.8	1.0	6.7	6.4	0.8
18:1(*n*-7)	0.7	0.4	0.1	0.3	0.3	.	.	.
18:2(*n*-6)	1.4	1.1	0.6	1.2	1.0	1.9	7.5	1.7
18:3(*n*-6)	0.5	.	.	.	0.3	.	1.7	0.3
18:3(*n*-3)	.	.	.	.	0.2	.	1.0	0.3
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	1.4	.	.	.	.	.	0.6	0.5
20:4(*n*-6)	6.1	6.3	3.0	1.3	5.0	13.6	14.8	5.5
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	34.2	60.9	70.0	45.2	21.5	8.9	25.5	35.9
Unidentified :	0.2	2.1	1.4	1.1	2.4	1.7	5.5	1.6
Total sats :	22.8	13.1	17.3	27.3	31.6	37.6	9.1	22.8
Total monos :	33.6	16.5	7.6	23.9	37.5	36.2	31.8	31.2
Total PUFA :	43.5	68.3	73.7	47.7	28.5	24.4	53.7	44.4
Total (*n*-9) :	2.7	1.7	1.0	0.8	1.0	6.7	6.4	0.8
Total (*n*-6) :	7.9	6.3	3.0	1.3	5.3	13.6	17.1	8.0
Total (*n*-3) :	34.2	60.9	70.0	45.2	21.8	8.9	26.5	36.2
(*n*-3)/(*n*-6) :	4.3	9.7	23.3	34.8	4.1	1.5	1.5	4.5
16:1/16:0 :	1.7	1.7	0.7	1.0	1.3	0.9	3.4	1.7
20:5/16:0 :	1.9	7.0	7.6	2.0	0.8	0.3	3.4	2.0
ΣC_{16}:	48.0	14.9	15.6	45.0	64.1	62.0	35.3	48.5
ΣC_{18}:	6.3	5.1	2.6	2.7	3.1	11.1	17.1	3.6
ΣC_{20}:	41.7	67.2	73.0	46.5	26.5	22.5	40.9	41.9

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

TABLE 6.3.12 : Individual lipid class FAME composition of *N. oculata* grown in batch culture for 408 hrs in S88 medium modified to contain treble normal NaCl with excess nitrate and phosphate.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.8	3.2	4.3	4.5	2.2	3.4	0.7	3.7
15:0	0.5	0.3	0.2	0.3	0.3	0.4	0.1	0.3
16:0	30.4	16.8	13.2	24.0	29.4	31.1	10.2	25.9
16:1(*n*-7)	36.0	21.6	6.9	21.8	24.9	30.3	19.7	29.7
16:1(*n*-13)x	.	.	.	.	3.2	.	.	.
16:2	0.8	.	.	.	.	.	.	.
17:0	.	.	.	.	.	.	.	.
16:3	.	.	.	.	.	.	.	0.2
16:4	.	.	.	.	.	.	.	.
18:0	1.1	2.6	2.2	0.5	0.5	1.2	0.5	0.9
18:1(*n*-9)	10.7	6.4	3.0	1.0	2.1	6.2	27.2	10.4
18:1(*n*-7)	.	.	.	0.2	0.2	.	.	.
18:2(*n*-6)	1.4	1.5	0.7	0.8	0.6	0.6	9.5	2.2
18:3(*n*-6)	.	.	.	.	.	.	1.5	0.3
18:3(*n*-3)	.	.	.	.	.	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.8	0.4	.	.	0.4	0.4	0.5	0.7
20:4(*n*-6)	2.6	7.7	4.6	1.1	7.3	15.5	10.3	4.4
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	8.9	38.6	63.4	45.6	27.4	9.2	14.9	18.4
Unidentified :	2.0	0.8	1.5	0.3	1.3	1.8	4.7	2.9
Total sats :	36.8	22.9	19.9	29.2	32.4	36.2	11.6	30.8
Total monos :	46.7	28.1	9.9	23.0	30.4	36.4	46.9	40.1
Total PUFA :	14.5	48.2	68.8	47.5	35.8	25.6	36.8	26.2
Total (*n*-9) :	10.7	6.4	3.0	1.0	2.1	6.2	27.2	10.4
Total (*n*-6) :	3.5	8.1	4.6	1.1	7.8	15.9	12.3	7.6
Total (*n*-3) :	8.9	38.6	63.4	45.6	27.4	9.2	14.9	18.4
(*n*-3)/(*n*-6) :	2.5	4.8	13.8	41.5	3.5	1.7	1.2	2.4
16:1/16:0 :	1.2	1.3	0.5	0.9	0.8	1.6	1.9	1.1
20:5/16:0 :	0.3	2.3	4.8	1.9	0.9	0.3	1.5	0.7
ΣC_{16}:	67.2	38.4	20.1	45.8	57.5	61.4	29.9	55.8
ΣC_{18}:	13.2	10.5	5.9	2.5	3.4	8.0	38.7	14.0
ΣC_{20}:	12.3	46.7	68.0	46.7	35.1	25.1	25.7	23.5

Abbreviations & Symbols
Data expressed in terms of % mass individual lipid class FAME.
tr.,<0.1%; ., not detected; ‡, including SQDG.

The polar lipid classes demonstrated increases in their mean acyl chain lengths and proportions of PUFA. The galactolipid MGDG from the culture grown in half salinity S88 yielded 77.4 % 20:5(*n*-3), the highest proportion of this fatty acid recorded in any individual lipid class FAME purified during the course of this thesis. In contrast, the supposedly biosynthetically related galactolipid DGDG (see Section 1) again showed remarkably little variation in its PUFA composition with salinity and culture growth phase. The phospholipid class PC showed the same complex pattern of changes in the distribution patterns of the fatty acids of different chain lengths and unsaturations. The evidence supports a theory implicating PC in the mechanism whereby salinity affected the acyl composition of the total lipid. Numerous other studies have linked PC with positionally specific lipid-linked fatty acyl desaturation (see Section 1). The fact that PC was the only class to contain significant proportions of C_{18} fatty acids suggested that it was also important in the elongation reactions, thereby controlling the flux between the quantitatively more important C_{16} and C_{20} fatty acids. Because PC was the class that showed the most extreme variation in its proportion of C_{18} fatty acids with salinity, it also suggests that changes in the C_{18} metabolism of PC was the source of the variations observed in the acyl compositions of the remaining lipid classes. This will be discussed further in later sections of this thesis.

This section has dealt with two environmental variables, temperature and salinity, which did not change with the growth phase of a batch culture. The next phase of experimentation involved the study of two environmental factors which were particularly involved with the determintion of the growth phase of a culture. As a consequence a change of culture technique, from batch to continuous culture, was introduced to overcome some of the effects of the culture's growth-phases on their acyl composition. These effects were particularly well illustrated in the present section and required the frequent sampling of a culture to observe variations within a pattern of variation. As a consequence this technique also generated large masses of compositional data with a recurrent, underlying pattern. To overcome this, the use of a continuous culture strategy allowed analysis to be cut to the minimum by generating fewer samples. In essence, the data above have shown that after

five doubling periods cell division was at its peak for the culture conditions imposed. Therefore the optimal point for sampling was after this period of culture growth. Continuous culture using the cage culture turbidostat enabled cultures to be maintained under the required environmental conditions for this sampling strategy to be employed effectively to investigate the effect of culture illumination level and culture medium nitrate concentration upon the lipid composition of the algal cells, without the requirement for time course sampling.

SECTION 7: VARIATION IN LIPID COMPOSITION AS A CONSEQUENCE OF GROWTH DEPENDENT EFFECTERS IN CONTINUOUS CULTURES.

7.1 INTRODUCTION.

The data recorded in the two previous sections (see sections 5 & 6) monitored the effects of several factors on the content and composition of algal lipids through the lifespan of batch cultures. Because the cultures never attained steady-state growth it was by necessity that the results were presented in these sections in a time-course format. The observations made in section 5 were due in effect to a combination of a multiplicity of factors each dependent upon the age of the cultures and the applied conditions. The complexity of the interrelationships between the various factors was such that it was not possible to determine which were causes and which were effects. In the literature there is very little algal culture work with the emphasis upon the lipid component of the cells, based upon continuous culture techniques. There is an apparent trend, however, in the literature of the late 1980's to adopt continuous culture for the study of algal physiology (see Richardson *et al.*, 1969; Parrish & Wangersky, 1987; Rezanka *et al.*, 1987; Mortensen *et al.*, 1988).

In the following section attempts were made to observe the effects of factors which, in batch culture, were subject to modification as a result of the growth of the cultures themselves. By applying a continuous culture strategy using the cage culture turbidostat, it was possible to control the chemical environment of the cells more rigorously than would have been possible with batch culture. Because the culturing apparatus allowed for the continuous flow through of fresh culture medium, the chemical environment of the cells could be said to approximate the original, predetermined composition of the medium in the supply reservoir. Having allowed the cells to attain steady-state growth under the applied conditions over a period of weeks, they were harvested and analysed. The reliability of the analytical material is much greater than with that derived from batch culture, because the cells have been maintained under the same conditions for a long period. As a consequence it may be stated that the cells are dividing at their maximal rate for the applied conditions (*i.e.* those

predetermined by the culture medium, illumination and temperature). Because all conditions, with the exception of the condition under investigation, are the same in each culture set-up, any variation observed is due either directly, or indirectly, to the test factor.

7.2 VARIATION IN LIPID COMPOSITION WITH ILLUMINATION INTENSITY.

7.2.1 Experimental.

Prior to the first experiment in this series, a small scale (< 500 ml) batch culture vessel) was set up containing 400 ml S88 medium, modified with respect to both nitrate and phosphate components. This culture was inoculated to an arbitrary cell density and incubated under the proposed illumination and temperature regime for a period of 4 to 5 days. The cage culture turbidostat was set up concurrently as described in sections 4.2.4 to 4.2.7. Having set up the medium reservoirs *etc.*, and optimised the alignment of the laser and photodiode, the vessel was inoculated. Once the batch culture had reached a cell density of 30 to 40 x 10^6 cells ml^{-1} it was aseptically inoculated into the turbidostat using the reversed dilution pump technique described in section 4.2.6.

In the illumination intensity experiments the medium composition was that of unmodified S88 (see Appendix C). During early trials it was found that sparging the medium reservoir with 5 % CO_2 in air at a rate of 5 ml min-1 was required for good algal growth in the turbidostat. In this way the medium was maintained in equilibrium with the gas mixture, with the influent gas mixture always being at a slight positive pressure. The medium pumping rate was such that the medium in the culture chamber would be exchanged during one period of twenty-four hours. This was equivalent to a net flow rate of *ca.* 900 ml day^{-1}. The actual pumping rate of the medium was considerably higher to allow for the dead volume of the medium inflow / outflow tubing. In the common inflow and outflow tubes, the dead volume was reduced as far as possible by the use of narrow bore (1.6 mm i.d.) siiicone rubber tubing. It was not possible to use this type of tubing universally on the device because of constraints originating from the requirements of the peristaltic pump and other connections.

The various illumination intensities used were governed by the lighting bank described in Section 4.3.5 and pictured in Plates 4.3.1 and 4.3.3. The ancillary equipment

was arranged in such a way so as not to obscure the culture vessels' illumination. The intensity of illumination falling upon the culture vessel was varied by the number of fluorescent tubes switched on at any particular time. This was translated into a numerical value using a light meter placed at the diametric axis of the culture vessel perpendicular to the plane of the lighting bank. Only the photosynthetically active radiation (PAR) was measured. This was expressed in the form of photon flux (units: $\mu E\ m^{-2}\ sec^{-1}$). The range of illumination thus achieved varied between 45 $\mu E\ m^{-2}\ sec^{-1}$ with only one tube illuminated and 170 $\mu E\ m^{-2}\ sec^{-1}$ with all nine tubes illuminated. The tubes were removed in sequence from the upper and lower edges of the lighting board to decrease illumination intensity. Finally, the central tube remained providing illumination along the central axis of the culture vessel of the turbidostat yielding the lowest photon flux used.

The culture period varied dependent upon the time required for 5 litres of culture medium to accumulate in the cell harvest reservoir as a result of the action of the dilution pump triggered by increasing turbidity. This indicated that the original cell population had increased more than five fold. Earlier batch cultures had shown this number of divisions to result in a plateau of fatty acyl unsaturation for exponentially growing cultures. Alternatively this could be described as 'steady-state'. The cells, in their suspending medium, were then withdrawn axenically, *en masse*, from the culture vessel *via* the dilution mechanism and harvested according to the protocol described in Figure 5.3.1. Because of the arrangement of the cannula attached to the dilution pump (see Figure 4.3.1) it was not possible to completely empty the vessel in this manner. Sample size was therefore limited to *ca.* 800 ml, a further 100 ml remaining in the culture vessel.

In this series of experiments the subsequent inoculations of the culture vessel were achieved by allowing the surplus cells from the previous culture to remain whilst 'topping up' the culture medium. This was done axenically and eliminated the requirements for the culture vessel to be dismantled, and resterilised. Added advantages were that the cells used were gradually acclimated to decreasing light intensities, and turn-around time for the turbidostat and the use of presterilised consumables were reduced considerably.

Following 'topping-up' the culture was allowed to grow at a reduced medium pumping rate until its turbidity reached the preset threshold for the turbidostat to start functioning. This point was regarded as 'inoculation' and therefore the beginning of a new experimental culture.

7.2.2 Results & Discussion.

From the data shown in Table 7.2.1 it was clear that the illumination intensity under which a culture was grown had a significant effect upon the total FAME composition of its acyl lipid component. However, at first sight the trend in the data was not that which was expected. In the light of the results presented in Sections 5 and 6, an increased cell division rate as a result of increased photosynthetic fixation of carbon would be expected to promote an increase in unsaturation. This was not the case at the level of total FAME. Increased illumination intensity resulted in decreased unsaturation of total FAME of *N. oculata* when grown in continuous culture. As with the data incorporated into earlier sections, the general pattern of variation in the fatty acyl composition resulted from complementary changes in the proportions of 16:0 and 20:5(*n*-3). Minor percentage changes also occurred in the acids 18:1(*n*-9) and 18:2(*n*-6) although, at the total FAME level, these were of small consequence. The acyl chain length distribution of the total FAME was within the ranges observed in the preceding section, the C_{16} and C_{20} acids predominating, with a lesser proportion of C_{18} acids which consisted of 18:0, 18:1 and 18:2. The proportions of C_{16} chain length fatty acids decreased with illumination from 56.2 % at 170 $\mu E\, m^{-2}\, sec^{-1}$ to 44.6 % at 45 $\mu E\, m^{-2}\, sec^{-1}$. The proportion of C_{20} chain length fatty acids increased from 29.5 % at 170 $\mu E\, m^{-2}\, sec^{-1}$ to 36.9 % at 45 $\mu E\, m^{-2}\, sec^{-1}$. The proportions of C_{18} chain length fatty acids showed no real variation with illumination. The proportion of PUFA increased at the expense of saturated fatty acids and, to a lesser extent, monounsaturated fatty acids in the illumination range 170 $\mu E\, m^{-2}\, sec^{-1}$ to 65 $\mu E\, m^{-2}\, sec^{-1}$. This trend was reversed slightly in the intensity region between 65 $\mu E\, m^{-2}\, sec^{-1}$ and 45 $\mu E\, m^{-2}\, sec^{-1}$. The proportion of the monounsaturate 16:1 increased slightly between these two intensities as did the proportion of 18:2(*n*-6). As a consequence the proportion of 20:5(*n*-3) decreased by a corresponding amount. Thus the

TABLE 7.2.1: Total FAME composition of *N. oculata* grown in continuous culture at various illumination intensities in unmodified S88 medium.

	ILLUMINATION INTENSITY ($\mu E\ m^{-2}\ sec^{-1}$)			
FATTY ACID	170	140	65	45
14:0	2.9	4.7	2.7	3.4
15:0	0.4	0.4	0.2	0.4
16:0	27.8	24.3	16.8	16.9
16:1(*n*-7)	28.4	28.1	24.6	26.6
16:1(*n*-13)t.	.	.	.	.
16:2	.	0.1	0.2	0.4
17:0	.	.	.	.
16:3	.	0.1	0.7	0.7
16:4	.	.	.	.
18:0	1.1	1.1	0.5	0.4
18:1(*n*-9)	5.0	5.7	3.7	3.4
18:1(*n*-7)	.	.	0.2	.
18:2(*n*-6)	1.9	2.1	3.8	4.4
18:3(*n*-6)	0.4	0.2	0.2	0.4
18:3(*n*-3)	.	.	.	.
18:4(*n*-3)	.	.	.	.
20:0	.	.	.	.
20:2	.	.	.	.
20:3(*n*-6)	0.6	0.6	0.5	0.4
20:4(*n*-6)	4.1	4.1	5.0	4.2
20:3(*n*-3)	.	.	.	.
20:4(*n*-3)	.	.	.	.
20:5(*n*-3)	24.8	25.8	35.4	32.3
Unidentified:	2.6	2.4	5.7	6.4
Total sats :	32.2	30.5	20.2	21.1
Total monos :	33.4	33.8	28.3	30.0
Total PUFA :	31.8	33.3	45.8	42.5
Total (*n*-9) :	5.0	5.7	3.7	3.4
Total (*n*-6) :	7.0	7.0	9.5	9.4
Total (*n*-3) :	24.8	25.8	35.4	32.7
(*n*-3)/(*n*-6) :	3.5	3.7	3.7	3.5
16:1/16:0 :	1.0	1.2	1.5	1.6
20:5/16:0 :	0.9	1.1	2.1	1.9
ΣC_{16}:	56.2	52.6	42.3	44.6
ΣC_{18}:	8.4	9.1	8.4	8.6
ΣC_{20}:	29.5	30.5	40.9	36.9

Abbreviations & Symbols
tr.,<0.1%, ., not detected

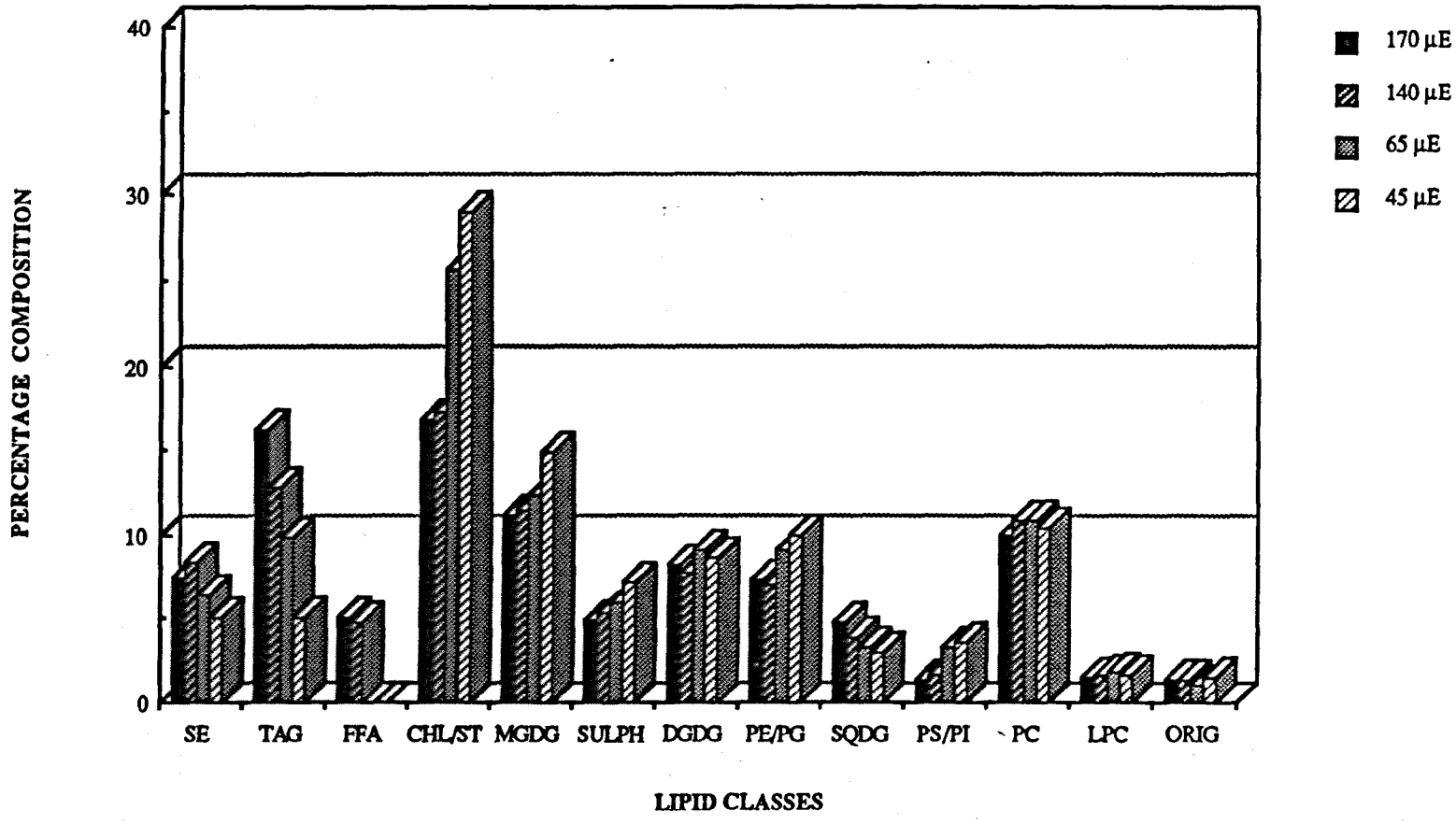


Figure 7.2.1 : Changes in the relative proportions of the various lipid classes of *N. oculata* grown in continuous culture at different illumination intensities[1].

acyl chain length distribution profile was modified:- C_{16} acyl chain lengths increased from 42.3 % to 44.6 % and C_{20} acyl chain lengths decreased in proportion from 40.9 % to 36.9 %. This change, when linked with the data obtained from HPTLC-densitometric analyses of the samples, gave an important clue as to what was occurring.

The levels of illumination employed were well within those likely to be encountered by the alga in its natural environment. Therefore it could not be argued that the cells were subject to photoinhibition at the 'higher' intensities, particularly as they had been maintained under each respective illumination regime for a period well in excess of 14 days (see above).

Figure 7.2.1 shows the results of analysis of the total lipid extract using HPTLC-densitometry. The point to which attention should be directed is the change in the proportion of total lipid present as TAG. In this particular series of experiments it must not be forgotten that the cultures were all in the exponential-phase and all the cells were actively dividing and were not limited by nutrient supply. Clearly, the most likely explanation was that the accumulation of TAG was due to an excess of fixed carbon over and above that required for maximal cell division. This became more evident when the series of cultures was considered as a whole. Each decrement in the illumination intensity resulted in a decrease in the margin of excess fixed carbon. Therefore TAG, which was acting as a 'sink' for the excess photosynthetically fixed carbon, was reduced in proportion. It must be noted that the increasing proportions of the polar lipid classes were not wholly due to the effects of the decreasing proportions of TAG. Increased pigment content in the total lipid extracted from cells grown at the lower illumination intensities was the cause of poor resolution of the unesterified fatty acid and chlorophyll / sterol components for the samples from the cultures grown at 65 $\mu E\ m^{-2}\ sec^{-1}$ and 45 $\mu E\ m^{-2}\ sec^{-1}$. This accounts for the discrepancies encountered in the proportions of these components at these illumination intensities.

Despite the unexpected changes in the total FAME composition of *N. oculata* as a result of illumination regime, the pattern ultimately proved to fit the predicted response in a

manner which was correlated with the findings of the previous sections. This became apparent from analyses of the acyl compositions of the individual lipid classes. The data contained in Tables 7.2.2 to 7.2.5 illustrate the point and repeat the data for the total FAME for ease of comparison. At the highest illumination intensity used for culturing algae, 170 μE m^{-2} sec^{-1}, the TAG component of the cells contained high proportions of C_{16} (and C_{18}) monounsaturated and saturated fatty acids at 43.6 % and 37.9 % respectively. These were counter-balanced by a correspondingly low proportion of PUFA at 14.6 %, of which only 10.8 % was accounted for by 20:5(*n*-3). However, in the case of the galactolipid classes there was a high degree of unsaturation with MGDG and DGDG having 71.0 % and 49.9 % of their respective fatty acid masses as 20:5(*n*-3). This fatty acid accounted for the major portion of PUFA in these two classes, as observed in the data presented in previous sections. Amongst the phospholipids, most notably PC which had 20.5 % of its fatty acyl mass as 20:5(*n*-3), there was generally a higher proportion of PUFA in the lipids of cultures grown at higher illumination intensities. The inverse seemed to apply for the neutral lipid classes. Both TAG and unesterified fatty acids displayed lower proportions of PUFA in the extracts from cultures grown at the higher illumination intensities (170 and 140 μE m^{-2} sec^{-1}) compared with those from the lower intensity range (65 and 45 μE m^{-2} sec^{-1}). This parallelled the findings presented in Section 5 regarding the partitioning of fatty acids between neutral or polar lipid biosynthesis during the lag-phase, the present difference being in the origins of the excess carbon flux. In the case of the lag-phase this was due to a suspension of cellular division. In the case of high illumination intensity carbon was incorporated into fatty acids at a greater rate than it could be used in membrane lipid biosynthesis (ultimately to be consumed in cellular division) and was therefore shunted into TAG biosynthesis. The hallmark of this occurrence, in the absence of HPTLC-densitometric analyses, being TAG with high proportions of C_{16} fatty acids.

The phospholipid PC showed wide variation in its fatty acyl profile with illumination intensity. This differed with respect to the other major classes in that the variation pattern involved C_{18} acids as well as those of C_{16} and C_{20} chain lengths. A phenomenon similar to

TABLE 7.2.2 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture at a light intensity of 170 μE m^{-2} sec^{-1} in unmodified S88 medium.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.6	2.9	7.8	3.6	2.0	2.1	0.9	2.9
15:0	0.4	0.9	.	0.2	0.3	0.4	0.1	0.4
16:0	31.6	23.8	9.1	20.2	24.4	29.7	13.0	27.8
16:1(*n*-7)	38.0	18.0	5.0	16.3	22.3	30.2	22.2	28.4
16:1(*n*-13)t	.	.	.	.	5.0	.	.	.
16:2	.	0.2	.	.	0.1	.	0.6	.
17:0	.	0.9	0.2	.	.	.	0.5	.
16:3	.	1.2	0.2	.	.	.	.	.
16:4	.	.	.	.	.	.	.	.
18:0	1.3	9.3	1.1	0.6	0.4	2.3	0.5	1.1
18:1(*n*-9)	5.6	7.7	1.3	0.7	1.4	5.6	12.7	5.0
18:1(*n*-7)	.	.	0.2	0.1	0.3	.	.	.
18:2(*n*-6)	0.9	1.1	0.1	0.6	.	0.8	10.4	1.9
18:3(*n*-6)	0.2	0.6	0.1	.	.	0.2	.	0.4
18:3(*n*-3)	.	.	.	.	0.1	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.6	0.3	.	.	0.4	0.4	0.7	0.6
20:4(*n*-6)	2.1	3.9	1.2	1.7	6.3	13.0	10.0	4.1
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	10.8	15.5	71.0	49.9	32.4	9.3	20.5	24.8
Unidentified :	3.9	13.7	2.7	6.1	4.6	6.0	7.9	2.6
Total sats :	37.9	37.8	18.2	24.6	27.1	34.5	15.0	32.2
Total monos :	43.6	25.7	6.5	17.1	29.0	35.8	34.9	33.4
Total PUFA :	14.6	22.8	72.6	52.2	39.3	23.7	42.2	31.8
Total (*n*-9) :	5.6	7.7	1.3	0.7	1.4	5.6	12.7	5.0
Total (*n*-6) :	3.8	5.9	1.4	2.3	6.7	14.4	21.1	7.0
Total (*n*-3) :	10.8	15.5	71.0	49.9	32.5	9.3	20.5	24.8
(*n*-3)/(*n*-6) :	2.8	2.6	50.7	21.7	4.9	0.6	1.0	3.5
16:1/16:0 :	1.2	0.8	0.5	0.8	0.9	1.0	1.7	1.0
20:5/16:0 :	0.3	0.7	7.8	2.5	1.3	0.3	1.6	0.9
ΣC_{16}:	69.6	43.2	14.3	36.5	51.8	59.9	35.8	56.2
ΣC_{18}:	8.0	18.7	2.8	2.0	2.2	8.9	23.6	8.4
ΣC_{20}:	13.5	19.7	72.2	51.6	39.1	22.7	31.1	29.5

Abbreviations & Symbols
tr.,<0.1%, . not detected, ‡, including SQDG.
Data expressed in terms of percentage of individual lipid class FAME mass

TABLE 7.2.3 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture at a light intensity of 140 μE m^{-2} sec^{-1} in unmodified S88 medium.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	4.1	2.1	7.1	2.9	1.9	2.2	0.9	4.7
15:0	0.5	0.5	0.1	1.9	0.3	0.3	0.1	0.4
16:0	31.3	13.9	8.0	21.0	22.2	26.9	10.2	24.3
16:1(*n*-7)	38.3	19.1	4.1	17.1	21.8	33.3	19.6	28.1
16:1(*n*-13)*t*	.	.	.	.	5.1	.	.	.
16:2	.	.	.	.	.	.	0.9	0.4
17:0	0.8	0.7	0.1	0.1	.	.	0.1	.
16:3	0.2	.	.	.	.	.	.	0.1
16:4	.	.	.	.	.	.	.	.
18:0	1.4	2.8	0.7	0.4	0.4	1.1	0.4	1.1
18:1(*n*-9)	7.1	5.0	0.8	0.7	1.7	5.4	16.4	5.7
18:1(*n*-7)	.	.	0.1	0.2	0.2	.	.	.
18:2(*n*-6)	0.8	1.4	0.3	.	0.6	0.8	11.7	2.1
18:3(*n*-6)	.	.	.	.	.	.	2.0	0.2
18:3(*n*-3)	.	.	.	.	.	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.5	0.3	0.1	.	0.5	.	0.3	0.6
20:4(*n*-6)	2.3	7.9	1.6	0.9	7.1	12.8	11.1	4.1
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	10.8	44.3	74.6	53.9	34.6	10.2	21.9	25.8
Unidentified :	1.9	2.0	2.4	0.9	3.6	7.0	4.4	2.4
Total sats :	38.1	20.0	16.0	26.3	24.8	30.5	11.7	30.5
Total monos :	45.4	24.1	5.0	18.0	28.8	38.7	36.0	33.8
Total PUFA :	14.6	53.9	76.6	54.8	42.8	23.8	47.9	33.3
Total (*n*-9) :	7.1	5.0	0.8	0.7	1.7	5.4	16.4	5.7
Total (*n*-6) :	3.6	9.6	2.0	0.9	8.2	13.6	25.1	7.0
Total (*n*-3) :	10.8	44.3	74.6	53.9	34.6	10.2	21.9	25.8
(*n*-3)/(*n*-6) :	3.0	4.6	37.3	59.9	4.2	0.8	0.9	3.7
16:1/16:0 :	1.2	1.4	0.5	8.1	1.0	1.2	1.9	1.2
20:5/16:0 :	0.3	3.2	9.3	2.6	1.6	0.4	2.1	1.1
ΣC_{16}:	69.8	33.8	12.1	38.1	49.1	60.2	30.7	52.6
ΣC_{18}:	9.3	9.2	1.9	1.3	2.9	7.3	30.5	9.1
ΣC_{20}:	13.6	52.5	76.3	54.8	42.2	23.0	33.3	30.5

Abbreviation & Symbols
tr.,<0.1%, ., not detected, ‡, including SQDG.

TABLE 7.2.4 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture at a light intensity of 65 μE m^{-2} sec^{-1} in unmodified S88 medium.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	5.5	2.7	8.5	6.0	4.0	4.6	7.4	2.7
15:0	0.4	0.3	0.2	0.3	0.4	0.2	0.8	0.2
16:0	17.6	7.0	6.7	22.5	29.8	30.5	10.6	16.8
16:1(*n*-7)	26.1	16.2	8.8	22.7	24.3	40.0	23.3	24.6
16:1(*n*-13)*t*	.	.	.	.	3.8	.	.	.
16:2	0.3	0.3	.	0.5	0.2	.	0.6	0.2
17:0	0.6	0.3	0.2	.	.	.	.	.
16:3	0.4	0.5	0.2	.	.	.	1.4	0.7
16:4	.	.	.	.	.	.	.	.
18:0	1.1	1.4	0.5	0.2	0.4	0.8	0.8	0.5
18:1(*n*-9)	3.3	2.8	0.9	0.6	1.6	4.9	11.8	3.7
18:1(*n*-7)	.	.	0.1	0.2	0.3	0.1	.	.
18:2(*n*-6)	1.3	3.0	1.3	1.8	1.3	1.5	12.7	3.8
18:3(*n*-6)	.	.	.	.	0.1	.	0.9	0.2
18:3(*n*-3)	.	.	.	.	0.3	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.4	0.2	.	.	0.2	.	0.4	0.5
20:4(*n*-6)	4.8	6.7	1.1	1.0	5.4	6.6	7.7	5.0
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	34.6	51.3	68.9	40.8	24.8	6.3	12.2	35.4
Unidentified :	3.6	7.3	2.6	3.4	3.1	4.5	9.4	5.7
Total sats :	25.2	11.7	16.1	29.0	34.6	36.1	19.6	20.2
Total monos :	29.4	19.0	9.8	23.5	30.0	45.0	35.1	28.3
Total PUFA :	41.8	62.0	71.5	44.1	32.3	14.4	35.9	45.8
Total (*n*-9) :	3.3	2.8	0.9	0.6	1.6	4.9	11.8	3.7
Total (*n*-6) :	6.5	9.9	2.4	2.8	7.0	8.1	21.7	9.5
Total (*n*-3) :	34.6	51.3	68.9	40.8	25.1	6.3	12.2	35.4
(*n*-3)/(*n*-6) :	5.3	5.2	28.7	14.6	3.6	0.8	0.6	3.7
16:1/16:0 :	1.5	2.3	1.3	1.0	0.8	1.3	2.2	1.5
20:5/16:0 :	2.0	7.3	10.3	1.8	0.8	0.2	1.1	2.1
ΣC_{16}:	44.4	24.0	15.7	45.7	57.9	70.5	35.9	42.3
ΣC_{18} :	5.7	7.2	2.8	2.8	4.0	7.3	26.2	8.4
ΣC_{20}:	39.8	58.2	70.0	41.8	30.4	12.9	20.3	40.9

Abbreviations & Symbols
tr.,<0.1%, ., not detected, ‡, including SQDG.

TABLE 7.2.5 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture at a light intensity of 45 $\mu E\ m^{-2}\ sec^{-1}$ in unmodified S88 medium.

	LIPID CLASSES								
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG	SQDG	PS/PI	PC	TOTAL
14:0	5.2	2.3	7.5	3.4	2.0	2.9	2.8	1.5	3.4
15:0	0.5	0.3	0.2	0.3	0.5	0.4	0.6	0.2	0.4
16:0	12.9	5.9	11.6	19.7	34.1	11.9	29.0	5.4	16.9
16:1(*n*-7)	16.1	11.7	10.8	27.4	30.2	25.0	38.3	30.4	26.6
16:1(*n*-13)*t*	.	.	.	.	5.8	.	.	.	.
16:2	0.2	0.2	0.2	1.0	0.2	0.4	0.3	1.8	0.4
17:0	0.3	0.1	.	0.1	.	0.2	0.2	0.7	.
16:3	0.5	0.2	0.2	0.3	0.3	0.7	0.7	0.1	0.4
16:4	.	.	.	.	.	.	.	0.5	.
18:0	1.3	1.0	0.5	0.4	0.2	0.9	0.9	0.3	0.4
18:1(*n*-9)	2.8	1.4	0.2	0.8	1.1	3.2	5.1	13.9	3.4
18:1(*n*-7)	0.3	0.3	.	0.4	0.2	0.2	.	.	.
18:2(*n*-6)	2.0	2.7	2.6	3.2	1.4	3.5	2.4	17.5	4.4
18:3(*n*-6)	0.2	0.2	.	.	.	0.3	.	1.9	0.4
18:3(*n*-3)	.	.	.	0.4	.	.	.	0.7	.
18:4(*n*-3)	.	.	.	.	.	.	.	0.6	.
20:0	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	2.0	0.3	.	.	0.4	0.6	0.4	0.7	0.4
20:4(*n*-6)	6.7	6.4	2.3	1.3	3.1	7.9	5.9	7.5	4.2
20:3(*n*-3)	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	42.9	63.2	60.7	37.7	18.6	36.5	7.8	10.3	32.3
Unidentified :	6.1	3.8	3.2	3.6	1.9	5.4	5.6	6.0	6.4
Total sats :	20.2	9.6	19.8	23.9	36.8	16.3	33.5	8.1	21.1
Total monos :	19.2	13.4	11.0	28.6	37.3	28.4	43.4	44.3	30.0
Total PUFA :	54.5	73.2	66.0	43.9	24.0	49.9	17.5	41.6	42.5
Total (*n*-9) :	2.8	1.4	0.2	0.8	1.1	3.2	5.1	13.9	3.4
Total (*n*-6) :	12.9	9.6	4.9	4.5	4.9	12.3	8.7	27.6	9.4
Total (*n*-3) :	42.9	63.2	60.7	38.1	18.6	36.5	7.8	11.0	32.7
(*n*-3)/(*n*-6) :	3.3	6.6	12.4	8.4	3.8	3.0	0.9	0.4	3.5
16:1/16:0 :	1.2	2.0	0.9	1.4	0.9	2.1	1.3	5.6	1.6
20:5/16:0 :	3.3	10.7	5.2	1.9	0.5	3.1	0.3	1.9	1.9
ΣC_{16}:	29.7	18.0	22.8	48.4	70.6	38.0	68.3	38.2	44.6
ΣC_{18}:	6.6	5.6	3.3	5.2	2.9	8.1	8.4	34.9	8.6
ΣC_{20}:	51.6	69.9	63.0	39.0	22.1	45.0	14.1	18.5	36.9

Abbreviations & Symbols
tr.,<0.1%, ., not detected.

that observed for the same fraction from the cells cultured in media of various salinities was noted, although in this case the data were not as clear cut. Based upon of the proportion of 20:5(*n*-3) in the total FAME sample, there was due to some doubt as to whether or not 140 $\mu E\ m^{-2}\ sec^{-1}$ was in fact the optimal illumination intensity for *N. oculata* . The data for the two samples were so similar that it was concluded that they were for all intents and purposes identical, resulting from a plateau in photosynthetic activity in the illumination intensity range 170 $\mu E\ m^{-2}\ sec^{-1}$ to 140 $\mu E\ m^{-2}\ sec^{-1}$.

At an illumination intensity of 170 $\mu E\ m^{-2}\ sec^{-1}$ the proportion of saturates in PC was 15.0 %, comprising mainly 16:0; that of monounsaturates was 34.9 %, accounted for by 16:1 and 18:1 and the proportion of PUFA was 42.2 %, this being present mainly as 10.4 % 18:2, 10.0 % 20:4(*n*-6) and 20.5 % of 20:5(*n*-3). The decrease in illumination intensity from 170 $\mu E\ m^{-2}\ sec^{-1}$ to 140 $\mu E\ m^{-2}\ sec^{-1}$ brought about changes in the acyl chain length distribution profile of the PC fraction. The proportion of C_{16} acids decreased from 35.8 % to 30.7 %, that of the C_{18} acids increased from 23.6 % to 30.5 % whilst the proportion of C_{20} acyl chain length fatty acids increased only slightly from 31.2 % to 33.3 %. In terms of unsaturation the C_{20} fatty acids were relatively unaffected. The major change resulted from a redistribution amongst the C_{16} and C_{18} chain length acids such that a decrease in the proportion of 16:1 resulted in increases in the proportions of 18:1 and 18:2. The accumulation of 18:2 at the expense of 16:0 and 16:1 accounted for the decrease in the proportion of saturates from 15.0 % to 11.7 % and the increase in the proportion of PUFA from 42.2 % to 47.9 % whilst the proportion of monounsaturates changed relatively little.

The remaining data from the lower illumination intensity cultures gave a clear trend of decreasing acyl chain length and unsaturation with decreasing illumination intensity amongst the polar lipid classes. The trend amongst the neutral lipid classes was, as observed previously, one of increasing chain length and unsaturation . This was particularly the case for the TAG component as its proportion in the total lipid decreased (see Figure 7.2.1). The proportion of C_{20} fatty acids in the galactolipid MGDG decreased from 76.3 % at 140 μE

m^{-2} sec^{-1} to 63.0 % at 45 μE m^{-2} sec^{-1}. Similarly the other galactolipid class DGDG showed a decrease in its proportion of C_{20} fatty acids from 54.8 % to 39.0 % over the same illumination range. In the case of MGDG this decrease was compensated for by an increase in the proportion of C_{16} fatty acids although there was also a slight concurrent elevation in the proportion of C_{18} fatty acids present at lower illumination intensity. This was also the case for DGDG. In the instances of both MGDG and DGDG it was interesting to note that the changes in the proportions of the C16 fatty acids showed the same asymmetric pattern of increase. With the first decrement in illumination intensity from 140 μE m^{-2} sec^{-1} to 65 μE m^{-2} sec^{-1} the major increase was in the proportion of 16:1 from 17.1 % to 22.7 % for DGDG and from 4.1 % to 8.8 % in the case of MGDG. The change in the proportion of 16:0 was an increase from 21.0 % to 22.5 % for DGDG and a decrease from 8.0 % to 6.7 % in the case of MGDG. With the next decrement in the illumination intensity, from 65 μE m^{-2} sec^{-1} to 45 μE m^{-2} sec^{-1}, the proportion of 16:0 decreased from 22.5 % to 19.7 % for DGDG and that of 16:1 increased still further from 22.7 % to 27.4 %. The proportions of the same two fatty acids in the MGDG fraction both increased slightly with decreased illumination intensity, 16:0 increased from 6.7 % to 11.6 % and 16:1 increased from 8.8 % to 10.8 %. The total proportion of C_{16} chain length fatty acids increased with decreasing illumination intensity in both the galactolipid classes. The relative proportions of the two major C16 acids did not remain constant as was shown by the changes in the 16:1/16:0 ratios for these classes. The pattern of change did not follow a consistent pattern of increase or decrease either.

The more complex situation involving PC and its higher proportion of C_{18} fatty acids gave rise to a pattern of compositional changes different from those described for the galactolipids. The proportion of C_{16} fatty acids increased with decreasing illumination intensity whereas that of 16:1 increased. In this case the ratio of 16:1/16:0 showed an overall trend of increase with decreasing illumination intensity. The proportion of 16:0 decreased from 10.2 % to 5.4 % over the illumination range 140 μE m^{-2} sec^{-1} to 45 μE m^{-2} sec^{-1}. That of 16:1 increased from 19.6 % to 30.4 % over the same range. The proportion of

C_{20} fatty acids (both 20:5(*n*-3) and 20:4(*n*-6)) decreased consistently with decreasing illumination. The proportions of the C_{18} acyl chain length fatty acids decreased between the illumination intensities 140 μE m^{-2} sec^{-1} and 65 μE m^{-2} sec^{-1} and then increased again between 65 μE m^{-2} sec^{-1} and 45 μE m^{-2} sec^{-1} with a shift in the distribution of the fatty acids. As can be seen from the data in Table 7.2.5, at 45 μE m^{-2} sec^{-1}, 18:2(*n*-6) had become the predominating PUFA in the PC fraction. Unusually, the major fatty acids in this fraction were 16:1, 18:1 and 18:2. The trend observed through the data in Tables 7.2.3 to 7.2.5 indicates a decreasing proportion of C_{20} PUFA and an increasing proportion of C_{18} PUFA. The accumulation of monounsaturates of decreasing chain length is also evident with the transition from the accumulation of 18:1 to accumulation of 16:1 occurring steadily over the experimental illumination range.

7.3 VARIATION IN LIPID COMPOSITION WITH NITRATE CONCENTRATION.

7.3.1 Experimental.

The culture related experimental details of this section are essentially very similar to those of the previous section. Because the factor under test was different, being a chemical rather than a physical variable, the procedures by which the variations were achieved also differed. There was a basic assumption, as with all the continuous culture experimentation recorded in this thesis. This was that the composition of the medium in the culture vessel was similar, if not identical, to that supplied from the medium reservoir. Obviously, this is most probably not the case when the nutrients approach limiting concentrations as they will be taken up rapidly by the cells in the culture vessel. In such cases it is the limiting nutrient *supply rate* which is important. This is a standard concept in chemostat theory and will be discussed in relation to the cage culture turbidostat experiments in a later section.

The aim of this series of continuous cultures was to elucidate the effects of decreasing the supply rate of the nutrient nitrate. The turbidostat was set up and inoculated in a similar manner to that described for the investigation of illumination intensity in Section 7.2. In the first instance the medium used was the standard unmodified S88 (see Appendix D). Being primarily a batch culture medium this was formulated to possess a high nitrate concentration in relation to that found naturally in the marine environment. This concentration, 1.0 mM NO_3^-, was used as the upper limit in these cultures. Because the medium was supplied continuously the amount of nitrate available to the culture was considerably higher than in batch culture, being replenished at 1.0 mMoles NO_3^- per day. The first culture in this series of experiments served to illustrate the difference between the growth of a culture in unmodified S88 medium using either batch or continuous culture. In the following series of cultures the nitrate concentration was decremented by factors of ten (*i.e.* 1.0, 0.1, 0.01 & 0.001 mM NO_3^-). This was achieved in a similar manner to that described for illumination in the previous subsection. Surplus cells from previous cultures

were used to inoculate immediately successive cultures in the turbidostat. In this way the cells used had a nitrogen status closer to that which they were likely to achieve in the next batch of medium.

All other factors in the culture medium were maintained as described in the recipe included in the appendices to this thesis. The incubation temperature used was again set at the standard of 20 ^{o}C and the illumination regime was 65 μE m^{-2} sec^{-1} as detailed in Section 7.2.1. The turbidostat mechanism was maintained at the same level as for the illumination experiment series.

7.3.2 Results & Discussion.

As was expected, in the light of the data from previous batch culture experiments, the variation in medium nitrate concentration produced significant effects upon the lipid class and fatty acyl composition of *N. oculata.* Table 7.3.1 shows the variation in the total FAME composition of lipid extracted from cultures grown using nitrate supplied to the culture vessel at 1.0 mMoles, 0.1 mMoles, 0.01 mMoles and 0.001 mMoles per day.

Decreasing the nitrate supply resulted in a decrease in the proportion of PUFA in the total lipid extract from 45.8 % at the maximum nitrate concentration of 1.0 mM NO_3^- to 17.5 % at the minimum concentration of 0.001 mM NO_3^-. The variation between cultures grown using S88 containing 1.0 mM and 0.1 mM NO_3^- was limited to almost complementary changes in the proportions of 16:0 and 20:5(*n*-3). No real variation occurred in the proportion 16:1 over the experimental range of culture medium nitrate concentrations. However, minor changes in the proportions of 18:1(*n*-9) and 18:2(*n*-6) resulted in a slight increase in the total proportion of monounsaturates and C_{18} chain length fatty acids in the culture grown in 0.1 mM NO_3^- in comparison with that grown in unmodified S88.

When the medium nitrate concentration was reduced by a further factor of ten, to 0.01 mM, the proportion of C_{16} fatty acids rose still further to 61.1 % as a result of the

TABLE 7.3.1: Total FAME composition of *N. oculata* grown in continuous culture in S88 medium modified to provide various nitrate concentrations.

	NITRATE CONCENTRATION (mM)			
FATTY ACID	1.0	0.1	0.01	0.001
14:0	2.7	4.0	2.8	3.3
15:0	0.2	0.2	0.2	0.2
16:0	16.8	31.5	35.0	35.8
16:1(*n*-7)	24.6	24.7	26.1	26.2
16:1(*n*-13)t.	.	.	.	.
16:2	0.2	.	.	.
17:0	.	.	.	.
16:3	0.7	0.4	.	0.2
16:4	.	.	.	.
18:0	0.5	1.5	1.2	1.5
18:1(*n*-9)	3.7	5.8	14.2	11.1
18:1(*n*-7)	.	.	.	.
18:2(*n*-6)	3.8	2.5	3.3	2.4
18:3(*n*-6)	0.2	0.6	0.4	0.5
18:3(*n*-3)	.	.	.	.
18:4(*n*-3)	.	.	.	.
20:0	.	.	.	.
20:2	.	.	.	.
20:3(*n*-6)	0.5	1.6	0.8	1.1
20:4(*n*-6)	5.0	2.9	1.7	2.2
20:3(*n*-3)	.	.	.	.
20:4(*n*-3)	.	.	.	.
20:5(*n*-3)	35.4	18.7	10.9	11.0
Unidentified :	5.7	5.6	3.4	4.5
Total sats :	20.2	37.2	39.2	40.8
Total monos :	28.3	30.5	40.3	37.3
Total PUFA :	45.8	26.7	17.1	17.5
Total (*n*-9) :	3.7	5.8	14.2	11.1
Total (*n*-6) :	9.5	7.6	6.2	6.2
Total (*n*-3) :	35.4	18.7	10.9	11.1
(*n*-3)/(*n*-6) :	3.7	2.5	1.8	1.8
16:1/16:0 :	1.5	0.8	0.7	0.7
20:5/16:0 :	2.1	0.6	0.3	0.3
ΣC_{16}:	42.3	56.6	61.1	62.2
ΣC_{18}:	8.2	10.4	19.1	15.5
ΣC_{20}:	40.9	23.2	13.4	14.3

Abbreviations & Symbols
tr.,<0.1%, ., not detected.

accumulation of 16:0. The proportion of C_{18} fatty acids also rose from 10.4 % at 0.1 mM NO_3^- to 19.1 % at 0.01 mM NO_3^- as 18:1 accumulated; whilst the proportion of C_{20} fatty acids decreased from 23.2 % to 13.4 % over the same concentration range. A reduction of the nitrate concentration still further to 0.001 mM NO_3^- gave no further changes in the acyl composition of the total lipid extract. The slight variations in acyl composition observed between the two lowest nitrate concentration were attributed to natural variations or minor differences in the batches of culture medium used for each culture set-up. Clearly the major changes in the acyl composition of *N. oculata* occur within the nitrate concentration range 1.0 mM NO_3^- to 0.01 mM NO_3^-.

The most significant quantitative changes were those which occurred in the fatty acids 16:0 and particularly 20:5(*n*-3). In the nitrate concentration range 1.0 mM NO_3^- to 0.01 mM NO_3^- the proportion of 16:0 increased from 16.8 % to 31.5 %. The proportion of C_{16} fatty acids underwent a concomitant increase from 42.3% to 56.6 %. The C_{20} fatty acid 20:5(*n*-3) showed a decrease in its proportion from 35.4 % to 18.7 % over the same nitrate concentration range. The proportion of 20:4(*n*-6) also decreased and contributed to the overall decrease in the proportion of C_{20} fatty acids from 40.9 % to 23.2 % with the first decrement in nitrate concentration. Within this particular nitrate concentration window the variation observed in the proportions of the C_{18} fatty acid chain lengths was limited to a slight increase in the proportion of 18:1 from 3.7 % to 5.8 %. The summaries at the foot of Table 7.3.1 show the apparent stability of the proportion of monounsaturated acids in comparison with those of the saturates and polyunsaturates. The trend of increasing saturation at the expense of PUFA continued with the progression of nitrate concentration to 0.01 mM NO_3^-.

The proportion of 20:5(*n*-3) decreased further from 18.7 % to 10.9 % with the next ten-fold decrement in the medium nitrate concentration from 0.1 mM NO_3^- to 0.01 mM NO_3^- This decrease was not associated with a concomitant increase in the proportion of 16:0. The change in this acid was much smaller increasing by 3.5 % within this nitrate concentration

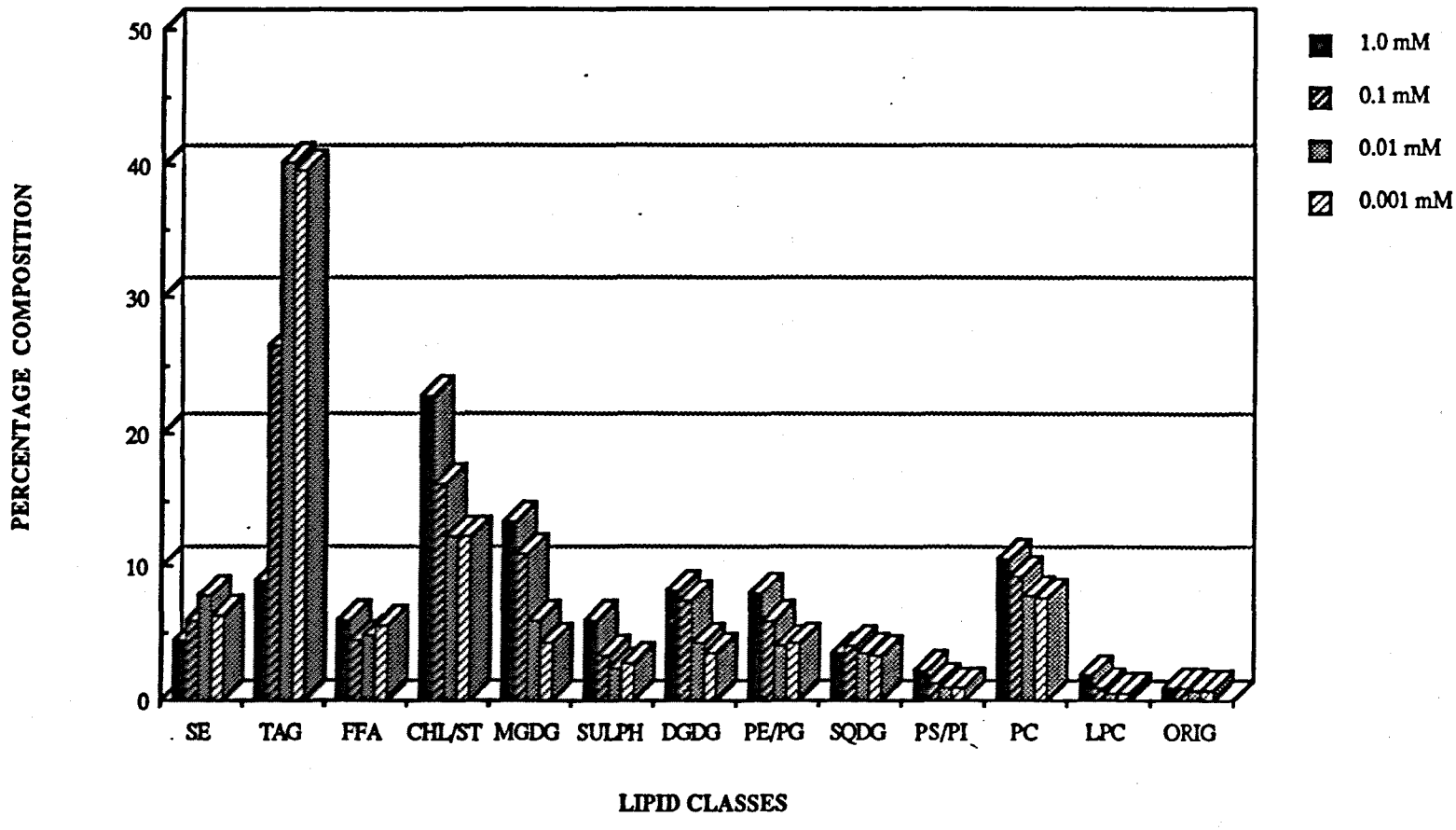


Figure 7.3.1 : Changes in the relative proportions of the various lipid classes of *N. oculata* grown in continuous culture at different nitrate concentrations[1].

window. The decrease in proportion of 20:5(*n*-3), which may practically be regarded as analogous to PUFA, was balanced by an increase in the proportion of monounsaturates. Although the proportion of 16:1(*n*-7) showed an increase from 24.7 % to 26.1 % the major contribution to the general increase in monounsaturation was due to an accumulation of 18:1(*n*-9).

The data yielded by HPTLC-densitometry is shown in Figure 7.3.1. It is obvious that the lipid class TAG played an important role in determining the acyl composition of the total lipid extracts of the nitrate depleted cultures. All the other classes show as minor components by comparison. As has been observed on several previous occasions, the relatively minor changes which occurred in the proportions of the remaining lipid classes were most probably artifacts of the form of data expression. However, the quantities of photosynthetic pigments showed an obvious reduction in the crude total lipid extracts of the nitrate depleted cultures which was plainly visible with the naked eye prior to the staining and charring of the developed thin-layer chromatograms.

Tables 7.3.2 to 7.3.5 contain the data from analyses of the individual lipid class FAME components from each culture grown under a different nitrate concentration regime. Each lipid class retained its characteristic pattern of fatty acid distribution, although these generally became distorted under the influence of nitrogen stress induced by the decrements in the nitrate supply. The general pattern of decreasing unsaturation and acyl chain length with decreasing medium nitrate concentration shown at the total FAME level was echoed at the individual lipid class FAME level. In the case of the culture grown in unmodified S88 there were generally higher levels of PUFA amongst the chloroplast lipids and in particular the neutral lipid class TAG. As the nitrate supply was decreased then the unsaturation of the TAG decreased. This was correlated with the sharp increase in the proportion of TAG observed during the HPTLC-densitometric analysis.

TABLE 7.3.2 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture using S88 medium modified to provide 1.0 mM NO_3^-.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	5.5	2.7	8.5	6.0	4.0	4.6	7.4	2.7
15:0	0.4	0.3	0.2	0.3	0.4	0.2	0.8	0.2
16:0	17.6	7.0	6.7	22.5	29.8	30.5	10.6	16.8
16:1(*n*-7)	26.1	16.2	8.8	22.7	24.3	40.0	23.3	24.6
16:1(*n*-13)t	.	.	.	.	3.8	.	.	.
16:2	0.3	0.3	.	0.5	0.2	.	0.6	0.2
17:0	0.6	0.3	0.2	.	.	.	.	.
16:3	0.4	0.5	0.2	.	.	.	1.4	0.7
16:4	.	.	.	.	.	.	.	.
18:0	1.1	1.4	0.5	0.2	0.4	0.8	0.8	0.5
18:1(*n*-9)	3.3	2.8	0.9	0.6	1.6	4.9	11.8	3.7
18:1(*n*-7)	.	.	0.1	0.2	0.3	0.1	.	.
18:2(*n*-6)	1.3	3.0	1.3	1.8	1.3	1.5	12.7	3.8
18:3(*n*-6)	.	.	.	.	0.1	.	0.9	0.2
18:3(*n*-3)	.	.	.	.	0.3	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.4	0.2	.	.	0.2	.	0.4	0.5
20:4(*n*-6)	4.8	6.7	1.1	1.0	5.4	6.6	7.7	5.0
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	34.6	51.3	68.9	40.8	24.8	6.3	12.2	35.4
Unidentified :	3.6	7.3	2.6	3.4	3.1	4.5	9.4	5.7
Total sats :	25.2	11.7	16.1	29.0	34.6	36.1	19.6	20.2
Total monos :	29.4	19.0	9.8	23.5	30.0	45.0	35.1	28.3
Total PUFA :	41.8	62.0	71.5	44.1	32.3	14.4	35.9	45.8
Total (*n*-9) :	3.3	2.8	0.9	0.6	1.6	4.9	11.8	3.7
Total (*n*-6) :	6.5	9.9	2.4	2.8	7.0	8.1	21.7	9.5
Total (*n*-3) :	34.6	51.3	68.9	40.8	25.1	6.3	12.2	35.4
(*n*-3)/(*n*-6) :	5.3	5.2	28.7	14.6	3.6	0.8	0.6	3.7
16:1/16:0 :	1.5	2.3	1.3	1.0	0.8	1.3	2.2	1.5
20:5/16:0 :	2.0	7.3	10.3	1.8	0.8	0.2	1.1	2.1
ΣC_{16}:	44.4	24.0	15.7	45.7	57.9	70.5	35.9	42.3
ΣC_{18}:	5.7	7.2	2.8	2.8	4.0	7.3	26.2	8.2
ΣC_{20}:	39.8	58.2	70.0	41.8	30.4	12.9	20.3	40.9

Abbreviations & Symbols
tr.,<0.1%, ., not detected, ‡, including SQDG

TABLE 7.3.3 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture using S88 medium modified to provide 0.1 mM NO_3^-.

	LIPID CLASSES							
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG‡	PS/PI	PC	TOTAL
14:0	5.0	2.9	8.0	6.0	2.9	3.2	0.9	4.0
15:0	0.4	0.3	.	.	.	0.4	.	0.2
16:0	37.4	22.7	6.6	22.7	27.0	28.7	16.4	31.5
16:1(*n*-7)	29.3	22.1	3.3	13.4	18.2	23.3	12.1	24.7
16:1(*n*-13)t	.	.	.	.	3.7	.	.	.
16:2	1.1	.	.	.	.	.	.	.
17:0	.	.	.	.	.	.	0.3	.
16:3	1.0	.	.	.	.	.	.	0.4
16:4	.	.	.	.	.	.	.	.
18:0	1.7	2.8	1.4	1.1	0.9	2.3	1.1	1.5
18:1(*n*-9)	6.4	4.0	0.4	0.5	1.5	6.8	12.1	5.8
18:1(*n*-7)	.	0.6	.	.	0.4	.	0.3	.
18:2(*n*-6)	1.7	1.6	0.3	0.6	0.7	1.2	12.4	2.5
18:3(*n*-6)	.	.	.	.	0.2	.	2.6	0.6
18:3(*n*-3)	.	.	.	.	.	.	0.4	.
18:4(*n*-3)	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.
20:3(*n*-6)	2.0	1.5	0.2	.	0.7	0.4	1.6	1.6
20:4(*n*-6)	2.1	6.8	1.6	0.7	6.1	6.2	11.0	2.9
20:3(*n*-3)	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.
20:5(*n*-3)	8.9	31.5	75.5	54.5	34.9	23.2	24.3	18.7
Unidentified :	3.0	3.2	2.7	0.5	2.8	4.3	4.5	5.6
Total sats :	44.5	28.7	16.0	29.8	30.8	34.6	18.7	37.2
Total monos :	35.7	26.7	3.7	13.9	23.8	30.1	24.5	30.5
Total PUFA :	16.8	41.4	77.6	55.8	42.6	31.0	52.3	26.7
Total (*n*-9) :	6.4	4.0	0.4	0.5	1.5	6.8	12.1	5.8
Total (*n*-6) :	5.8	8.6	2.1	1.3	7.7	7.8	28.0	7.6
Total (*n*-3) :	8.9	31.5	75.5	54.5	34.9	23.2	24.3	18.7
(*n*-3)/(*n*-6) :	1.5	3.7	36.0	41.9	4.5	3.0	0.9	2.5
16:1/16:0 :	0.8	1.0	0.5	0.6	0.7	0.8	0.7	0.8
20:5/16:0 :	0.2	1.4	11.4	2.4	1.3	0.8	1.5	0.6
ΣC_{16}:	68.8	44.8	9.9	36.1	44.1	52.0	28.5	56.6
ΣC_{18}:	9.8	9.0	2.1	2.2	3.7	10.3	28.9	10.4
ΣC_{20}:	13.0	39.8	77.3	55.2	41.7	29.8	36.9	23.2

Abbreviations & Symbols
tr.,<0.1%, -, not detected, ‡, including SQDG.

TABLE 7.3.4 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture using S88 medium modified to provide 0.01 mM NO_3^-.

	LIPID CLASSES								
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG	SQDG	PS/PI	PC	TOTAL
14:0	4.3	3.6	7.4	3.1	2.3	3.4	3.5	1.4	2.8
15:0	0.3	0.5	.	.	0.3	0.4	0.5	0.2	0.2
16:0	38.0	34.9	14.2	26.3	30.9	20.6	32.2	18.1	35.0
16:1(*n*-7)	30.1	29.7	10.0	14.1	25.1	9.3	26.9	13.8	26.1
16:1(*n*-13)t	.	.	.	.	3.2	.	.	.	.
16:2	1.0	.	.	.	.	.	.	.	.
17:0	.	.	.	0.9	.	.	.	.	.
16:3	0.5	.	.	.	.	.	.	.	.
16:4	.	.	.	.	.	.	.	.	.
18:0	1.2	3.6	2.3	3.1	2.5	2.4	4.7	1.1	1.2
18:1(*n*-9)	10.3	7.3	3.0	3.9	2.7	3.2	11.2	19.1	14.2
18:1(*n*-7)	.	1.0	0.5	1.1	0.4	0.5	.	0.4	.
18:2(*n*-6)	1.8	1.4	0.5	0.7	1.0	0.7	1.3	16.2	3.3
18:3(*n*-6)	0.3	.	.	.	.	.	.	2.7	0.4
18:3(*n*-3)	.	.	.	.	.	.	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	1.2	1.6	.	.	1.1	0.3	.	0.6	0.8
20:4(*n*-6)	1.6	2.4	1.9	0.6	9.7	4.5	7.1	8.0	1.7
20:3(*n*-3)	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	7.6	12.3	55.5	46.3	18.4	53.0	9.8	15.0	10.9
Unidentified :	1.8	1.7	4.7	.	2.4	1.7	2.8	3.4	3.4
Total sats :	43.8	42.6	23.9	33.4	36.0	26.8	40.9	20.8	39.2
Total monos :	40.4	38.0	13.5	19.0	31.4	13.0	38.1	33.3	40.3
Total PUFA :	14.0	17.7	57.9	47.6	30.2	58.5	18.2	42.5	17.1
Total (*n*-9) :	10.3	7.3	3.0	3.9	2.7	3.2	11.2	19.1	14.2
Total (*n*-6) :	7.6	5.4	2.4	1.3	11.8	5.5	8.4	27.5	6.2
Total (*n*-3) :	7.6	12.3	55.5	46.3	18.4	53.0	9.8	15.0	10.9
(*n*-3)/(*n*-6) :	1.0	2.3	23.1	35.6	1.6	9.6	1.2	0.5	1.8
16:1/16:0 :	0.8	0.9	0.7	0.5	0.8	0.5	0.8	0.8	0.7
20:5/16:0 :	0.2	0.4	3.9	1.8	0.6	2.6	0.3	0.8	0.3
ΣC_{16}:	69.6	64.6	24.2	40.4	59.2	29.9	59.1	31.9	61.1
ΣC_{18}:	13.6	13.3	6.3	8.8	6.6	6.8	17.2	39.5	19.1
ΣC_{20}:	10.4	16.3	57.4	46.9	29.2	57.8	16.9	23.6	13.4

Abbreviations & Symbols
tr.,<0.1%, ., not detected.

TABLE 7.3.5 : Individual lipid class FAME composition of *N. oculata* grown in continuous culture using S88 medium modified to provide 0.001 mM NO_3^-.

	LIPID CLASSES								
FATTY ACID	TAG	FFA	MGDG	DGDG	PE/PG	SQDG	PS/PI	PC	TOTAL
14:0	4.4	2.6	6.3	2.7	1.5	3.0	3.0	0.7	3.3
15:0	0.3	0.4	0.5	.	.	.	.	.	0.2
16:0	35.0	35.0	16.2	26.7	31.2	18.8	32.5	17.2	35.8
16:1(*n*-7)	31.4	31.8	12.7	13.7	25.6	8.1	23.5	10.3	26.2
16:1(*n*-13)*t*	.	.	.	.	2.0	.	.	.	.
16:2	0.8	.	.	.	.	.	.	.	.
17:0	.	.	1.6	.	.	1.3	.	.	.
16:3	0.5	.	.	.	.	.	.	.	0.2
16:4	.	.	.	.	.	.	.	.	.
18:0	1.0	2.9	2.6	3.9	3.7	4.3	7.4	2.2	1.5
18:1(*n*-9)	13.1	9.8	2.3	1.1	3.9	1.9	11.9	25.1	11.1
18:1(*n*-7)	.	.	4.6	.	0.4	3.0	.	.	.
18:2(*n*-6)	1.7	2.0	0.7	0.6	1.3	0.7	1.2	18.0	2.4
18:3(*n*-6)	0.2	.	.	.	.	.	.	1.8	0.5
18:3(*n*-3)	.	.	2.6	.	.	2.3	.	.	.
18:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:0	.	.	.	.	.	.	.	.	.
20:2	.	.	.	.	.	.	.	.	.
20:3(*n*-6)	0.7	0.3	1.4	.	1.2	.	.	0.4	1.1
20:4(*n*-6)	1.1	2.2	2.4	0.7	11.1	6.0	5.4	7.7	2.2
20:3(*n*-3)	.	.	.	.	.	.	.	.	.
20:4(*n*-3)	.	.	.	.	.	.	.	.	.
20:5(*n*-3)	7.0	13.6	36.7	50.5	17.5	44.2	13.8	15.1	11.1
Unidentified :	2.8	0.4	9.4	0.1	0.6	6.4	1.3	1.5	4.4
Total sats :	40.7	40.9	27.2	33.3	36.4	27.4	42.9	20.1	40.8
Total monos :	44.5	40.6	19.6	14.8	31.9	40.0	35.4	35.4	37.3
Total PUFA :	12.0	18.1	43.8	51.8	31.1	53.2	20.4	43.0	17.5
Total (*n*-9) :	13.1	9.8	2.3	1.1	3.9	1.9	11.9	25.1	11.1
Total (*n*-6) :	3.7	4.5	4.5	1.3	13.6	6.7	6.6	27.9	6.2
Total (*n*-3) :	7.0	13.6	39.3	50.5	17.5	46.5	13.8	15.1	11.1
(*n*-3)/(*n*-6) :	1.9	3.0	8.7	38.8	1.3	6.9	2.1	0.5	1.8
16:1/16:0 :	0.9	0.9	0.8	0.5	1.2	0.4	0.7	0.6	0.7
20:5/16:0 :	0.2	0.4	2.3	1.9	0.6	2.4	0.4	0.9	0.3
ΣC_{16}:	67.7	66.8	28.9	40.4	58.8	26.9	56.0	27.5	62.2
ΣC_{18}:	16.0	14.7	12.8	5.6	9.3	12.2	20.5	47.1	15.5
ΣC_{20}:	8.8	16.1	40.5	51.2	29.8	50.2	19.2	23.2	14.3

Abbreviations & Symbols
tr.,<0.1%, ., not detected.

The variation with medium nitrate concentration observed in the TAG component in both quantitative and qualitative terms was fairly predictable. Analysis of the FAME composition of this fraction showed that it echoed the changes which were observed during the analysis of the total FAME. The general trend was one of decreasing chain length and polyunsaturation whilst both monounsaturated and saturated fatty acids increased in proportion. At the highest nitrate concentration examined, there were high proportions of 20:5(*n*-3) and 20:4(*n*-6), accounting for 34.6 % and 4.8 % of the FAME mass respectively, the total proportion of C_{20} fatty acids being 39.8 %. The C_{16} acyl chain lengths accounted for 44.4 % of the FAME in total, this being divided between 16:0 (17.6 %) and 16:1 (26.1 %). The C_{18} chain length fatty acids gave only a minor contribution to the FAME mass at 5.7 %. This was divided between 18:0 (1.1 %), 18:1 (3.3 %) and 18:2 (1.3 %). When the nitrate concentration was reduced to 0.1 mM NO_3^- the TAG fraction showed a marked change in its FAME profile. The proportion of PUFA, which was essentially accounted for by 20:5(*n*-3) at 1.0 mM NO_3^-, was drastically reduced from 41.8 % at 1.0 mM NO_3^- to 16.8 % at 0.1 mM NO_3^-. The counter trend in the proportions of C_{16} acyl chain length acids resulted in an accumulation of 16:0 (from 17.6 % to 37.4 %) and therefore a concomitant increase in the proportion of saturates. The proportion of 16:1 increased slightly from 26.1 % to 29.3 %. This increase, combined with an increase in the proportion of 18:1 resulted in a slight increase in the proportions of monounsaturates from 29.4 % to 35.7 % and C_{18} chain lengths from 5.7 % to 8.9 %. Over the remaining decreases in culture medium nitrate there was little further change in the proportions of the C_{16} fatty acids. This is not to say that no further changes in the acyl profile of this lipid class occurred.

With the remaining two decrements in the medium nitrate concentration the changes involved the accumulation of 18:1. This fatty acid changed from 6.4 % at 0.1 mM NO_3^- to 13.1 % at 0.001 mM NO_3^-. The change was also observed as an increase in the total proportion of C_{18} fatty acids from 9.8 % to 16.0 %. Over the same concentration range the proportion of C_{20} fatty acids decreased from 10.4 % to 8.8 % whilst that of the C_{16} chain length fatty acids decreased from 69.6 % to 67.7 % to accommodate the increase in the C_{18}

chain lengths. A similar pattern of change was observed in the FFA fraction although the accumulation of both 16:0 and 16:1, and the decrease in proportion of C_{20}, PUFA were more marked.

The polar classes showed similar trends of decreasing proportions of PUFA with decreasing medium nitrate concentration. There was however a point of interest in the variation of the FAME composition of the polar lipid classes in the nitrate concentration window 1.0 mM NO_3^- to 0.1 mM NO_3^-. By comparing the data for the MGDG and DGDG fractions from these two cultures (Tables 7.3.2 and 7.3.3) an increase in the proportion of PUFA is observed with a *decrease* in nitrate concentration. Similarly for the phospholipid class PC, the total proportion of PUFA increased dramatically from 35.9 % to 52.3 % over this concentration range. In the case of PC the increased proportion of PUFA was again due to an increased proportion of the C_{20} chain lengths at the expense of C_{16} chains, with the C_{18} acids increasing only slightly in proportion. This anomalous finding is linked to the FAME composition of the neutral lipid classes which, as previously detailed, decreased sharply in their proportion of PUFA over the same nitrate concentration range. In previous cultures such a change had never been observed, since the neutral lipid classes had always followed the same trend as the polar classes. When the proportions of PUFA were high in the polar classes then they were also relatively high in the neutral classes. Therefore the implication was that this anomaly must have been a consequence of the changes occurring in the partitioning of fatty acids between the polar and neutral lipid classes in this particular nitrate concentration window. Figure 7.3.1 shows clearly that the cells were accumulating TAG to a far greater extent at 0.1 mM NO_3^- than they were at 1.0 mM NO_3^-. The changes observed in the FAME composition of TAG and FFA fit the previous data with respect to decreased proportions of PUFA and decreased acyl chain length due to the partitioning of *de novo* acyl biosynthesis into TAG biosynthesis. Whilst the polar lipid classes of the 0.1 mM NO_3^- culture were increasing their proportions of C_{20} PUFA, compared to the culture grown at 1.0 mM NO_3^-, its total FAME composition showed an overall decrease in its proportion of PUFA. This demonstrates the importance of TAG accumulation in determining

the total FAME composition of the algal cells. The hypothetical reasons for this phenomenon will be discussed in the light of the data from previous sections in the concluding general discussion section of this thesis.

At nitrate concentrations of 0.01 mM NO_3^- and below, when the cellular proportion of TAG had attained its maximum value, the FAME composition of the polar lipid classes had obviously become affected (Tables 7.3.4 & 7.3.5). At 0.01 mM NO_3^- the FAME composition of the galactolipid classes MGDG and DGDG, MGDG in particular, had become much less unsaturated. The proportion of C_{20} fatty acids in MGDG had decreased from 77.3 % at 0.1 mM NO_3^- to 57.4 % at 0.01 mM NO_3^-. This decrease was countered by increased proportions of both C_{18} chain length fatty acids (from 2.1 % to 6.3 %) and C_{16} chain length fatty acids (from 9.9 % to 24.2 %). As would be expected from the previous statement, the proportions of saturated and monounsaturated fatty acids also increased. The next decrement in nitrate concentration, from 0.01 mM NO_3^- to 0.001 mM NO_3^-, resulted in the same trend in MGDG which showed a decrease in its proportion of 20:5(*n*-3) from 55.5 % at 0.01 mM NO_3^- to 36.7 % at 0.001 mM NO_3^-. The consequent changes in the remaining FAME were further increases in the proportions of C_{16} and C_{18} saturates and mono-unsaturates. The proportions of the total C_{18} fatty acyl chain lengths increased from 6.3 % to 12.8 % whilst that of the C_{16} chain length FAME increased from 24.2 % to 28.9 %. DGDG did not follow its previous trend, instead, its proportions of C_{20} PUFA increased whilst those of the C_{18} chain lengths declined and those of the C_{16} chains remained the same. This may have been due to natural inter-culture variation especially since in previous cultures DGDG was observed to have an unusually stable FAME profile. If this was not the case then the variation in DGDG was at an extremely subtle level. The observed compositional stability of DGDG may have reflected a low turnover rate. This should have become more obvious as the cultures division rates decreased and the net biosynthesis rates of the polar lipid classes decreased accordingly. Interestingly, this appeared to be the case - and will be discussed in context with the previous experiments in the concluding discussion section of this thesis.

The major phospholipid class PC demonstrated a relatively straightforward pattern of change compared with those of previous cultures. In the nitrate range 0.1 mM NO_3^- to 0.001 mM NO_3^- the PUFA content of the FAME decreased from 52.3 % to 43.0 %. Over the same nitrate concentration range the proportion of saturated fatty acids increased slightly from 18.7 % to 20.1 % . The major change to account for the drop in the proportion of PUFA was, therefore, an increase in the proportion of monounsaturated fatty acids from 24.5 % to 35.4 %. This increase was due to accumulation of 18:1 (from 12.1 % to 25.1 %), the proportion of the other major monounsaturated fatty acid 16:1 decreasing concurrently from 12.1 % to 10.3 %.

The decrease in the proportion of PUFA in the PC component was not accounted for by the simple decrease in the proportions of all PUFA. Instead there was a restructuring of the FAME profile of the class. The proportion of C_{20} FAME, both 20:5(*n*-3) and 20:4(*n*-6), decreased with decreasing nitrate concentration, from 36.9 % to 23.2 %. The proportion of 20:5(*n*-3) decreased from 24.3 % to 15.1 % and that of 20:4(*n*-6) decreased from 11.0 % to 7.7 %. To some extent this was offset by an increase in the proportion of 18:2 from 12.4 % to 18.0 %. However, the minor proportions of other C_{18} PUFA, such as 18:3(*n*-3), declined slightly and reduced the impact of the increase in the proportion of 18:2(*n*-6). Overall the proportion of C18 FAME increased from 28.9 % to 47.1 % with the decrease in nitrate concentration from 0.1 mM NO_3^- to 0.001 mM NO_3^-. The variation in the proportion of C_{16} FAME played a much reduced role in the overall pattern of variation. The total proportion of the C_{16} chain length FAME decreased from 28.5 % to 27.5 %. The proportions of the two major fatty acids 16:0 and 16:1 changed slightly in this nitrate concentration window as was demonstrated by the change in the 16:1/16:0 ratio from 0.7 to 0.6. the proportion of 16:0 increased from 16.4 % to 17.2 % whilst that of 16:1 decreased from 12.1 % to 10.3 %.

Amongst the remaining phospholipid fractions, PE/PG and PS/PI, the pattern was again very similar to that described for PC. It was difficult to judge the overall pattern

because of the poor resolution of the sulpholipid SQDG from the PE/PG fraction for some of the samples. It appeared that, in the nitrate concentration range 0.01 mM NO_3^- to 0.001 mM NO_3^- little variation in the FAME profile of the PE/PG fraction occurred. The SQDG fraction showed slight variation following the general pattern displayed by PC. On the whole, these lipid classes seemed to be remarkably conservative in their FAME composition when compared to TAG, FFA, MGDG, and PC. This suggested that the latter four classes were the major players in the patterns of variation observed in the FAME profile of *N. oculata.*

SECTION 8: THE POTENTIAL FOR VARIATION OF POLAR LIPID CLASS MOLECULAR SPECIES COMPOSITION WITH CHANGES IN ENVIRONMENTAL CONDITIONS.

8.1 INTRODUCTION

Because the fatty acid composition of the lipid extracted from *N. oculata* was so unusual with respect to the almost complete absence of C_{18} fatty acids from MGDG and DGDG, the logical progression for further analysis was to investigate their molecular species composition. A cursory analysis of the galactolipid classes MGDG and DGDG was conducted, along with a similar analysis for the phospholipid class PC. Although the work included in this section did not form a major part of the project, analysis of the molecular species of the major polar lipid classes of *N. oculata* did however introduce some interesting and unusual information, the implications of which are relevant to the overall perspective of the present thesis.

Several recently published analytical methodologies were combined to give an analytical protocol for the analysis of the molecular species of diglycerides liberated from microalgal phospholipids and galactolipids (Heinze *et al.*, 1984; Takamura *et al.*, 1986; Bell, 1989). The methods applied here differ from those published previously (Norman & St. John, 1986) because, in combination, they enable direct separation *and* quantification of the molecular species. Previous techniques relied upon either the absorption of U.V. light of wavelength 205 nm by the double bonds of the PUFA or detection of the intact lipid by FID. Due to the variablity of the fatty acyl unsaturation, direct quantification of complex lipid class molecular species using this technique is not practicable. The techniques used here substitute the head-group at the *sn*3 position of the glycerol backbone of the complex lipid class with a U.V. absorbing chromophore (dinitrobenzoyl), detection using U.V. absorbance at 254 nm is thus made possible. Since the ratio of DAG to chromophore is 1:1, and the fatty acyl moieties do not absorb significantly at this wavelength, its relative quantity is directly proportional to U.V. absorbance and may therefore be quantified using peak areas calculated by the recording integrator. Accurate quantification using the U.V. spectrophotometer, a

relatively standard analytical tool, is made possible.

The following sub-section, although brief, presents some of the data gathered during a pilot study to assess the feasibility of the analysis of the complex lipid class molecular species of *N. oculata.* Various cultures of *N. oculata*, grown in batches of S88 medium under otherwise undefined culture conditions, were analysed with respect to the molecular species of their MGDG, DGDG and PC. The DNB-DAG derivatives were prepared and analysed using HPLC - U.V. detection in accordance with the protocols described in Section 3.

8.2 RESULTS & DISCUSSION.

The data in Tables 8.2.1, 8.2.2 and 8.2.3 show the proximate *major* molecular species composition of MGDG, DGDG and PC from several cultures of *N. oculata*. These tables illustrate the type of variation observed between cultures and between individual lipid classes. Such differences may have significant implications for the formulation of pathways for fatty acyl elongation and polydesaturation. As discussed in Section 1, algae differ from higher plants in that the long chain PUFA of their phospholipid and galactolipid classes occur predominantly at the *sn*-1 position of the glycerol backbone. Positional specificity of desaturation and elongation reactions appeared, potentially, to be central to the pattern of changes in the molecular species of galactolipids of *N. oculata.*

Due to the time limitations upon the duration of the project it was not possible to fully test this theory as a part of the current thesis. It is with this in mind that the author suggests that such investigations should be made a priority in any future studies of this type. The following discussion section (Section 9) contains speculation regarding the linkage between the eukaryotic and prokaryotic pathways of PUFA biosynthesis and interactions of MGDG and PC and MGDG and DGDG.

The data from GLC analysis of the galactolipid classes show that MGDG and DGDG differ in their proportions of PUFA, MGDG having a higher proportion of 20:5(*n*-

Table 8.2.1 : The molecular species composition of MGDG from *N. oculata* batch cultured under various, but undefined conditions.

Molecular Species	Culture Reference Number 1	2	3
20:5/20:5	63.1	43.7	61.8
20:5/20:4	.	2.2	6.8
20:5/18:2	0.6	.	3.3
14:0/20:5	11.5	13.9	7.8
16:1/20:5	4.9	4.2	0.7
20:4/14:0	1.8	.	.
16:1/20:4	.	.	1.4
16:1/14:0	0.2	1.7	0.2
16:1/16:1	1.0	0.3	0.3
16:0/20:5	10.3	30.1	8.1
18:1/20:5	.	.	1.2
16:1/16:0 & 18:1/14:0	0.1	2.3	0.6

., not detected.

Data expressed in terms of % mass of individual lipid class molecular species normalised to 100.0 %.

No implication as to the *sn* position of the fatty acids is made by their ordering in the molecular species pair.

Table 8.2.2 : The molecular species composition of DGDG from *N. oculata* batch cultured under various, but undefined conditions.

Molecular Species	Culture Reference Number 1	2	3
20:5/20:5	5.1	2.7	10.0
20:5/20:4	.	2.2	.
20:5/18:2	.	.	.
14:0/20:5	9.6	5.8	9.1
16:1/20:5	29.1	21.2	24.0
20:4/14:0	1.8	.	.
16:1/20:4	.	.	1.4
16:1/14:0	5.2	1.3	.
16:1/16:1	6.5	4.0	1.9
16:0/20:5	36.8	59.7	35.6
18:1/20:5	.	.	.
16:1/16:0 & 18:1/14:0	4.6	.	0.6

., not detected.

Data expressed in terms of % mass of individual lipid class molecular species normalised to 100.0 %.

No implication as to the *sn* position of the fatty acids is made by their ordering in the molecular species pair.

3). Molecular species analysis showed that MGDG had a greater proportion of the di-PUFA species 20:5/20:5, whereas DGDG possessed higher proportions of the two species 16:0/20:5 and 16:1/20:5. For samples 1 and 3, greater than 60 % of the mass of molecular species of MGDG was accounted for 20:5/20:5, on the other hand, the combined species 16:0/20:5 and 16:1/20:5 only accounted for *ca.* 15 % of the total mass. However, in the case of sample 2, the proportion of 20:5/20:5 was reduced to 44 % whilst the proportion of 16:0/20:5 had increased to *ca.* 30 %. One might surmise that the MGDG of sample 2 had a much lower proportion of 20:5 in its FAME. In fact this was not the case, the proportion of 20:5 in sample 1 was 77 % whilst that in sample 2 was 73 % (results not shown). The proportion of 16:0 was higher in sample 2 (at 14 %) than in sample 1 (at 5 %). Because molecular species are associated as pairs of acyl moieties, a reduction in a di-PUFA species, such as di-20:5, is proportionally equivalent to the same reduction in the FAME composition of these acyl moieties. In contrast, an increase in the proportion of 16:0/20:5 would need to be double the decrease in di-20:5 to have a compensatory effect upon the total FAME composition. The conclusion from this was that, not only was there the potential for variation in the proportion of the fatty acyl moieties at the FAME level, as elucidated for *N. oculata* in the previous sections of this thesis, but that the positional distribution of the fatty acids may have as dramatic an effect upon the properties of biomembranes as changes in the quantitative and qualitative distribution of the acyl moieties. The lack of an observable effect of culture incubation temperature upon the fatty acyl composition of the membrane lipid classes of *N. oculata* may have been a consequence of a more subtle change at the molecular species level.

The reciprocal relationship of 16:0 and 20:5 was preserved at the molecular species level indicating a potential biosynthetic relationship between 16:0/20:5 and 20:5/20:5 specifically associated with MGDG. The biosynthetically closely related galactolipid class DGDG, thought to be formed by the direct galactosylation of MGDG (see Section 1), has a quite different molecular species composition. In DGDG the major PUFA containing molecular species are 16:0/20:5 and 16:1/20:5 accounting for up to 80 % of the total molecular species mass whilst 20:5/20:5 accounts for as little as 3 % of the total mass. This

Table 8.2.3 : The molecular species composition of PC from *N. oculata* batch cultured under various, but undefined conditions.

Molecular Species	Culture Reference Number 1	2	3
20:5/20:5	0.5	0.5	0.3
20:4/20:5	.	.	.
18:2/20:5	.	.	.
14:0/20:5	.	.	.
16:1/20:5	10.4	3.8	3.0
14:0/20:4	.	.	.
16:1/20:4	.	.	.
14:0/16:1	3.0	2.0	1.9
16:1/16:1	14.6	1.5	1.5
16:0/20:5	29.7	22.0	25.5
18:1/20:5	5.1	6.7	3.5
16:1/18:1	19.8	31.0	21.7
16:0/18:1	3.2	17.5	11.8

., not detected.

Data expressed in terms of % mass of individual lipid class molecular species normalised to 100.0 %.

No implication as to the *sn* position of the fatty acids is made by their ordering in the molecular species pair.

suggested that the elongation and / or desaturation reactions, *via* which the C_{16}/C_{20} molecular species were thought to be synthesised, were specific to the chloroplast and therefore a part of the 'prokaryotic' pathway of lipid biosynthesis.

Analysis of the positional specificity of the C_{20} moieties in the galactolipid classes would serve to test such a theory. Such analyses are readily conducted and have been carried out for several microalgal species (Safford & Nichols, 1970; Arao *et al.*, 1987;Giroud *et al.*, 1988; Arao & Yamada, 1989; Greca *et al.*, 1989). These studies have shown that the longer chain fatty acyl moiety of the pair is preferentially located at the *sn*-1 position of the glycerol backbone of the galactolipid molecular species.

Phospholipid classes, other than PG which is chloroplast associated, reportedly differ from the glycolipids in that they have the longer chain fatty acid at the *sn*-2 position of their glycerol backbone (Harwood *et al.*, 1988). This type of distribution of the fatty acyl moieties in typically of the 'eukaryotic' pattern. It has been shown, for a species of *Dunaliella* that, ^{14}C-palmitate was incorporated at the *sn*-2 position by chloroplasts, and in the *sn*-1 position by microsomal ER (Norman & Thompson, 1985).

In the light of the previous information, the fact that the MGDG of *N. oculata* has a molecular species composition displaying characteristics of both prokaryotic and eukaryotic pathways suggests that the component acyl moieties may be derived from *both* these pathways. This is not exactly a new idea, however, the dynamics of the molecular species composition observed under different culture conditions points to a novel mechanism accounting for their patterns of change. The switch between the accumulation of di-20:5 (characteristically 'eukaryotic'; thought to be derived *via* cytosolic pathways) and 16:0/20:5 (with the C_{20} acyl moiety at the *sn*-1 position is characteristically 'prokaryotic') suggests that, under optimal cell division conditions, exchange of DAG between cellular compartments may occur. The result is the accumulation of di-20:5 due to complementary desaturation of the acyl moieties at the *sn*-1 position in the chloroplast and at the *sn*-2 position in the cytosol. Under poor conditions, the exchange may be prevented resulting in a

glycolipid molecular species distribution more reminiscent of the cyanobacteria (*cf.* Section 1) and a phospholipid molecular species profile more reminiscent of the eukaryotic pathway with C_{16} fatty acids at the *sn*-1 position and $C_{18/20}$ fatty acids at the *sn*-2 position. Such a scheme is tentatively proposed to account for the observed variations in the molecular species profiles presented here as well as questions raised by earlier authors (Appleby *et al.*, 1971) who concluded that the 'eukaryotic', or lecithin pathway, did not operate in *Anabaena* although it was able to incorporate ^{14}C-oleate and linoleate into its MGDG. Arao & Yamada (1989) reported that the galactolipids of *P. tricornutum* contained little C_{18} fatty acid, whereas PC possessed these acids concentrated at the *sn*-2 position *and* 20:5 at both positions. A schematic representation of the proposed relationship is shown in Figure 8.2.1 below.

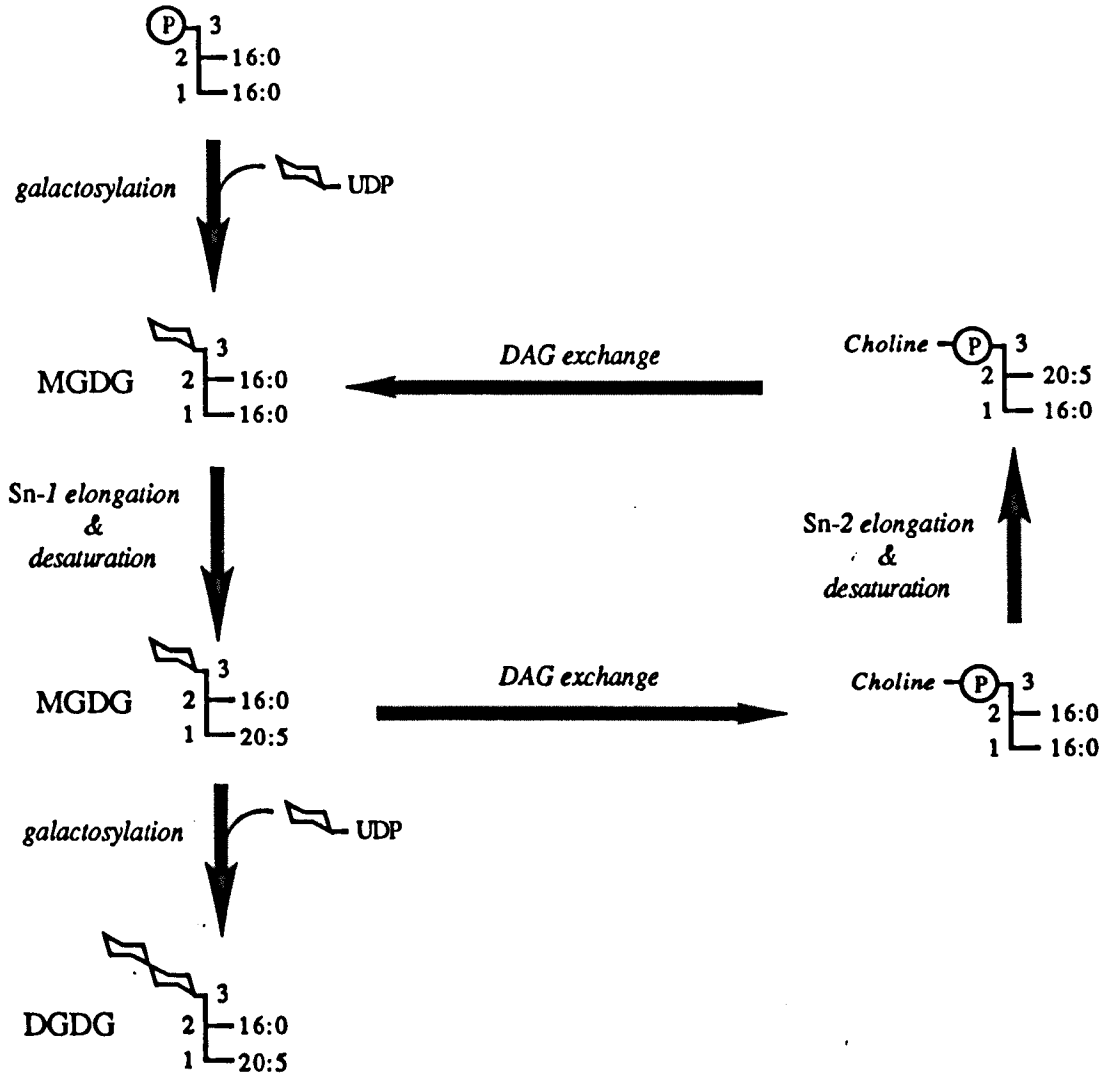


Figure 8.2.1 : Schematic relationship between the prokaryotic and eukaryotic pathways for fatty acyl elongation and desaturation in *N. oculata*[4]

SECTION 9: DISCUSSION, CONCLUSIONS & FUTURE PROSPECTS.

9.1 Introductory remarks.

In the 'Results and Discussion' sub-sections at the ends of the previous experimental sections the findings most relevant to an overview of the lipid metabolism of *Nannochloropsis oculata* were highlighted. The aim of the present section is to link these observations together to form an overall impression of the various effects that the interactions and interrelationships of the culture conditions had upon the lipid composition of the alga. It has been necessary to use generalised terms carried over from previous sections to avoid becoming bogged down in the numerical data.

Because of the lack of algal-related, supporting literature in this poorly investigated field, much of the data included in this thesis must stand alone. In part, the *lack* of good supporting data suggested that the root of the effect of environmental conditions upon individual polar lipid classes may be due to 1) changes in the total lipid levels of the cells *and* 2) the growth phase distribution of neutral and polar lipid classes in batch culture. The present thesis indicates that such effects originate from changes in the relative rates of polar and neutral lipid biosynthesis accompanying the changes in the growth phases of the cultures. This is a concept which many other workers appear to have shied away from, tending by omission to abstract several factors which are in fact closely inter-linked. The backgrounds of the researchers influence the emphasis placed upon the culturing of the algal cells. Those with a biochemical interest often ignore the the full implications of culture dynamics and treat the culturing simply as a means of obtaining samples for analysis, as demonstrated by the simplistic view of 'the growth phase' discussed by James *et al.*, (1989).

To set the initial background, a summary of some of the basic concepts regarding the effects of culture dynamics and the significant analytical observations will be presented. These will be developed progressively, through the subsequent sub-sections into the main conclusion of the present thesis this being that the *major*.changes in the lipid and fatty acyl composition of *N. oculata* stem from the changes which occur during the normal cell cycle.

9.2 Considerations linking culture dynamics with the overall lipid composition of algal cultures.

In the case of the batch culture experiments the over-riding influence upon the lipid class and fatty acid composition of the cells of *N. oculata* was that of their enclosed culturing environment. This is not to say that the compositions of cells grown in batch culture were not a true reflection of the culture conditions employed. The cells themselves responded consistently to a given set of culture conditions. Rather, it was the lack of *true* control over, and unpredictability of, the culture conditions that were the major problems when attempting to correlate 'cause and effect'.

One can consider the 'dynamics' of batch cultures in terms of two cases; 1) the case of the individual cell or 2) the culture as a whole. For the purposes of nutritional and ecological studies the latter form is probably the more valid of the two because in practical terms one is unable to influence a single cell, only the population as a whole. The logic behind the previous statement is based upon the fact that, in an asynchronous culture, there are cells representative of all stages of the cellular growth cycle (Lorenzen & Hesse, 1974). In other words, from newly divided cells through a spectrum of intermediate growth stages to cells about to undergo division. The intermediate stages have specific lipid compositions which represent the progression between lipid compositions of the cells immediately after, and immediately prior to, cell division.

The duration of the cell division event is relatively short. In a synchronised culture it would be expected to account for only 10 % of the generation time (Lorenzen & Hesse, 1974). The remaining time is taken up by the accumulation of biomass by the cells in preparation for division. Otsuka & Morimura (1966) reported the accumulation of TAG, DAG and MAG for *C. ellipsoidea* with a concomitant increase in cell volume during the period prior to cell division. The neutral lipid was consumed during the process of cell division which usually occurred in the dark period. Sicko-Goad *et al.* (1988) also suggested cyclical changes in the fatty acyl composition of the diatom species *Cyclotella meneghiniana.* Although reported in an ambiguous style their findings appear to agree with those of the

former authors with respect to lipid accumulation and consumption patterns during light / dark cycles.

The relative proportions of the cells in a culture at each stage of growth determine the lipid composition of the culture at any specific time-point. For example, at the end of the exponential growth phase all the cells do not stop dividing at the same time. There is a gradient during which the number of non-dividing cells increases progressively, until little or no cell division occurs. This phenomenon is reflected in the changing shapes of the cultures growth curves. The chemical composition of the samples removed during a time course also reflect this distribution change. Otsuka & Morimura (1966) pointed out that, in the absence of nitrogen, the cells of *C. ellipsoidea* were able to progress up to, but not beyond, the point in their division cycle immediately prior to the division event. This was possible due to the importance of the accumulation of carbon in this part of the growth cycle rather than the presence of nitrogen, although nitrogen seems to be required for division.

The previous point was well illustrated by the data included in Section 5. The lipid class and total FAME composition of the cultures changed following a pattern which was closely correlated to their growth characteristics. A scheme accounting for the gross level changes was proposed in Section 5.3 to account for the rapid short term accumulation of TAG. The scheme was based upon a repartitioning of the products of the fatty acyl biosynthetic pathway between the polar lipid classes and the neutral lipid classes which characterised the division and non-division phases of culture growth respectively. Data from the literature (see Sections 1 & 2) suggested that TAG were characterised by a highly saturated FAME composition. This suggested *a priori* that the increasing saturation of the total FAME at the end of the exponential-phase of cellular division was simply due to an accumulation of TAG which had been found to characterise the cells of *N. oculata* when their cell division rate declined at the end of a batch culture's lifespan. Relative to the polar lipid classes, the TAG of *N. oculata* were indeed more saturated. However, the FAME composition of the individual lipid classes was found to be quite variable. This was also true of TAG, which was found to have the widest range of fatty acyl composition of all the

individual lipid classes. The 20:5(*n*-3) content of *N. oculata*'s TAG component varied between *ca.* 7 % and 43 %. Generally this was inversely correlated with the proportion of TAG in the total lipid extract.

In early batch culture experiments (Section 5.3) the variations in the FAME profile of TAG were correlated to those of the polar lipid classes. Because several of the polar lipid classes have been implicated in the desaturation processes (Nichols *et al.*, 1966; Gurr *et al.*, 1969; Gurr & Brawn, 1970; Safford & Nichols,1970; Appleby *et al.*, 1971; Jones & Harwood, 1980; Lem & Williams, 1981; Ohnishi & Yamada, 1982; Norman & St. John, 1986; Sato *et al.*, 1986; Andrews & Heinz, 1987; Jaworski, 1987; Norman & St. John, 1987), it was assumed that their fatty acyl profiles consequently affected the general level of unsaturation within cells. However, the data yielded later in the course of the present thesis demonstrated that a relationship existed between the rate of fatty acyl biosynthesis and the rate of cellular division. This relationship, essentially that which was outlined in Figure 5.4.2, was found to be the single most important factor in determining the total FAME composition of *N.oculata.*

The biochemical pathways by which the acyl moieties incorporated into the TAG of *N. oculata* were derived are unknown (Yuen *et al.*, 1987; Roessler, 1988). The experimental data suggests that at least two pathways exist. One is a direct route from the FAS complex omitting the stages of elongation and desaturation, the other involves acyl exchange with the polar lipid classes, possibly *via* an unesterified fatty acyl intermediate. The latter pathway accounts for the C_{18} and C_{20} PUFA content of the TAG as detailed earlier (Sections 1 & 5).

If one were able to analyse the lipid composition of the individual cells in a sample one would find cells with lipid compositions resembling that of the culture as a whole in the exponential phase and those with a composition more representative of a stationary phase culture. The cells containing a higher proportion of their biomass as fatty acids influence the FAME composition of culture as a whole to an extent which is disproportionately high in comparison to their numbers. Similarly, during the lag-phase the short term change which

occurs in the growth characteristics of the cultures produces a major change in the lipid and FAME composition of the cultures. The gross lipid composition of a culture is thus greatly influenced by factors which precipitate a shift towards net lipid accumulation, even if this is in only a small proportion of the cells. For example, the case in hand: TAG accumulation is particularly effective in this type of situation because one mole of TAG contains three moles of fatty acid. In contrast one mole of the quantitatively important phospholipid or galactolipid classes contains only two moles of fatty acid. Thus, variations in the total FAME composition of *N. oculata* were highly influenced by the proportion of TAG relative to the polar lipid classes and by the fact that when TAG accumulated, its FAME composition became more saturated with shorter chain lengths.

In the situations described above the culture was not in steady-state. The relative proportions of cells at each stage of growth were in a state of continual flux. The pattern of flux complemented the changes occurring in the culture medium chemistry which were driven by the growth of the culture. The data presented in this thesis showed that non-dividing *N. oculata* cells still accumulated biomass, but in a different form to that accumulated during the division phase. The culture's biomass was increasing and so could still be said to be growing. Therefore it was not in the stationary-phase. Essentially, the major distinction between the two types of growth at the gross level is a transition between biomass dissemination amongst numerous, small daughter cells and biomass accumulation by fewer, but larger, individual cells.

The data in Section 5.3 showed that lipid accounted for an increasing proportion of the biomass of cells of *N. oculata* as individual cell mass increased (Figure 5.3.5). Further analysis showed that the increased lipid mass was due to the increasing proportion of TAG in the total lipid extract particularly during the stationary-phase (Figure 5.3.6). Similar data have also been recorded for natural populations (Morris *et al.*, 1985; Nichols *et al.*, *in press*)

The stationary-phase proper was only observed in the initial batch culture experiments. In this case the rate of biomass accumulation was not only greatly reduced but there was also evidence of repartitioning of the existing biomass. Similar phenomena have been discounted for other microalgal species (Suen *et al.*, 1987). The proportion of biomass accounted for by TAG increased, whereas the total biomass per unit of culture volume did not undergo any significant increase. Whether TAG would, in fact, begin to decline in the extreme stationary phase remains untested by the work included in the present thesis. The occurrence of the phases of growth in batch culture must therefore be regarded as much a function of the biomass accumulation rate as of the cell division rate. For the purposes of this thesis it is more convenient to redefine the classical phases of batch culture to avoid confusion later in this discussion. Because growth rate may be defined in terms of biomass and cell numbers, it was necessary to distinguish between the terminologies used.

Growth in the present context refers specifically to the accumulation of biomass regardless of changes in cell numbers. However, the phases of growth (*i.e.* the lag-, exponential- and stationary phases) although most commonly associated with cellular division, in practical terms describe the divisional and non-divisional types of biomass accumulation detailed in the two preceding paragraphs: with divisional biomass accumulation occurring in the 'exponential-phase' and non-divisional biomass accumulation occurring in the 'lag-' and 'stationary-phases'. The distinction is particularly important in the context of the present thesis because, as previously described, the lipid composition of the cells differs drastically depending upon which type of biomass accumulation mechanism is operating.

In the case of continuous culture there are by necessity still specific proportions of cells at various points in the cellular growth cycle. The *modus operandi* of this type of culture, in contrast to batch culture, is to hold those proportions constant throughout the culture period. This reduces the complexity of the deductions one has to make when sampling the culture and the relationship of cause to effect is clarified. This may be regarded as a standardising procedure analogous to the 'equivalence points' used during the series of batch cultures to investigate the effects of temperature upon the lipid composition of *N*.

oculata. Although the cause and effect relationship may have been clarified, the means *via* which the variation occurred were still obscured by the potential for variation in the growth phase distribution profile of the cultures. The ideal strategy for such experiments would be one which used synchronised, continuous cultures. In this way the potential changes in the growth phase distribution of the cells in the cultures would be truly eliminated by all cells being in the same growth phase.

9.3 Modification of the growth-event time-scale by culture incubation temperature.

The effect of culture temperature upon the lipid composition of *N. oculata* was due to the effect that it had upon the phases of the batch cultures used in its investigation. The experimental work was planned in such a way that, should this be the case, it would be easily detectable. Cross comparison of the compositional data with the growth data recorded over the incubation period enabled correlations to be made between the phases of growth and the lipid class and FAME compositions. These showed that the period of greatest variation between the compositions of the cultures incubated at different temperatures was during the lag-phase and *early* exponential-phase. The proportions of cells at each growth stage stabilised as the culture settled into a 'virtual' but short-lived steady-state. Once the cells had undergone a specific number of divisions, usually between four and five, the compositional data hinted at temperature specific changes in the proportions of saturated and mono-unsaturated fatty acids. However, their PUFA compositions stabilised at similar proportions. This was a recurrent phenomenon during the batch culture experiments which also occurred when salinity was the test variable. After five cell divisions the cultures were said to have reached their exponential-phase of growth. This phase was associated with the minimum observed total lipid yield per cell, the lowest proportion of TAG and the highest proportion of polyunsaturated FAME. This was significant in relation to the facts discussed in the previous sub-section. The fact that such a phenomenon exists serves to underline the necessity for a tighter definition of what constitutes the 'exponential-phase'. Prior to the virtual steady-state period the biochemical composition of the culture is still in flux although cell division shows an exponential pattern.

Throughout the literature, temperature specific changes in the FAME composition cell membrane lipids have been recorded and occasionally these observations have included both the micro and macro-algae (Patterson, 1969; Aaronson, 1973; Teshima *et al.*, 1983; Seto *et al.*, 1984; Kayama *et al.*, 1985; Cohen *et al.*, 1987; Rezanka *et al.*, 1987; Henderson & Mackinlay, 1989; James *et al.*, 1989; Kawabata & Kaneyama, 1989, Pettitt *et al.*, 1989). The general trend is one of increasing proportions of PUFA with decreasing temperature. The type of temperature effect observed in the present thesis could not be attributed to a misinterpretation of data by earlier workers due to the differences in the acyl composition of cultures at different points on their growth curves. For example, if a slow growing culture at 10 °C were to be compared with a faster growing culture at 20 °C, after a *fixed time interval* the growth of the 10 °C culture would have lagged behind that of the 20 °C culture. The 10 °C culture would still be in its 'lag-phase' when the 20 °C culture had reached its 'log-phase'. Because the 20 °C would have reached its maximum fatty acyl unsaturation, the 10 °C culture would have a relatively more saturated acyl composition. Because the cell division rate had not been taken into account during the course of the current work the apparent effect would have been one of decreasing proportions of PUFA with decreasing temperature. This was due to the extension of the lag-phase, during which predominantly saturated TAG was accumulated by the cells. However, James *et al.* (1989) working with a strain of *Nannochloropsis* different from that used in the present thesis recorded total FAME data which is consistent with the type of misinterpretation described above. Thus, *Nannochloropsis* species may be less susceptible to the effects of temperature variation than other species. By contrasting the analytical data (Section 6.2) from the early stages of the high temperature cultures with those from lower temperatures, the effect of the changes in the rate of progression through the lag-phase upon the lipid composition of each culture was evident. At high incubation temperature, and high growth rate, the lag phase was so short that it was poorly chronicled in the analytical data. It appeared as a sharp increase in the mass per cell. The evidence provided by analysis showed a rapid increase in the proportion of lipid per cell, of which a large proportion could be accounted for by TAG. The sharpness of this peak represented the rapidity of the changes as all the cells attained peak mass and

divided almost simultaneously. At lower incubation temperatures, and growth rates, the effect of the lag-phase was extended in time and so a shallower, bell-shaped distribution of mass was observed as the cells accumulated biomass and then resumed cellular division much more gradually. This obviously results from temperature-specific changes in the growth phase distribution of the population and accounts for the inter-culture variations observed during the early culture period.

9.4 Possible explanations for the effects of salinity variation.

Of all the effecters examined during the course of the present thesis, salinity is the most poorly documented by the published literature. Pugh (1971) using the diatom *Coscinodiscus eccentricus* showed an indistinct pattern of variation over the salinity range 20 ‰ to 35 ‰. The peak proportion of 20:5(*n*-3) was recorded in the late exponential-phase for a salinity of 25 ‰. At salinities higher and lower than this the proportion of 20:5 was several percent lower. In a more recent study (Lee *et al.*, 1989) *P. cruentum* was grown over a NaCl concentration range of 12 g l^{-1} to 87 g l^{-1}. These workers found that at the two extremes of salinity, higher proportions of the cellular biomass were present as fatty acids and that a greater proportion of these was PUFA. The effects were correlated with lower rates of cellular division, although the increase in PUFA yield was tenfold whilst the depression of cellular division rate was by a factor of three. Hence, these authors stated that the observed variation in acyl composition was not a growth rate effect. Cohen *et al.* (1988) also working with *P. cruetum* observed increased proportions of 20:5 and decreased proportions of 20:4 with decreasing salinity. These authors, in contrast to the authors of the more recent study (Lee *et al.*, (1989), reported this phenomenon to be growth rate dependent. The ratio of 20:4 / 20:5 being inversely proportional to the specific growth rate (μ). The findings of the present study are in agreement with those of Cohen *et al.* (1988) with respect to the trends in the proportions of 20:5. Cohen *et al.* (1988) also reported the accumulation of neutral lipid classes, evident from the increased proportion of the neutral lipid class marker fatty acid; 18:2, for the lower division rate cultures. However, in contrast to the findings of the present study, this resulted in a decreased yield of fatty acids as a percentage of the dry weight.

None of the authors cited above were able to propose a substantive reason for the effect of changing salinity upon acyl composition. Much of the data yielded by previous studies using temperature as the test variable had been discussed in terms of homeoviscous adaptation to maintain membrane fluidity. This led Lee *et al.* (1989) to discuss their findings in this light. However, salinity is not known to have any effect upon membrane viscosity. Substantial evidence exists to demonstrate that the means of osmoregulation of microalgal species involves the intracellular accumulation of glycerol (Evans & Kates, 1984; Ahmad & Hellebust, 1985). It seems equally probable that the changes in acyl composition are due to changes in the solubility of oxygen which is required as a proton acceptor in the fatty acyl desaturation reaction (see Figure 1.4.1). Theories have been proposed in relation to the temperature effect whereby increases in acyl unsaturation are often correlated with decreasing temperature. Similarly the effect of increasing salinity is to decrease the solubility of oxygen in water. The data yielded by the present study seems to fit such a pattern although in the absence of other data it would be rash to assume that this was the case.

An alternative, but rather confusing suggestion proposed by Lee *et al.* (1989) was that the increased fatty acyl content of the membrane bilayer found at lower salinities would act to impede the movement of water molecules across it. Considering that both influx and efflux of water may occur dependent upon the culture medium salinity, little would be gained by altering the membrane acyl composition in a salinity-dependent manner unless this was in the form of an asymmetric acyl distribution between the internal and external faces of the cell membrane. It is not the direction of the water flux that is important but that neither influx nor efflux should occur in an uncontrolled manner. Thus on this basis the ideal acyl composition for the membrane acting as a water barrier would be long chain (*ca.* $C_{20/22}$) fatty acids. The increased hydrocarbon chain length would increase the relative hydrophobicity of the membrane. This does not explain why the acyl moieties should be more unsaturated, unless this is simply a function of the ability of the cells to synthesise C_{20} PUFA.

Although the fatty acyl composition of the membrane lipid classes of *N. oculata* varied when the NaCl concentration of the culture medium was modified, it is difficult to

attribute the variation to a single cause. Decreasing unsaturation of TAG has been linked to increasing net synthesis of this lipid class due either to high carbon assimilation rates or low nitrogen assimilation rates. An effect of salinity upon the relative rates of cell division and carbon / nitrogen assimilation of two marine diatom species *Dityllum brightwellii* and *Sketetonema costatum* has been reported (Rijstenbil *et al.*, 1989). It seems unlikely that this is the case for *N. oculata* given the apparent high affinity of the genus for nitrate (see below). In the light of the present experimental data, if a salinity decrease depressed cell division to a greater extent than carbon fixation, one would expect a response similar to that observed during the lag-phase where TAG was accumulated rapidly and the proportion of PUFA decreased. Instead, as described above, the opposite was observed.

Changes in the salinity of the culture medium clearly do affect the processes of fatty acyl biosynthesis, elongation and desaturation. The acyl composition of the membrane phospholipid class PC is particularly affected. The mean chain length of PC was affected such that the proportion of C_{18} mono and di-unsaturated fatty acids increased with salinity at the expense of both C_{16} and C_{20} acids. These changes were echoed to a lesser extent in the other phospholipids and the galactolipids. This was probably due to the lesser proportion of C_{18} fatty acids in the other classes.

9.5 The effects of environmental factors modifying the available carbon / available nitrogen ratio.

The major effect of temperature upon the lipid composition of *N. oculata* was to modify the growth rate characteristics of the cultures. As discussed above, despite the apparent effect of temperature upon the lipid and fatty acyl composition of the cultures, its real effect was to complicate the inter-sample comparison process. The remaining three test factors were found to have more of an impact upon the culture's lipid composition. Unlike the effects of temperature, those of culture illumination intensity and medium nitrate concentration modified the biochemical composition of the culture rather than the rate of reactions. Ultimately it was not possible to determine whether or not these effects were due to changes in the proportions of cells at the various stages of growth in each respective

culture. However, the weight of the evidence was in favour of this being the case, because the quantitative changes in the polar lipid classes were far outweighed by those in TAG.

The variation in total FAME composition due to changes in both the illumination intensity and nitrate concentration of continuous cultures was for all intents and purposes determined by variations in the proportion of TAG. Changes were, however, observed as a result of the effects of the test factors upon the pathways of fatty acyl desaturation and elongation amongst the polar lipid classes. These were quantitatively far less significant in terms of the cultures as a whole. The reasons for this were consequences of the pathway *via* which the fatty acids incorporated into TAG were derived (as proposed in Section 5.3 these are independent of polar lipid class biosynthesis).

The cause of the effects observed in the cases of changing both culture illumination and the medium nitrate concentration originated from changes in the availabilities of carbon and nitrate to the cells. It is in this respect that the two factors are linked to the phases of growth observed during batch culture. The rationale for this summation is that, during batch culture, the medium nitrate became depleted as it was abstracted from solution by the cells and, as the cells divided, the intensity of illumination throughout the culture was reduced and therefore the carbon supply to the cells also began to decline. Because the two factors varied concurrently it was difficult to attribute specific variations to either one. The changing relationship between these two factors, the C/N ratio, is one of the major driving forces behind the changes occurring in the batch culture environment, the ultimate situation being when medium nitrate is fully depleted. Cellular division ceases but carbon, still available in solution *via* the equilibrium between the atmosphere and the culture medium, may possibly be incorporated into TAG for as long as carbon fixation continues. In continuous culture such phenomena were controlled by the culture apparatus. The observations made with respect to these two factors were somewhat paradoxical and had significant implications with regard to the expected partitioning of carbon by the cells in their natural environment. Two patterns of TAG accumulation were observed. At the higher illumination intensities TAG was accumulated in response to a near optimal environment, whereas at low nitrate

concentrations the same phenomenon occurred but because of poor environmental conditions. In both cases the accumulated TAG was highly saturated and monounsaturated. Although their total FAME profiles were similar, a distinction could be drawn between cells showing each type of TAG accumulation by examining their polar lipid class FAME profiles. Under near optimal growth conditions the polar lipid class FAME was highly unsaturated whereas at sub-optimal conditions there were higher proportions of saturates and monounsaturates. This observation provides further evidence that *de novo* TAG biosynthesis is indeed independent of polar lipid synthesis.

9.5.1 The effect of illumination intensity.

The assumption is often made that, whilst the cells are supplied with an adequate supply of nitrate and phosphate the culture will grow exponentially. During such growth it is assumed that all the fixed carbon is utilised in the biosynthesis of structural molecules; notably proteins and phospholipid classes (Sicko-Goad *et al.*, 1988). Storage compounds, such as TAG, are often assumed not to accumulate. The results of the present study show clearly that this is not the case. Whilst nitrate is readily available the cells may still accumulate carbon faster than they are able to utilise it in cell division. Because TAG accumulation was observed at the relatively low artificial illumination intensity of 65 $\mu E\ m^{-2}\ sec^{-1}$, it seems probable that such an effect should also be common in the natural marine environment, particularly the shallow pool environment from which *N. oculata* was isolated.

The overall effect of the accumulation of TAG was that the total FAME composition of the culture became increasingly saturated with increasing illumination intensity. This was contrary to the predictions based upon early batch culture data. By extrapolating the batch culture data it was predicted that the unsaturation of the total FAME would increase with increasing growth rate which would in turn increase as illumination intensity increased. The basis for this was the observed decline in the proportion of the TAG during the exponential growth phase of batch cultures. It was further predicted that the increased illumination intensity would induce increased biosynthesis of the highly polyunsaturated galactolipid classes. A similarly anomalous pattern, by comparison to much of the published data, was

observed for the diatom species *Nitzschia closteridium* by Orcutt & Patterson (1974) and for several other commonly cultured microalgal species by Thompson *et al.* (1990). The former authors reported that the total FAME profile of *N. closteridium* became increasingly saturated with increased illumination. This was accounted for by the accumulation of 16:0 and 16:1 in the TAG fraction which showed an increased proportion in the total lipid extract. In general the polar lipid class FAME had a higher PUFA content at high illumination intensity. The data for the individual polar lipid classes were not included. Thompson *et al.* (1990) correlated the changes in the proportion of 16:0 with the specific growth rate, μ. The proportions of other fatty acids, 16:1 and 20:5(*n*-3), were negatively correlated with μ. These authors concluded that the diatoms were able to adjust their proportions of 16:0 and 16:1 in response to illumination intensity. Yet again no analyses of the proportions of the various lipid classes were reported so it was not possible to conclude whether or not this could be accounted for by accumulation of TAG although this is what the authors implied.

Analysis of the individual lipid class FAME compositions of *N.oculata* showed that the galactolipids became more unsaturated with a higher proportion of 20:5(*n*-3) at higher illumination intensity. However, any increase in the quantity of the polar lipid classes was far outweighed by the increase in proportion of TAG. These findings support the previous theories regarding the partitioning of fatty acids between the neutral and polar lipid classes at a stage prior elongation and desaturation. The fatty acid synthesis rate is obviously increased by the increasing illumination intensity and, as a result, carbon is partitioned into TAG which acts as a sink for the excess.

The implications in terms of the current global warming problems would probably not be significant. If microalgal cells accumulating carbon as neutral lipid under growth limiting nutrient or silicate concentrations were subjected to grazing pressure by secondary producers, then the population would crash and carbon fixation would be reduced. It seems therefore that the effect of TAG accumulation observed under near optimal growth conditions would be more favourable in this respect. It is possible that these effects may be important in high latitude phytoplankton populations. During the austral spring and summer months

when daylight hours are at their optimum, the neutral lipid content of sea ice diatoms was, contrary to expectation, found to be increased during a bloom (Nichols *et al.*, 1988). These authors reported that, whilst the overall incorporation of ^{14}C into the lipid fraction of such sea ice communities was not altered over the course of a bloom, the partitioning of the carbon within the lipid component was altered dramatically. Such effects point to a constant rate of fatty acyl biosynthesis but that somehow the partitioning of the acyl moieties is modified during the cell cycle which is modified by the various environmental parameters.

9.5.2 The effect of nitrate concentration.

The basic premise upon which interpretation of the data from the illumination intensity cultures was made can also be applied in the case of variation of the nitrate supply. The effects of the two variables being analogous to two sides of the same coin. In the illumination experiments the nitrate supply was maintained at a constant level whilst the carbon input was varied by the effects upon photosynthetic carbon fixation. In this series of cultures the carbon input was maintained at a consistent rate by fixed illumination intensity whilst the medium nitrate supply was varied. Thus, in effect, the ratio of C/N was modified, but by changes in nitrate rather than carbon. The effects upon the basic lipid class composition of the cultures were also analogous in many respects. The most obvious effect of changing the nitrate concentration was again on the proportion of TAG present in the culture. In the case of this series of cultures the predicted pattern of change was substantiated by the experimental data. As the culture medium nitrate concentration was decreased so the amount of carbon available to the cells was increased in relative terms. Again, the excess carbon entering the fatty acyl biosynthesis pathway was partitioned into TAG as 16:0 and 16:1. Thus TAG accumulated resulting in a decreased relative proportion of PUFA. A similar effect was recorded for *Monodus subterraneous* by Fogg (1959) (Fogg & Thake, 1987) upon transfer to a nitrogen-free culture medium. Fogg & Thake (1987) also observed that the relationship between nutrient deficiency and photosynthetic efficiency is complex and that the published data contains many contradictions. It is generally observed that photosynthetic efficiency decreases with nitrogen limitation. Thus, the observed effect of

'excess' carbon fixation under such conditions is more probably due to a lesser degree of biosynthesis of other components such as protein which require nitrogen amongst their components. The supposition of such an effect is supported by the effect of silicate deficiency upon lipid biosynthesis in diatoms. There are several instances where the rate of lipid accumulation during silicate deficiency has been reported to be much higher than under nitrate deficiency. Thus it seems that silicate deficiency, as one might expect, has a lesser effect upon photosynthesis than that of nitrogen deficiency whilst being equally effective in preventing cellular division.

Pohl (1974) reported that the most important environmental factors determining the PUFA content of various species of marine microalgae were nitrogen supply and illumination intensity. Nitrogen concentrations higher than 400 μM favoured the biosynthesis of PUFA whilst lower concentrations resulted in biosynthesis of saturates and monounsaturates. This is in agreement with the data presented in this thesis. The culture grown at a nitrate concentration of 1 mM had a much higher proportion PUFA in its total FAME when compared to those grown at concentrations of 0.1 mM to 0.001 mM nitrate. This was also correlated with the nitrate concentration range in which the net accumulation of TAG, and its concommitant decrease in unsaturation began. However, it was not until nitrate concentrations as low as 0.01 mM that a decrease in the PUFA content of the polar lipid classes was noted. Skoglund & Jensen (1976) reported that the division rate of the diatom *S. costatum* in dialysis culture was nitrate concentration independent over the range 886 to 0.25 μM. This was obviously not the case for *N.oculata* which showed the effects of nitrate concentrations below 10 μM, in the form of changes in its lipid composition. Yuen *et al.* (1987) working on a different strain of *Nannochloropsis*, *Nannochloropsis* (QII) which showed characteristics very similar to *N. oculata*, compared the partitioning of photosynthetically fixed $^{14}CO_2$ at nitrate concentrations of 2 mM and 0.2 mM corresponding to N sufficiency and N deficiency respectively. They obtained figures showing that TAG accounted for 79 % of the radiolabel at 0.2 mM NO_3^- whilst it only accounted for < 1 % at 2 mM NO_3^-. *De novo* lipid biosynthesis was enhanced and the total FAME were more saturated

saturated under nitrogen deficient conditions. Other authors have reported similar findings for nutrient stressed microalgae (Tornabene *et al.*, 1983; Ben-Amotz *et al.*, 1985).

9.6 Conclusions.

Temperature did not affect the lipid metabolism of *N. oculata* in any significant way. The compositional data hinted at decreased and increased proportions of saturates and monounsaturates respectively with decreased temperature. Salinity increase was found to decrease the polyunsaturation index of the polar lipid classes. In practical terms (*i.e.* considering the culture as a whole) the environmental factors having the most significant effect upon the lipid and fatty acyl composition of *N. oculata* were those affecting the growth stage distribution of the cells in the culture. Clearly, the significant variations in the lipid component of *N. oculata* were derived from those originating in the growth cycle of the cells. Variations in the acyl composition of the polar lipid classes, although equally interesting in terms of biosynthetic pathways, were not major influences upon the acyl composition of the algal cells. The environmental factors causing the bulk effects have, for obvious reasons, the greatest *potential* impact upon the food chain, be it in the natural or artificial culture environment. Because the artificial culture environment used in the present study was very different from that which the alga would encounter in a natural environment the extrapolations one can justifiably make are limited.

The most striking analogy between the experimental data presented here and those from the natural marine environment concern the diatoms. Although *N. oculata* is not a member of the Bacillariophyceae it shows a remarkably similar fatty acyl and lipid class profile to the members of this group. Diatoms are also characterised by their high proportions of 16:0, 16:1 and 20:5(*n*-3) and low proportions of the C_{18} fatty acids in artificial culture (Kates & Volcani, 1966; Opute, 1974; Pohl & Zurheide, 1979). The studies of wild diatom populations (discussed below) have revealed that they also have similar properties.

The dynamics of a natural phytoplankton population are much more complex case than those of artificial culture. The introduction of species succession, a factor touched upon by Rijstenbil *et al.* (1989) in their study of *D. brightwellii* and *S. costatum*, introduces an

additional variable, namely that of species compositional variation as one species outcompetes another. Mayzaud *et al.* (1989) reported such an effect in their experiments upon the effect of nutrient pulses upon an enclosed natural phytoplanktonic assemblage. The fatty acyl composition of their samples changed as the proportion of diatom species rose. The composition of the culture assumed a profile characteristic of diatoms. Nichols *et al.* (1988) formed similar conclusions in their account of variation in the FAME profiles of several antarctic sea-ice diatom communities, as did Morris *et al.* (1985) in their study of a bloom in an enclosed sea loch water column. It seems that in the natural environment species succession and a mixed population will always result in a nutritionally adequate food supply for herbivores in terms of essential fatty acids as these seem only to be required in relatively small amounts. This is because the (*n*-3) series of fatty acids are characteristic of marine organisms (Ackman *et al.*, 1964; Williams, 1965; Ackman *et al.*, 1968) and the C_{18} PUFA may be readily elongated to $C_{20/22}$ PUFA by zooplankton. Despite the variation in their supply rate *via* the effects of local or large scale environmental variables upon the primary producers it does not seem probable that these acids would become the limiting nutrient in the food chain. The relative importance of diatom blooms in temperate and polar marine primary production gives an additional significance for the findings presented here with respect to the marine food chains. Factors as diverse as nitrogen, phosphate and silicate supply and illumination are known to affect the lipid and fatty acyl composition of microalgae. It is also possible that any other factor affecting the division rate of the cells may have effect similar to those described in this thesis. Factors such as those discussed in recent proposals for a solution of the current greenhouse gas / global warming problems by supplementation of the iron content of polar waters (Christoper, 1990; Davies, 1990) may, at a theoretical level, be supported by the findings presented in this thesis. Photosynthetic carbon fixation has been shown to be stimulated by increasing the iron content of polar waters from the Ross Sea which were sufficient in other nutrients such as nitrates and phosphates (Martin *et al.*, 1990). Such supplementation may have increased the biosynthesis of ferredoxins, thus enabling carbon fixation even if other nutrients became division limiting, therefore ridding the atmosphere of CO_2 *via* the equilibrium between atmosphere

and ocean. Martin *et al.* (1990) suggested that supplementation of iron deficient polar waters may have significant impact upon global CO_2 levels.

How sudden changes in the algal fatty acyl compositions may affect the fatty acyl flow from the phytoplankton through the zooplankton into the higher trophic levels is not known. Of the several publications considering such topics, transfer of marker fatty acids seemed to be a major concern, (Mayzaud *et al.*, 1976) implying that the source of origin of particular fatty acids does have some significance to the composition of the remainder of the trophic levels in the food chain . The effects of variations in the lipid and fatty acyl composition of the primary producers is poorly documented. This is apparently due to the lack of knowledge regarding the nutritional requirements of primary consumers (*e.g.* copepods). The importance of long chain PUFA has been implied for natural populations by Mayzaud *et al.* (1989). Lee *et al.* (1971) demonstrated that the quantities lipid and fatty acyl composition of the quantitatively important wax ester component of the copepod *Calanus helgolandicus* resembled that of its algal food. Other workers have established that the consumer organisms in higher trophic levels, such as cod larvae, herring larvae and menhaden, show variations in their fatty acyl composition originating in the phytoplankton (Gatten *et al.*, 1983; Klungsøyr *et al.*, in press). The relative decrease in the proportion of (*n*-3) series fatty acids occurring as a result of a perturbation in the growth characteristics of a culture may or may not have a significant effect. Mixed populations and species succession means that the (*n*-3) series fatty acids are almost ubiquitous in the marine plankton although the specific acyl chain length may vary between C_{16} and C_{22} in a species specific fashion. Because the quantities of the (*n*-3) series acids required by the consumer organisms are unknown it is difficult to say whether there is any significant nutritional effect from the fatty acyl variation in their natural environment.

The variations in the patterns of fatty acyl biosynthesis during natural phytoplankton blooms are not as drastic in their effect as a poor quality lipid diet when introduced into an artificial environment. Thus the effect of environmental culture conditions becomes far more important in a commercial fin or shellfish hatchery. It is here that considerable scope exists

for the manipulation of the environmental conditions to optimise the production of a larval diet of the correct nutritional value (Volkman *et al.*, 1989). The solutions, which may also apply to the commercial production of PUFA by microalgae, do not necessarily require costly measures. The findings of the current thesis suggest that economical use of illumination may in fact increase the relative proportion of PUFA in the total lipid extract, a philosophy echoed by Thompson *et al.* (1990). It seems essential that the correct balance between the growth limiting inorganic nutrients NO_3^-, PO_4^{3-} and SiO_4^{2-} and photosynthetic carbon fixation in artificial culture should be made. Excess energy if made available to a microalgal species such as *N. oculata* is converted into saturated and monounsaturated fatty acids which are both nutritionally and commercially less valuable than the C_{20} PUFA that would otherwise be produced.

A species with a high proportion of its fatty acids made up by a marketable fatty acid such as 20:5(*n*-3) is potentially exploitable on a commercial scale. Realistically this would have to be the artificially inflated healthfood market bearing in mind the comparative production costs between the traditional fish oil products and the high productivity fungal systems (Yongmanitchia & Ward, in press). The current study has shown that the algae do not require unusually high illumination intensities or low temperatures to produce high proportions of PUFA. Such findings mean reduced operating costs and thus may be presented as counter-arguments to the fashion for heterotrophic culture of algae using inorganic carbon sources to eliminate the requirement for illumination. Dark-heterotrophic culture strategies often result in a decrease in the proportion of PUFA (as discussed in Section 1). The commercial potential of cultured microalgae is the topic of many of the volumes and reviews cited in Sections 1 & 2.

9.7 Future prospects.

The clarification of several important areas would fill some of the grey areas in the overview presented in this thesis. Bearing in mind that the significant variations in lipid and fatty acyl composition originate from those inherent in the cellular growth cycle, it seems that attention should be paid to the investigation of the variations occurring in synchronous

cultures of *N. oculata*. The limited study of Otsuka & Morimura (1966), in combination with the current data, provides evidence to support the line of reasoning that changes in the proportions of TAG occur as part of the division cycle of microalgal cells. Investigation of synchronous cultures would enable the extrapolation of division cycle data to test the hypothesis that the variation in the growth stage distribution pattern of cells in a population or culture could be responsible for the variations in lipid and fatty acyl composition observed during the course of this thesis. Visible increases in mean cell volume occurred during the non-division phases of growth. Measurement of the size distribution profile of the cultures as part of the counting protocol may have yielded useful information, enabling the correlation of the growth phase distribution profile of the culture with the changes in its lipid composition.

The cursory investigation of the polar lipid class molecular species composition of *N. oculata* conducted as part of this project indicated the potential for positional redistribution of the acyl moieties of these classes. Further investigation of such phenomena would be expected to yield clues to various biosynthetic pathways leading from 16:0 to 20:5(*n*-3), so clarifying the potential biosynthetic relationship between these two fatty acids. As has been discussed above, the polar lipid classes, notably MGDG and PC, have been examined in the past for a relationship linking them with desaturation and elongation. If a relationship between the positional distribution of fatty acids in these classes at various points in the cellular division cycle of *N. oculata* could be demonstrated, this would go some way toward explaining the specific variations observed during the current work. This may also lead to the identification of a specific step, or pathway (such as the prokaryotic or eukaryotic pathways of galactolipid biosynthesis, or transacylation with TAG) which is affected by environmental factors. The apparent lack of an effect of temperature upon the PUFA content of *N.oculata* leads the author to ask questions regarding the possibility of temperature specific variation in the positional distribution and acyl combination patterns of molecular species as discussed in Section 8.

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APPENDICES

APPENDIX A : Listings of B.B.C. - B.A.S.I.C. computer programmes written & used during the course of the project.

```
10  REM. MASS DETECTOR DATA INTERPRETATION PROG. (PART A)
20  CLS
30  REM. TO SET UP MAIN MENU
40  meancnt=0:M=0
50  MODE3
60  PRINT TAB(35,8) "MAIN MENU"
70  PRINT TAB(35,9) "=========="
80  PRINTTAB(23,11) "1.) CALIBRATION CURVE ALTERATIONS."
90  PRINTTAB(23,12) "2.) DATA INPUT."
100 PRINTTAB(23,13) "3.) DATA PROCESSING."
110 PRINTTAB(23,14) "4.) REVIEW DATA FILE."
120 PRINTTAB(23,15) "5.) END."
130 PRINTTAB(23,17) "TYPE REQUIRED OPTION No. ";
140 menopt=GET
150 IF menopt=49 THEN 210
160 IF menopt=50 THEN 850
170 IF menopt=51 THEN 1120
180 IF menopt=52 THEN 1290
190 IF menopt=53 THEN CLS:END
200 GOTO 30
210 REM. TO SET UP CALIB. MENU
220 CLS:PRINTTAB(31,8)"CALIBRATION MENU"
230 PRINTTAB(31,9)"================"
240 PRINTTAB(23,11)"1.) SUMMARY OF AVAILABLE CURVES"
250 PRINTTAB(23,12)"   AND FILE CODES."
260 PRINTTAB(23,13)"2.) INPUT NEW CALIBRATION CURVE."
270 PRINTTAB(23,14)"3.) REVIEW CALIBRATION CURVE."
280 PRINTTAB(23,15)"4.) MAIN MENU."
290 PRINTTAB(23,18)"TYPE REQUIRED OPTION No. ";
300 calopt=GET
310 IF calopt=49 THEN 360
320 IF calopt=50 THEN 520
330 IF calopt=51 THEN 670
340 IF calopt=52 THEN 30
350 GOTO 210
360 REM. AVAILABLE CURVES + CODES
370 CLS:PRINTTAB(25,3)"AVAILABLE CALIBRATION CURVE LIST"
380 PRINTTAB(25,4)"============================="
390 PRINTTAB(25,6)"TRIACYLGLYCEROLS (TAG)."
400 PRINTTAB(25,7)"FREE FATTY ACIDS (FFA)."
410 PRINTTAB(25,8)"CHOLESTEROL (CH)."
420 PRINTTAB(25,9)"MONOGALACTOSYLDIACYLGLYCEROL (MGDG)."
430 PRINTTAB(25,10)"DIGALACTOSYLDIACYLGLYCEROL (DGDG)."
440 PRINTTAB(25,11)"PHOSPHATIDYLETHANOLAMINE (PE)."
450 PRINTTAB(25,12)"PHOSPHATIDYLGLYCEROL (PG)."
460 PRINTTAB(25,13)"PHOSPHATIDYLINOSITOL (PI)."
470 PRINTTAB(25,14)"PHOSPHATIDYLCHOLINE (PC)."
480 PRINTTAB(25,23)"HIT 'SPACE-BAR' TO CONTINUE.";
490 J=GET
500 IF J=32 THEN 30
510 GOTO 360
520 REM. CALIBRATION INPUT
530 CLS:PRINTTAB(27,5)"CALIBRATION INPUT OPTION."
```

```
540  PRINTTAB(27,6)"========================="
550  INPUTTAB(24,8)"CALIBRATION CURVE FILE CODE  "A$
560  Z=OPENOUT A$
570  REPEAT
580  PRINTTAB(23,18)"TYPE '999' AS AREA CO-ORD. TO STOP."
590  INPUTTAB(23,12)"AMOUNT CO-ORDINATE ",Y
600  INPUTTAB(23,14)"  AREA CO-ORDINATE ",X
610  PRINTTAB(23,12)"                    "
620  PRINTTAB(23,14)"                    "
630  PRINT#Z,X,Y
640  UNTIL X=999
650  CLOSE#Z
660  GOTO 210
670  REM. CALIBRATION REVIEW
680  CLS:PRINTTAB(28,5)"REVIEW CALIBRATION CURVE."
690  PRINTTAB(28,6)"========================="
700  INPUTTAB(27,8)"CALIBRATION CURVE CODE "B$
710  D=OPENIN B$
720  calrev=0
730  REPEAT
740  INPUT#D,N,V
750  IF N=999 THEN 790
760  calrev=calrev+1
770  PRINTTAB(30,calrev+9);V
780  PRINTTAB(42,calrev+9);N
790  UNTIL N=999
800  CLOSE#D
810  PRINTTAB(27,calrev+11)"HIT 'SPACE-BAR' TO CONTINUE "
820  chcont=GET
830  IF chcont<>32 THEN 810
840  GOTO 210
850  REM. DATA INPUT OPTION
860  CLS:PRINTTAB(31,2)"DATA INPUT OPTION"
870  PRINTTAB(31,3)"================"
880  incnt=0
890  INPUTTAB(33,5)"RUN No.  "Q$
900  INPUTTAB(28,6)"INJECTION MASS (ug)  "P
910  PRINTTAB(27,8)"PEAK    CODE      AREA "
920  PRINTTAB(25,23)"TYPE 'END' AS CODE TO STOP"
930  L=OPENOUT Q$
940  PRINT#L,P
950  REPEAT
960  incnt=incnt+1
970  PRINTTAB(27,incnt+8);incnt;".)"
980  INPUTTAB(37,incnt+8)I$
990  INPUTTAB(49,incnt+8)S
1000 PRINT#L,I$,S
1010 UNTIL I$="END" OR incnt=12
1020 CLOSE#L
1030 PRINTTAB(24,incnt+10)"HIT 'SPACE-BAR' TO CLEAR SCREEN"
1040 flclr=GET
1050 IF flclr<>32 THEN 1030
1060 CLS:PRINTTAB(20,10)"TYPE  1.) TO INPUT ANOTHER RUN."
1070 PRINTTAB(20,11)"     2.) TO RETURN TO MAIN MENU."
1080 runcnt=GET
1090 IF runcnt=49 THEN 850
1100 IF runcnt=50 THEN 30
1110 GOTO 1060
```

```
1120  REM. DATA PROCESSING OPTION
1130  CLS:PRINTTAB(29,3)"DATA PROCESSING OPTION"
1140  PRINTTAB(29,4)"======================"
1150  T=OPENOUT "ANALIST"
1160  anacnt=0
1170  PRINTTAB(19,6)"TYPE IN RUN Nos. FOR ANALYSIS (max. 10 nos.)"
1180  PRINTTAB(31,21)"TYPE 'END' TO STOP."
1190  REPEAT
1200  anacnt=anacnt+1
1210  PRINTTAB(35,anacnt+8);anacnt".)"
1220  INPUTTAB(42,anacnt+8)U$
1230  PRINT#T,U$
1240  UNTIL U$="END" OR anacnt=10
1250  CLOSE#T
1260  PRINTTAB(23,23)"TO BEGIN PROCESSING HIT 'SPACE-BAR'."
1270  prcont=GET
1280  IF GET<>32 THEN 1260
1285  CHAIN "PARTB"
1290  REM. TO REVIEW A DATA FILE
1300  CLS:PRINTTAB(31,5)"REVIEW DATA FILE"
1310  PRINTTAB(31,6)"================"
1320  INPUTTAB(31,7)"DATA FILE No.  "N$
1330  N=OPENIN N$
1340  datrev=0
1345  INPUT#N,imass
1346  PRINTTAB(28,9)"INJECTION MASS  ";imass" ug."
1350  REPEAT
1360  INPUT#N,X$,I
1370  IF X$="END" THEN 1410
1380  datrev=datrev+1
1390  PRINTTAB(31,datrev+10);X$
1400  PRINTTAB(41,datrev+10);I
1410  UNTIL X$="END"
1420  CLOSE#N
1430  PRINTTAB(25,datrev+12)"HIT 'SPACE-BAR' TO CONTINUE"
1440  rdfcont=GET
1450  IF rdfcont<>32 THEN 1430
1460  GOTO 30

  10  REM. MASS DETECTOR DATA INTERPRETATION PROG. (PART B)
  15  MODE7
  20  REM. TO DIMENSION ARRAYS
  30  DIM tag(10):DIM ffa(10):DIM chol(10):DIM mgdg(10):DIM dgdg(10):DIM pe(10):DIMpg(10)
      :DIM pi(10):DIM pc(10):DIM sumlip(10):DIM imass(10):DIM perlip(10):DIMptag(10):DIM
      pmtag(10) :DIM pffa(10):DIM pmffa(10):DIM pchol(10)
  40  DIM pmchol(10):DIM pmgdg(10):DIM pmmgdg(10):DIM pdgdg(10):DIM pmdgdg(10): DIM
      ppg(10):DIM pmpg(10):DIM ppe(10):DIM pmpe(10):DIM ppi(10):DIM pmpi(10):DIM
      ppc(10):DIM pmpc(10):DIM plip(10)
  50  R=OPENIN "ANALIST"
  60  M=0:meancnt=0
  70  REPEAT
  80  REM. TO INPUT RUN NO. FROM ANALYSIS FILE
  90  INPUT#R,E$
 100  IF E$="END" THEN 430
 110  K=OPENIN E$
 120  M=M+1:meancnt=meancnt+1
 130  INPUT#K,O
 140  imass(M)=O
```

```
150  REPEAT
160  INPUT#K,R$,W
170  IF R$="END" THEN 380
180  REM. TO CHECK AREA VS. CALIB CURVE
190  G=OPENIN R$
200  A=0:B=0:E=0:F=0:H=0
210  REPEAT
220  INPUT#G,A,B
230  IF A=999 THEN 260
240  IF A>=W AND E<=W THEN H=F+(((B-F)/(A-E))*(W-E)):A=999
250  E=A:F=B
260  UNTIL A=999
270  CLOSE#G
280  REM. TO STORE CALCULATED MASSES FOR RE-ANAL
290  IF R$="TAG" THEN tag(M)=H
300  IF R$="FFA" THEN ffa(M)=H
310  IF R$="CH" THEN chol(M)=H
320  IF R$="MGDG" THEN mgdg(M)=H
330  IF R$="DGDG" THEN dgdg(M)=H
340  IF R$="PE" THEN pe(M)=H
350  IF R$="PG" THEN pg(M)=H
360  IF R$="PI" THEN pi(M)=H
370  IF R$="PC" THEN pc(M)=H
380  UNTIL R$="END"
390  CLOSE#K
400  REM. TO SUM CLASS MASSES, ACCOUNT FOR TOTAL LIPID
410  sumlip(M)=tag(M)+ffa(M)+chol(M)+mgdg(M)+dgdg(M)+pe(M)+pg(M)+pi(M)+pc(M)
420  perlip(M)=(INT((sumlip(M)/imass(M))*100))/100
430  UNTIL E$="END"
440  CLOSE#R
450  REM. TO CALCULATE CLASS MEANS +/- SD
460  FOR M= 1 TO meancnt
470  ttag=ttag+tag(M)
480  tffa=tffa+ffa(M)
490  tchol=tchol+chol(M)
500  tmgdg=tmgdg+mgdg(M)
510  tdgdg=tdgdg+dgdg(M)
520  tpe=tpe+pe(M)
530  tpg=tpg+pg(M)
540  tpi=tpi+pi(M)
550  tpc=tpc+pc(M)
560  tsumlip=tsumlip+sumlip(M)
570  timass=timass+imass(M)
580  NEXT M
590  mtag=(INT((ttag/meancnt)*100))/100
600  mffa=(INT((tffa/meancnt)*100))/100
610  mchol=(INT((tchol/meancnt)*100))/100
620  mmgdg=(INT((tmgdg/meancnt)*100))/100
630  mdgdg=(INT((tdgdg/meancnt)*100))/100
640  mpe=(INT((tpe/meancnt)*100))/100
650  mpg=(INT((tpg/meancnt)*100))/100
660  mpi=(INT((tpi/meancnt)*100))/100
670  mpc=(INT((tpc/meancnt)*100))/100
680  msumlip=(INT((tsumlip/meancnt)*100))/100
690  mimass=(INT((timass/meancnt)*100))/100
700  REM. TO CALCULATE SD FROM MEANS
710  FOR M= 1 TO meancnt
720  dtag=dtag+(tag(M)-mtag)^2
```

```
730  dffa=dffa+(ffa(M)-mffa)^2
740  dchol=dchol+(chol(M)-mchol)^2
750  dmgdg=dmgdg+(mgdg(M)-mmgdg)^2
760  ddgdg=ddgdg+(dgdg(M)-mdgdg)^2
770  dpe=dpe+(pe(M)-mpe)^2
780  dpg=dpg+(pg(M)-mpg)^2
790  dpi=dpi+(pi(M)-mpi)^2
800  dpc=dpc+(pc(M)-mpc)^2
810  dsumlip=dsumlip+(sumlip(M)-msumlip)^2
820  dimass=dimass+(imass(M)-mimass)^2
830  NEXT M
840  sdtag=(INT((SQR(dtag/meancnt)*100)))/100
850  sdffa=(INT((SQR(dffa/meancnt)*100)))/100
860  sdchol=(INT((SQR(dchol/meancnt)*100)))/100
870  sdmgdg=(INT((SQR(dmgdg/meancnt)*100)))/100
880  sddgdg=(INT((SQR(ddgdg/meancnt)*100)))/100
890  sdpe=(INT((SQR(dpe/meancnt)*100)))/100
900  sdpg=(INT((SQR(dpg/meancnt)*100)))/100
910  sdpi=(INT((SQR(dpi/meancnt)*100)))/100
920  sdpc=(INT((SQR(dpc/meancnt)*100)))/100
930  sdsumlip=(INT((SQR(dsumlip/meancnt)*100)))/100
940  sdimass=(INT((SQR(dimass/meancnt)*100)))/100
950  REM. TO CALCULATE % COMPOSN LIPID
960  FOR M=1 TO meancnt
970  ptag(M)=(INT((tag(M)/sumlip(M))*10000))/100:tptag=tptag+ptag(M):
     pmtag(M)=(INT((tag(M)/imass(M))*10000))/100:tpmtag=tpmtag+pmtag(M)
980  pffa(M)=(INT((ffa(M)/sumlip(M))*10000))/100:tpffa=tpffa+pffa(M):
     pmffa(M)=(INT((ffa(M)/imass(M))*10000))/100:tpmffa=tpmffa+pmffa(M)
990  pchol(M)=(INT((chol(M)/sumlip(M))*10000))/100:tpchol=tpchol+pchol(M):
     pmchol(M)=(INT((chol(M)/imass(M))*10000))/100:tpmchol=tpmchol+pmchol(M)
1000 pmgdg(M)=(INT((mgdg(M)/sumlip(M))*10000))/100:tpmgdg=tpmgdg+pmgdg(M):
     pmmgdg(M)=(INT((mgdg(M)/imass(M))*10000))/100:
     tpmmgdg=tpmmgdg+pmmgdg(M)
1010 pdgdg(M)=(INT((dgdg(M)/sumlip(M))*10000))/100:tpdgdg=tpdgdg+pdgdg(M):
     pmdgdg(M)=(INT((dgdg(M)/imass(M))*10000))/100:tpmdgdg=tpmdgdg+pmpg(M)
1020 ppg(M)=(INT((pg(M)/sumlip(M))*10000))/100:tppg=tppg+ppg(M):
     pmpg(M)=(INT((pg(M)/imass(M))*10000))/100:tpmpg=tpmpg+pmpg(M)
1030 ppe(M)=(INT((pe(M)/sumlip(M))*10000))/100:tppe=tppe+ppe(M):
     pmpe(M)=(INT((pe(M)/imass(M))*10000))/100:tpmpe=tpmpe+pmpe(M)
1040 ppi(M)=(INT((pi(M)/sumlip(M))*10000))/100:tppi=tppi+ppi(M):
     pmpi(M)=(INT((pi(M)/imass(M))*10000))/100:tpmpi=tpmpi+pmpi(M)
1050 ppc(M)=(INT((pc(M)/sumlip(M))*10000))/100:tppc=tppc+ppc(M):
     pmpc(M)=(INT((pc(M)/imass(M))*10000))/100:tpmpc=tpmpc+pmpc(M)
1060 plip(M)=(INT((sumlip(M)/imass(M))*10000))/100:tplip=tplip+plip(M)
1070 NEXT M
1080 mptag=(INT((tptag/meancnt)*100))/100:mpmtag=(INT((tpmtag/meancnt)*100))/100
1090 mpffa=(INT((tpffa/meancnt)*100))/100:mpmffa=(INT((tpmffa/meancnt)*100))/100
1100 mpchol=(INT((tpchol/meancnt)*100))/100:mpmchol=(INT((tpmchol/meancnt)*100))/100
1110 mpmgdg=(INT((tpmgdg/meancnt)*100))/100:mpmmgdg=(INT((tpmmgdg/meancnt)
     *100))/100
1120 mpdgdg=(INT((tpdgdg/meancnt)*100))/100:mpmdgdg=(INT((tpmdgdg/meancnt)
     *100))/100
1130 mppg=(INT((tppg/meancnt)*100))/100:mpmpg=(INT((tpmpg/meancnt)*100))/100
1140 mppe=(INT((tppe/meancnt)*100))/100:mpmpe=(INT((tpmpe/meancnt)*100))/100
1150 mppi=(INT((tppi/meancnt)*100))/100:mpmpi=(INT((tpmpi/meancnt)*100))/100
1160 mppc=(INT((tppc/meancnt)*100))/100:mpmpc=(INT((tpmpc/meancnt)*100))/100
1170 mplip=(INT((tplip/meancnt)*100))/100
1180 REM. TO DUMP OUTPUT TO DFS
```

```
1190  INPUTTAB(0,10)"SAMPLE DESCRIPTION "S$
1200  INPUTTAB(0,12)"DATE          "P$
1210  L=OPENOUT"DTADMP"
1220  PRINT#L,S$,P$,mimass,msumlip,mtag,mffa,mchol,mmgdg,mdgdg,mpg,mpe,mpi,
      mpc,sdimass,sdsumlip,sdtag,sdffa,sdchol,sdmgdg,sddgdg,sdpg,sdpe,sdpi,sdpc,
      mptag,mpffa,mpchol,mpmgdg,mpdgdg,mppg,mppe,mppi,mppc,mplip,mpmtag,
      mpmffa,mpmchol,mpmmgdg
1230  PRINT#L,mpmdgdg,mpmpg,mpmpe,mpmpi,mpmpc
1240  CLOSE#L
1250  CHAIN"PARTC"


  10  REM. MASS DETECTOR DATA INTERPRETATION PROG. (PART C)
  15  MODE3
  20  REM. RESULT DISPLAY/PRINTOUT
  30  L=OPENIN"DTADMP"
  40  INPUT#L,S$,P$,mimass,msumlip,mtag,mffa,mchol,mmgdg,mdgdg,mpg,mpe,
      mpi,mpc,sdimass,sdsumlip,sdtag,sdffa,sdchol,sdmgdg,sddgdg,sdpg,sdpe,sdpi,
      sdpc,mptag,mpffa,mpchol,mpmgdg,mpdgdg,mppg,mppe,mppi,mppc,mplip,
      mpmtag,mpmffa,mpmchol,mpmmgdg
  50  INPUT#L,mpmdgdg,mpmpg,mpmpe,mpmpi,mpmpc
  60  CLOSE#L
  70  CLS:PRINTTAB(0,12)"DO YOU WANT A PRINTED COPY ?  (Y/N)."
  80  prntcnt=GET
  90  IF prntcnt=78 THEN 180
 100  IF prntcnt<>89 THEN 20
 110  CLS
 120  *FX5,1
 130  *FX6
 140  VDU2
 150  PRINT:PRINT:PRINT:PRINT:PRINT:PRINT
 160  PRINTTAB(0,3)"SAMPLE :- "; S$ ;"   DATE :- "; P$
 170  PRINT
 180  PRINTTAB(0,5)"LIPID CLASS              !  MASS (ug)  +/- s.d.   PERCENT
      PERCENT
 190  PRINTTAB(0,6)"=============================================
      ================================"
 200  PRINTTAB(0,4)"                    !"
 210  PRINTTAB(0,7)"INJECTION MASS          !" mimass" "sdimass"    100.00"
 220  PRINTTAB(0,8)"TOTAL LIPID             !" msumlip" "sdsumlip" "mplip"   100.00"
 230  PRINTTAB(0,9)"                    !"
 240  PRINTTAB(0,10)"TRIACYLGLYCEROL         !" mtag" "sdtag" "mpmtag" "mptag
 250  PRINTTAB(0,11)"FREE FATTY ACIDS        !" mffa" "sdffa" "mpmffa" "mpffa
 260  PRINTTAB(0,12)"CHOLESTEROL             !" mchol" "sdchol" "mpmchol" "mpchol"
 270  PRINTTAB(0,13)"                    !"
 280  PRINTTAB(0,14)"MONOGALACTOSYLDIACYLGLYCEROL !" mmgdg"
      "sdmgdg" "mpmmgdg" "mpmgdg
 290  PRINTTAB(0,15)"DIGALACTOSYLDIACYLGLYCEROL  !" mdgdg" "sddgdg"
      "mpmdgdg" "mpdgdg
 300  PRINTTAB(0,16)"                    !"
 310  PRINTTAB(0,17)"PHOSPHATIDYLGLYCEROL      !" mpg" "sdpg" "mpmpg"
      "mppg
 320  PRINTTAB(0,18)"                    !"
 330  PRINTTAB(0,19)"PHOSPHATIDYLETHANOLAMINE   !" mpe" "sdpe"
      "mpmpe" "mppe
 340  PRINTTAB(0,20)"PHOSPHATIDYLINOSITOL       !" mpi" "sdpi" "mpmpi" "mppi
 350  PRINTTAB(0,21)"PHOSPHATIDYLCHOLINE        !" mpc" "sdpc" "mpmpc" "mppc
```

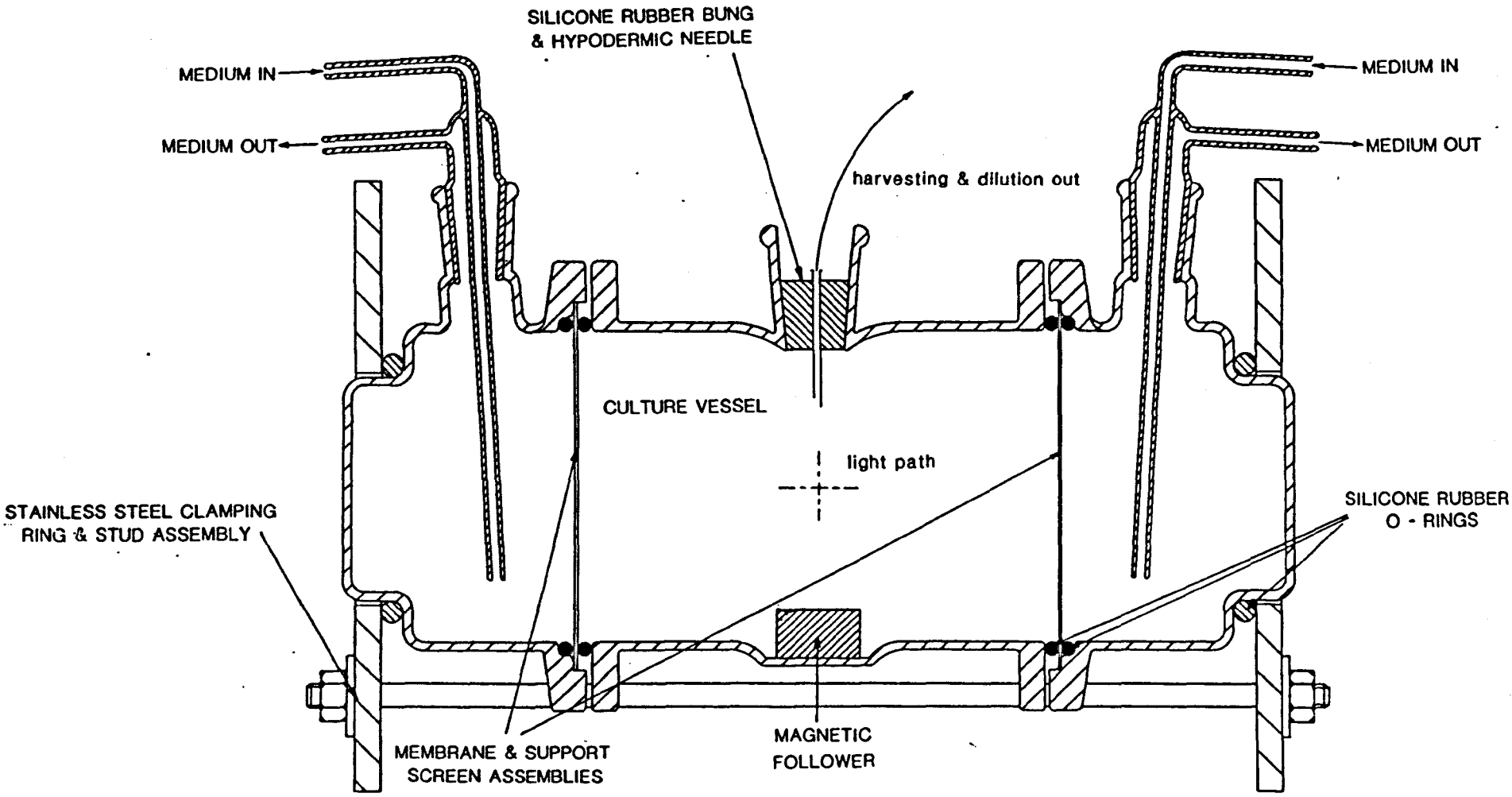
```
360  PRINT
370  VDU3
380  PRINT"HIT 'SPACE-BAR' FOR MAIN MENU"
390  econt=GET
400  IF econt<>32 THEN 380
410  CHAIN "PARTA"
```

```
10  REM GC TRACE CORRECTION PROG.
20  *KEY 9 VDU 23,1,1;0;0;0;:*FX 4,0
30  @%=&20209:*KEY 0 X
40  *FX 4,1
50  MODE3
60  DIM A(50):DIM B(50):DIM C(50):DIM D(10):DIM E(10)
70  CLS:PRINTTAB(34,5)"MAIN MENU":PRINTTAB(34,6)"=========":
    PRINTTAB(31,8)"1.) DATA INPUT.":
    PRINTTAB(31,9)"2.) ARTIFACT INPUT":PRINTTAB(31,10)"3.) INTERNAL
    STANDARD":PRINTTAB(31,11)"4.) ALTER EXISTING DATA":PRINTTAB(31,12)"5.)
    RECALCULATE"
80  PRINTTAB(31,13)"6.) OUTPUT OPTIONS":PRINTTAB(31,14)"7.)
    END":PRINTTAB(31,16)"TYPE OPTION No. "
90  VDU 23,1,0;0;0;0;
100 Z$=GET$
110 IF Z$=CHR$ 49 THEN VDU 23,1,1;0;0;0;
    :PROCtitle:PROCscreenlayout:PROCinput:PROCredisplaydata:
    PROCcorrections:PROCartifactinput:PROCintstdinput:PROCcalculations:
    PROCvduout:GOTO 70
120 IF Z$=CHR$ 50 THEN PROCartifactinput:GOTO 70
130 IF Z$=CHR$ 51 THEN PROCintstdinput:GOTO 70
140 IF Z$=CHR$ 52 THEN
    PROCscreenlayout:PROCredisplaydata:PROCcorrections:GOTO 70
150 IF Z$=CHR$ 53 THEN PROCcalculations:GOTO 70
160 IF Z$=CHR$ 54 THEN PROCoutputopt
170 IF Z$<>CHR$ 55 THEN 100 ELSE CLS:END
180 DEFPROCoutputopt
190 CLS:PRINTTAB(30,9)"OUTPUT
    OPTIONS":PRINTTAB(30,10)"==============":PRINTTAB(30,12)"1.) TO VDU
    ONLY":PRINTTAB(30,13)"2.) TO PRINTER ONLY":PRINTTAB(30,15)"TYPE
    OPTION No. ":Y$=GET$
200 IF Y$=CHR$ 49 THEN PROCvduout:GOTO 70
210 IF Y$=CHR$ 50 THEN PROCprinter:GOTO 70
220 GOTO 190
230 DEFPROCscreenlayout
240 REM TO DISPLAY FATTY ACID PROMPT LIST FOR INPUT
250 CLS:PRINTTAB(18,1)Q$:PRINT TAB(18,3)"14:0     " :PRINT TAB(45,3)"20:0    "
260 PRINT TAB(18,4)"15:0     " :PRINT TAB(45,4)"20:1 (n-9) "
270 PRINT TAB(18,5)"16:0     " :PRINT TAB(45,5)"20:1 (n-7) "
280 PRINT TAB(18,6)"16:1 (n-??)" :PRINT TAB(45,6)"20:2     "
290 PRINT TAB(18,7)"16:1 (n-7) " :PRINT TAB(45,7)"20:3 (n-6) "
300 PRINT TAB(18,8)"16:2     " :PRINT TAB(45,8)"20:4 (n-6) "
310 PRINT TAB(18,9)"17:0     " :PRINT TAB(45,9)"20:3 (n-3) "
320 PRINT TAB(18,10)"16:3     " :PRINT TAB(45,10)"20:4 (n-3) "
330 PRINT TAB(18,11)"16:4     " :PRINT TAB(45,11)"20:5 (n-3) "
340 PRINT TAB(18,12)"18:0     " :PRINT TAB(45,12)"22:0     "
350 PRINT TAB(18,13)"18:1 (n-9) " :PRINT TAB(45,13)"22:1     "
360 PRINT TAB(18,14)"18:1 (n-7) " :PRINT TAB(45,14)"22:2     "
370 PRINT TAB(18,15)"18:2     " :PRINT TAB(45,15)"22:3     "
380 PRINT TAB(18,16)"18:3 (n-6) " :PRINT TAB(45,16)"22:4     "
390 PRINT TAB(18,17)"18:3 (n-3) " :PRINT TAB(45,17)"22:5     "
400 PRINT TAB(18,18)"18:4 (n-3) " :PRINT TAB(45,18)"22:6 (n-3) "
410 ENDPROC
420 DEFPROCinput
430 REM TO INPUT DATA TO ARRAY + DISPLAY ON SCREEN
440 B=0 : C=2
450 FOR cnt = 1 TO 32
460 IF cnt>16 THEN B=26 : C=-14
```

```
470  INPUTTAB(B+30,cnt+C) A(cnt)
480  NEXT cnt
490  ENDPROC
500  DEFPROCcorrections
510  REM TO INPUT CORRECTIONS TO DATA
520  *FX 4,0
530  PRINTTAB(18,20) "MOVE CURSOR USING ARROW KEYS TO MAKE
     CORRECTION":PRINTTAB(23,21) "THEN HIT SPACE BAR AND MAKE NEW
     ENTRY":PRINTTAB(15,22)"PRESS 'f0' KEY TO END & ENTER ARTIFACT / INT.
540  A$=GET$ :IF A$=CHR$88 THEN ENDPROC
550  IF A$<>CHR$32 THEN 510
560  arr=0 : hei=VPOS-2 :X=POS : Y=VPOS
570  IF POS>45 THEN arr=16
580  IF X>45 THEN PRINTTAB(56,VPOS)"        " :INPUTTAB(56,Y) A(arr+hei)
590  IF X<45 AND X>16 THEN PRINTTAB(30,Y)"        ": INPUTTAB(30,Y) A(arr+hei)
600  GOTO 510
610  DEFPROCcalculations
620  LOCAL cnt,N:*FX 4,1
625  asum=0
630  REM CONVERSION OF % VALUES TO ELIMINATE ARTIFACTS
640  FOR cnt= 1 TO 32
650  asum=asum + A(cnt)
651  B(cnt)=A(cnt)*factor
652  C(cnt)=B(cnt)*E(4)
660  NEXT cnt
670  A(39)=100-(asum+sumd):A(40)=A(1)+A(2)+A(3)+A(7)+A(10)+A(17)+A(26):
     A(41)=A(4)+A(5)+A(11)+A(12)+A(18)+A(19)+A(27):
     A(42)=A(6)+A(8)+A(9)+A(13)+A(14)+A(15)+A(16)+A(20)+A(21)+A(22)
     +A(23)+A(24)+A(25)+A(28)+A(29)+A(30)+A(31)+A(32)
680  A(43)=A(11)+A(18):A(44)=A(14)+A(21)+A(22):A(45)=A(15)+A(16)
     +A(23)+A(24)+A(25)+A(32):A(46)=A(45)/A(44)
681  FOR N=39 TO 46
682  B(N)=A(N)*factor:C(N)=B(N)*E(4)
683  NEXT N
690  ENDPROC
1160 DEFPROCvduout
1170 LOCAL A$,B$,N,X,Y,cnt
1180 FOR cnt=1 TO 3
1190 X=28:Y=0
1200 CLS:PROCscreenlayout:PROCscreenbottom
1210 IF cnt=1 THEN PRINTTAB(33,1)"ORIGINAL DATA"
1220 IF cnt=2 THEN PRINTTAB(30,1)"PERCENTAGE ADJUSTED DATA"
1230 IF cnt=3 THEN PRINTTAB(30,1)"ABSOLUTE ADJUSTED DATA"
1240 FOR N=1 TO 32
1250 Y=N+2:IF N>16 THEN Y=N-14:X=56
1260 IF cnt=1 THEN PRINTTAB(X,Y) A(N)
1270 IF cnt=2 THEN PRINTTAB(X,Y) B(N)
1280 IF cnt=3 THEN PRINTTAB(X,Y) C(N)
1290 NEXT N
1300 X=28
1310 FOR M=39 TO 46
1320 Y=M-19:IF M>42 THEN Y=M-23:X=56
1330 IF cnt=1 THEN PRINTTAB(X,Y) A(M)
1331 IF cnt=2 THEN PRINTTAB(X,Y) B(M)
1332 IF cnt=3 THEN PRINTTAB(X,Y) C(M)
1333 NEXT M
1334 VDU 21:INPUTTAB(1,23) W:VDU 6
1335 IF W<>0 THEN 1334
```

```
1336  NEXT cnt
1337  ENDPROC
1340  DEFPROCprinteroutput
1350  REM TO PRINT ORIGINAL AND ADJUSTED VALUES
1360  CLS:PRINT:PRINT:PRINT
1370  PRINTTAB(10)"FATTY ACID      ORIGINAL      ADJUSTED      INT. STD."
1380  PRINT
1390  DEFPROCartifactinput
1400  sumd=0:D(1)=0:D(2)=0:D(3)=0:D(4)=0:D(5)=0:D(6)=0:D(7)=0:D(8)=0:
      D(9)=0:D(10)=0:LOCAL cnt:CLS:PRINTTAB(32,5)"ARTIFACT INPUT"
      :PRINTTAB(32,6)"==============":PRINTTAB(25,20)"TYPE '0' OR 'RETURN'
      TO DEFAULT"
1410  FOR cnt=1 TO 10
1420  INPUTTAB(35,cnt+7) E
1430  IF E=0 THEN 1455
1440  D(cnt)=E:sumd=sumd+D(cnt)
1450  NEXT cnt
1455  factor=100/(100-sumd)
1460  ENDPROC
1470  DEFPROCredisplaydata
1480  LOCAL cnt,B,C:B=0:C=2
1490  FOR cnt=1 TO 32
1500  IF cnt>16 THEN B=26:C=-14
1510  PRINTTAB(B+30,cnt+C) A(cnt)
1520  NEXT cnt
1530  ENDPROC
1540  DEFPROCintstdinput
1550  LOCAL A,B:E(1)=0:E(2)=0:E(3)=0:E(4)=0:E(5)=0:E(6)=0:E(7)=0
      :E(8)=0:E(9)=0:E(10)=0:CLS:PRINTTAB(30,8)"INTERNAL
      STANDARD":PRINTTAB(30,9)"================":PRINTTAB(25,16)"TYPE '0'
      OR 'RETURN' TO DEFAULT"
1560  INPUTTAB(25,11)"AMOUNT OF LIPID (ug)    " A:IF A=0 THEN ENDPROC ELSE E(1)=A
1570  INPUTTAB(25,12)"AMOUNT INTERNAL STD (ug)  " B:IF B=0 THEN ENDPROC
      ELSE E(2)=B
1580  INPUTTAB(25,13)"PERCENT INTERNAL STD    " E(3)
1590  E(4)=E(2)/E(3):ENDPROC
1600  DEFPROCscreenbottom
1610  PRINTTAB(18,20)"UNKNOWNS      ":PRINTTAB(45,20)"TOT. (n-9)   "
1620  PRINTTAB(18,21)"TOT. SATS     ":PRINTTAB(45,21)"TOT. (n-6)   "
1630  PRINTTAB(18,22)"TOT. MONOS    ":PRINTTAB(45,22)"TOT. (n-3)   "
1640  PRINTTAB(18,23)"TOT. PUFA     ":PRINTTAB(45,23)"(n-6)/(n-3)   "
1650  ENDPROC
1660  DEFPROCprinter
1666  @%=&2020C:VDU 2:*FX 6
1669  PRINTTAB(10);Q$:PRINT
1670  PRINTTAB(10)"FATTY ACID      ORIGINAL  ADJUSTED  INT.STAND":PRINT
1680  PRINTTAB(10)"14:0     ",A(1),B(1),C(1):PRINTTAB(10)"15:0
      ",A(2),B(2),C(2):PRINTTAB(10)"16:0     ",A(3),B(3),C(3):PRINTTAB(10)"16:1 (n-
      ?)",A(4),B(4),C(4):PRINTTAB(10)"16:1 (n-7)",A(5),B(5),C(5)
1690  PRINTTAB(10)"16:2     ",A(6),B(6),C(6):PRINTTAB(10)"17:0
      ",A(7),B(7),C(7):PRINTTAB(10)"16:3     ",A(8),B(8),C(8):PRINTTAB(10)"16:4
      ",A(9),B(9),C(9):PRINTTAB(10)"18:0     ",A(10),B(10),C(10)
1700  PRINTTAB(10)"18:1 (n-9)",A(11),B(11),C(11):PRINTTAB(10)"18:1 (n-
      7)",A(12),B(12),C(12):PRINTTAB(10)"18:2     ",A(13),B(13),C(13):PRINTTAB(10)
      "18:3 (n-6)",A(14),B(14),C(14):PRINTTAB(10)"18:3 (n-3)",A(15),B(15),C(15)
1710  PRINTTAB(10)"18:4 (n-3)",A(16),B(16),C(16):PRINTTAB(10)"20:0
      ",A(17),B(17),C(17):PRINTTAB(10)"20:1 (n-9)",A(18),B(18),C(18)
      :PRINTTAB(10)"20:1 (n-7)",A(19),B(19),C(19):PRINTTAB(10)"20:2    "
```

```
      ,A(20),B(20),C(20)
1720  PRINTTAB(10)"20:3 (n-6)",A(21),B(21),C(21):PRINTTAB(10)"20:4 (n-
      6)",A(22),B(22),C(22):PRINTTAB(10)"20:3 (n-3)",A(23),B(23),C(23)
      :PRINTTAB(10)"20:4 (n-3)",A(24),B(24),C(24):PRINTTAB(10)"20:5 (n-
      3)",A(25),B(25),C(25)
1730  PRINTTAB(10)"22:0     ",A(26),B(26),C(26):PRINTTAB(10)"22:1
      ",A(27),B(27),C(27):PRINTTAB(10)"22:2     ",A(28),B(28),C(28):
      PRINTTAB(10)"22:3     ",A(29),B(29),C(29):PRINTTAB(10)"22:4
      ",A(30),B(30),C(30)
1740  PRINTTAB(10)"22:5 (n-6)",A(31),B(31),C(31):PRINTTAB(10)"22:6 (n-
      3)",A(32),B(32),C(32):PRINT
1750  PRINTTAB(10)"UNKNOWNS  ",A(39),B(39),C(39):PRINTTAB(10)"TOT.SATS.
      ",A(40),B(40),C(40):PRINTTAB(10)"TOT.MONOS",A(41),B(41),C(41):
      PRINTTAB(10)"TOT.PUFA. ",A(42),B(42),C(42):PRINTTAB(10)"TOT.(n-9)
      ",A(43),B(43),C(43)
1760  PRINTTAB(10)"TOT.(n-6) ",A(44),B(44),C(44):PRINTTAB(10)"TOT.(n-3)
      ",A(45),B(45),C(45):PRINTTAB(10)"n-3/n-6   ",A(46),B(46),C(46):PRINTTAB
      (10)"ARTIFACTS ",D(1)+D(2)+D(3)+D(4)+D(5)+D(6)+D(7)+D(8)+D(9)+D(10)
1765  PRINTTAB(48)C(39)+C(40)+C(41)+C(42):PRINTTAB(48)((C(39)+C(40)+
      C(41)+C(42))/E(1))*100
1770  VDU 3:ENDPROC
1780  DEFPROCtitle
1790  CLS:INPUTTAB(18,10)"TITLE   " Q$
1800  ENDPROC
```



Appendix B : Diagram showing the detail of the MK I CCT culture vessel.

APPENDIX C: Densitometry scanning parameters for Shimadzu CS9000 densitometer used in this study.

*** LIST OF PARAMETER ***

PHOTO MODE	; ABS REF	TRACE	; OFF
DIFFERENCE	; OFF	ZERO SET MODE	; B.C
SCAN MODE	; ZIG-ZAG	SWING WIDTH	; 10.0
λ ; SINGLE	λ = 370		
OUTPUT	; AREA		
STAGE CONTROL	; AUTO	DELTA "Y"	; 0.02
START "Y"	; 4.0	START "X"	; 40.0
END "Y"	; 98.0	TOTAL LANE	; **
LANE DIST.	; 10.0		
SIGNAL PROCESS	PEAK DETECTION		
B.C ACCUM	; 8	PKF FILTER	; 5
ACCUM NO.	; 1	DRIFT LINE	; 0.0000
LINEARISER	; OFF	MINI WIDTH	; 2.0
SMOOTHING	; 7 POINTS	MINI AREA	; 1000.0

REMARK ; SCANNING PARAMETERS FOR N.OCULATA TOTAL LIPID ON HPTLC PLATES AFTER DD-TLC & CU ACETATE.

ANALYST ; PAUL. DATE 89/08/15 03:15

APPENDIX D : Recipe for S88 algal culture medium (Droop, 1968).

Trace metal solution - TM 11.
(Made up in 1litre distilled water. Solution made acid prior to adding $FeSO_4.7H_2O$.)

$FeSO_4.7H_2O$	25.0 g	$CuSO_4.5H_2O$	190.0 mg
$MnSO_4.8H_2O$	1.5 g	$CoSO_4.7H_2O$	24.0 mg
$ZnSO_4.7H_2O$	220.0 mg	$MoO_4Na_2.H_2O$	12.0 mg

S88 medium
(Made up in 1litre distilled water)

NaCl	16.0 g
KCl	0.4 g
$CaSO_4.2H_2O$	0.5 g
$MgSO_4.7H_2O$	2.5 g
EDTA	50.0 mg
K_2HPO_4	10.0 mg
KNO_3	100.0 mg
KBr	32.5 mg
RbCl	100.0 µg
LiCl	50.0 µg
KI	25.0 µg
$AlCl_3.6H_2O$	250.0 µg
$SrCl_3.6H_2O$	6.5 mg
Glycine	250.0 mg
Glycylglycine	500.0 mg
Vitamin B_{12}	100.0 ng
Thiamine	50.0 µg
TM 11	0.1 ml